Systematics, Morphology, Phylogeny and Historical Biogeography of the Mayfly family Prosopistomatidae (Ephemeroptera: Insecta) of the World

by

Helen M. Barber-James

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Department of Entomology

Rhodes University

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Abstract

The diversity, classification and historical biogeography of the mayfly family Prosopistomatidae are explored. First, the higher classification of the Ephemeroptera is reviewed, focussing on the phylogenetic placement of the Prosopistomatidae relative to other mayfly families. All relevant literature from 1762 to 2010 is synthesized. Baetiscidae are established as the probable sister lineage of Prosopistomatidae, the two constituting the superfamily Baetiscoidea.

Next, qualitative morphological variation within the Prosopistomatidae is reviewed and revised, emphasizing nymphs because imaginal specimens are few. The labium and associated structures and the hypopharynx of nymphs, and the highly-derived wing venation of the imaginal stages, are re-interpreted. The structure of the male tarsal claws changes considerably between subimago and imago, which, together with deeply scalloped ridges on male imaginal forelegs and unusual pits on the female thorax, are interpreted as providing an unusual mating mechanism. These structures provide morphological characters for species definition and phylogenetic analyses.

Two approaches to species delimitation are explored. First, morphometric variation is analysed using Principal Component Analysis, revealing groupings that can be interpreted as species, although there is some overlap between them. Discriminant Function Analysis shows that head width and carapace shape have the most value in identifying nymphs of different species. The carapace of *Prosopistoma* nymphs is shown to grow allometrically and gradually, in contrast with that of *Baetisca*, indicating a difference in early ontogeny. Second, an Artificial Neural Network algorithm applied to nymphal morphological characters accurately identified species. This computer-driven artificial intelligence method has power to provide future easy-to-use electronic identification aids.

Phylogenetic analysis of nymphal morphology using the parsimony method shows two clades of Prosopistomatidae, one sharing characters with the type species, *Prosopistoma variegatum* and the other predominating in Africa, although also occurring in Asia; these

clades are named the "*P. variegatum*" and "*African*" clades, respectively. Parsimony analysis of adult morphology supports these two clades, but supertree analysis obscures the relationships, nesting the "*P. variegatum*" lineage within the other clade.

Preliminary molecular phylogenetic analysis of the 16S rRNA, (mitochondrial) 18S rRNA and Histone-3 genes using Bayesian Inference methods does not support the two clades shown by morphology. Instead, there is a strong relationship between the European species and one African species, with the single Asian representative being most distantly related. These results are limited by lack of fresh material, patchy taxon sampling, and problems with finding suitable primers. A molecular clock program, BEAST, calibrated using fossils, suggests divergence times for the oldest crown-group *Prosopistoma* clade, represented by the Asian *P. wouterae*, of about 131 Ma, with the youngest species, the African *P. crassi*, of 1.21 Ma.

Stem-group relationships are analysed using parsimony analysis, focussing on wing characters of the Baetiscoidea, other extant mayfly lineages, and extinct stem-group lineages. This suggests that the Baetiscoidea diverged from main-line Ephemeroptera earlier than any other extant mayfly lineage. This approach expands upon ideas hinted at by earlier scientists. Finally, historical biogeographical analysis of the distribution of known Baetiscoidea *s.s.* stem-group fossils implies a once Pangean distribution of the lineage. Changing palaeo-climate, catastrophic extinction events and plate tectonic movements in relation to the distribution of crown-group species are reviewed. Other approaches to historical biogeography that build on both morphological and molecular phylogenies are used to interpret disperalist and vacarianist arguments. Distribution patterns of eight unrelated freshwater organisms which share a similar distribution pattern are compared, assuming that shared patterns indicate similar historic biogeographic processes. The distribution of recent *Prosopistoma* species is seen to be the product of evolution resulting from both vicariance and dispersal.

In conclusion, this thesis encompasses a variety of disciplines. It successfully recognises new characters and distinguishes previously unknown species. It uses new approaches to delimiting species and known methods to determine phylogeny from several angles. The analysis of stem-group relationships offers an insight into possible early lineage splitting within Ephemeroptera. Interpretation of historical biogeography allows for both a Gondwanan origin of Prosopistomatidae, with rafting of species on the Deccan plate to Asia, and for subsequent dispersal from Asia down to Australia and across to Europe, and possibly back to Africa.

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Preface

To facilitate the reading of this thesis, data that were distracting to the flow of the thesis were included in appendices. Thus, material examined, details of collecting sites, tables of morphometric measurements, nymphal and adult characters and coding for phylogenetic analysis, specimens used for molecular analysis and GenBank numbers of downloaded sequences, river ecosystem parameters for each species, are all included in appendices. In addition, the characters used for the Artificial Neural Networks computer-driven species identification (Chapter 4) and the mathematics behind it, are included in the appendices. This was a collaborative chapter. As this is not a mathematical PhD, and the mathematics is not my own work, examiners are not expected to judge the mathematical aspect.

Some of the work presented here has already been published. As this work has been written as a full thesis rather than as a series of papers, the content of the published sections is included in the main body of the thesis, not necessarily in the same format as in the publications.

The following chapters contain data which have been published:

Chapter 2

Barber-James, H.M. 2010a. Two new species of *Prosopistoma* (Ephemeroptera: Prosopistomatidae) from South Africa and Swaziland. *African Entomology* 18(1): 147-165.

Barber-James, H.M. 2010b. Neotype erection and redescription of the larva and first description of the winged stages of *Prosopistoma variegatum* Latreille, 1833 (Insecta, Ephemeroptera) from Madagascar. *Aquatic Insects* 32(3): 215-243.

Chapter 5

Barber-James, H.M., 2009. A preliminary phylogeny of Prosopistomatidae (Ephemeroptera) based on morphological characters of the larvae, and an assessment of their distribution. *In*: Staniczek, A.H. (ed). International Perspectives in Mayfly and Stonefly Research. *Proceedings of the 12th International Conference on Ephemeroptera and the 16th International Symposium on Plecoptera.* Stuttgart 2008. *Aquatic Insects* 31(1): 149-166.

Other publications anticipated from the work are outlined below:

1.) Barber-James, H.M. (in prep, a). New species of *Prosopistoma* (Ephemeroptera: Prosopistomatidae) from West and Central Africa and Namibia.
Intended Journal: *Cimbebasia* or *African Invertebrates*

2.) Barber-James, H.M. (in prep, b). New *Prosopistoma* species (Ephemeroptera: Prosopistomatidae) from East Africa.
Intended Journal: *African Entomology*

3.) Barber-James, H.M. (in prep, c). An overview of the Prosopistomatidae (Ephemeroptera) of Madagascar and the Comores, with descriptions of new species.
Intended Journal: *Zootaxa*

4.) Burton, M.H. and Barber-James, H.M. (in prep). An artificial Neural Network Approach to species identification in Mayflies (Ephemeropera), using the family Prosopsitomatidae as an example.
Intended Journal: *BioSystems*

5.) Barber-James, H.M. and Monaghan, M. T. (in prep). Fossils, molecules and morphology: an in-depth look at the historical biogeography of the mayfly family Prosopistomatidae (Ephemeroptera).
Oral paper: 9th Conference of South African Society for Systematic Biology, Grahamstown, January 2011.
Intended Journal: *Systematic Entomology*

6.) Barber-James, H.M. and de Moor, F.C. (in prep). Preliminary investigation of unusual mating behaviour in the mayfly family Prosopitomatidae, derived from a study of morphology.
Oral paper: to be presented at XIIIth International Conference on Ephemeroptera, XVIIth International Symposium on Plecoptera, Kiyosato, Japan. June 5-11th 2011.
Written paper: to be published in the Conference Proceedings.

7.) Barber-James, H.M., Monaghan, M. T. and Schletterer, M. (in prep). Is *Prosopistoma pennigerum* really only one species? A preliminary molecular assessment.
Intended Journal: *European Journal of Entomology*

8.) Barber-James, H.M. and Villet, M.H. (proposed). Species delimitation in the mayfly family Prosopistomatidae (Ephemeroptera) using morphometric techniques.
Intended Journal: *African Entomology*

9.) Barber-James, H.M. (proposed). The palaeo-history of the Ephemeroptera, and proposed origin of stem-group Prosopistomatidae based on fossil evidence
Intended Journal: *Evolutionary Biology*

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Declaration

The thesis presented here has not been submitted to a university other than Rhodes University, Grahamstown, South Africa. All the work presented here is done by the author unless otherwise stated.

Dedication

To my family; my mother and father for allowing me the freedom to be who I wanted to be, and supporting the career of my choice; my children, for their unfailing love and support.

Chapter 1

Introduction, with a review of the systematics of Ephemeroptera, focusing on the Prosopistomatidae

"Taxonomy (the science of classification) is often undervalued as a glorified form of filing – with each species …… in its prescribed place in an album; but taxonomy is a fundamental and dynamic science, dedicated to exploring the causes of relationships and similarities among organisms. Classifications are theories about the basis of natural order, not dull catalogues complied only to avoid chaos"

Stephen Jay Gould (1991, p. 98)

Synopsis

This chapter outlines the expectations of the thesis as a whole, giving a brief account of each section. It then provides a detailed historical account of the classification of the Prosopistomatidae. The various approaches taken to the systematics of modern Ephemeroptera are synthesized, with emphasis on the relationship between the Prosopistomatidae and their putative sister group, the Nearctic Baetiscidae. This sets the scene for the later use of the Baetiscidae for outgroup comparison in the phylogenetic studies carried out on the Prosopistomatidae species. A summary of all known *Prosopistoma* species with species authors, country occurrence and known life history stage is provided at the end of this chapter, and *Prosopistoma* species author names are not mentioned again when discussing species in the following chapters.

1.1 Overview of thesis

This thesis aims to give a global review of the mayfly family Prosopistomatidae, a relatively small, currently monogeneric family of mayflies, which has its largest diversity in the Afrotropical and Oriental regions. The aim is to investigate nymphal and adult morphology for characters which distinguish it as a family, and for species-defining characters. These are then used to establish the phylogenetic relationship between the Afrotropical species and those found in the Palaearctic, Oriental and Australasia regions, based on morphology. Molecular phylogenetic analysis is undertaken to evaluate if this is supported. The thesis has

been divided into three main sections, each containing several chapters, and each section encompassing a particular group of aims (Figure 1.1).

The first section includes this introductory chapter and looks at the systematics of the Prosopistomatidae in relation to other mayflies, and the history of the systematics of the group itself. This places the family in context before moving on to Chapter 2, where detailed studies of morphology are undertaken. Nymphal morphology includes the reinterpretation of certain structures, as well as providing the first detailed account of the hypopharynx, which is highly modified in this family. In the imaginal stages, wing venation and homology of the veins is discussed, and detailed investigation of the structure of male forelegs, tarsal claws and the female thorax offer an unusual explanation for mating behaviour in the family. Both life history stages in the Prosopistomatidae have several autapomorphies with respect to other mayfly families, and variations in these provide sound characters for morphological phylogenetic analyses between species.

The next section, consisting of four chapters, looks at different approaches to species delimitation and determining relationships between species. What are the indicators of relationship in Prosopistomatidae? Do different morphological characters show lack of independence for phylogenetic analysis due to adaptation? Do independent sources of data (nymphal and adult morphology versus DNA) provide the same interpretation of relationships? Are the two clades implied in the morphological phylogeny sound, and do they support the creation of a separate genus? These are core questions addressed in this thesis.

Simple geometric morphometric measurements analyzed using principal component analysis (PCA), discriminant function analysis (DFA) and scatter plots are used to see whether there are trends which show species grouping; this looks at clustering of species both in the nymphs, using a variety of measurements, ratios and trusses, and in the imaginal stages looking at truss measurements between points on the wings only. In both nymphs and adults, such measurements are shown to be helpful in separating species.

A new approach using artificial neural networks to classify and identify species is then investigated. This is based on phenetic characters, and produces a computer-aided partitioning of species for identification purposes, with applied implications aimed at a practical solution to species identification problems, rather than looking for relationships.

After empirically categorising species in these ways, the relationships between all known *Prosopistoma* species are investigated. This is done by selecting shared, derived

characters based on nymphal and adult characters for morphologically determined phylogenies, and analyzing these using both Maximum Parsimony and Bayesian Inference analyses. The phylogenetic analysis of mitochondrial and nuclear genes aims to corroborate the morphology-based phylogenies. The intention is to use multiple genes to investigate both the relationships between species, and to estimate ages of lineage splitting, based on dating nodes using fossils. The study of relationships delves into truly understanding what a species is, which is more than an arbitrary assignation of characters, but rather an interpretation of the evolutionary processes that have shaped each lineage.

The third section of the thesis consists of two chapters that look at relationships between the Prosopistomatidae plus Baetiscidae lineage, fossil taxa and other recent mayflies, and also between recent *Prosopistoma* species and their global and regional distributions in time and space. Fossil evidence, gained from a synthesis of literature on fossil insects, suggests an early split of the lineage now comprising the Prosopistomatidae and Baetiscidae from the stem group leading to modern mayflies. The historical biogeography is investigated using six different approaches, and the role played by vicariance and dispersal in the speciation of the mayfly species seen today is discussed.

1.2 History of classification of the family Prosopistomatidae

The first known species of the monogeneric ephemeropteran family Prosopistomatidae was discovered in France by Geoffroy (1762), who collected and described a nymph from the streams near Paris as one of three organisms he thought were Crustacea. He called one “le binocule à queue en plumet” (roughly translated as “the binocule with a plume”), the plume presumably referring to the caudal filaments. This was the first prosopistomatid mayfly collected, although it was not recognised for over a century as a member of the Ephemeroptera, until Joly (1871) managed to rear a nymph through to the winged stage. The other two organisms, which were genuinely Crustacea, were redescribed by Müller (1776), who applied binomial nomenclature to these, introducing the generic name *Binoculus* but he did not include “le binocule à queue en plumet” within this new genus. In listing the genus *Binoculus*, de Fourcroy (1785) included “le binocule à queue en plumet” as *Binoculus foliaceum*. He gave this specific epithet because Geoffroy (1762) had indicated that *Monoculus foliaceum* Linnaeus (1758) was the same species as *Binoculus foliaceum*. Müller (1785) realised that this organism was a different species to Linnaeus’s *Monoculus*

foliaceum and placed it and one of the other of Geoffroy's "three Crustacea" into the new genus *Limulus*, as *Limulus pennigerus*. At this point, no types were yet designated. Latreille (1802) realised that it was different to *Limulus* and renamed it *Binoculus pennigerus* (*Binocule permigère* according to Vayssiére, 1881), designating it as the type species of *Binoculus*. It was around this time that Latreille (1802) introduced the concept of the family as a rank, a very important step in classification as it allowed genera to be placed as subordinate groups below this. However, the family Prosopistomatidae was only erected in 1917 by Lameere (1917c) (as Prosopistomidae – Prosopistomatidae as we know it today was erected by Ulmer (1920); this is important to note as it gives priority to the name Prosopistomatidae over Binoculidae Demoulin 1954).

Duméril (1816) renamed it *Binoculus piscinus*, though no reason was given for this change and the name was not subsequently used. Latreille (1833) then separated it from the other Crustacea with which it had been grouped by Geoffroy, and erected the new genus *Prosopistoma*, renaming it as *Prosopistoma punctifrons*. He included in this genus a species from Madagascar that he named *Prosopistoma variegatum*, although these were not yet recognised as mayflies; it was still incorrectly thought that the *Prosopistoma* nymphs were the adult of an unusual branchiopod Crustacean. The name *Prosopistoma* was derived from the Greek word for mask, *prosopion*, and *stoma* for mouth, due to the masked appearance of the mouth or "masque bouche", which he noted was different from the other crustacea with which it was placed. Latreille did not nominate a type species. Joly (1871), Joly and Joly (1872a,b,c) and Joly and Joly (1895) were the first to realise that these were in fact nymphs belonging to the insect order Ephemeroptera. They proposed the name *Chelysentomon pennigerum*, as they argued that the name *Prosopistoma* could no longer be used for this animal, but thereafter they and other authors at that time continued to refer to it as *Prosopistoma*. These names were widely accepted until Demoulin (1954) indicated that the valid name should be *Binoculus*, and he proposed the family name Binoculidae, although this was unnecessary as the genus *Binoculus* can be in the family Prosopistomatidae, according to the Code. Indiscriminate use of either generic name, and several of the species names for the European species, followed. Hubbard (1979) reinvestigated the nomenclature and attempted to resolve this issue. Following the rules of the ICZN (version not stated), Hubbard deduced that the valid generic name should be *Prosopistoma*, and the species name for the European species should be *pennigerum* and not *foliaceum* (*Binoculus* is still a valid

name for a Crustacean, and *foliaceus* refers to Linnaeus's *Monoculus folicaeus*, also a crustacean).

Eaton (1884) designated the Madagascan species *P. variegatum* as the "type" of the genus, without actually seeing the material or designating a holotype. The original material has long since been lost, and a neotype has been designated (Barber-James, 2010b). The original *P. variegatum* material was collected by a travelling naturalist, M. Goudot junior (Latreille, 1833), along with some Coleoptera. Goudot was an ardent collector of insects and plants in Madagascar in the 1830s, though there seems to be scant record of where Goudot travelled. Correspondence with a botanist currently at the Paris Museum (Peter Phillipson, Museum National d'Histoire Naturelle, Paris, France, pers. comm., 2007) revealed several sites where Goudot collected plants, but this did not help to clarify the collecting site for the original Prosopistomatidae material. With regard to the type locality, Latreille (1833), Westwood (1877) and Eaton (1884), who all examined Goudot's material, gave only "Madagascar" as the locality. It seems that Westwood (1877) was the last person to see the material used by Latreille (1833); he stated that he examined "the wreck" of Latreille's collection in Paris. Westwood (1877) provided four diagrams of *P. variegatum* of limited diagnostic value but the shape and proportions of the animal he illustrated support the neotype designation (Barber-James, 2010b).

In her efforts to trace this material, Josette Fontaine (then based at the University of Lyon, France) corresponded in March 1971 with D.E. Kimmins at the Natural History Museum, London. He referred her letter to another curator, P.H. Ward, who replied that the material was not in London, but might be in the Hope-Westwood Collection of Entomology at the Oxford University Museum of Natural History. Following this up, it could not be traced in that collection (James Hogan, Oxford University Museum, Oxford, U.K., pers. comm., November 2007). Kimmins's (1971) list of type specimens of Ephemeroptera in the BMNH, and personal examination of material in the museum (September 2004), further confirmed the absence of this material. Latreille's material is thus declared to be lost.

Latreille's (1833) description involved comparison with Entomostraca and Branchiopoda. He said that the antennae were small, five-segmented, and broken in many individuals. It seems the material studied by Latreille was in poor condition with various parts described as mutilated. Comparing *P. variegatum* with *P. pennigerum*, he noted that *P. variegatum* is more raised, and oval in shape (like a gyrinid beetle), while *P. pennigerum* is more rounded (like a coccinellid beetle). He described *P. variegatum* as "… almost six

millimetres long. Its body is a dark brown, dull, mixed with yellow on the top and at the sides" (in French). Given this size, it is probable that the nymphs examined by Latreille had not reached maturity, as the maximum length measured among the more recently collected larvae examined for this study was over 8 mm.

Vayssière (1890a,b) mentioned that M. Fr. Sikora (an Austrian naturalist living in Madagascar) found three *P. variegatum* larvae in a small tributary of the Mangoro River, in a river with rapid current, near the village of Ambodinangano, about 50 km south east of Antananarivo. These were found in October (year not stated) and were mature, with well developed wing buds, and measured 7-9 mm in length (compared to Latreille's 6 mm). This record is consistent with the present known distribution of the species, but there is no reason to think that it corresponds to the locality from which Goudot collected the earlier material used by Latreille. However, this influenced the choice of neotype locality, and material collected in 1998 from the closest site to this, a small tributary of a river in the Mangoro River basin, was chosen for the neotype (Barber-James, 2010b). Demoulin (1954), in reasoning that *Binoculus* should be the generic name rather than *Prosopistoma*, pointed out that in this scenario, *P. variegatum* would lose its status as the type species. However, since Hubbard (1979) reinstated the name *Prosopistoma*, this argument falls away.

Many other species of *Prosopistoma* have been discovered since these early days. The distribution of *Prosopistoma* species subsequently discovered in the Afrotropical realm, Middle East, Oriental and Australasian realms, is dealt with in Chapter 8. A full list of names and synonyms is provided in Table 1.1, which includes the species characterized for this thesis but not yet formally described. Coden abbreviations for museums housing specimens are given in Appendix Table A1.1.

1.3 Introduction to higher classifications and phylogeny of families within the modern Ephemeroptera

A number of different classifications have been proposed for the Ephemeroptera at various times. Before trying to understand the classification of the Prosopistomatidae, it is necessary to examine the history of the higher classification of Ephemeroptera as a whole, to see the Prosopistomatidae in context. This is not a comprehensive review of every classification that has ever been done, but rather an attempt to understand classifications and implied relationships pertaining to the Prosopistomatidae. The placement of the Prosopistomatidae

in relation to their purported sister group, the Nearctic family Baetiscidae, by different authors over time has varied according to which criteria have been used to determine the relationships. The understanding of this relationship is critical to the choice of outgroup for the phylogenetic analyses addressed in this study.

The first formal classification of the Ephemeroptera was introduced by Linnaeus (1758), who created the genus *Ephemera*, at that time within the order Neuroptera, and included six genera in this. These were further divided into those with three caudal filaments (cauda triseta), and those with two (cauda biseta). The rank of "order" has been attributed to the German botanist, Augustus Quirinus Rivinus (1690s), but Linnaeus was the first person to use it consistently. The order Ephemeroptera was introduced after the Neuroptera of Linnaeus was divided into smaller groups. This was done in several stages. Leach (1815) divided Linnaeus's group with two caudal filaments into those with four wings and those with two. Burmeister (1839), Packard (1886) and Handlirsch (1903) proposed further divisions. Several other authors made small changes and introduced new genera and species, most notably Pictet (1843-1845). Packard (1886) introduced the rank "Plectoptera", which included all mayflies and stoneflies known at that time. Hyatt and Arms (1890) split the Plectoptera, introducing the modern terms Ephemeroptera and Plecoptera (stoneflies).

Latreille (1802) introduced the concept of the family as a rank, a very important step in classification as it allowed genera to be placed as subordinate groups below this. The family Prosopistomatidae was erected in 1917 by Lameere, as Prosopistomidae, and included *Baetisca*, *Prosopistoma* and the extinct †*Hexagenites*, as a subfamily †Hexagenitinae, which was subsequently recognized as a family in its own right (†Hexagenitidae) by Tshernova (1961). The name Prosopistomidae continued to be used by Lestage (1917). Ulmer (1920) referred to Prosopistomidae in the text of his paper, but introduced the term Prosopistomatidae in his classification (including only *Prosopistoma* in this), although Lameere (1917c) is still given credit for the name Prosopistomatidae. It is not clear why there was a transition from Prosopistomidae to Prosopistomatidae. Spieth (1933) introduced the taxon Baetiscoidea, referring in this instance to only the Baetiscidae. Peters and Hubbard (1989) showed that as Baetiscidae was described by Banks (1900), Baetiscoidea should be attributed to Banks 1900, and therefore takes priority over the Prosopistomatoidea of Edmunds and Traver, 1954a. The name Baetiscoidea should therefore also take precedence over other more modern names, and implies that other high

ranking taxon names (Carapacea *sensu* McCafferty (1997), Posteritorna (Kluge *et al*., 1995)) may be considered unnecessary, although they are not synonyms.

Joly (1871) and Joly and Joly (1872a,b,c) were the first to recognize *Prosopistoma* as a mayfly (discussed earlier), and they proposed that there were three categories of mayflies, those with operculate gills such as Caenidae, those with purely external gills, such as *Ephemera*, *Palingenia*, *Baetis* and others, and those with gills which are completely protected as in *Prosopistoma*. The most comprehensive earlier work on the systematics of mayflies was that of Eaton (Eaton 1883-1888), where he summarised all the preceding classifications, recognising many synonyms, and described some 300 species in 55 genera. His division of the mayflies (which he called "family Ephemeridae") into sections (3 groups, 9 series and 14 sections) constituted the basis of modern classification and remained almost unchanged for a century except for the hierarchical rank of the sections. It is worth noting that he did not place *Prosopistoma* and *Baetisca*, the two groups with a nymphal carapace, in the same grouping, instead putting *Prosopistoma* with *Caenis* and *Baetisca* with *Siphlurus*, based largely on wing venation.

Lestage (1917) divided the mayflies into two groups based on nymphal characters, namely whether the gills were visible from the exterior (nudibranches) or concealed (cryptobranches), where he included only the Prosopistomatidae (as Prosopistomidae); he did not mention the Baetiscidae. While the earlier classifications were based mainly on adult characters, from the time of Lestage (1917) onwards, nymphal characters became preferred as they were seen to produce natural groupings. Unlike many of the earlier descriptions of other mayflies, *Prosopistoma* was known only from the nymphal stage for over a hundred years. This continues to be the case for many of the *Prosopistoma* species known today.

Ulmer (1920) proposed three suborders (Unterordnung) (closely following Eaton's three sections); Ephemeroidea, Baëtoidea (which included Prosopistomatidae) and Heptagenioidea (which included Baetiscidae). Needham *et al.* (1935) modified Eaton's classification to include three families (Ephemeridae, Heptageniidae and Baetidae), which were further divided into 17 subfamilies. *Prosopistoma* and *Baetisca* were both included in the Baetidae, in Prosopistomatinae (subfamily 17) and Baetiscinae (subfamily 12), along with eight other subfamilies. No mention was made of Lameere's (1917c) Prosopistomatidae, even though this paper is quoted in the references. From a modern perspective, Needham *et al.*'s system can be seen to be highly polyphyletic.

Vayssière (1934) and Gillies (1954) placed the Prosopistomatidae and the Baetiscidae as sister lineages, based on nymphal characteristics, particularly the fused, carapace-like thorax. Fontaine (1958), based on detailed anatomical studies of the two families, concluded that they had too many characteristics in common to be explained purely by convergence, agreeing with associating these two families as sister groups.

Demoulin (1954) proposed the family name Binoculidae instead of Prosopistomatidae, due to the original misplacement of the group as an entomostracan, trying to tie in the family name with the generic name (Binoculidae with *Binoculus*). Edmunds and Traver (1954a) proposed a higher classification of the Ephemeroptera in which they restored the name Prosopistomatidae instead of Binoculidae, creating the new rank Prosopistomatoidea (instead of Baetiscoidea), containing the Baetiscidae and Prosopistomatidae. Demoulin (1958), trying to link fossil and modern mayflies, produced a classification which grouped the Ephemeroptera into eight superfamilies, including two superfamilies containing extinct taxa (fossils), believing these to have arisen from two stem groups already present in the late Palaeozoic. Under his classification, the Prosopistomatidae fell under the Ephemerelloidea with the Ephemerellidae, Tricorythidae and Caenidae, while Baetiscidae fell under the Oligoneurioidea which also included the families Iconychiidae, †Paedephemeridae and Oligoneuriidae.

Edmunds (1962) attempted the first "phylogenetic" construction of the order, although this was deduced intuitively, not based on coding and analysis such as parsimony as this concept was not yet widely used. Thus he did not provide justification from a true phylogenetic perspective. His grouping was different considerably to Demoulin's, with Prosopistomatidae and Baetiscidae placed together under the superfamily Prosopistomatoidea, following his and Traver's earlier ranking. Edmunds, Allen and Peters (1963) continued to keep these two families as sister groups.

The importance of a classification representing a phylogeny was highlighted during a conference in Tallahassee, Florida in 1970. Different classifications were presented, derived from different evidence (e.g. Landa (1973), using internal tracheation (based on the results in Landa, 1969); Riek (1973), largely using fossil evidence; Koss (1973), using egg external morphology; Edmunds (1973), based on external morphology of nymphs and adults, as well as fossils and deriving insight from Landa's study of internal morphology). This illustrated the importance of using as many different characters as possible to get the best phylogeny. Landa (1973) placed Prosopistomatidae with Caenidae, and Baetiscidae

with Neoephemeridae. Edmunds (1973), referring to Landa's work, pointed out that the Malphigian tubule system of Caenidae and Prosopistomatidae are both reduced, but concluded that this is not an indication of phyletic relationship but rather of size. Edmunds preferred to group the Prosopistomatidae and Baetiscidae, based on other shared characters such as the last ganglion of the nervous system being in the metathorax in both families, and the existence and placement of the carapace. Riek (1973), in discussing the placement of these families, pointed out that in Baetiscidae, the caudal filaments have hairs arranged in a lateral fringe, while these are in whorls in the Caenoidea. Personal observation of these hairs in Prosopistomatidae shows that they are arranged in a lateral fringe as in Baetiscidae. Riek pointed out that this is the primitive state, with representatives from the lower Permian having short cerci with lateral hair fringes. He maintains that hairs in whorls are associated with the habit of a nymph, with whorls being associated with the sprawling habit and slower flow, and fringes being present in nymphs which actively swim (not to be confused with habitat, as Baetiscidae are sand dwellers, while Prosopistomatidae are associated with rocky substrates). Looking at adult features, particularly wing venation, Riek kept Baetiscidae and Prosopistomatidae together, but with reservations based on several marked differences at both adult and nymphal stages.

Other authors also contributed to the progression of the systems of classification e.g. Tshernova (1970), placed the Caenidae and Prosopistomatidae in the Caenoidea. Koss and Edmunds (1974), based on detailed egg structure evaluation, found several areas which strongly disagreed with Demoulin's 1958 classification (and subsequent - e.g. Demoulin 1961, 1968, 1969); of relevance here was that they found that Baetiscidae eggs show no affinities with Siphlonuroidea species (with which Demoulin (1969) associated the Baetiscidae), and that Prosopistomatidae did not fit with the Ephemerelloidea (in which Demoulin (1958) included Ephemerellidae, Tricorythidae, Prosopistomatidae and Caenidae).

A significant change in classification came about when McCafferty and Edmunds (1979) divided the mayflies in two suborders, Pannota (nymphal wingpads fused with the mesonotum for more than half their length) and Schistonota (nymphal wingpads unfused for more than half their length, which is considered the ancestral condition). In this system, the carapace was seen to represent the extreme state of fusion of the nymphal wingpads in the Pannota, and Baetiscidae and Prosopistomatidae were placed together in the superfamily Prosopistomatoidea. To try and get round the problem of the Schistonota concept being

paraphyletic, Kluge (1989) proposed the Furcatergalia (including Pannota and part of the Schistonota) and Costatergalia (including the rest of Schistonota). The latter was still seen as paraphyletic. McCafferty (1991) then proposed three new suborders, Rectracheata (equivalent to Kluge's Furcatergalia), Setisura and Pisciforma. Here, he kept the Pannota as an Infraorder in the Suborder Rectracheata. However, within the Pannota, he separated the Prosopistomatidae and Baetiscidae, putting Prosopistomatidae within the Caenoidea, with Caenidae, Ephemerellidae and Tricorythidae, based on wing venation and flight behaviour, and the fact that Caenidae and Prosopistomatidae females are reproductively mature as subimagos. Baetiscidae were put into Baetiscoidea as a sister group to the Neoephemeridae, based on shared internal anatomical apomorphies.

In contrast to these views, Kluge (1992-unpublished, 1998) and Kluge *et al.* (1995) placed the Baetiscidae and Prosopistomatidae together in a suborder of their own, the Posteritorna, partially based on forewing venation. The Posteritorna have the tornus of the forewing behind CuP, while in the Anteritorna, the tornus is situated between CuA and CuP (Figure 7.5f,g). The potential importance of this distinction to understanding of the evolution of the mayflies is discussed in Chapter 7. McCafferty (1997), Wang *et al.* (1997), and McCafferty and Wang (2000) supported the removal of both of these families from the Pannota, placing them alone in a suborder, the Carapacea (= Baetiscoidea = Prosopistomatoidea = Posteritorna, depending on which nomenclature is followed).

Kluge (1998, 2004) proposed revisions at different levels of the phylogeny of the mayflies and summarized them in a comprehensive modern treatment including all mayfly lineages, available at http://www.insecta.bio.pu.ru/z/Eph-spp/Contents.htm. As he felt that the system of ranking zoological nomenclature developed by Linnaeus (1758) could no longer cope with the demands of modern systematics needs, he has developed a parallel, non-hierarchical ranking nomenclature (Kluge, 1999), which he calls a circumscription-based nomenclature. He introduced the rank Ephemera/fg3 to include all recent Ephemeroptera *s.s.* lineages, which he equated to Euplectoptera Tillyard 1932; Ephemera/fg2 to include modern Ephemeroptera *s.l.* plus fossils which were clearly ephemeroperan, which he equated to Euephemeroptera Kluge 2000, and Ephemera/fg1 which includes the broader spectrum of fossils including those which are of dubious placement, which is equivalent to Panephemeroptera Crampton 1928. Kluge (2010a) also summaries some of the earlier nomenclatures not covered here. Note that Euplectoptera

Fischer 1853 was used for Dermaptera, and is no longer in current use; Euplectoptera Tillyard 1932 is used for Ephemeroptera *s.str.* (Kluge, 2010b).

An alternative to Kluge's non-ranking system must be mentioned here, even though it has not yet been applied to the Ephemeroptera. Kluge's reasoning is similar to the thinking which produced the PhyloCode approach, initiated by de Queiroz (e.g. 1988, 1997; de Queiroz and Donoghe, 1988, de Queiroz and Gauthier, 1992, 1994). This proposes defining a name phylogenetically, looking at shared ancestors, rather than by shared characters. Nomenclature is based on evolutionary theory defined by members of a clade rather than by attributes of each member.

Although different in approach to more conventional Linnean-based systems, the outcome of Kluge's classification produced a grouping which is similar to the more recent classification summary of McCafferty's work. The latter was synthesized by Ogden and Whiting (2005) (Figure 1.2), based mostly on McCafferty's works after 1991, from a number of studies (McCafferty & Wang, 2000; Kluge, 2003; Molineri and Dominguez, 2003; McCafferty, 2004; Wang & McCafferty, 2004). These two systems divide the order into four main lineages: (1) Carapacea *sensu* McCafferty / Posteritorna *sensu* Kluge; (2) Furcatergalia; (3) Setisura; (4) Pisciforma *sensu* McCafferty / Tridentiseta *sensu* Kluge. Although the four main lineages are generally accepted, the relationships between and among them, the placement of some taxa and the rankings remain problematic. However, most important here is that the Prosopistomatidae and Baetiscidae are placed here as a sister lineage to the rest of the mayflies, and have many shared characteristics which are taken as derived.

Several phylogenies based on molecular data have been proposed to help understand the higher relationships within the order. Ogden & Whiting (2005), using 18S rDNA, 28S rDNA, 16S rDNA, 12S rDNA and Histone three protein coding for the nucleosome, produced a phylogeny including 31 out of 42 currently recorded mayfly families (Barber-James *et al.*, 2010). Ogden and Whiting (2005) concluded, amongst other things, that the Baetiscoidea are monophyletic, suggesting that this supports the homologous nature of the carapace, a concept questioned in Chapter 3.

Sun *et al.* (2006) sequenced the 18S rDNA of 20 families. Unlike Ogden and Whiting (2005), who found the Baetidae to be the basal ephemeropteran lineage, Sun *et al.* (2006) place *Baetisca* as the most basal clade. They did not include Prosopistomatidae in this study, so, from the perspective of this thesis, their results are not very useful, but one

would assume that Prosopistomatidae would group with them. Ogden *et al*. (2008) re-analysed the data of Sun *et al.* (2006), showing that their methodology was flawed, and expanded the study by adding more sequences to the analysis. The Baetiscidae were not basal in this revised analysis. Using both molecular and morphological data, Ogden *et al.* 2009 found several opposing options. In a parsimony consensus, Baetiscidae and Prosopistomatidae were recovered as monophyletic, with a suggestion of Oligoneuriidae being a close sister lineage, although this was not well supported. Using likelihood analysis of the molecular dataset alone, Oligoneuriidae were not in the same clade, while in the combined tree, this relationship was re-established. These analyses also did not place Baetiscoidea as basal.

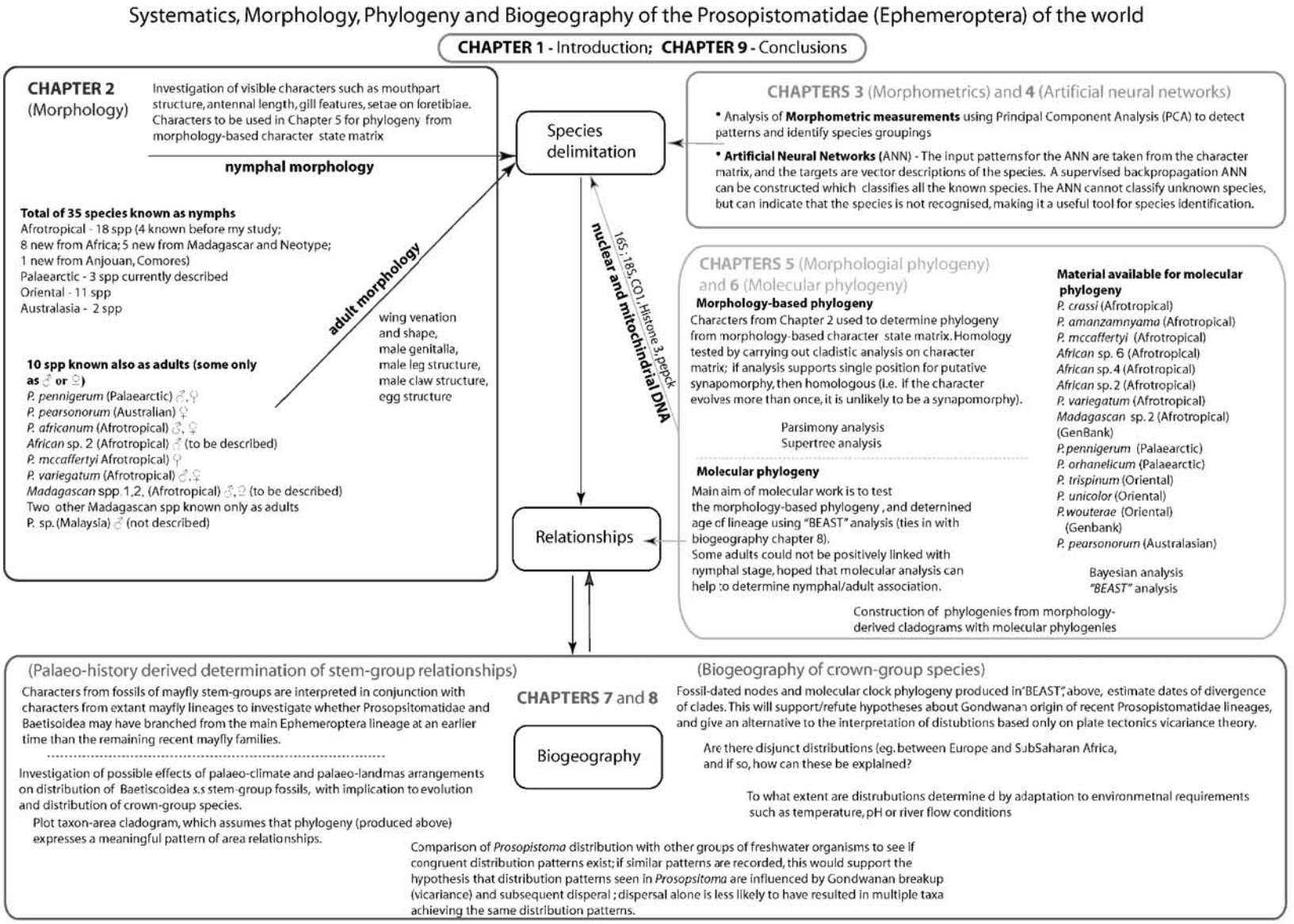
Overall, current thinking places the family Prosopistomatidae as being most closely related to the Nearctic family Baetiscidae. While one strives to find the most strongly supported relationships between groups of organisms, it is inevitable that the details of phylogenies will continue to be debated in the light of further new information. It is hoped that this thesis will contribute further to the understanding of the Prosopistomatidae, and thereby to their relationships with other mayflies.

Table 1.1. World list of named or soon to be named *Prosopistoma* species (in alphabetical order, by region). n = nymph, ♂ = male subimago or imago, ♀ = female subimago. Abbreviations of species names as in Appendix Table A5.1, 5.2 and 5.3.

Current species known	Biogeographic region	Distribution	Life history stage known	Abbr
P. orhanelicum Dalkiran, 2009	Palaearctic	Turkey	n	*P. orh*
P. pennigerum (Müller, 1785)	Palaearctic	Europe	n, ♂, ♀	*P. pen*
P. oronti Alouf, 1977	Palaearctic	Israel, Lebanon, Syria	n	*P. oro*
P. annamense Soldán & Braasch, 1984	Oriental	Vietnam, China	n	*P. ann*
P. boreus Peters, 1967	Oriental	Philippines	n	*P. bor*
P. funanense Soldán & Braasch, 1984	Oriental	Vietnam; China (Hong Kong and Guangdong Province)	n	*P. fun*
P. indicum Peters, 1967	Oriental	Kerala, India	n	*P. ind*
P. lieftincki Peters, 1967	Oriental	Sri Lanka	n	*P. lief*
P. olympus Sartori & Gattolliat, 2003	Oriental	Borneo	n	*P. oly*
P. palawana Peters, 1967	Oriental	Philippines	n	*P. pala*
P. sinense Tong & Dudgeon, 2000	Oriental	China (Guangdong Province)	n	*P. sine*
P. trispinum Zhou & Zheng, 2004	Oriental	Southwestern China	n	*P. tri*
P. unicolor Zhou & Zheng, 2004	Oriental	Southwestern China	n	*P. uni*
P. wouterae Lieftinck, 1932	Oriental	Thailand, Malaysia, Java, Sumatra	n	*P. wou*
P. sedlaceki Peters, 1967	Australasian	New Guinea, Solomon Islands	n	*P. sedl*
P. pearsonorum Campbell & Hubbard, 1998	Australasian	Northern Australia	n, ♀	*P. pear*
P. africanum Gillies, 1954	Afrotropical	Tanzania	n, ♂, ♀	*P. afr*
P. amanzamnyama Barber-James, 2010a	Afrotropical	KwaZulu-Natal, South Africa	n	*P. aman*
P. crassi Gillies 1954	Afrotropical	South Africa	n	*P. cras*
P. deguernei (Vayssière, 1893)	Afrotropical	Senegal	n	*Excluded from phylogeny*
P. mccaffertyi Barber-James, 2010a	Afrotropical	Mpumalanga, South Africa	n, ♀	*P. mccaf*
P. variegatum Latreille, 1833	Afrotropical	Madagascar	n, ♂, ♀	*P. var*
African sp. 2	Afrotropical	Namibia	n, ♂	Af sp. 2

Current species known	Biogeographic region	Distribution	Life history stage known	Abbr
African sp. 3	Afrotropical	Democratic Republic of Congo	n	Af sp. 3
African sp. 4	Afrotropical	Kenya	n	Af sp. 4
African sp. 6	Afrotropical	Democratic Republic of Congo	n	Af sp. 6
African sp. 7	Afrotropical	West Africa	n	Af sp. 7
African sp. 8	Afrotropical	Botswana, Zimbabwe	n	Af sp. 8
Comores sp. 1	Afrotropical	Anjouan	n	Anj
Madagascan sp. 1	Afrotropical	Madagascar	n, ♂, ♀	Mad sp. 1
Madagascan sp. 2	Afrotropical	Madagascar	n, ♂, ♀	Mad sp. 2
Madagascan sp. 3	Afrotropical	Madagascar	n	Mad sp. 3
Madagascan sp. 4	Afrotropical	Madagascar	n	Mad sp. 4
Madagascan sp. 5	Afrotropical	Madagascar	n	Mad sp. 5
Madagascan sp. 6 (unknown adult 1)	Afrotropical	Madagascar	♂	P0249
Madagascan sp. 7 (unknown adult 2)	Afrotropical	Madagascar	♂, ♀	P0400
Madagascan sp. 8 (molecular only)	Afrotropical	Madagascar	n	Mad sp. 8
Total no of species and life history stage known			**37 total 35 n, 8♂, 8♀**	

Figure 1.1. Structure and content of the thesis.



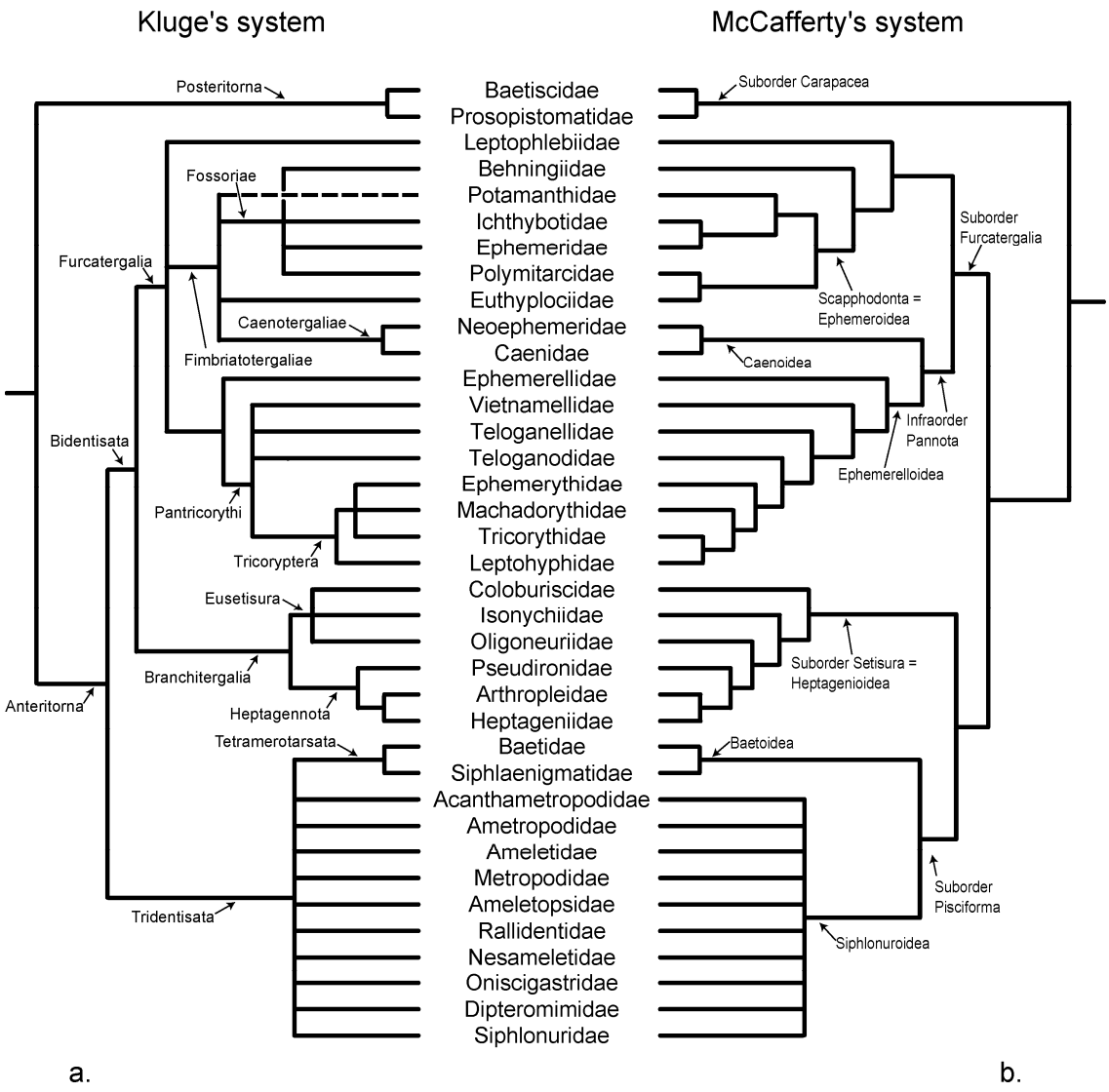


Figure 1.2. Comparison of (a) Kluge's and (b) McCafferty's systems, from Ogden and Whiting (2005), with permission from Ogden (19 March 2008). Both classifications group the Prosopistomatidae and Baetiscidae as sister lineages.

Chapter 2

Assessment of the morphology of the nymphs and winged stages of the Prosopistomatidae

"Morphology, in the biological sense, is the science of form in living organisms. Anatomy is the determination of structural facts… In the study of insect morphology we cannot confine ourselves to the limits of entomology… the basic structure of the Arthropoda is much older than the arthropods themselves."

Snodgrass, 1935 (p.1)

"To understand the structural organisation of any animal, it is necessary to know that animal's history, for no living creature has arrived at its present organization by a direct line of development from its beginning. Structure is generally an adaptation to function; but many of the organs of complex animals have served a series of different functions during the course of their evolution and, as a consequence, have had their structure many times remodelled by way of adaptation to their changing functions or to new function."

Snodgrass, 1935 (p.14)

Synopsis

Details of the morphology of the nymphs and winged stages of the Prosopistomatidae are presented. The methods used for preparing specimens, both for light microscopy and scanning electron microscopy (SEM) are provided, including some adaptations of the more standard preparation techniques used for larger mayflies. Although the basic morphology of the family, especially of the nymphs, was previously known, several of the structures are reinterpreted, especially the labium and hypopharynx, which are both described in detail for the first time. Details are also given for the imaginal stages, with an in-depth discussion on homologies in wing venation.

Unlike all other mayflies, the male forelegs of *Prosopistoma* are found not extend in length during the moult between subimago and imago. Furthermore, the claws change from being terminally hooked in the male subimago to being pad-like in the imago, a structure which is apomorphic to this family. Newly discovered structures on the female thorax are interpreted as providing slots for males to insert their tarsal claws and grip the female during copulation. A unique structure at the base of the male head may also play a significant role in an unusual mating ritual. These structures may play an important role in

mate recognition of conspecifics and could be important in determining species distinctions. The egg structures of a few species of *Prosopistoma* are investigated under SEM, and interpreted in relation to egg structures known for other species. All observations are presented in the form of combined results and discussion about each structure.

2.1 Introduction

An elaborate, detailed monograph on the morphology of the nymph of the European species, *Prosopistoma pennigerum* (Müller 1785), was produced by Vayssière (1890b), preceded by several shorter works (Vayssière, 1881a,b, 1882, 1890a). These remarkable studies included illustrations and descriptions of the external morphology, the gills, the Malpighian tubules, nervous and tracheal systems, and musculature of the retractile caudal filaments. Various other authors discussed the morphology of *Prosopistoma*, focusing mostly on *P. pennigerum* which was the main species available for study at that time. These include the authors who developed the nomenclature of the group (covered in Chapter 1), and those who purely noted their distribution patterns e.g. Trägårdh (1911), or studied life history aspects, e.g. Lafon (1952), who reared nymphs of *P. pennigerum* in a tank in her laboratory in Lyon, providing further illustrations of morphology, and information on their biology and ecology. Gillies (1954) and Peters (1967) produced the first detailed descriptions of nymphal morphology of species from Africa and the Oriental region respectively.

This chapter builds on the foundations laid by these earlier researchers, providing a synthesis of morphology for the morphometrics and neural networks chapters, and morphology-based phylogeny. Some of the findings corroborate the results of previous authors, and some introduce new interpretations of morphological structures in Prosopistomatidae. The neotype for *P. variegatum* was selected, defining the family and genus (Barber-James, 2010b), with the imaginal stages of this species being described for the first time. The nymphs of 13 species hitherto unknown to science have been characterised, with the winged stages for six species also being recognized (Tables 1.1, 2.1), although in most cases only the male or the female is known, rarely both. Two of these new species have been formally described (Barber-James, 2010a), with other descriptions in preparation.

2.2 Materials and methods

2.2.1 Material examined

Material used for this and subsequent chapters came from a number of sources, and was gathered over many years, some specifically for this research, and some as a by-product of other freshwater invertebrate surveys. The details of the sources of material and locality information are provided in the Appendices, and in publications such as the neotype redescription (Barber-James, 2010b) and other new species descriptions (Barber-James, 2010a; Barber-James, in prep, a, b, c).

2.2.2 Collection and preservation of material

Material examined for this thesis is listed in Appendix Table A2.1. Specimens of two species (*P. crassi* and *P. mccaffertyi*) were collected by the author. Other material studied was from museum collections, or was specially collected by colleagues during river survey work. The nymphs were found clinging to the underside of large rocks and boulders, often on a bedrock substrate, and were picked off with forceps and placed directly into tubes of 80 % ethanol. Although a range of instars was evident, some of the nymphs were mature. As is typical of the Prosopistomatidae species known so far, adult emergence occurred at dawn, when female subimagos were collected at light traps at two sites (Appendix Table A2.1); these were also preserved in ethanol.

Earlier collections tended to be preserved in 4% formaldehyde (e.g. collections in South African rivers surveys (Chutter, 1963, 1967, 1970, 1971 and Oliff, 1960a,b). More recent collections (e.g. survey of the Cunene River (de Moor *et al*., 2000); surveys of rivers in KwaZulu-Natal (de Moor and Barber-James, 1998; de Moor *et al*. 1998, 1999) have been preserved in 70–80% ethanol.

2.2.3 Slide Mounting Techniques and Light Microscopy

Mounting of specimens on permanent slides was tested using several techniques before the most appropriate technique was decided upon. These are outlined below. With many mounting media substances, clouding is caused by minute amounts of water or other substances not completely miscible with the medium. To prevent such clouding, material may be run through an alcohol dehydration series to remove water prior to mounting. Alternatively, the material can be transferred to a medium which is miscible with the mounting medium; for example, Cellosolve or Euparal Essence is miscible with Euparal.

The slide should be made using a mounting medium that will allow it to be preserved for viewing long into the future, and this criterion was used in assessing which mounting method to use. A summary of mounting techniques investigated is given in Appendix Table 2.2.

One of the most common mounting resins is Canada Balsam, a yellow, resinous exudate obtained from the balsam fir (*Abies balsamea*) of North American boreal forests. Canada Balsam is valued as an optical mounting cement for microscope slides, since it yields, when dissolved in an equal volume of xylene, a non-crystallizing cement with a refractive index nearly equal to that of ordinary glass (Oldroyd, 1958). To successfully make slides using Canada Balsam, the dehydration approach, using a series of progressively stronger percentages of ethanol up to absolute, is required. The specimens must then be impregnated with an oily liquid, such as clove oil, cedar-wood oil or xylol, which mixes with the balsam (Oldroyd, 1958). Some researchers clear specimen parts in creosote, without dehydration, and then mount them directly in Canada Balsam (Sartori and Gattolliat, Museum of Zoology, Lausanne, Switzerland, pers. comm., 2005). On testing this method, it was found that for Prosopistomatidae, the soft tissues (e.g. hypopharynx) were dissolved by the creosote, so for more fragile material, this method is not as effective.

Hoyer's solution, another popular mountant, was avoided as recent evidence has pointed to this water-miscible medium, made up of gum from *Acacia arabica*, chloral hydrate, water and glycerine, being a poor long-term mounting medium. Crosskey (1987), in his obituary to the dipterist David Lewis, pointed out that many of the slides made by Lewis using this medium have become "heavily blackened and opaque". Upton (1993) noted that its longevity is dubious, recommending that it is not used for permanent mounts. The implications are that deterioration of the medium would result in the long-term loss of slide collections.

Another favoured mountant is Euparal, also a resin based substance. This was devised in 1904 by Professor G. Gilson of Louvain University, Belgium. The solvent is 'Euparal Essence', which is also a clearing agent. It is a mixture of natural resins, eucalyptol, camsal and sandarac, and paraldehyde (a solvent). Parts to be mounted can be transferred directly from absolute alcohol. There are several advantages in using Euparal above other media. It avoids the use of xylene, which is known to be toxic; if material is fixed and stained, colours are well preserved; it is quick drying and permanent. One

option is to pass the material through graded increasing concentrations of alcohol, then clear it in Euparal Essence before mounting, or pass more delicate material through a graded series of alcohol mixed with Euparal Essence, ending with pure Euparal Essence, before mounting in Euparal mountant. For parts that do not need clearing, direct transferral from 70-80% ethanol to Cellosolve (ethylene glycol mono-ethyl ether) before mounting in Euparal is also effective for avoiding clouding in the mountant. This was the method chosen in this thesis.

Clearing of *Prosopistoma* parts to be mounted was generally unnecessary as they are already small and thin. From a health perspective, the use of Cellosolve is undesirable, but it produced better slides than using Euparal Essence because parts transferred from Euparal Essence tended to cause the Euparal mountant to lose too much viscosity. Material stored in 70-80% ethanol was dissected in Cellosolve and mounted in Euparal on standard glass slides in Euparal, with a small piece of tissue paper being used to absorb any extra liquid between the tips of the forceps before placement of the part. The mounted parts were covered with a small glass coverslip and oven-dried at around 50˚C for at least 36 hours. Mounted structures were examined under a Leica DM 1000 compound microscope.

Due to their delicate nature, gills and wings became invisible or crumpled if mounted in Euparal. Michel Sartori (Museum of Zoology, Lausanne, Switzerland pers. comm., 2005) recommended mounting them in a drop of ethanol on a slide, and covering them with a cover slip. Once the ethanol had evaporated, the slide was then sealed with clear nail varnish. Although these soft tissues still shrivelled at times using this technique, it allowed viewing and photography of the parts before applying the varnish to hold the coverslip. Usually the coverslip held the parts in a flat position and allowed them to dry without loss of clarity. Care had to be taken not to allow the varnish to be sucked under the coverslip and onto the specimen part.

2.2.4 Scanning electron microscopy

The material used for scanning electron microscopy (SEM) was run through an alcohol dehydration series to absolute alcohol before dissection. The parts were then critical-point-dried, mounted, gold-sputtered, viewed and photographed under a Tescan Vega LMU scanning electron microscope. Where adequate material was available, whole mature larvae were also mounted, with one specimen placed dorsally and one ventrally. Eggs,

dissected from mature nymphs or subimagos, where available, were treated in a similar manner.

2.2.5 Drawing

Mounted structures were examined under a Leica DM1000 compound microscope and photographed using a Canon Powershot S70 digital camera. Drawings were made from the digital photographs by placing selected photographs as a template in Adobe Illustrator CS version II, and using a Wacom drawing tablet to trace outlines in digital electronic format, forming a high resolution vector graphic image. Further close comparisons were made with the mounted specimens during the drawing process.

Due to problems with depth of field, it was necessary to take several pictures at slightly different focal depths, and to compare them while drawing. At the start of this project, automontage software was not available locally. More recently, a freeware Z-stacking program has been used to try and get better resolution with photographs of whole nymphs.

2.3 Results and Discussion

2.3.1 Nymphal morphology and terminology

Some of the characters discussed in this section are investigated further in terms of their use as characters for phylogenetic analysis in Chapter 5.

2.3.1.1 The head and associated structures

External head morphology and antennal structure

The head in nymphs of *Prosopistoma* spp. (Figures 2.1, 2.2) is transversely broad, wider than long, widest at the level of the eyes, tapering inwardly posteriorly where it joins the thorax, narrowing axially, flattened, shaped to fit neatly into the notch in the upper portion of the carapace, giving the whole nymph a continuous oval appearance. There are a pair of compound eyes laterally, and three ocelli, a lateral pair almost as large as the eyes, and a median ocellus somewhat smaller and transversely narrower (Figures 2.1a,c; 2.2). The relatively large size of the lateral ocelli was first noted by Lafon (1952) for the European species, and this observation also holds true for all other known species (Figure 2.2a-g). The head capsule of mayfly nymphs is composed of a fused vertex, occiput and genae (Needham *et al*., 1935). A distinctive Y-shaped epicranial suture is visible across the head,

and either passes in front of the lateral ocelli (Figure 2.2a), or most commonly, through them (Figures 2.2b-g). The stem of the Y, the coronal suture, branches to form two frontal sutures that continue anterolaterally, extending to the margin of the head. The area posterior to the frontal sutures is known as the vertex (Needham *et al*., 1935) and the bears the eyes and lateral ocelli. The area anterior to the frontal sutures is known as the fronto-clypeus, and this bears the median ocellus and antennae.

The number of antennal segments in the nymph (Figure 2.3) and the relative sizes of some has been used as one of the features distinguishing species (e.g. Joly and Joly 1872; Trägårdh 1911; Gillies 1954; Peters 1967; Alouf 1977; Soldán and Braasch 1984; Tong and Dudgeon 2000; Sartori and Gattolliat 2003; Zhou and Zheng 2004; Dalkıran 2009). However, a consistent error has been perpetuated by all of these authors, who have named the predominantly longer segment as segment II, implying that they have counted the pedicel as segment I, which is clearly not correct. If the scape is segment I of the antenna, the pedicel is segment II, and the remainder of the segments, which constitute the flagellum, can be numbered consecutively. The first flagellar segment, which is consistently the longest and follows the scape and pedicel, should either be considered as segment III of the antenna, or as segment I of the flagellum. In this study, it is regarded as the former.

The relative length of the first flagellar segment compared to the remaining segments provides a useful diagnostic character in *Prosopistoma*. Antennal segment I of *P. pennigerum* has unusual articulation between it and the head capsule, and the antenna can lie flat against the head or stand erect (Lafon, 1952). In this case it is apparent that Lafon is referring to the scape. Her observation seems true of all the species that have been examined. Lafon (1952) noted that in the specimens of *P. pennigerum* that she was examining, six antennal segments were present, whereas Vayssière (1881, 1882, 1890) reported five. Both Eaton (1883-1888) and Vayssière (1890a) figured the antenna of *P. pennigerum*, but did not number the segments. The number of antennal segments in species of *Prosopistoma* has been reported to range from four to six depending on the species; in *P. variegatum* (Figure 2.3), seven segments are present in mature nymphs. According to some authors (Gillies, 1954; Peters, 1967), the number of segments can vary within a species; Dalkıran (2009) has shown that the number changes with stage of development of the nymph, with mature nymphs having more segments than immature nymphs. The number of segments is consistent in the mature nymphs of the undescribed

species discovered as a result of the research for this thesis. Less mature nymphs had fewer segments, in agreement with Dalkıran's findings. The distal segment is very small in some species, e.g. *P. variegatum* (Figure 2.3), and this may easily be overlooked at low magnification. The length of the antenna relative to the head capsule length provides another useful species-defining character when used in combination with other characters. This may be much shorter than the distance from the antennal base to the anterior margin of the head capsule in some species (e.g. *P. variegatum* (Figure 2.2a) and *P. pearsonorum* (Figure 2.2f)); equal (e.g. Comores sp. 1 (Figure 2.2d) and *P. orhanelicum* (Turkey) (Figure 2.2f)); or longer (e.g. *P. mccaffertyi* (Figure 2.2b) and African sp. 2 (Cunene River) (Figure 2.2c)). Further comparisons can be seen in Figure 5.4 (Chapter 5), where they are considered as characters for phylogeny.

The Mouthparts

Prosopistoma variegatum, the type species for the family and genus (Barber-James, 2010b) will be used as the main example in the discussion on mouthpart structure which follows. Other species will be mentioned for comparison when there is deviation from the basic structure seen in *P. variegatum.*

The labrum in *Prosopistoma* (Figure 2.4g) is small and recurved posteriorly at the distal margin when viewed dorsally. Unlike the labra of most other mayflies, which tend to lie in one plane, in *Prosopistoma* it is usually strongly flexed, and difficult to mount and view on a flat slide. The labrum is relatively much wider than long, and usually fringed with setae along the distal margin.

The mandibles (Figures 2.4a-e, 2.5a, 5.6a-c) are narrow and elongate, with the outer canine (more generally known as the incisor region in other mayflies) composed of three stout, chitinous apical teeth, the inner canine smaller (this may be homologous with the prostheca of other mayflies), with two or three apical teeth, and several long setae immediately proximal to this inner canine, the number varying between individuals; the molar region is absent. A single, stout, simple seta is present medially on the lateral margin (Figures 2.4e, 2.5b; 5.6a-c).

Unlike most other mayflies, *Prosopistoma* has mandibles and maxillae that are "virtually symmetrical" (Eaton, 1884; Kluge, 2004), which has been ascribed to their largely predatory feeding behaviour (Fontaine, 1980; personal observation). However, is asymmetry a derived state and symmetry a more primitive state? Mandibles in primitive ectognathous groups, such as Archeognatha (monocondylous) and Zygentoma

(dicondylous), are basically symmetrical. Prosopistomatid mandibles may have reverted to the primitive condition, or this may represent a secondary adaptation to predation. However, as only mature nymphs of some species are predatory (personal observation), with gut content analysis showing that several species apparently feed on detritus, what selective pressure would produce such mandibles? Possibly the shape of the mandibles in prosopistomatid nymphs is not due to selective pressure for predation, but represents rather a remnant of an ancient condition carried forward largely unchanged since the Triassic (see Chapter 7). A point to link in here is the observation of Landa (1969) that the hind gut was exceptionally well developed in *P. pennigerum*; however, a longer hind gut could be expected to indicate herbivory. Further investigation of gut length and content in a range of species, relating to feeding behaviour, may help to resolve this apparent paradox.

The maxilla (Figures 2.4f, 2.5c; 5.6d,e) has a slender three-segmented palp. In Ephemeroptera, the galea and lacinia are always fused, yet in *Prosopistoma* there is a demarcation between the two parts, the original lacinia apparently represented by a more sclerotised area. The galea-lacinia is crowned by a single, long canine, an extension of the maxilla itself, and surrounding this are three or four long, curved dentisetae (*sensu* Kluge, 2004), that are closely associated with the canine (Figure 2.5c). Three (occasionally two) fine pectinate setae (Figure 2.5d) are located proximal to this. A small single seta is always present about midway along the sclerotised area proximal to the crown of dentisetae; the length of the seta varies according to species. The palp is distinctive to the family; the demarcation between the basal and the second segments is often unclear, and the third segment is always small relative to the other two. The three well developed dentisetae are again an indication of carnivory, and are much more developed in *Prosopistoma* than in most other mayflies. A useful character for distinguishing certain species is whether the palp extends beyond the notch that marks the separation between the stipes and galea-lacinia, or only just reaches this notch (Figures 2.4f, 5.6d,e).

The structure of the hexapod labium can be best understood through its homology with the fused second maxillae of crustaceans (Mackerras, 1970a). In most insects the mentum itself is reduced or non-existent, perhaps fused. The terms submentum and postmentum are often, erroneously, used synonymously, and it is important to clarify the use of these terms. Snodgrass (1935) described the labium of an orthopteroid insect as "standard" for his terminology, with the postmentum secondarily divided proximally into

submentum and distally into mentum, However, it is not clear at which evolutionary stage the separation of the prementum into submentum and mentum occurred in insects, or if it even occurred several times. In mayflies, however, there is generally no separate "mentum" – the labium is clearly divided in only two parts, prementum and postmentum (Arnold Staniczek, Staatlisches Museum für Naturkunde, Stuttgart, Germany, pers. comm., 2009). This condition is also seen in the Apterygota, e.g. *Machilis* (Snodgrass, 1935), suggesting that it represents the ancestral condition. Thus in prosopistomatids, by definition, the mentum is part of the postmentum. The postmentum can be identified as it always articulates with the head capsule. The labium therefore consists of the prementum, including glossae and paraglossae (assumed to be fused), and bearing slender, three-segmented palps; the prementum fits into a greatly expanded postmental plate (Figures 2.4i, 2.5e; 5.6f,g). This postmental plate extends across base of the head (Figure 2.1b,d), protecting the other mouthparts. Several authors refer to this plate as the mentum (Eaton, 1884; Gillies, 1954), but Vayssière (1890b), Lafon (1952) and Kluge (2004) maintained that this is the submentum. Other authors refrained from naming it.

To investigate whether prosopistomatid glossae and paraglossae are actually fused within the prementum or lost, a comparison was made with the labium of *Baetisca rogersi* (Figure 2.8), a representative of the sister lineage, the Baetiscidae. The baetiscid labium also has an expanded postmentum, but the glossae and paraglossae are only partly fused with the prementum (Figure 2.8), which, assuming the structures seen in the two families are homologous, indicates that the glossae and paraglossae are probably fused in the Prosopistomatidae rather than lost. SEM investigation of the prementum of *Prosopistoma* for sensory cones (sensillae coeloconica, *sensu* Zacharuk and Shields (1991)), which are typically found on the glossae of mayflies, showed that these were present (Figure 2.5f). This indicates that the glossae and paraglossae are most likely fused within this structure rather than lost, but since such sensillae are also common on the postmentum (personal observation), this is not conclusive. Microscopic investigation of the musculature of the labium (not illustrated) shows the presence of the prementum abductor and adductor muscles, but there is no evidence of musculature that may have been associated with the glossae and paraglossae. Although it cannot be said conclusively whether the glossae and paraglossae are lost or fused with the prementum in Prosopistomatidae, the accumulated evidence points to fusion. Possibly a study of the early ontogeny of Prosopistoma species would be able to confirm this.

Gillies (1954) incorrectly indicated that the hypopharynx is absent in *P. africanum* and *P. crassi*. The hypopharynx is certainly not always easy to find due to the small size of many of the *Prosopistoma* species. It is easier to see in recently collected material, as in older material it seems to soften and is prone to disintegrate during dissection. The hypopharynx rests dorsally against the postmentum, opening in the region of the insertion of the labial palps. This concurs with the observation of Vayssiére (1890b), who figured the hypopharynx of *P. pennigerum*, situated at the base of the prementum. The dentisetae of the maxillae lie just dorsally to this, so the dentisetae and labial palps may work together to assist with food manipulation into the hypopharynx, which leads on to the pharynx, a bulbous, cylinder directly beyond the hypopharyx.

Examination of the hypopharynx of *Prosopistoma* species under both light microscopy and SEM shows that the inner surfaces of the two parts making up the hypopharynx are lined with long setae internally (Figure 2.6 a-c). Lafon (1952) noted in that *P. pennigerum*, the hypopharynx is "not formed of a single lobe; the hairs fairly long, inserted laterally on the side, curving back to the midline" (translated from French), which is consistent with observations made in this study. Kluge (2004) maintained that, as in other carnivorous mayflies, the superlinguae are lost in the hypopharynx of *Prosopistoma*. The evidence presented here (Figures 2.4h, 2.6a-c) indicates rather that the superlinguae of the hypopharynx are fused, and are in a ventral position instead of the lateral position seen in other mayflies. The surface view of a *Prosopistoma* hypopharynx (Figure 2.4h) does not give a clear perspective of the three dimensional nature of the hypopharynx. Figures 2.6a and 2.6c allow a visualisation of its three dimensional nature; it appears to have a mouth-like opening apically, lined with hairs, which leads into a roughly cylindrical oesophagus-like pharynx. The setae within the hypopharynx may aid swallowing and they are all aligned in one inwardly directed plane.

2.3.1.2 The thorax and abdomen

The thorax and abdomen are much fused. The nymphs of *Prosopistoma* species are characterized by a greatly enlarged mesonotum (Figures 2.1, 3.1, 5.1-5.3) forming a dorsally convex carapace (notal shield) that covers all but the last three abdominal segments. This apomorphy is shared with the Baetiscidae (Figure 2.7). Pescador and Peters (1974), in their detailed study of the ontogeny of nymphal Baetiscidae, found this carapace to be of mesothoracic origin, and it is assumed that this is homologous in their

sister lineage, the Prosopistomatidae. This is discussed further in Chapter 3. Nymphs of these two families are easily distinguished at a glance as the Baetiscidae nymphal carapace has spiny extensions (Figure 2.7).

The carapace in prosopistomatids is antero-medially notched to accommodate the head, which fits in tightly, giving the animal a disc-like appearance (Figures 2.1a,c). The carapace covers the abdominal gills and forms a respiratory chamber (Figure 2.11). The developing wing-buds are concealed by the carapace, and are usually visible through the carapace in mature nymphs just prior to emergence (Figure 2.10). The carapace has a median suture running from beneath the posterior margin of the head to the posterior end of the carapace, ending above the seventh abdominal segment.

The ventral surface of *Prosopistoma* nymphs (Figures 2.1b,d; 3.1b,d) is characterized by having the prosternum represented as a small semi-rectangular plate, and the meso- and metasterna fused to form a second plate, which ends in a triangular posterior portion, extending past the hind legs. Latreille (1833) termed this the "plastron" and this terminology was followed by others, e.g. Joly and Joly (1872a) and Vayssière (1890b). Lafon (1952) referred to the structure as a sclerotised plate. The term *plastron* is often used in French in relation to plates on the breast of certain animals (such as birds and turtles). This structure has nothing to do with the plastron gill, a morphologically specialized structure of many aquatic insects that holds a bubble of air. For clarity, this fused meso-and metasternal plate will be referred to as the sternal plate from here onwards. The sternal plate may be festooned with small scale-like structures in some species, e.g. *P. mccaffertyi*. A third sclerotised plate surrounds the sternal plate ventro-laterally and posteriorly, possibly formed from the fusion of the sterna of the first six abdominal segments and the metanotum; this may include the fusion of the episternum and sternum.

The segmentation of the anterior segments of the nymphal abdomen is not clearly visible in *Prosopistoma*, as dorsally it is covered by the carapace, and ventrally segments I - VI are fused into a sclerotised plate continuous with the thorax. The abdomen superficially appears to have nine rather than ten segments, as is the general case in Ephemeroptera. Many of the illustrations in early studies show clearly marked segmentation of the abdomen (e.g. Joly, 1875; Westwood, 1877; Eaton, 1884; Vayssière, 1890b), most indicating nine segments. In nearly all of the material examined during this study, including all of the new species, the abdominal segments within the carapace are not

clearly visible, neither from the exterior nor when dissected. The number of segments can only be determined by finding where the gills are placed and counting the segments in relation to these. Lafon (1952) states that "one can clearly distinguish nine abdominal segments but we see that, based on the number of pairs of gills, we must admit the presence of ten segments" (translated from French). The body terminates in three retractile caudal filaments (Figures 2.1a,c, 2.9) that are fringed laterally with setae. Gillies (1954) reported that the extended cerci of *P. crassi* measure approximately one sixth (0.16) of the length of the whole nymph; similar measurements of cercus-to-body length ratios in nymphs of *P. crassi* in this study concur with this (range 0.15 – 0.16, n = 10), while in *P. variegatum* this is closer to one fifth, ranging from 0.18 – 0.19 (n = 6). These measurements do not include the base of the cerci hidden within the nymphal body. The ability to retract the cerci is an autapomorphy of the Prosopistomatidae. The retractile nature of the caudal filaments was first noted by M. Audouin (in Latreille, 1833), long before they were recognized as insects. Joly (1872), Lafon (1952) and Gillies (1954) independently observed that the tenth abdominal segment is retractable within the ninth segment, so that is it often not visible. Eaton (1883) also drew attention to this phenomenon in his synthesis of the family. Kluge (2004) observed that abdominal segments I – VI inclusive are fused with each other and the thorax, though sutures between these are visible dorsally (i.e. when viewing the abdominal plate dorsally after removing the carapace) (Figure 2.11), while ventrally there is only a suture between segments V and VI. The three small retractile caudal filaments are connected to strong longitudinal muscles, which run down each side of the abdomen (Kluge, 2004). It is possible that when the tenth abdominal segment and caudal filaments are extended, they are used in swimming, and are retracted when the animals are clinging to rocks. While it has been reported that *Prosopistoma* nymphs are able to swim efficiently in captivity (Lafon, 1952), Gillies (1954) expresses doubts about this as a normal means of locomotion, except perhaps under stress. They are normally found clinging to the under-surface of stones, where they move about slowly (Vayssière, 1890b; Lafon, 1952; Gillies, 1954; personal observation). These stones are in flowing water and although current speed was not recorded, the flow is usually swift, so it seems likely that they would be swept away by the current if they tried to swim.

Forelegs (Figure 2.12) of the nymphs of all species have distinctive setation, especially on the tibiae, and the number of setae tends to be diagnostic for a species,

although some of the ranges in number may overlap between species. The setae along the ventral margin of the foretibia are pectinate, while small paired setae lie on the lateral surface of the tibia in some species (e.g. *P. variegatum*, *P. orhanelicum*). The detail of the pectination may vary between species, but this was not easily discernable under a light microscope. Dalkıran (2009) found that there are fewer of these pectinate setae in younger nymphs of *P. orhanelicum*. The details of the setation of the foretibiae in four different species, using SEM, shows that these setae are more deeply pectinate in some species (e.g. Figures 2.13b,d, 5.5). This provides a useful phylogenetic character, and is discussed further in Chapter 5. The tarsal claws of all three pairs of legs are without denticles. The middle and hindlegs offer no useful diagnostic characters, and are therefore not described further.

Six pairs of abdominal gills are present (Figures 2.11, 2.14, 2.15), concealed by the carapace in a gill chamber. The first gill (Figures 2.14a, 2.15a, 5.7) has a narrow, lamellate extension fringed with multiple ribbon-like filaments. The second gill is broadly lamellate and trapezoid, and the dominant trachea form a dorsally visible Y-shaped ridge (Figures 2.14d, 2.15b, 2.16a-d), similar to that seen in the operculate second gill of Caenidae (Figure 2.16e), although it may not be homologous as the orientation of the 'Y' is different in the two families. This character was first illustrated by Riek (1973), where he compared gills of Baetiscidae, Prosopistomatidae, Neoephemeridae and Caenidae, discussing the operculate nature of gill II. This tracheal ridge is more easily seen in some species than others, but appears to be present in all species examined to date. It is not seen in the Baetiscidae, where the tracheation in gill II consists of random branching (Pescador and Peters, 1974). This may indicate a closer relationship with the Caenidae than currently thought, but the different orientation suggests that it is more likely a product of convergent evolution. Many other characters would need to be considered in conjunction with this before speculating further about family relationships.

Gills III-V (Figures 2.14, 2.15) are composed of multiple branching filaments, and gill VI is a folded, conical lamella, smaller than the others. Gill VI is not in line with the bases of the preceding gills, being offset towards the centre (Figure 2.11). Lafon (1952) provided the first illustration of the VIth gill in *P. pennigerum*, which was first noted, but not illustrated, by Vayssière (1890b). Several authors (e.g. Lieftinck, 1932; Gillies, 1954) initially overlooked the VIth gill, but it has been found in this study to be present in all species. Tracheation is usually visible in all gills except gill VI. The bases of gills I to V

are close to one another, resulting in all except gill VI being more or less stacked one above the other. The gills are contained in a "brachial chamber" (*sensu* Eaton 1883; "chambre respiratoire" *sensu* Vayssière, 1890b) (Figure 2.11). Joly and Joly (1875) report that "Concerning the movement of the gills, we can perceive them very clearly, even through the carapace. They move up and down alternately all together and in a rhythmic way" (translated from French).

Water flows the respiratory chamber via two ventro-lateral orifices (Figure 2.1b,d) situated on either side of the upper abdominal sternites between the junction of the carapace and fused plate composed of abdominal sternites I-VI, at the position of the IVth and Vth sternites (Vayssière, 1890b; Lafon, 1952; Gillies, 1954). Water is exhaled through a single dorso-medially situated orifice just below the posterior margin of the carapace (Figures 2.1a, c). Joly and Joly (1875) suggested that the movement of the gills may bring about a flow of water through the chamber. Nymphs of *P. mccaffertyi* were generally found on the under-surface of large stones, facing into the current. Although the stones to which the nymphs cling are in fast-flowing current, the nymphs would be in a boundary layer of laminar water flow close to the stones because of their small, relatively flat disc-like shape. Boundary layer and laminar flow effects are an extensively researched subject and Schlichting and Gersten (2000) provide a good review of this. It is possible that the nymph, attached to the stone may be able to perform small up and down movements of the body to effect a current through the chamber. Such movement would create a circulation of water for respiration. Lifting the body could draw water through the ventro-lateral orifices on either side, and when the body is lowered, water could be expelled through the single posterior dorso-median orifice, having circulated over the gills. This may require a valve, and it is possible that gill II may form this. If it was not for the boundary layer effect, the strongly flowing water would counteract the lifting and make this form of respiration energetically expensive.

2.3.2 Subimaginal and imaginal stages

Winged stages are currently described for only five species, and characters are known for a further five as yet undescribed species (Table 2.1).

Table 2.1. Species in which at least one of the winged stages is known. Madagascan species 6 and 7 are not known as nymphs.

Species	**nymph**	**♂ sub- & imago**	**♀ subimago**	**Author**
P. africanum	X	X	X	Gillies, 1954, 1956
P. mccaffertyi	X		X	Barber-James, 2010a
P. pearsonorum	X		X	Campbell and Hubbard, 1988
P. pennigerum	X	X	X	Vayssière, 1881; Fontaine, 1955; Degrange, 1955
P. variegatum	X	X	X	Barber-James, 2010b
African sp. 2	X	X		Undescribed
Madagascan sp.1	X	X	X	Undescribed
Madagascan sp. 2	X	X	X	Undescribed
Madagascan sp. 6 (P0400)		X	X	Undescribed
Madagascan sp. 7 (P0249)		X		Undescribed

Joly (1871) reared the first recorded adult female of *P. pennigerum.* Vayssière (1881) reared through two females of this species and produced the first description and speculated that females may remain in the subimaginal stage, forgoing the final moult to imago. Vayssière (1890b: 30) was still under the impression that they should have the imaginal stage - "these individuals, all females, died before they were able to transform to the imago" (translated). He continued his efforts to rear through nymphs and nearly succeeded again (Vayssière, 1925), but the female was trapped in the nymphal shuck. Fontaine (1955) was the first to conclusively show that *P. pennigerum* females remain in the subimaginal stage and are sexually mature as subimagos.

2.3.2.1 Wing structure and venation

Prosopistomatids have two pairs of wings (Figures 2.17, 2.18). The forewing is much larger and has better defined venation than the hindwing. A synapomorphy shared with the Baetiscidae is the presence of the vein CuP, posterior to the tornus in the forewing (Figures 2.17a, 2.18b) (hence Kluge *et al*. (1995) introduced the term Posteritorna – see Chapter 1). Autapomorphies include the unique wing venation, with no crossveins in either fore or hindwings, and the absence of branching in MA. There is considerable sexual dimorphism in the venation and wing shape. Males' forewings (Figure 2.17a) have long intercalaries adjacent to the main veins, while females' lack intercalaries (Figure 2.18b), but their main veins are more strongly formed. Gillies (1954) suggested that the

male wings require the secondary veins to compensate for the weakness of their main veins. The hindwings in both sexes (Figure 2.17b, 2.18c) have fan-like venation, the homology of which is impossible to derive.

A number of notations have been used to name the veins in Prosopistomatidae (Degrange 1955, Kluge 1998, 2004), while Gillies (1954; 1956), Fontaine (1958) and Campbell and Hubbard (1998) refrained from naming them. To promote standardisation, Kukalová-Peck's (1983) wing venation terminology has been followed here. It is difficult to be sure of the homology of the wing veins in a group such as the Prosopistomatidae which has such reduced and sexually dimorphic venation, but the homologies at the base of the forewings of the females (Figure 2.18a) are clear. However, a branch off RP_1 (labelled RP_{1a} in Figure 2.18b) interrupts the regular fluting convex-concave pattern typical of mayfly wings, resulting in two positive veins lying adjacent to each other. A possible explanation is that this vein (which is less prominent in the forewings of females of other known species), is in the process of being lost to a varying extent in different species, and the countering negative vein may already have been lost through reduction. Although RP_1 and RP_{1a} are complete in *P. variegatum*, the figure of *P. pearsonorum* (Campbell and Hubbard 1998) shows RP_{1a} to be completely lost, while in *P. pennigerum*, a portion of RP_1 is shown to be absent in a section between its stem and the wing margin, and a second small intercalary lies between it and RP_{1a} (Fontaine 1955, 1958), providing the negative counter vein in this species. In *P. africanum* and *P. mccaffertyi* (Barber-James, 2010a), RP_1 is reduced and does not reach the wing margin.

Why is the venation in *Prosopistoma* wings so derived? What could be the driving force of the great reduction in venation seen in this group? Why is this even more evident in the female than in the male? It could be because the female has little need to fly, just needing the frame and support of the main vein to lift her during her brief mating flight, after which she floats down to deposit her eggs on the water surface. The long intercalaries seen in the male forewings (Figure 2.17a) may function to strengthen the wings for more sustained flight than that needed by the female. Edmunds (1972) noted that generally in mayflies, male have fewer cross veins and other minor veins than females of the same species, and that smaller species have fewer veins than larger species. In the case of *Prosopistoma,* the males have two more anal veins than the females, as well as the long intercalary veins, but lack the vein RP_{1a}. Vein RP_1 is variously reduced in the

females of smaller species, such as *P. mccaffertyi* (Barber-James 2010a), which agrees with Edmund's observation of fewer veins in smaller species.

Microtrichia are present on the wings in both subimagos (male and female) and the male imago, though the fringe of setae on the wing margin of the male imago are shorter than those on the wings of the male or female subimago. The hindwings of both sexes have longer microtrichia than the forewings (Figures 2.17b, 2.18c).

The wings of fossil insects show a complex network of veins (an archedictyon), still thought to be present in several families of the extant mayflies, especially the burrowers (e.g. Polymitarcyidae). A faint background reticulation is visible on prosopistomtid wings under certain lighting conditions (Figure 2.19). It is suggested that this is a trace of the old archedictyon, which may be of importance in understanding the evolution of wing venation in the Prosopistomatidae and is discussed further in Chapter 7.

The three axillary sclerites at the base of the forewing of Ephemeroptera (Figure 2.20) are considered homologous to the first, second, and the third axillary sclerites of Neoptera (Willkommen and Hörnschemeyer, 2007). These sclerites were examined in the wing of a male specimen of an undescribed species, and seem to be similar to other mayflies, but this was not repeated for other specimens, and this observation must be considered preliminary. Wing sclerites were not investigated further for this thesis.

2.3.2.2 Other characteristics of the winged stages

The male head is wide with small but prominent eyes, raised on small stems (stalks) and three prominent ocelli; the eyes are more pronounced in the imago (Figure 2.22) than in the subimago (Figure 2.21). The antennae are well developed and very characteristic; the first segment (scape) is small, subquadrangular; the second segment (pedicel) is swollen basally, tapering distally to meet the terminal flagellar segment, which consists of a fine filament. The antennae of the male subimago are fringed with setae and more robust than in the male imago, where they are glabrous and slightly wrinkled. Females' antennae are smaller, and the pedicel in particular is not swollen as in the male (Figure 2.23). The posterior margin of the male head is raised to form an upwardly projecting flange behind the eyes, which forms a rim along the distal margin of the head – this is more pronounced and notched medially in the imago (Figure 2.22b), while in the subimago it is unnotched (Figure 2.21b). This flange is less prominent in females than in males (e.g. *P. variegatum*), or absent (e.g. *P. africanum*). In general appearance, the female subimago is

larger and more robust that the male, with slightly smaller eyes. Mouthparts in both sexes are vestigial, with only remnant labium and labrum evident.

The pronotum is short, broader than long. The mesonotum of all winged stages is well developed, with a prominent, tapering dorso-median projection, (Figures 2.21a,b, 2.22b, 2.23). The mesosternum is broadly ovoid, the metanotum is inconspicuous and largely hidden by the cuticular projection from the mesonotum.

The mid and hind legs of the male imago of *P. africanum* are slightly atrophied (Gillies, 1954), but in *P. variegatum*, all three pairs of legs of the male imago are well developed (Barber-James 2010b). The tibiopatellar suture is absent. The forelegs do not increase in length in Prosopistomatidae as they do in the male imago of most other mayflies, and this applies to the males of all eight species examined to date. Forelegs in male imagos of *P. africanum* and *P. pennigerum* have an unusual conformation, twisting up over the head in life (Gillies, 1954, Fontaine, 1955), apparently to compensate for the relative shortness of these legs. This has been seen in some of the other species studied, e.g. Madagascan sp. 1 and sp. 2 (Figure 2.24). In *P. variegatium* (Barber-James, 2010b), the legs are all orientated ventrally and do not show this contortion, at least in the preserved state. In *P. pennigerum,* the male legs increase slightly in length from the foreleg to the hind leg, and the femur is slightly bent back in the hind leg (Fontaine, 1955). A similar size variation is observed in *P. variegatium* (Figure 2.25) in both the subimago and imago.

There is a significant difference in the claws of the two life cycle stages; the tarsal claws of the subimago (Figure 2.25a-c) end apically in a double hook, while in the imago (Figure 2.25d-f) the claws terminate in a bulbous structure, preceded by a small thumb-like apophysis (Figure 2.25d-f). Due to a shortage of male imago *P. variegatium* material, SEM investigation of the claws and legs could not be carried out for this species. However, male subimaginal material was available for Madagascan sp. 2 (Figure 2.26), and male imaginal material from Madagascan spp 1, 2 and 6 (Figure 2.27). In Madagascan sp. 2, the subimaginal protarsal claw also has a claw hook as seen in *P. variegatum*, but in this case there is a single hook (Figure 2.26a). The rest of the claw consists of an oval spatulate disk. The mid and hindclaws (Figure 2.26c,d), were unfortunately not well orientated to reveal the claws, although it is probable that their structure is similar to the foreclaw. The stout setae on the forefemur of this species (Figure 2.26b) are retained in

the imago, and may play a role in gripping the female during mating. It is possible that the hooked tarsal claws in the male subimago may have a function in moulting.

In the imago, the hooks on the tarsal claws are lost in of all species investigated (Figure 2.27a-e). Instead, the claws appear to be soft pad-like structures covered in setae. This is very unusual, as the in Ephemeroptera there is usually a loss in setae and microtrichia between the subimago and imago (Edmunds and McCafferty, 1988). These terminal tarsal pad "claws" and tarsi in the imago are densely fringed with setae, and Figure 2.27e reveals that the ventral section of the claw is almost cushion-like (arrowed). The evolution of these softly structured imaginal claws must be significantly correlated with the mating behaviour of the adults because, and a hypothesis about their function is developed below, in synthesis with other structures. A close investigation of the external structure of the imaginal forelegs of two species shows that the tibiae are covered with pronounced scalloped ridges (Figure 2.28 a-d), also seen as an adaptation to an unusual form of mating behaviour.

It is necessary to compare the claw structure seen in Prosopistomatidae with that seen in other mayfly families, in particular with the Baetiscidae, to determine homology and to see if there is a trend reflecting evolutionary progression. A range of possible claw states is shown in Figure 2.29. Kluge (2004) observed that the most common state of the pretarsus in Ephemeroptera is to have the anterior claw blunt and the posterior claw pointed, curved and sclerotised (Figure 2.29b). The final stage in this reduction is to have no claw, e.g. *Behningia* or *Campsurus*. *Baetisca* male imagos have typical ephemeropteran claws, and elongated forelegs, so the specialized system seen in Prosopistomatidae is unique to this family. The role of the male imaginal foreleg and claw structure in Prosopistomatidae and their evolutionary significance will be elaborated upon after discussion of the females' structures.

The postero-lateral margins of segments VII-IX of the male abdomen are produced to form tergal spurs (Figure 2.31, 2.32), which are slightly longer in the imago than in the subimago. The males have fused penes, which are terminally bifid; the claspers are distally distinctively mitten-shaped (Figures 2.31b,c, 2.32b, 2.33a, c, d, e, 2.34a-e). The penes and claspers are much shorter in the subimago (Figure 2.31) than in the imago (Figure 2.32), where both increase in length during the final moult. In the male imago of *P. variegatum*, the claspers are without setae and instead have a rugose outer margin (Figure 2.32b), compared to the setose claspers of the subimago (Figure 2.31b). This is

also seen in Madagascan sp. 6 (cf Figure 2.33e and Figure 2.34e). Madagascan sp. 7 seems to have retained setae and have the rough dorso-lateral surface in the male imaginal clasper (Figure 2.34c).

The caudal filaments are unsegmented and short in subimagos of both sexes (Figures 2.31a, 2.36a, 2.37), (approximately equal in length to two abdominal segments in male subimagos). In male imagos they are elongated (nearly equal to the length of the abdomen) and covered in relatively long hairs (Figure 2.32a) in all eight species studied. Why are the caudal filaments so hairy in the male imago? Do they have a function? Watching a male in flight may help to answer this.

Female prosopistomatids are always larger than their conspecific males, their bodies are broader and squatter compared to the more slender, fragile-looking males, and with the exception of the terminal segment, their entire abdominal cavity is tightly packed with eggs (Figure 2.37). A thin flange protrudes upwards at right angles from the posterior margin of the head (Figure 2.23), but is less prominent than in male. The antennae are less well developed than in the male. Legs in the female have well developed femora but atrophied tibiae and tarsi (Figure 2.30).

The morphology of the female subimago was studied with two-fold intentions. One was to search for characters which would be useful for the study of phylogeny. The other approach was to seek how the male imago manages to grasp the female during their nuptial flight. SEM has revealed a number of possible options. Dorso-lateral grooves (Figure 2.35) were seen on the female thorax (arrowed) in two species (Madagascan sp. 1 and sp. 2). It is proposed that these grooves offer a point of purchase for the male's short legs. The groove ends in an invagination (Figure 2.35b), and it is hypothesized that the male's blunt, pad-like foreclaw is inserted into this. The fine setation on the male tarsi, discussed earlier, may allow the legs to slide smoothly across these grooves, while the scalloped tibial ridges and stout femoral setae may help to temporarily lock the legs into place. The size of the thoracic groove is larger than the width of the male leg, and would accommodate the leg comfortably. Examination of the female's gonopore area (Figure 2.36) reveals the presence of a remarkable spine within the inter-segmental membrane between segment VII and VIII. This would offer a point of attachment for the mitten-shaped male claspers to grasp on either sides of the gonopore during copulation. Figure 2.38 offers an interpretation of this mating procedure. This is very different from other mayflies (Figure 2.39). In most other mayflies, the greatly elongated male forelegs grasp

the female around the bases of the forewings (e.g. Brinck, 1957; Kluge, 2004). Thus Prosopistomatidae appear to have derived both unusual structures and behaviour to achieve mating, which include the loss of the imaginal stage in the female. A separate study is required to observe the mating behaviour of several species to confirm or refute the hypotheses presented here.

Emergence and mating occur at dawn (Gillies 1954; Fontaine 1955; Campbell and Hubbard 1998; personal observation, Sabie River, South Africa (1990); J-M. Elouard, (Lauret, Languedoc-Roussillon, France, pers. comm., 2005). In *P. africanum*, the eggs are released as soon as the female touches the water surface Gillies (1954).

2.3.2.3 Egg structure

Viewed under a compound light microscope, the egg surface has a granular appearance. Scanning electron microscopy (Figures 2.40a-c, 2.41a-d)) shows the outer surface to have a tuberculate appearance, and that the eggs are without polar caps. The eggs of *P. variegatum* (Figure 2.40) are pentagonal or hexagonal rather than rounded, possibly due to the tight packing of the eggs within the abdominal cavity of the female subimago. In *P. variegatum*, there are an estimated 1600 eggs in one female subimago (counted through the abdominal wall). Eggs of *P. variegatum* are 143-150 μm long and 111-120 μm wide. The chorion in each species appears to be covered by a series of contiguous, more or less hexagonal ornamentations (Figures 2.40c, 2.41), in *P. variegatum* each ornamentation is ca 20 μm in diameter, with an inner spherical disc ca 13 μm in diameter, with a small central raised nipple-like projection, approximately 2.3 μm in diameter. These may be knob-terminated coiled threads, as described by Koss and Edmunds (1974). The small aperture situated in the vicinity of the posterior pole (Figure 2.40b), appears to be a funnelform micropyle (*sensu* Koss and Edmunds, 1974). Greater magnification is required to be certain of these interpretations. A sticky, possibly mucus-like secretion is sometimes evident, for example along the margins of the eggs where they abut other eggs (Figure 2.40b).

The eggs of *P. variegatum* (Figure 2.40), *P. africanum*, *P. crassi* and Madagascan sp. 2 (Figure 2.41) are similar in general appearance to those described for *P. pennigerum* and *P. pearsonorum*. The egg of *P. pennigerum* is somewhat larger than that of *P. variegatum* (206-234 μm in length, 175-183 μm in width) (Degrange, 1960), and the description of the micropyle indicates that it is funnelform in the European species.

Campbell and Hubbard (1998) describe the egg of *P. pearsonorum* from Australia, which is similar in size (150 long and 110 μm wide) and also has a funnelform micropyle. Koss and Edmunds (1974) describe the egg of *P. africanum*, indicating a tagenoform micropyle and a pair of irregularly coiled threads, although they do not mention dimensions. Tong and Dudgeon (2000) describe the egg of *P. sinense* (150 long and 110 μm wide), which they have interpreted as having knob-terminated coiled threads, similar to those as seen in *P. variegatum* and Madagascan sp. 2. *P. sinsense* has two micropyles, one tagenoform and one funnelform. It is unusual to have more than one kind of micropyle associated with an egg, and while the tagenoform micropyle is clearly evident, the interpretation of the funnelform micropyle is dubious (Elda Gaino, University of Perugia, Italy, pers. comm., 2009).

2.4 Conclusions

This chapter has contributed much to furthering the knowledge of morphology in the Prosopistomatidae in all life history stages. It has successfully clarified issues regarding mouthpart structure in the nymphs, especially in the labium and hypopharynx. A standardised wing venation nomenclature for the family is suggested. Many unusual structures have been identified in the imaginal stages, which have helped to suggest a unique mating procedure for the family. Kluge (2004) lists as a family autapomorphy the non-functionality of legs in both female subimago and male imago, suggesting that they are functional only in the male subimago to aid in moulting. The morphology presented here indicates that at least the front legs of *Prosopistoma* male imagos are functional, but behavioural studies are needed to verify this. Further research is needed in studying the mating behaviour of these insects, and linking this with differences in morphology.

This study has identified many characters that are used as the foundation for much of the remaining thesis. Morphology is also used in Chapter 3, where a morphometric approach to species delimitation is undertaken. In Chapter 4, a mathematical modelling approach to species identification using artificial neural networks is based on nymphal morphological characters. Phylogenetic analysis using many of the morphological characters is undertaken in Chapter 5, although in certain cases, there are not enough representatives of each species in all life history stages for comparison of all of the characters covered here. Finally, morphological comparisons with fossil wings allow the

development of a hypothesis about the origins of the lineage of mayflies leading to the crown-group Prosopistomatidae.

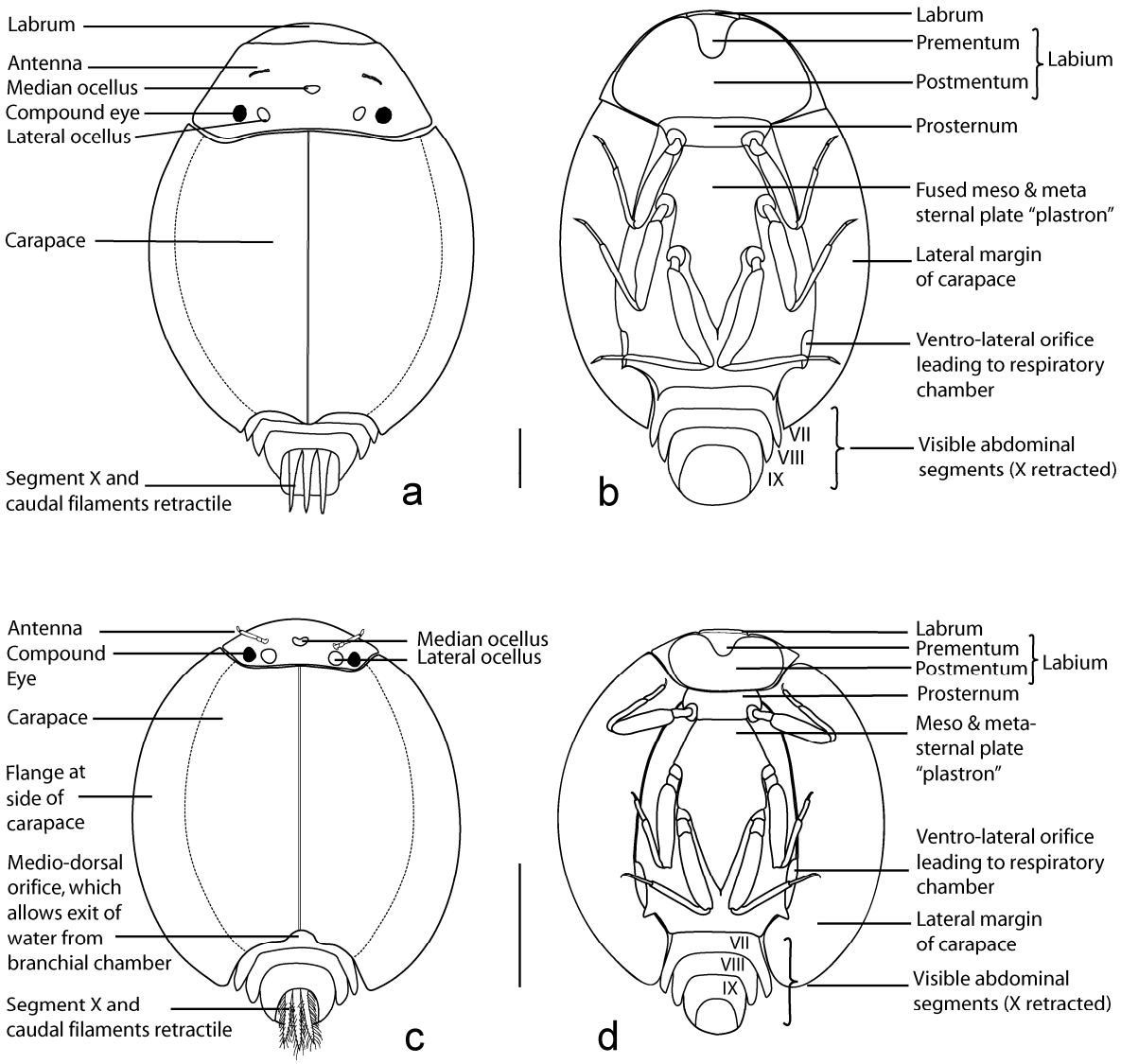


Figure 2.1. Nymphs of *P. variegatum*, (a) dorsal view, (b) ventral view, and *P. crassi*, (c) dorsal view, (d) ventral view. Scale line equals 1 mm.

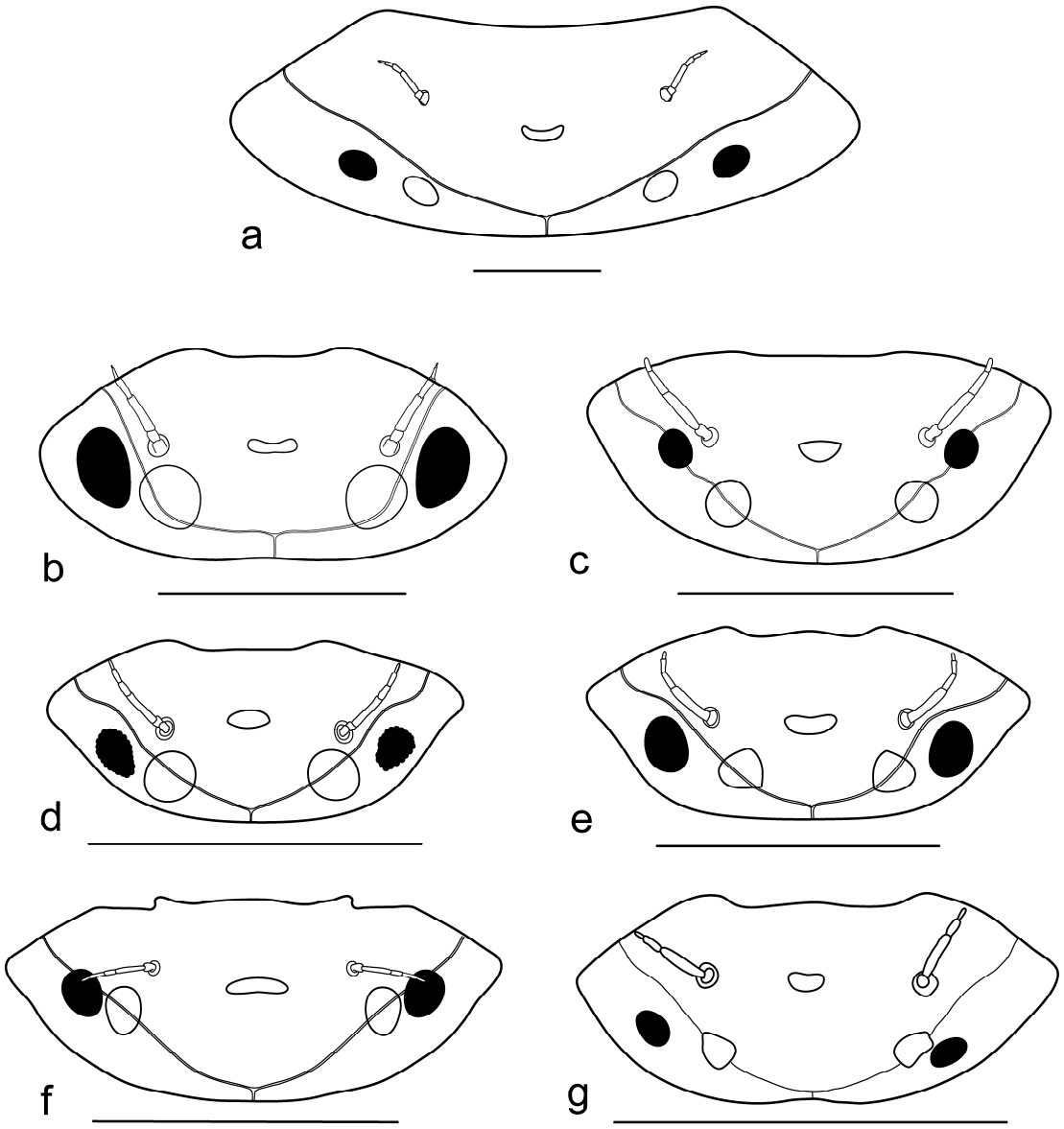


Figure 2.2. Heads of mature nymphs, flattened under a coverslip to show epicranial ecdysial sutures on their dorsal surface. (a) *Prosopistoma variegatum,* (b) *Prosopistoma mccaffertyi*, (c) African sp. 2, (d) Comores sp. 1, (e) Madagascan sp. 2, (f) *Prosopistoma pearsonorum*, (g) *P. orhanelicum* (after Dalkıran 2009). Scale bar = 1 mm. Note that due to flattening, the perspective of these heads is different to the unflattened dorsal in-situ view.

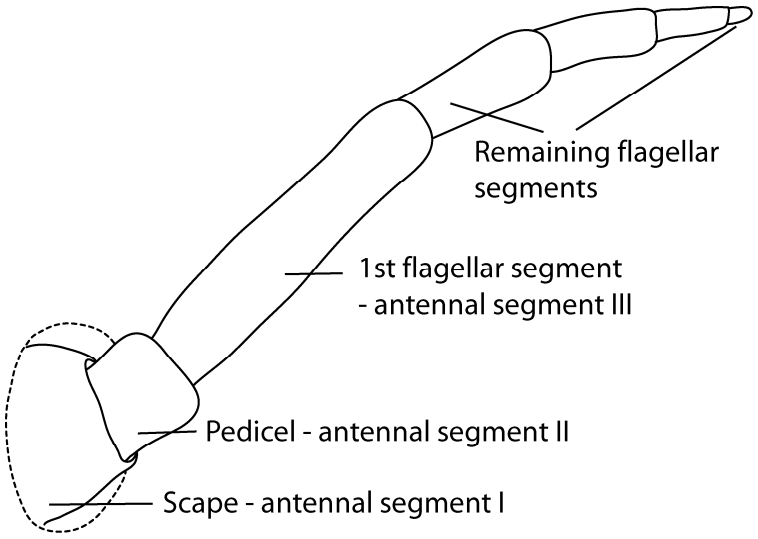


Figure 2.3. Antenna of *P. variegatum*, showing the numbering of segments in a mature nymph. The first flagellar segment is segment III.

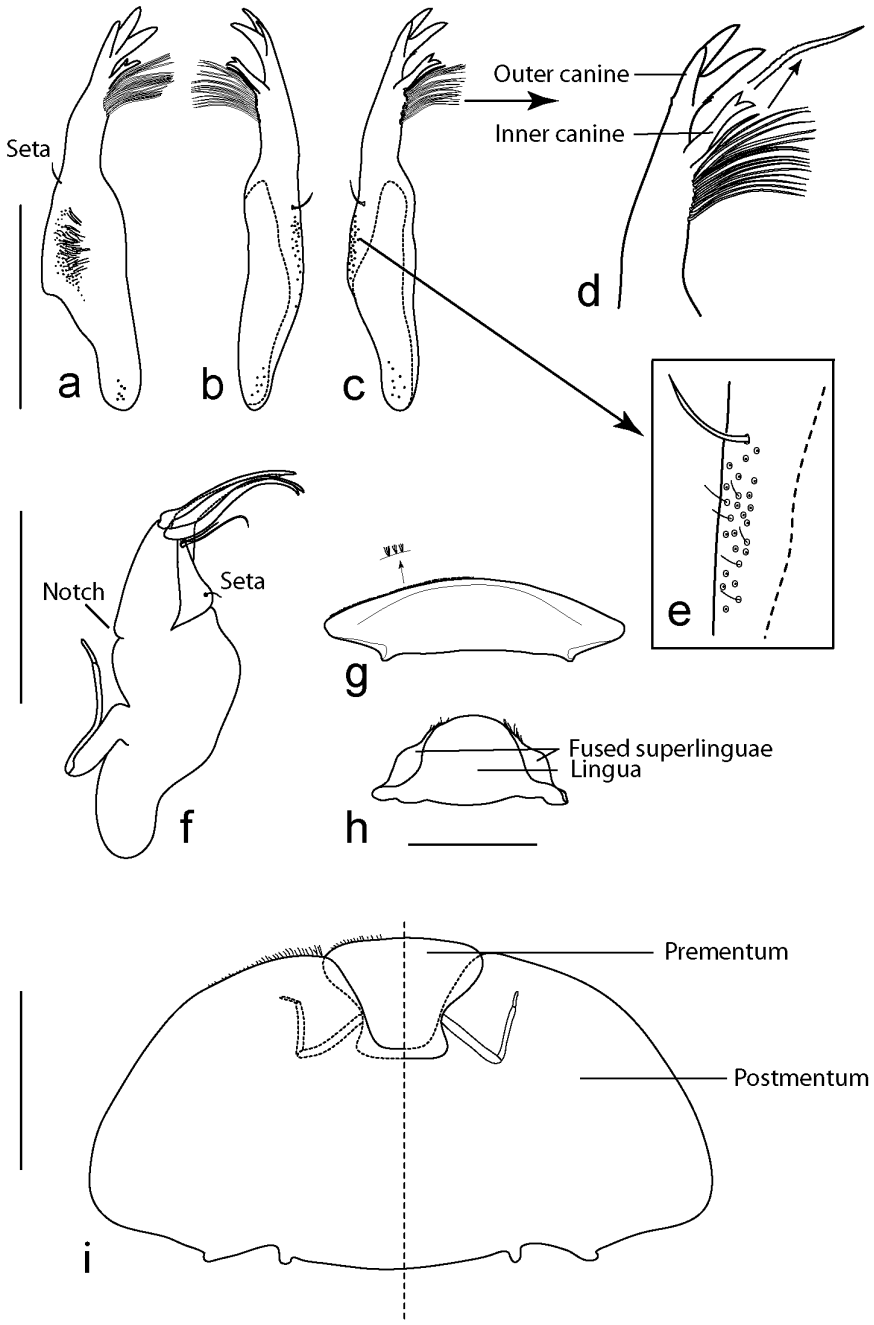


Figure 2.4. Mouthparts of mature larva of *P. variegatum* (a) right mandible, dorsal view, (b) right mandible, ventral view, (c) left mandible, ventral view, (d) apex of left mandible enlarged showing details of inner and outer canine and setae, and enlargement of thicker seta preceding the remaining setae below canines, (e) margin of middle section of mandible enlarged to show single stout seta and field of small setal bases and setae, (f) maxilla, dorsal view, (g) labrum, dorsal view, (h) hypopharynx, dorsal view, (i) labium, consisting of prementum within expanded postmentum; left side ventral, right dorsal. Scale lines equal 1 mm.

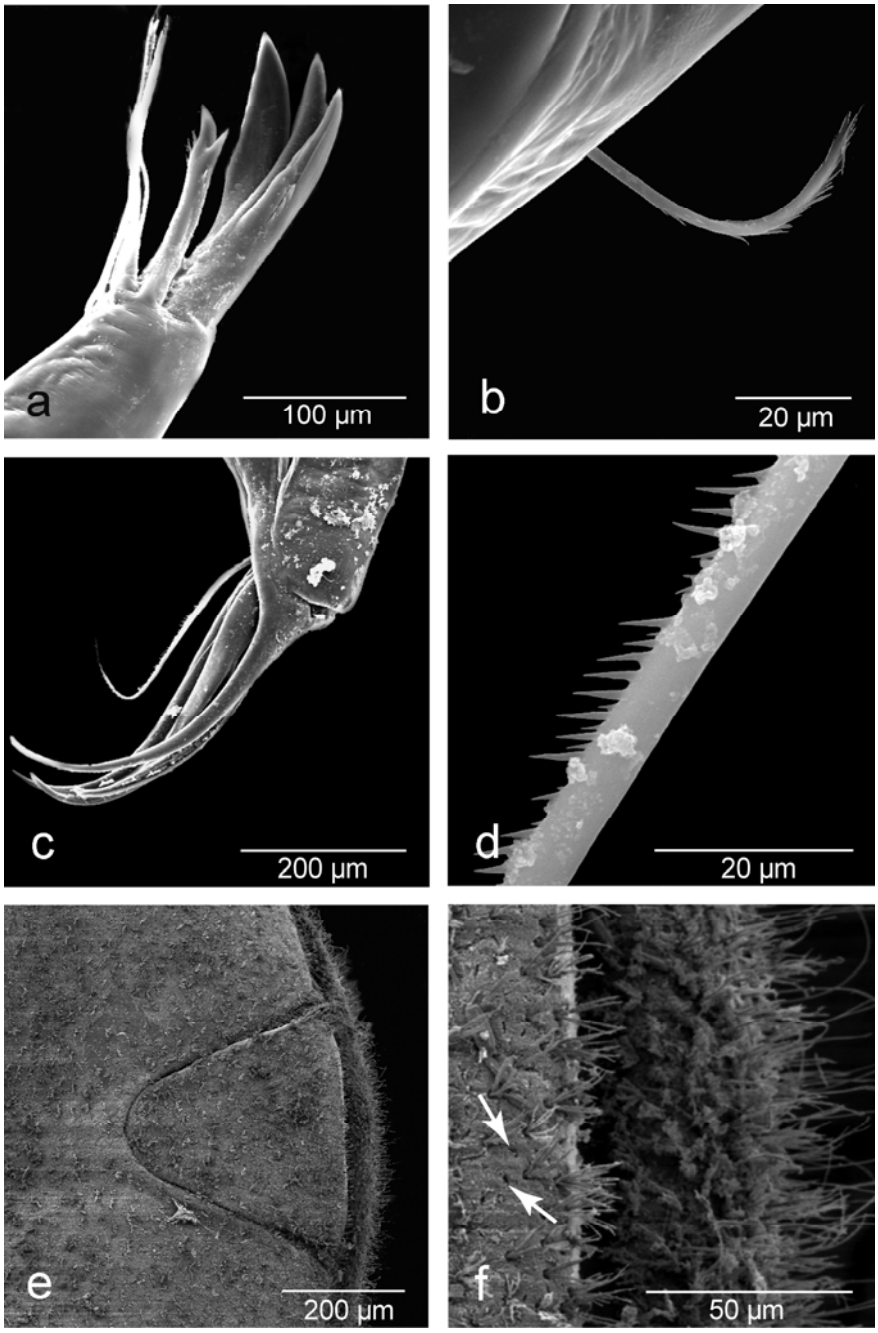


Figure 2.5. *Prosopistoma variegatum*, scanning electron micrographs (a) apex of mandible, (b) seta on mid dorso-lateral margin of mandible, (c) apex of maxilla, (d) enlargement of maxillary seta directly below dentisetae, (e) labial prementum, recessed within enlarged postmentum, ventral view, (f) edge of labium below labrum (on right), showing groups of setae arising from common basal point, and sensillae coeloconica (arrowed).

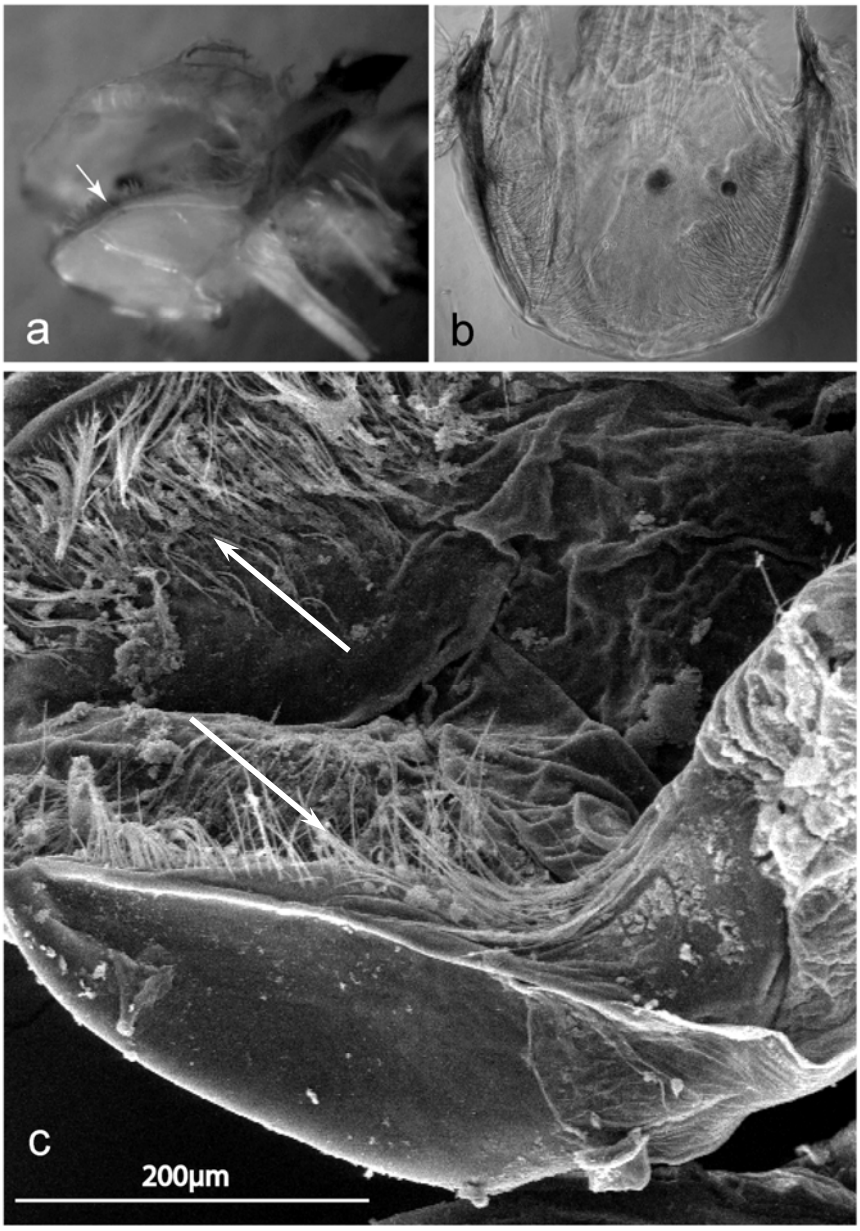


Figure 2.6. *Prosopistoma variegatum* hypopharynx. (a) ventro-lateral view from left side, showing mouth-like opening and internal setae (arrowed), viewed under dissecting microscope (x 50 magnification), (b) dorsal view of lingua under compound microscope (x400 magnification) showing setae, (c) SEM showing close up of fused superlinguae. Arrows point to the multiple setae within the hypopharynx.

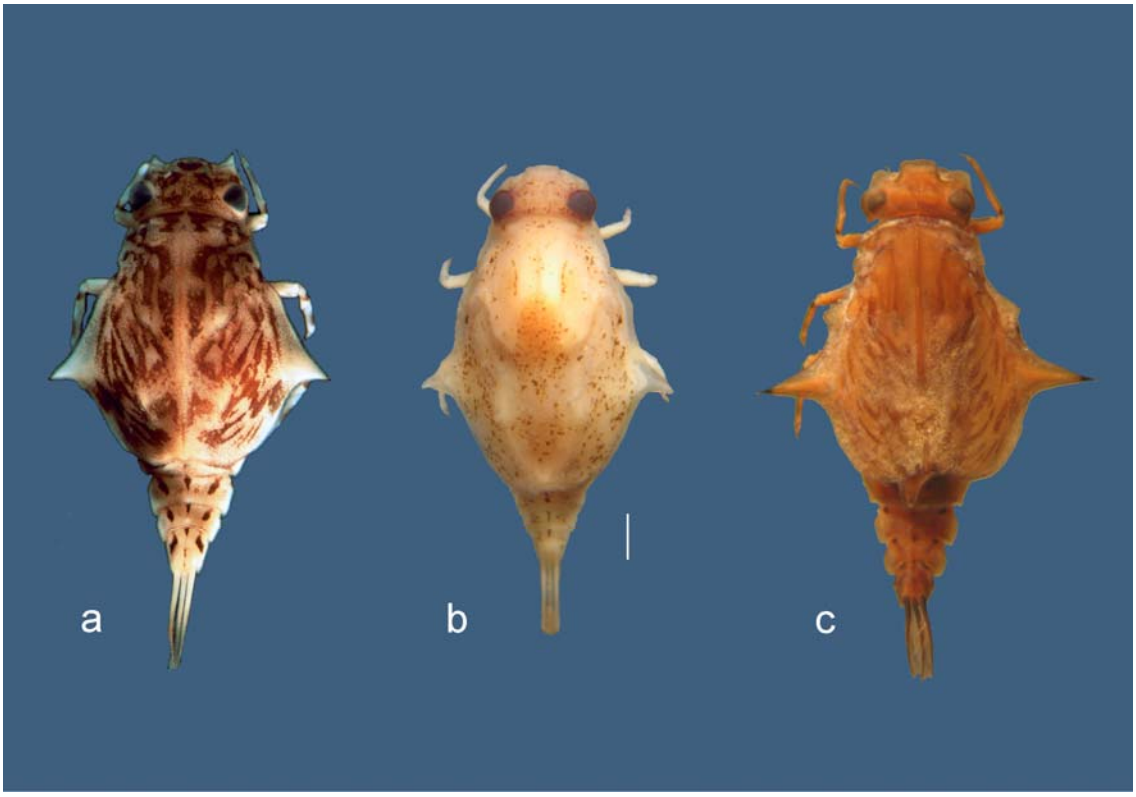


Figure 2.7. Nymphs of Baetiscidae. (a) *Baetisca carolina* (image modified from www.benthos.org), (b) *Baetisca lacustris*, (c) *Baetisca rogersi.* Scale line equals 1 mm.

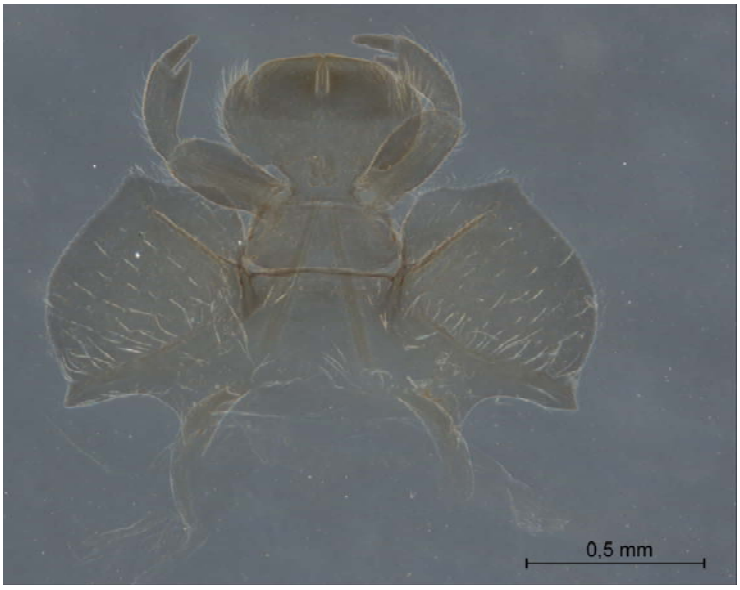


Figure 2.8. Labium of *Baetisca rogersi* for comparison with that of *Prosopistoma* (cf. Figure 2.4i). (Image prepared by Mr Milan Pallmann, Staatlisches Museum für Naturkunde, Stuttgart, Germany).

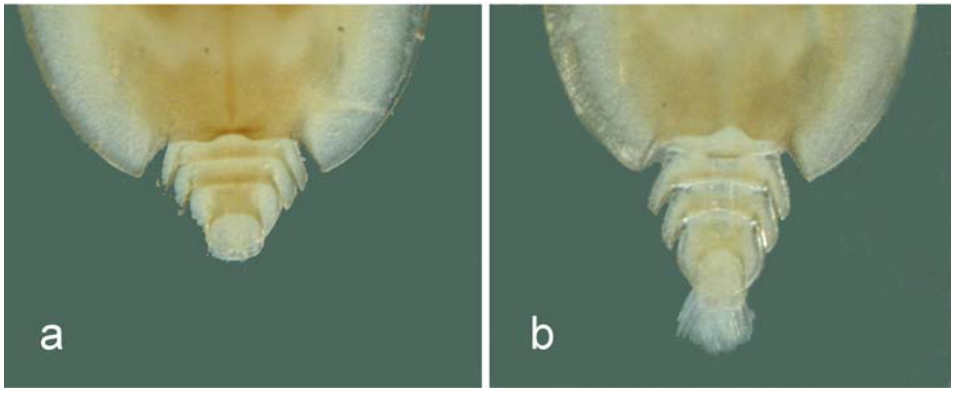


Figure 2.9. Dorsal view of lower end of abdomen and cerci of African sp. 2 showing segment X and cerci (a) retracted, (b) extended.

Figure 2.10. Mature nymph of *Prosopistoma crassi*, showing developing wing buds visible through the carapace.

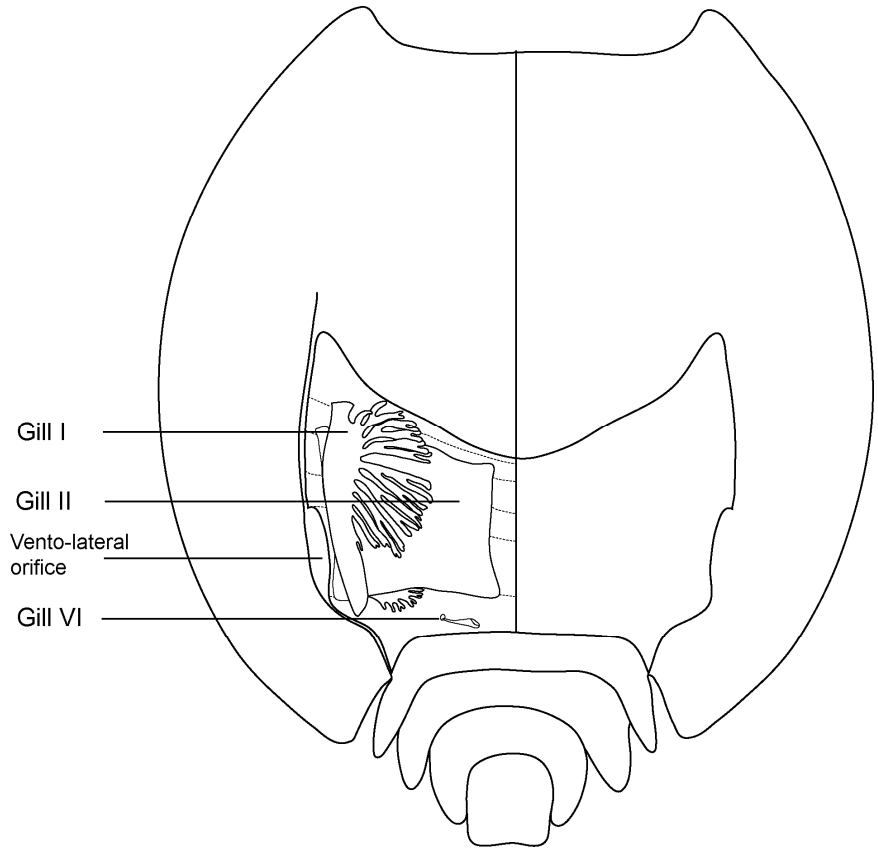


Figure 2.11. Dorsal view of *P. crassi* nymph showing gills in situ. Note placement of the large lamellate second gill in relation to the ventro-lateral orifice, which can be seen behind the gills; gill II may form a valve covering the ventro-lateral orifice when water is being expelled through the dorso-median orifice. A faint trace of ventral abdominal segmentation is visible with the carapace section removed. (Illustration after Vayssière, 1882; Kluge, 2004.)

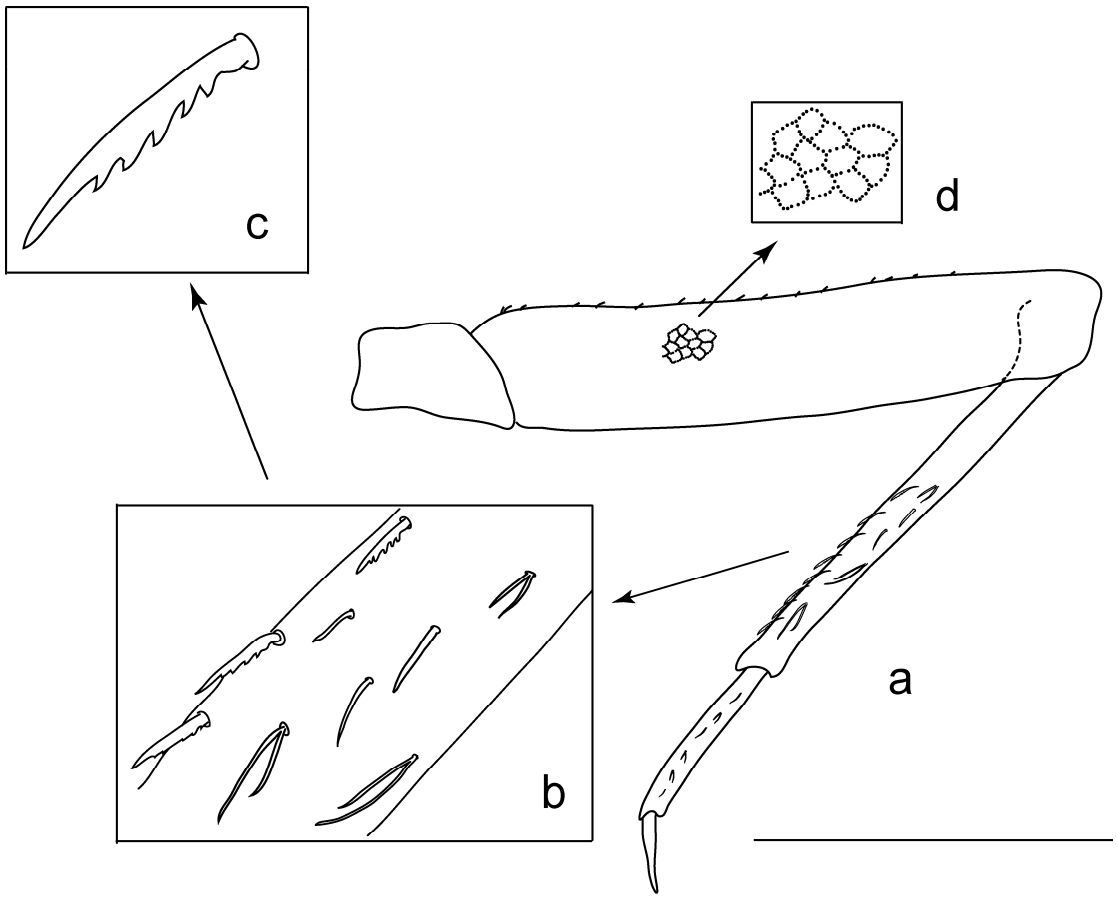


Figure 2.12. *Prosopistoma variegatum*, foreleg of nymph. (a) whole leg, lateral view, (b) enlargement of part of tibia to show setation, (c) enlargement of pectinate seta near lateral margin of tibia, (d) detail of scale pattern covering entire surface of coxa, trochanter and femur. Scale line equals 1 mm.

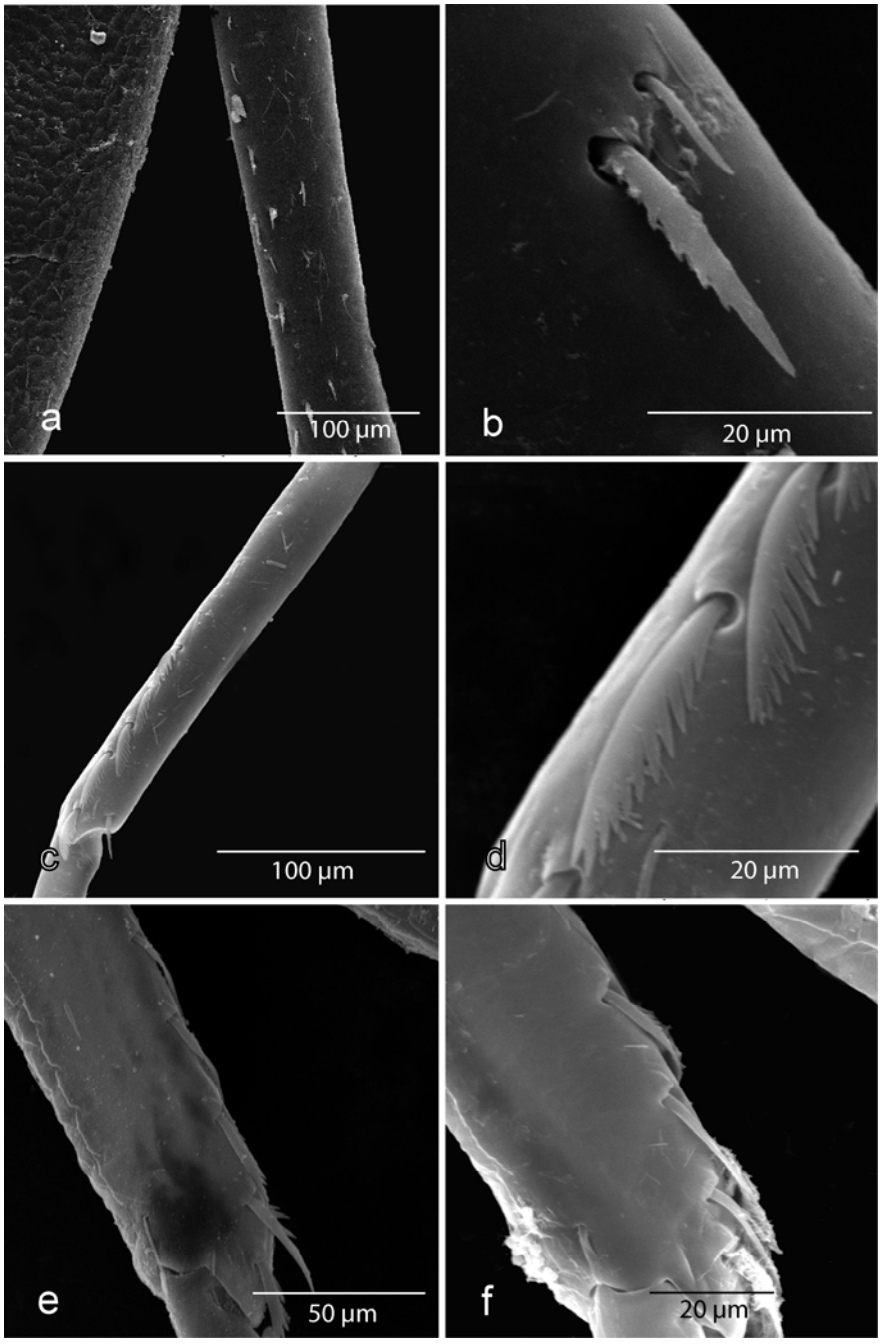


Figure 2.13. Details of foretibial pectinate setae, as seen under SEM. (a,b) *P. variegatum*, (c,d) Madagascan sp. 2, (e) *P. crassi*, (f) African sp. 2.

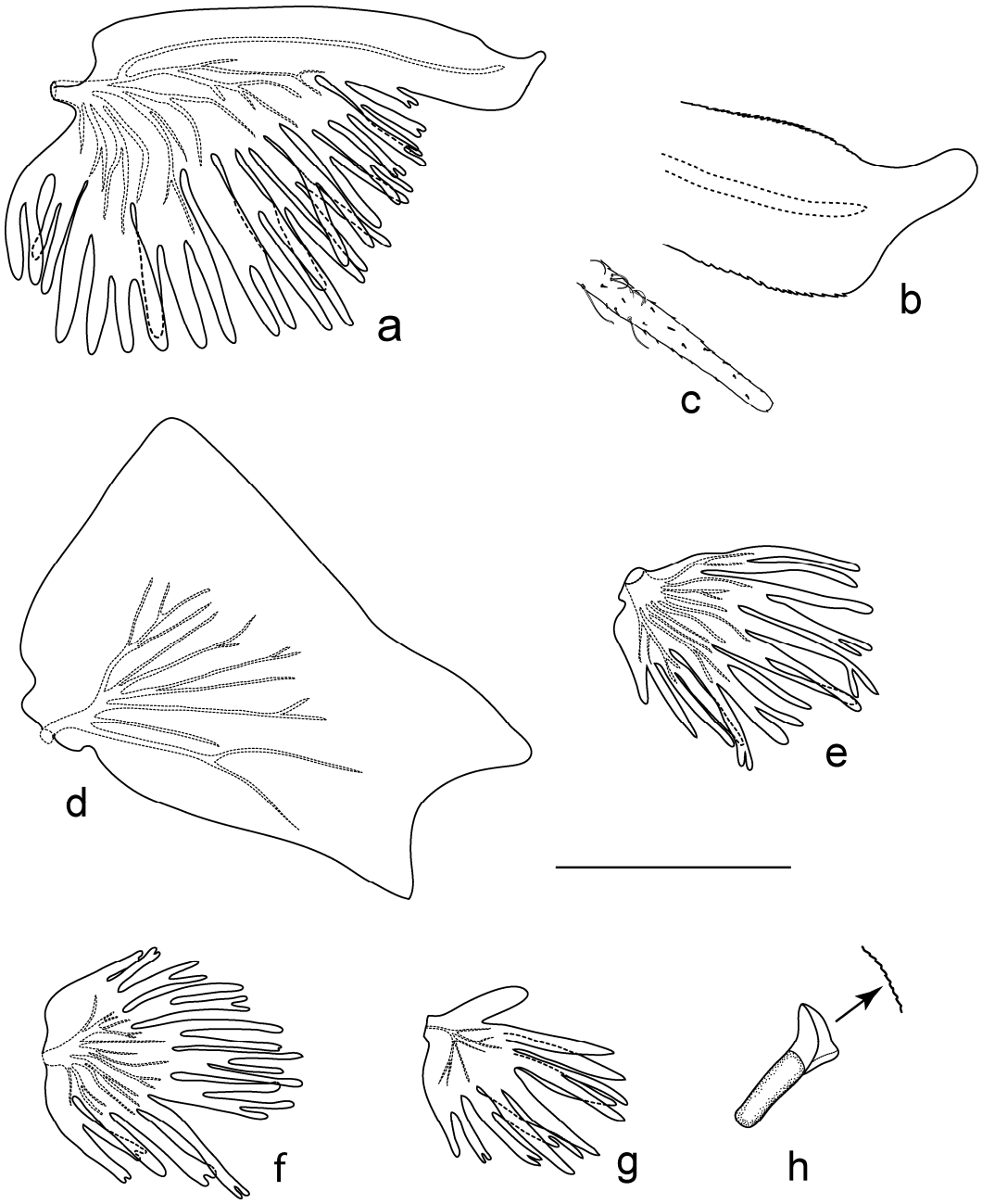


Figure 2.14. *Prosopistoma variegatum*, nymphal gills. (a) gill 1, (b) gill 1, apex of lamellate section magnified to show serrated upper and lower margins, (c) gill I, enlargement of one filament, (d) gill II, (e) gill III, (f) gill IV, (g) gill V, (h) gill VI, showing slight serration of margin (arrow). Scale line equals 1 mm.

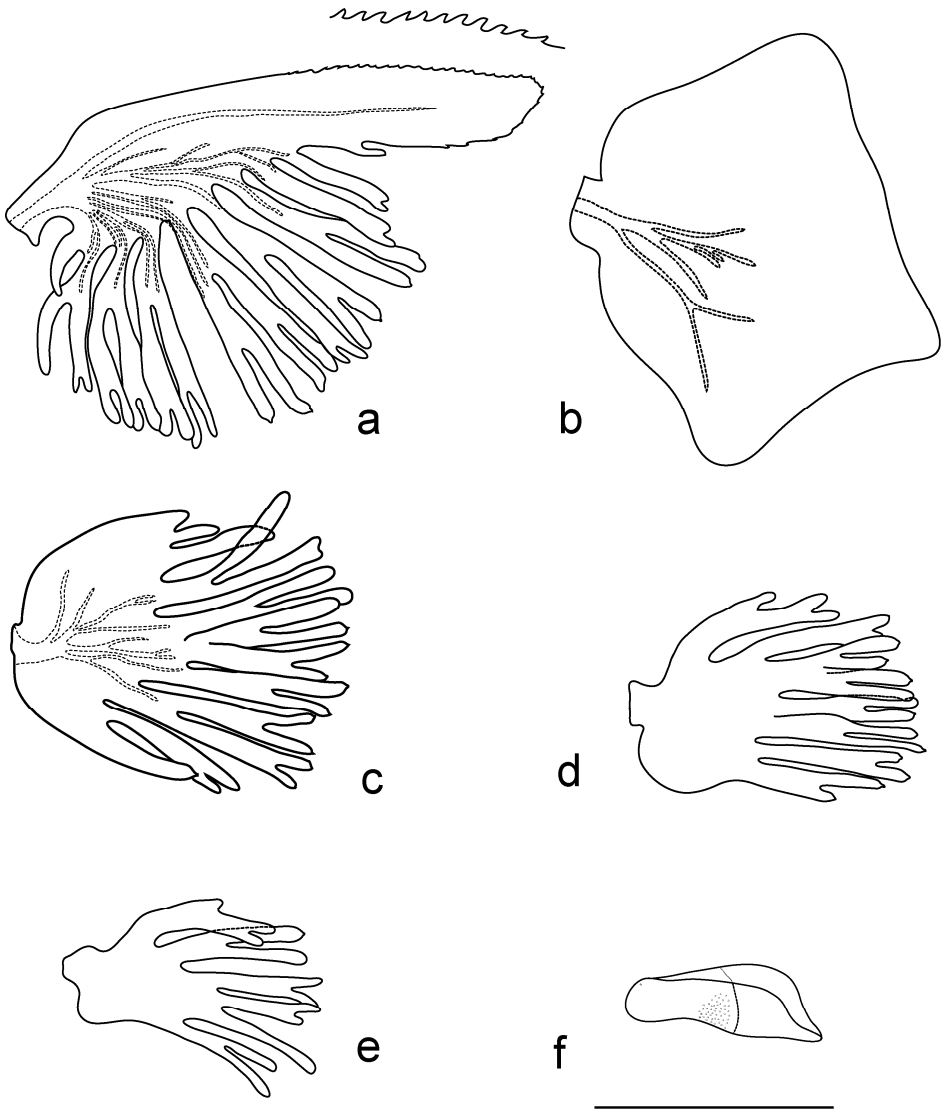


Figure 2.15. *Prosopistoma crassi*, nymphal gills. (a) gill 1, with apex of lamellate section magnified to show serrated upper and lower margins, (b) gill II, (c) gill III, (d) gill IV, (e) gill V, (f) gill VI. Scale line equals 1 mm.

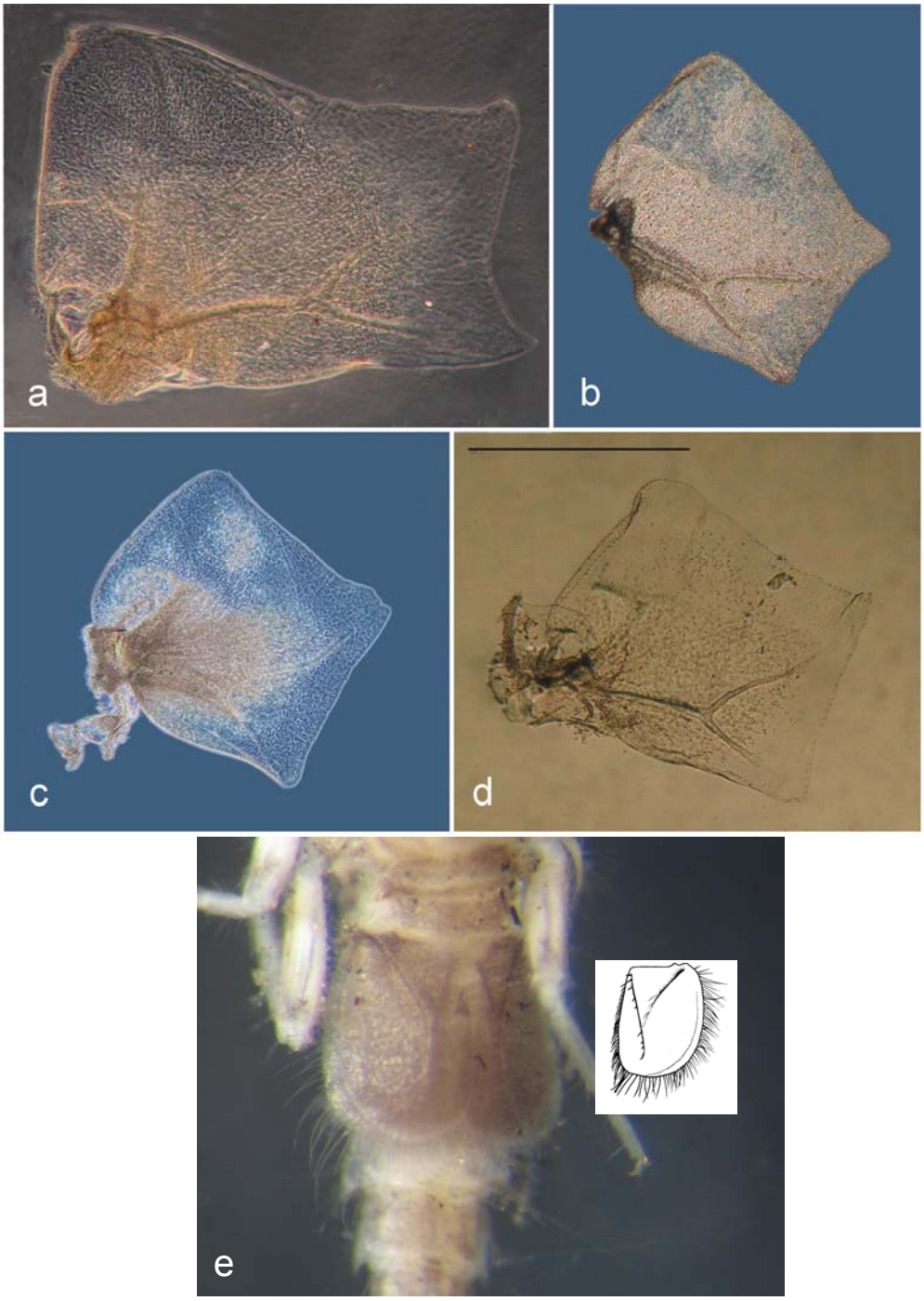


Figure 2.16. Gill 2 showing the Y-shaped tracheal pattern, from (a) *P. variegatum*, (b) Comores sp. 1, (c) *P. crassi*, (d) African sp. 2, (e) Caenidae (with inset to emphasize the Y-shaped tracheal pattern on gill II). Scale line equals 1 mm for *Prosopistoma* species gills.

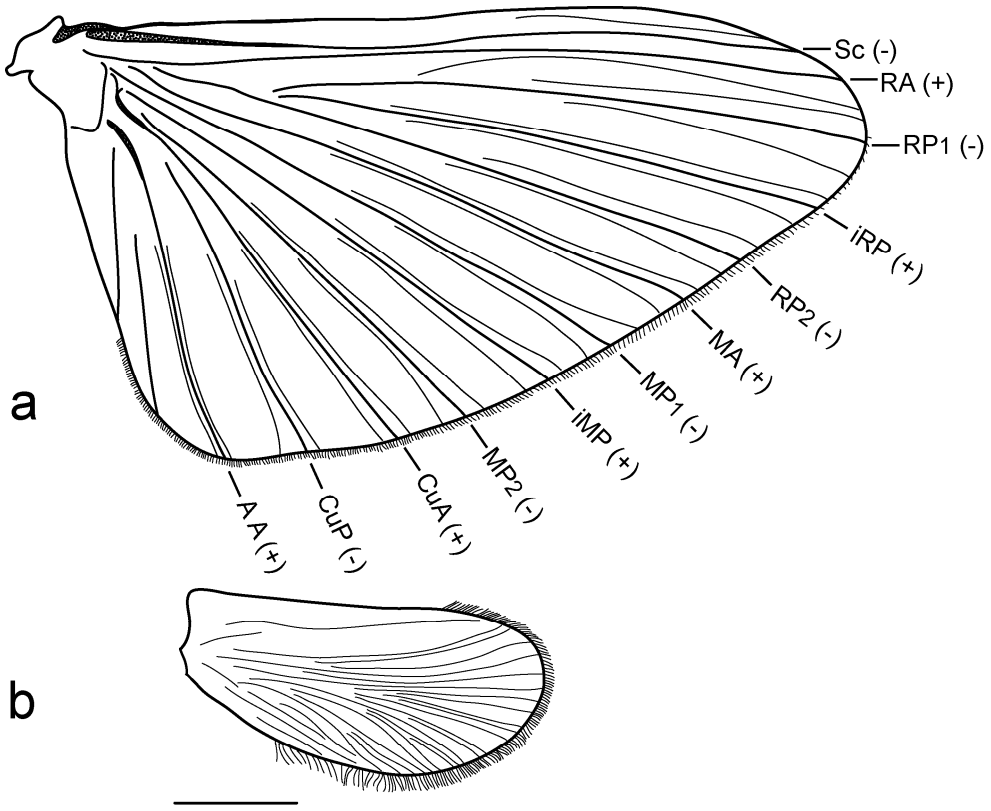


Figure 2.17. *Prosopistoma variegatum*, typical wings of male subimago and imago (a) forewing, (b) hindwing. Notation follows Kukalová-Peck (1983). Scale line equals 1 mm.

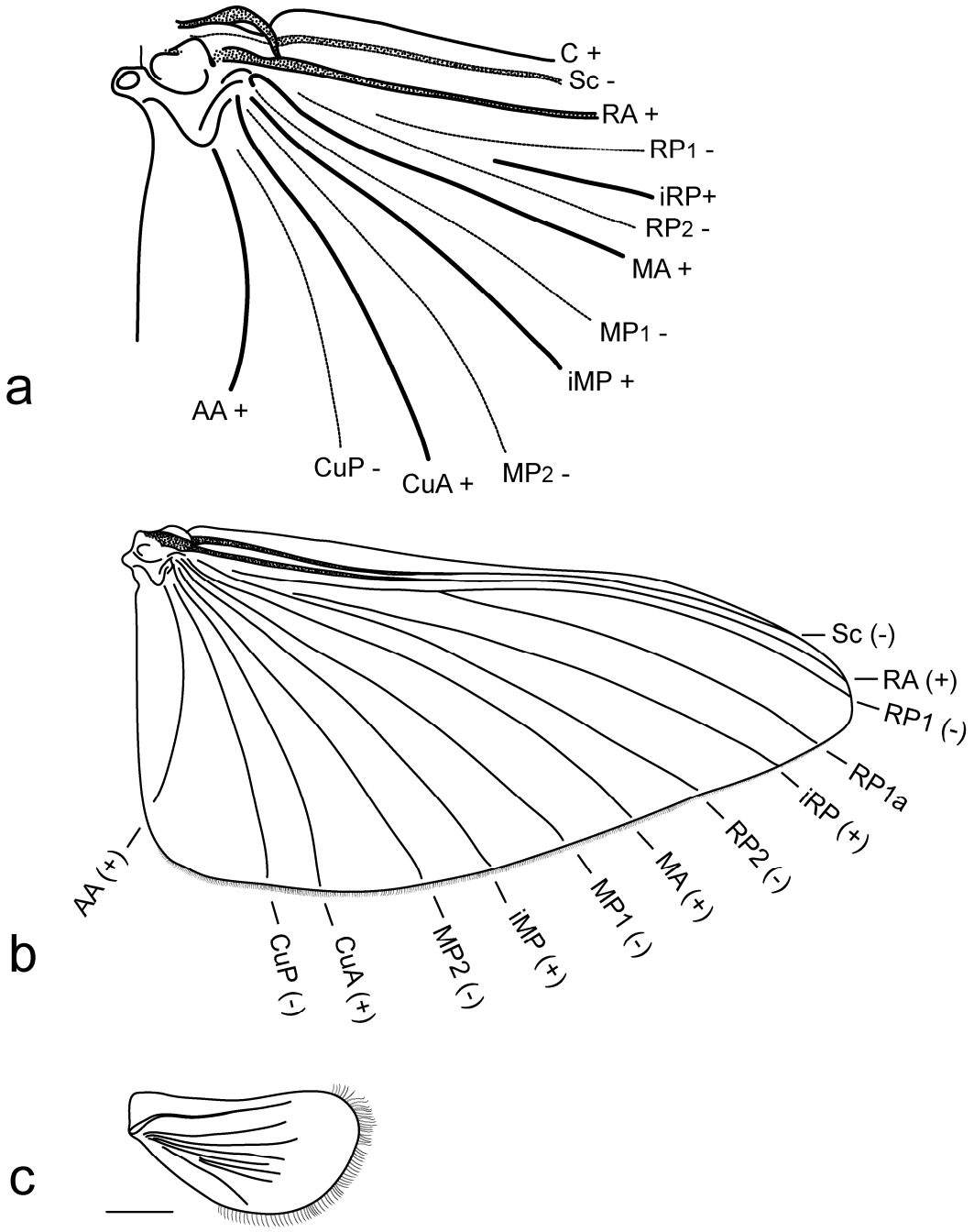


Figure 2.18. *Prosopistoma variegatum*, wings of female subimago (a) base of forewing, showing origin of veins, (b) forewing (c) hindwing. Notation follows Kukalová-Peck (1983). Scale line equals 1 mm.

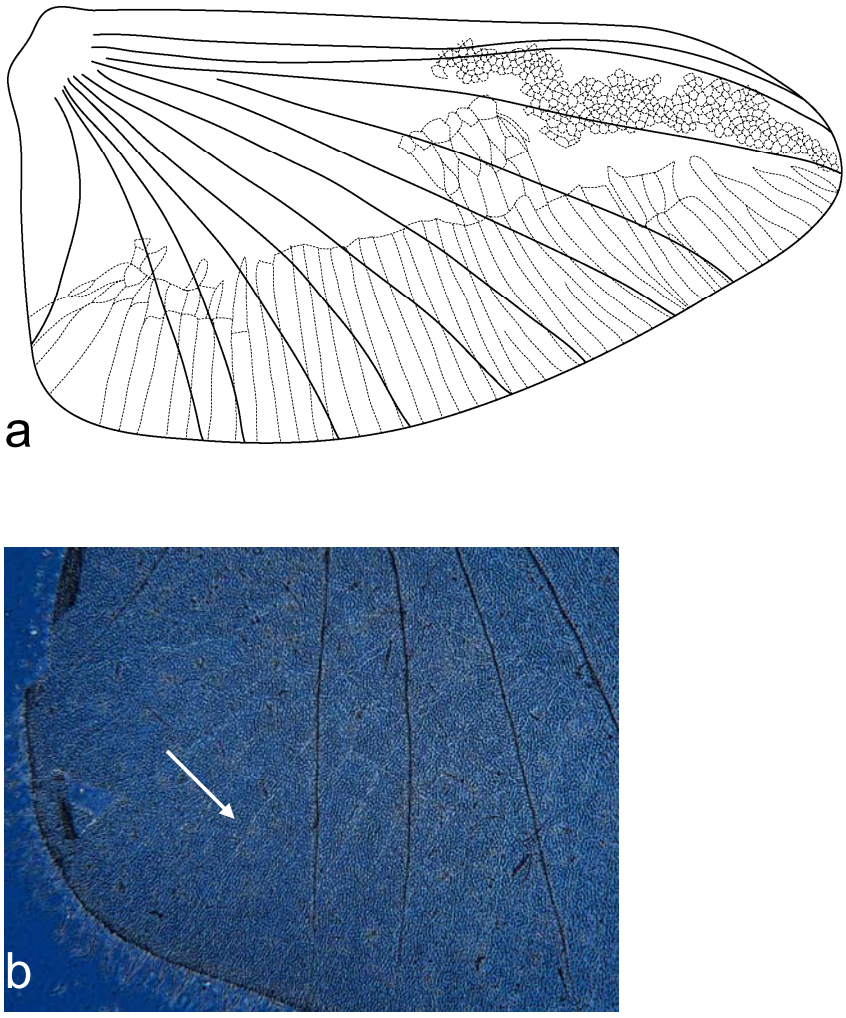


Figure 2.19. *Prosopistoma* forewing showing traces of archedictyon between the main veins, seen under phase contrast lighting conditions. (a) pattern of archedictyon traced from whole wing, where it was clearly seen; it was too faint in some parts to be drawn accurately, hence the blank areas, (b) anal region of wing enlarged, with arrow pointing to an example of the remnant archedictyon, visible as paler lines behind the main veins.

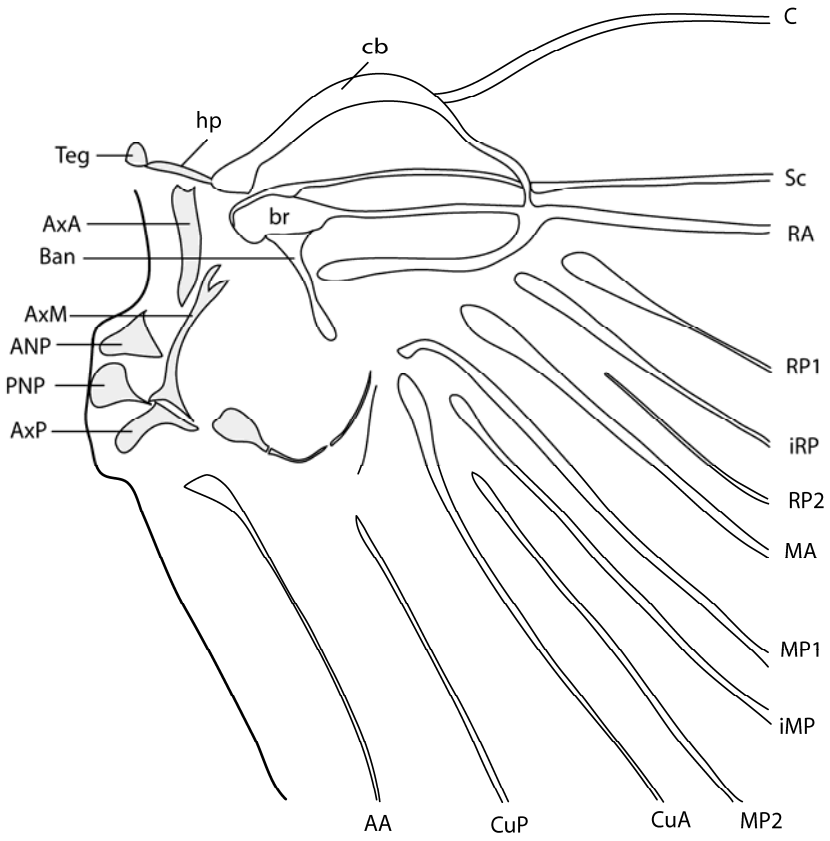


Figure 2.20. Forewing of *Prosopsitoma* showing the arrangement of the sclerites at the wing base (Madagascan sp. 2, male). AxA – anterior axillary sclerite; AxM- median axillary sclerite; AxP – posterior axillary sclerite; Ban – basanale; br – base of radius; cb – costal brace; hp – humeral plate; Teg – tegula; ANP – anterionotal wing process; PNP – posterior notal wing process.

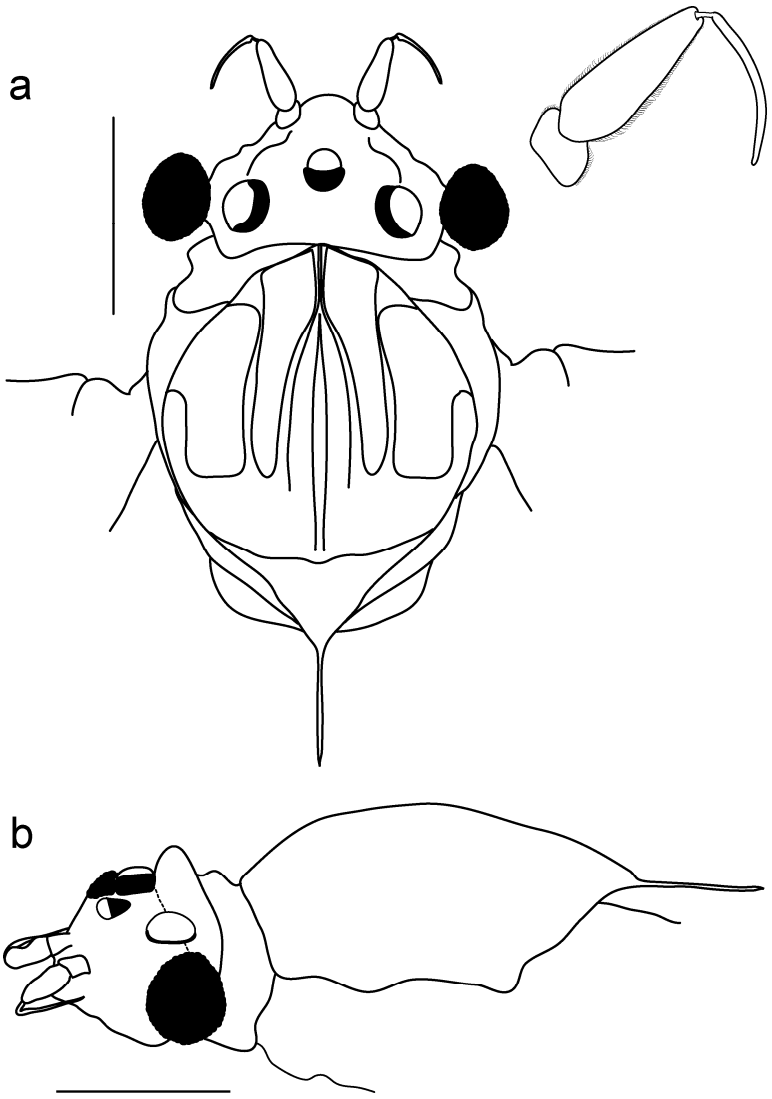


Figure 2.21. *Prosopistoma variegatum*, head of male subimago (a) dorsal view, with enlargement of antenna, showing scape, enlarged pedicel and flagellum, (b) lateral view, showing posterior margin of head everted to form narrow, almost vertical, flange-like extension; posterior, dorso-median projection from mesothorax evident in both diagrams. Scale line equals 1 mm.

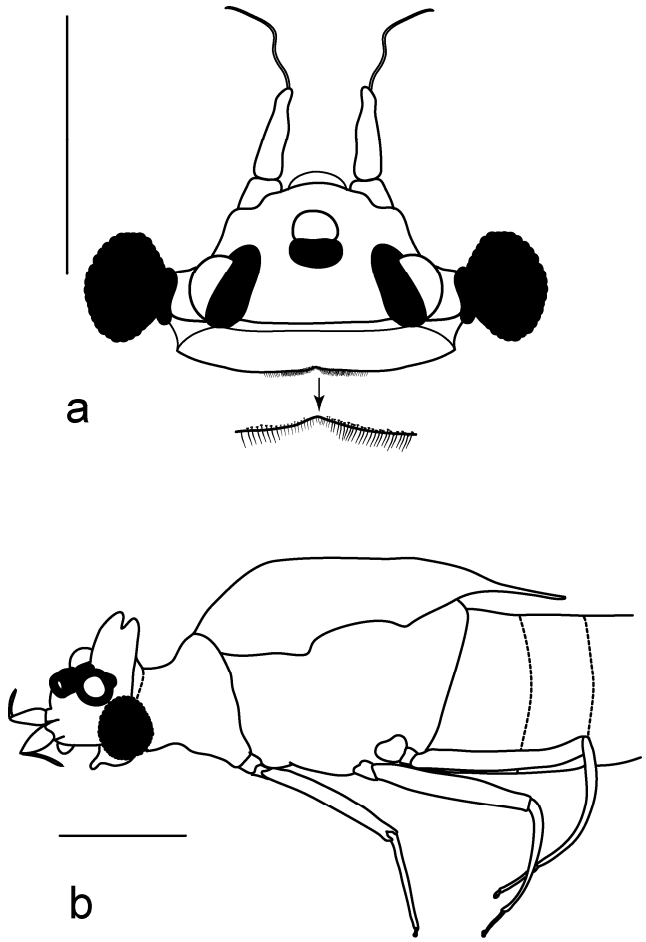


Figure 2.22. *Prosopistoma variegatum*, head of male imago, (a) dorsal view, showing enlargement of setae fringing the dorsal margin of the vertically protruding rear flange behind the head, (b) lateral view; note the flange at the rear of the head, and the posterior orientation of the legs. Scale lines equal 1 mm.

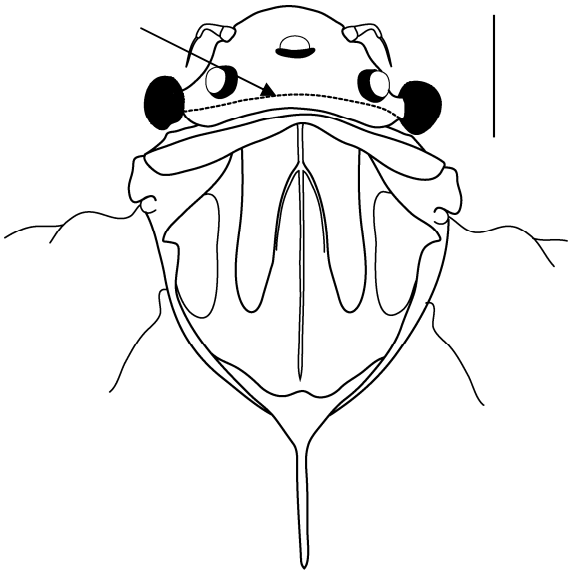


Figure 2.23 *Prosopistoma variegatum*, female subimago, dorsal view of head. Flange along posterior margin of head arrowed. Scale line equals 1 mm.

Figure 2.24. Oblique dorsolateral view of head and forelegs the male imago of Madagascan sp. 1, showing the position in which the forelegs are held, twisted up above the thorax.

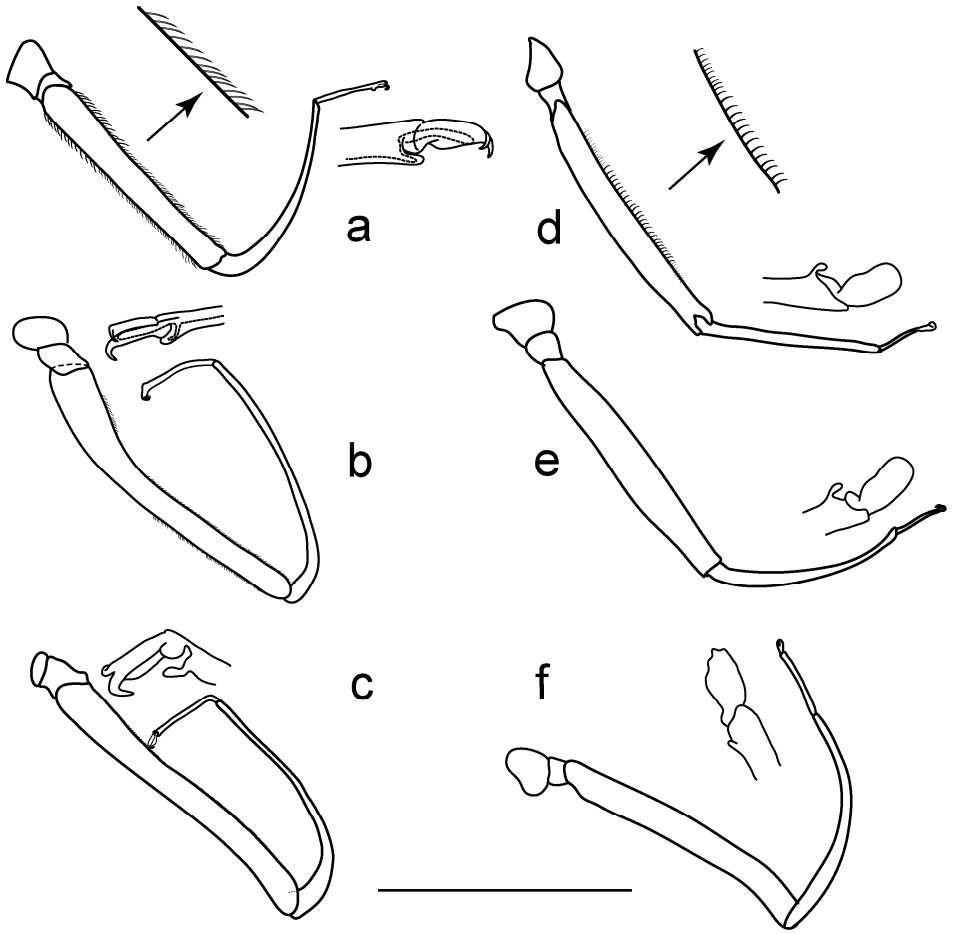


Figure 2.25. *Prosopistoma variegatum*, male subimago, (a) foreleg, (b) midleg, (c) hindleg, male imago, (d) foreleg, (e) midleg, (f) hindleg. Enlargement of each claw and terminal tarsal pad is shown. Scale line equals 1 mm.

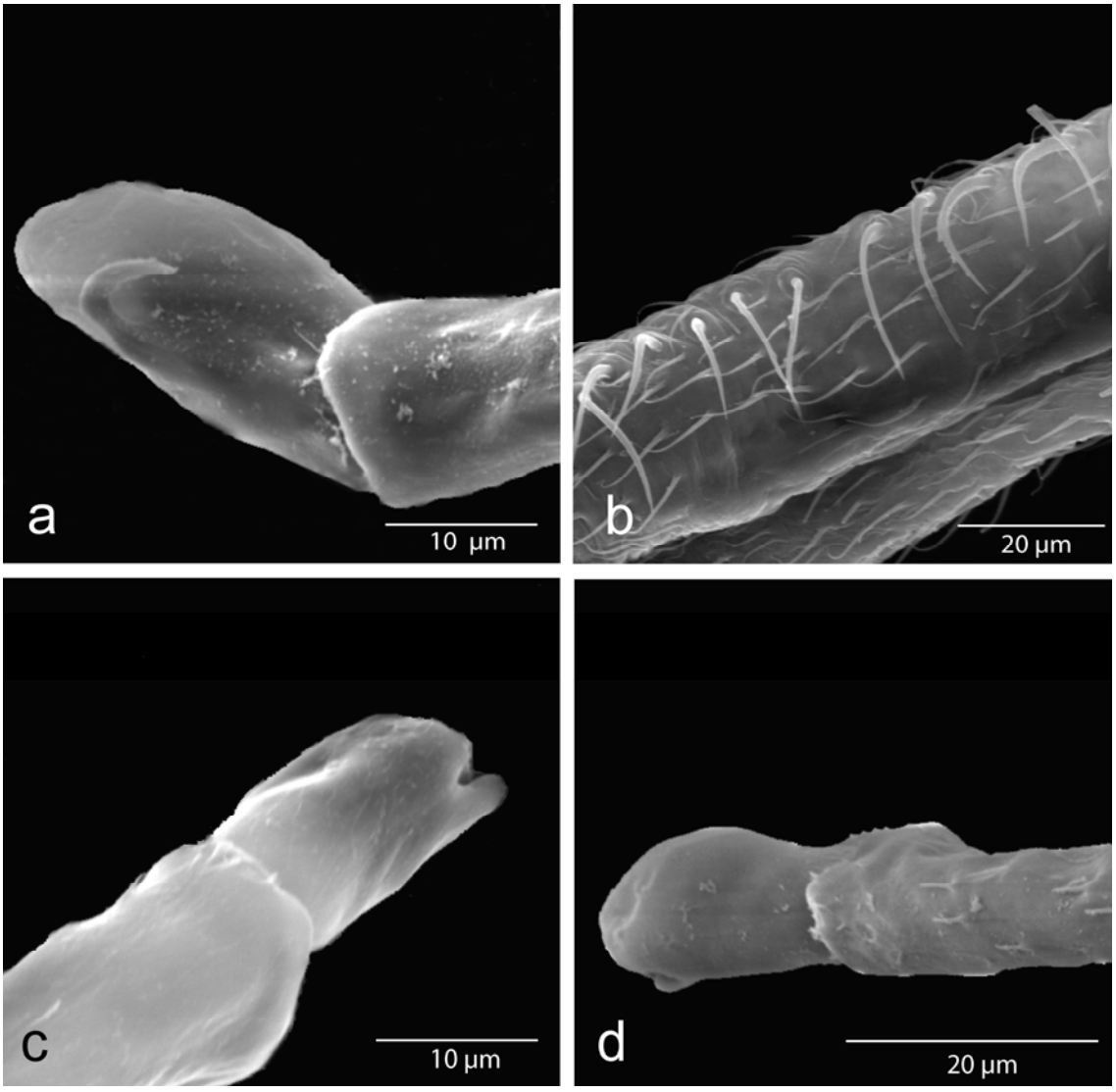


Figure 2.26. Claws and femoral setae of male subimago of Madagascan sp. 2. (a) foreclaw, (b) setae of forefemur, (c) midleg claw, (d) hindleg claw.

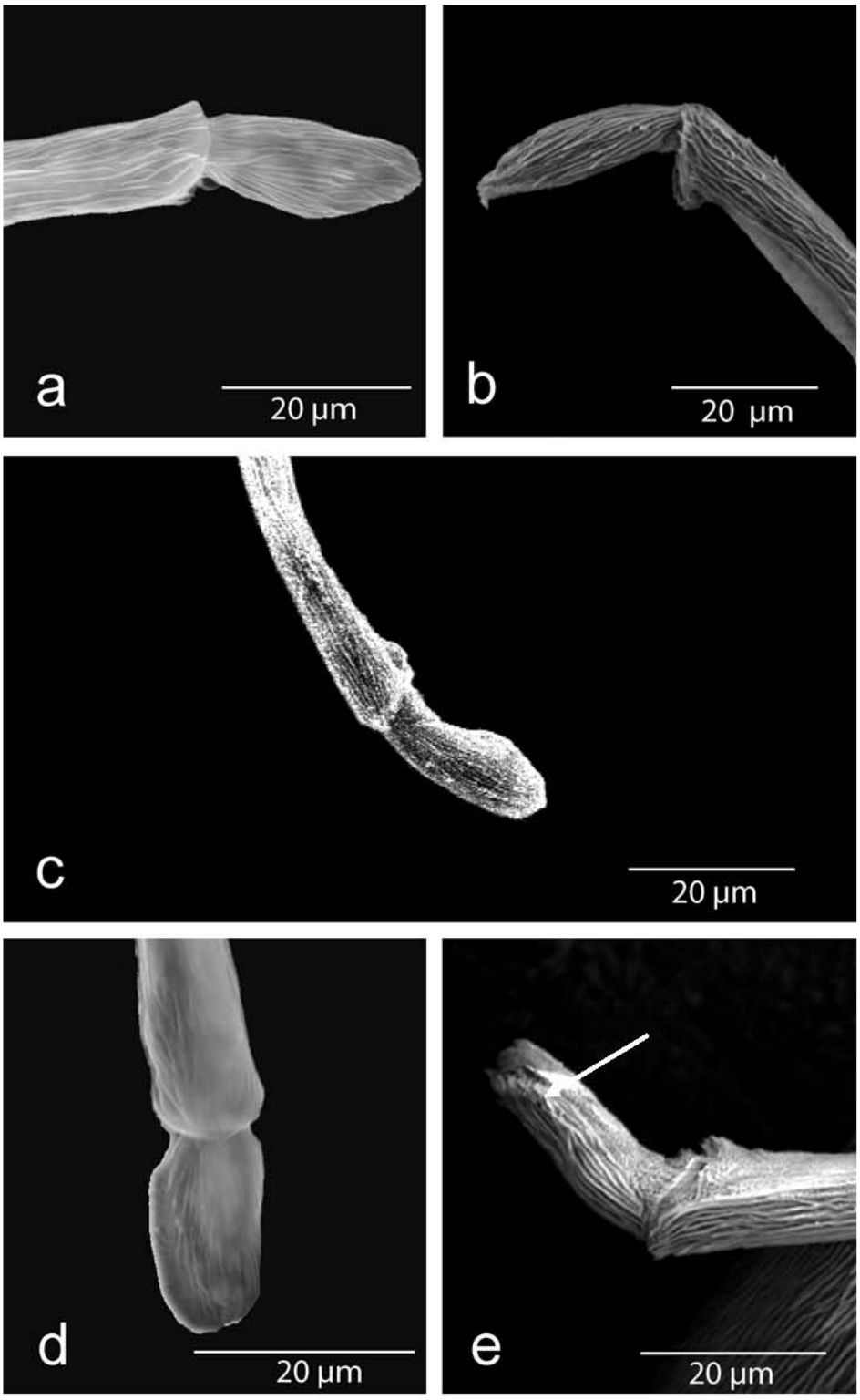


Figure 2.27. “Claws” consisting of terminal tarsal pads of male imago (a) Madagascan sp. 2, foreleg pad, (b) Madagascan sp. 6, foreleg pad, (c) Madagascan sp., 1 midleg pad, (d) Madagascan sp. 2, hindleg pad, (e) Madagascan sp. 6, hindleg pad.

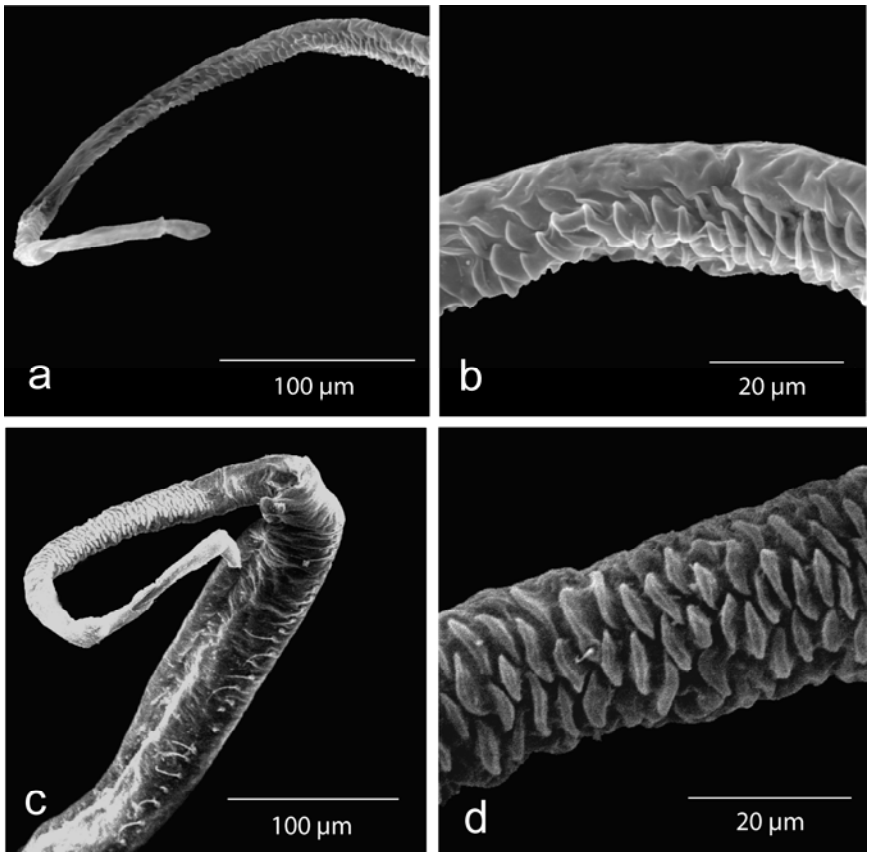


Figure 2.28. Foreleg of male imago, showing an overview of the leg, and closer detail of the scalloped structure seen on the fore tibiae. (a,b) Madagascan sp. 2, (c, d) Madagascan sp. 1.

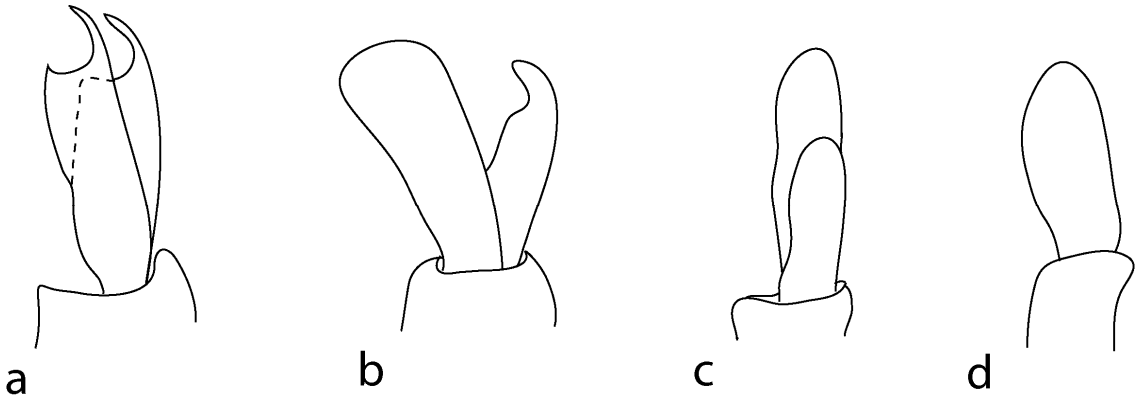


Figure 2.29. Comparison of claws in male imagos showing different types seen in mayflies, (a) double pointed claw (e.g. *Fittkaulus*) (after Domínguez *et al*., 2006), (b) one claw blunt, one claw pointed (e.g. *Ecuaphlebia*) (after Domínguez *et al*., 2006), (c) both claws blunt (e.g. *Baetisca*) (after Pescador and Peters, 1974), (d) single blunt claw (e.g. *Prosopistoma*).

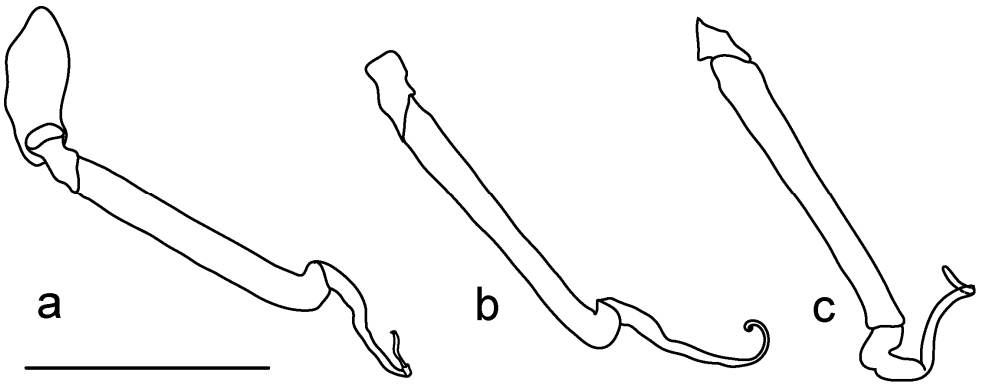


Figure 2.30. *Prosopistoma variegatum*, female subimago, (a) foreleg, (b) midleg, (c) hindleg. Scale line equals 1 mm.

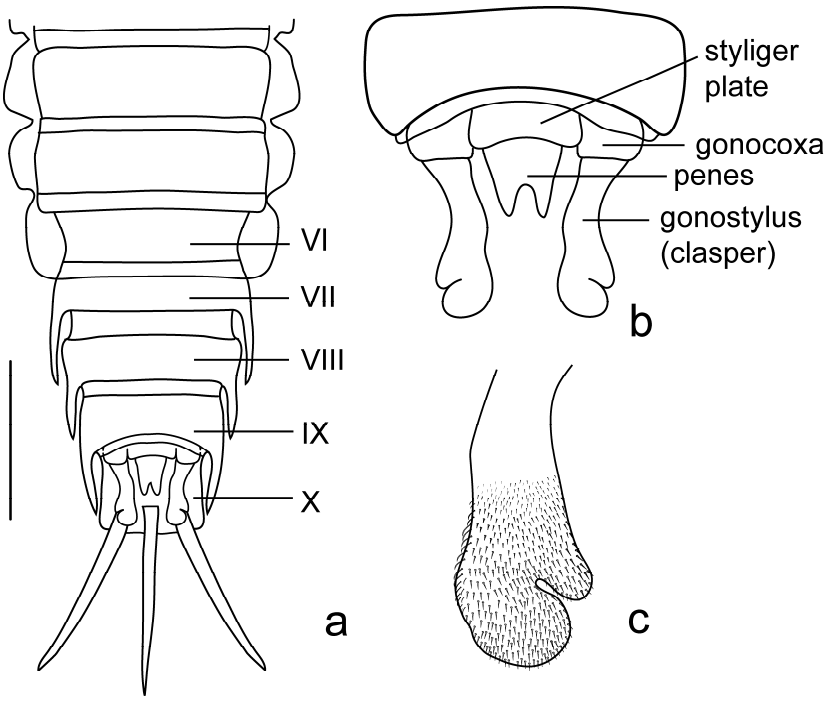


Figure 2.31. *Prosopistoma variegatum*, male subimago (a) ventral view of lower abdomen of male subimago, showing dorso-lateral extensions of segments VIII and IX (b) claspers and penes, (c) detail of clasper, showing fine covering of setae. Scale line equals 1 mm.

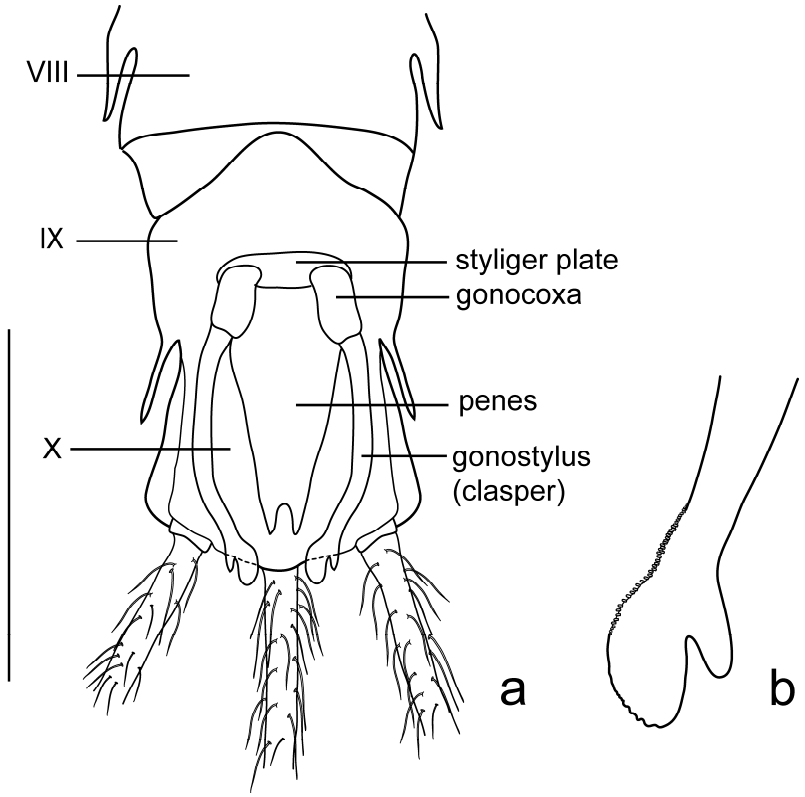


Figure 2.32. *Prosopistoma variegatum*, male imago (a) ventral view of lower abdomen of adult male, showing lateral extensions of posterior segments, claspers and penes, (b) detail of clasper, showing rough, granulated outer surface. Scale line equals 1 mm.

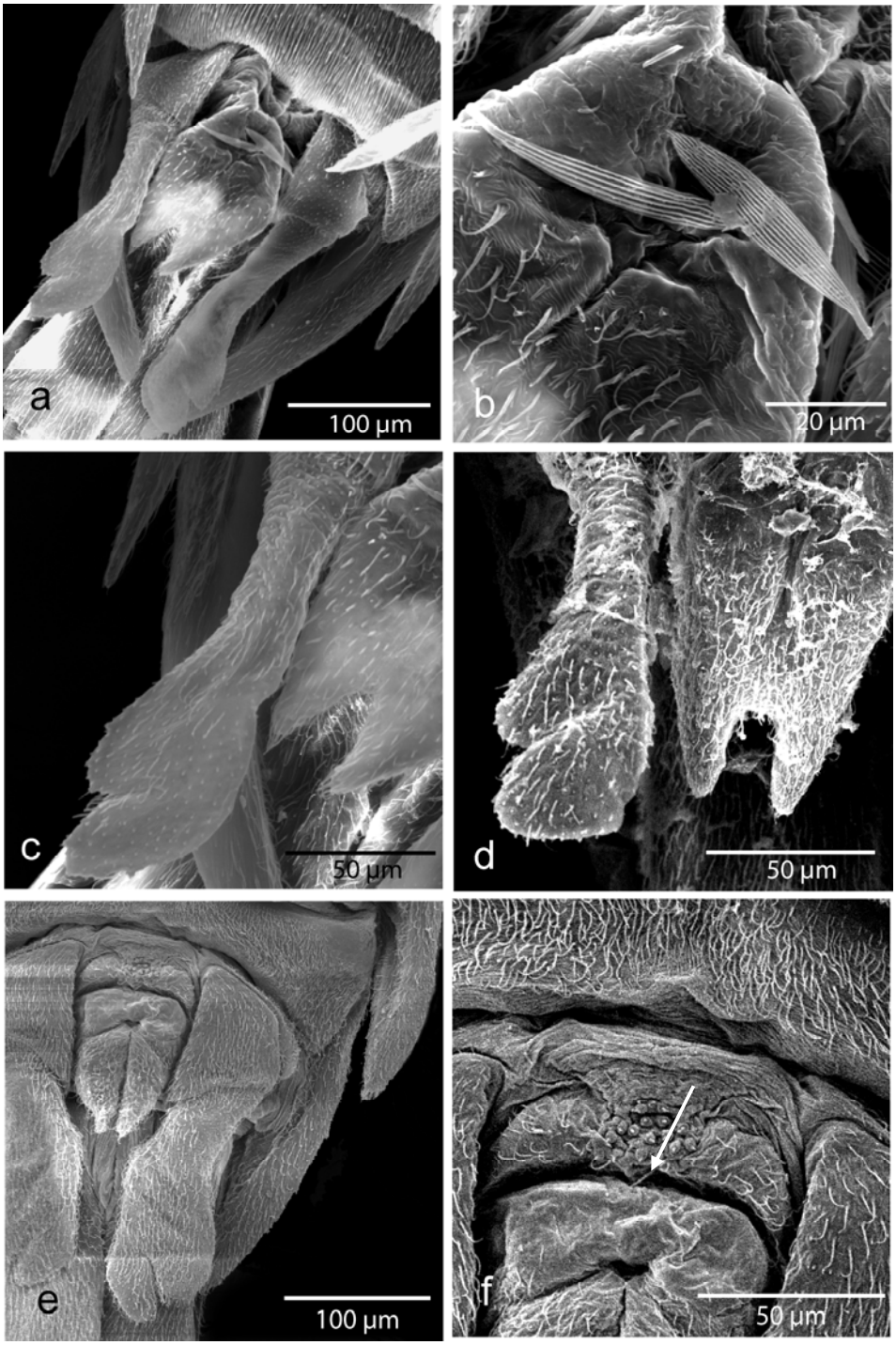


Figure 2.33. Subimaginal male genitalia. (a-c) Madagascan sp. 2, (a) clasper and penes, (b) styliger plate above penes (image contaminated with scales from another insect), (c) closer view of tip of penes and right clasper. (d) Madagascan sp. 1, penes and right clasper, (e,f) Madagascan sp. 6, (e) overview of genital apparatus, (f) focus on presumed sensory cones on styliger plate (arrowed).

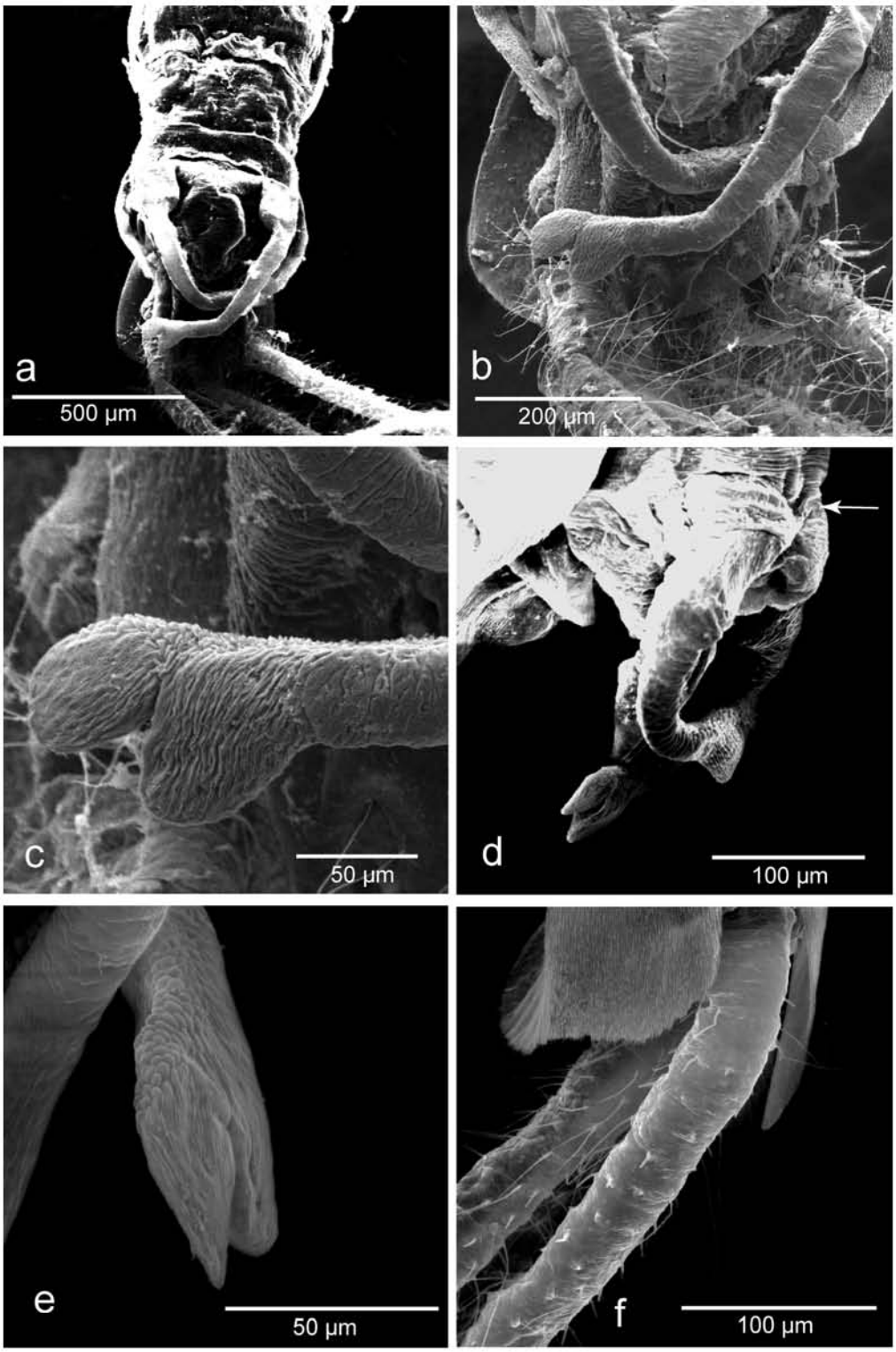


Figure 2.34. Details of male imaginal genitalia and cerci. (a-c) Madagascan sp. 7, showing progressively closer view of genitalia, (d) Madagascan sp. 2, with possible sensory hairs arrowed, (e,f) Madagascan sp. 6, lateral view of clasper, and cerci, showing setation.

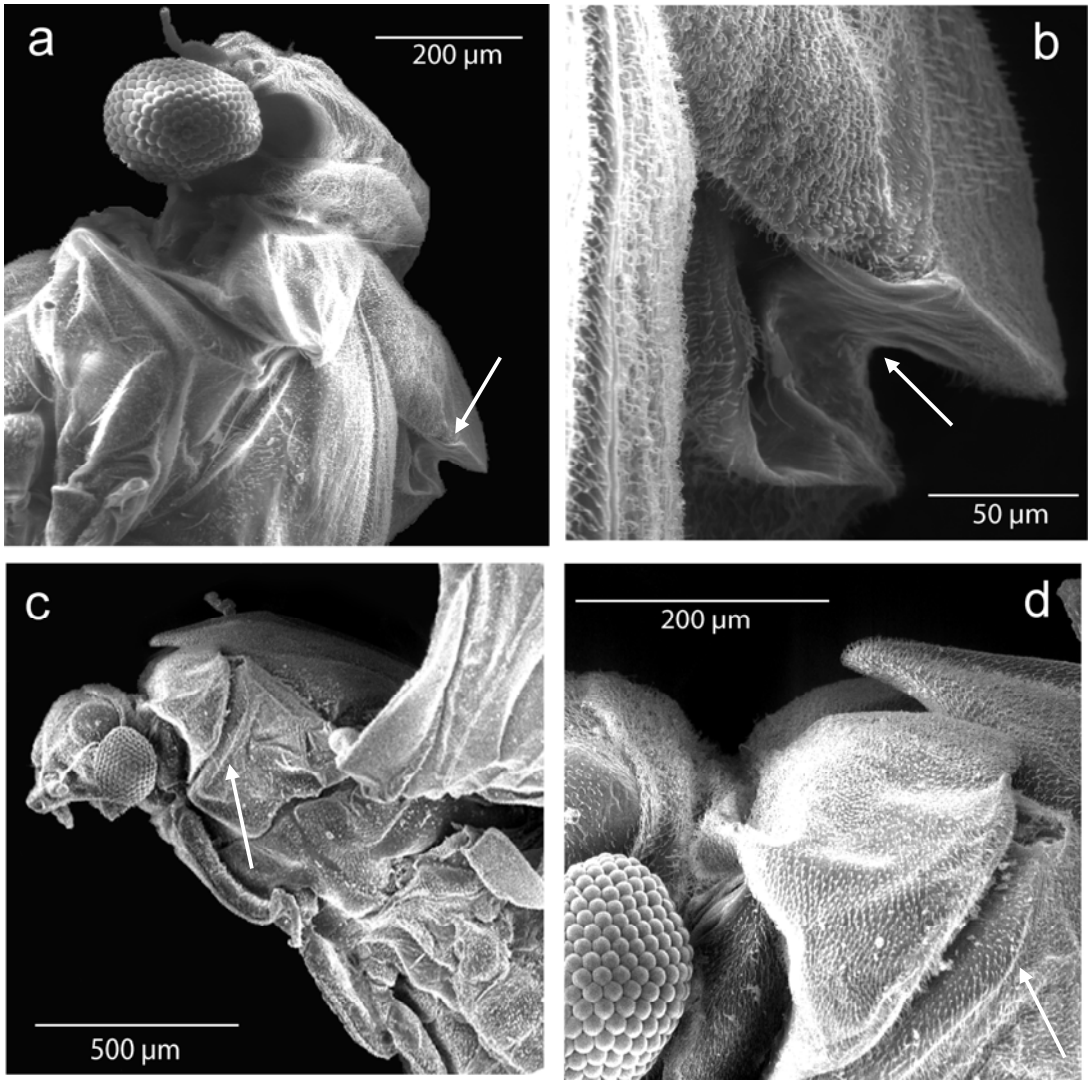


Figure 2.35. Thoracic structures of female subimago that may play a role in attachment between male and female during mating. (a, b) Madagascan sp. 2, (c,d) Madagascan sp. 1. Arrows show location of thoracic grooves, discussed in text.

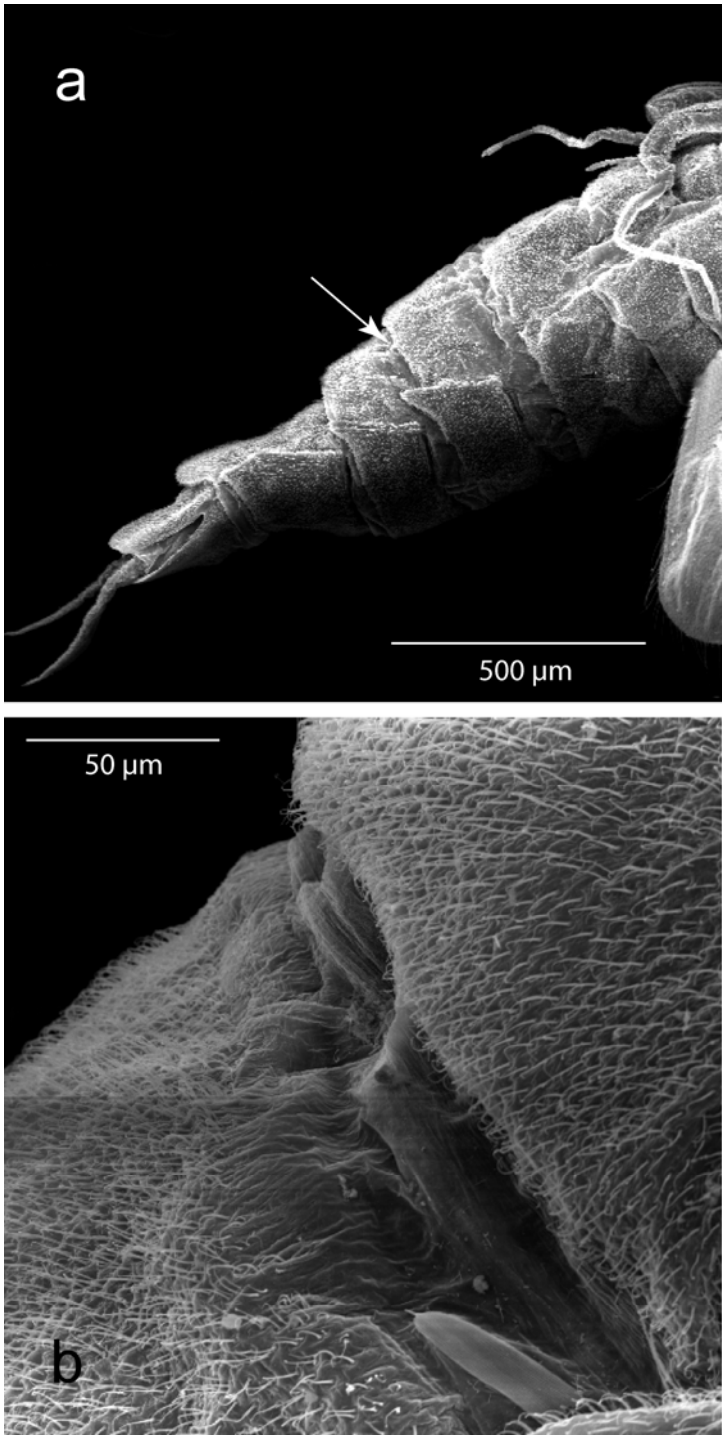


Figure 2.36. Madagascan sp. 1 female gonopore and associated structures, (a) ventral position of gonopore, (b) close up view of gonopore with spike-like structures adjacent to gonopore, postulated to be a site of attachment of male clasper during copulation.

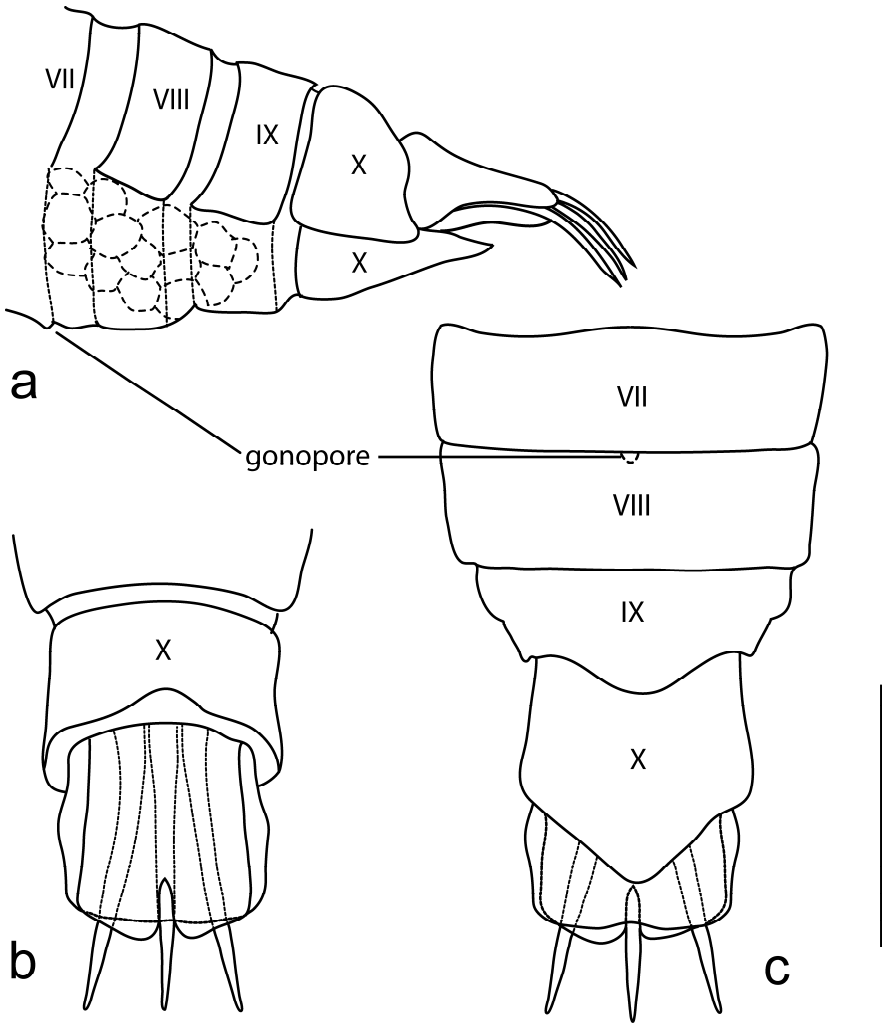


Figure 2.37. *Prosopistoma variegatum*, lower abdomen of female subimago (a) lateral view, (b) dorsal view, (c) ventral view. Scale line equals 1 mm.

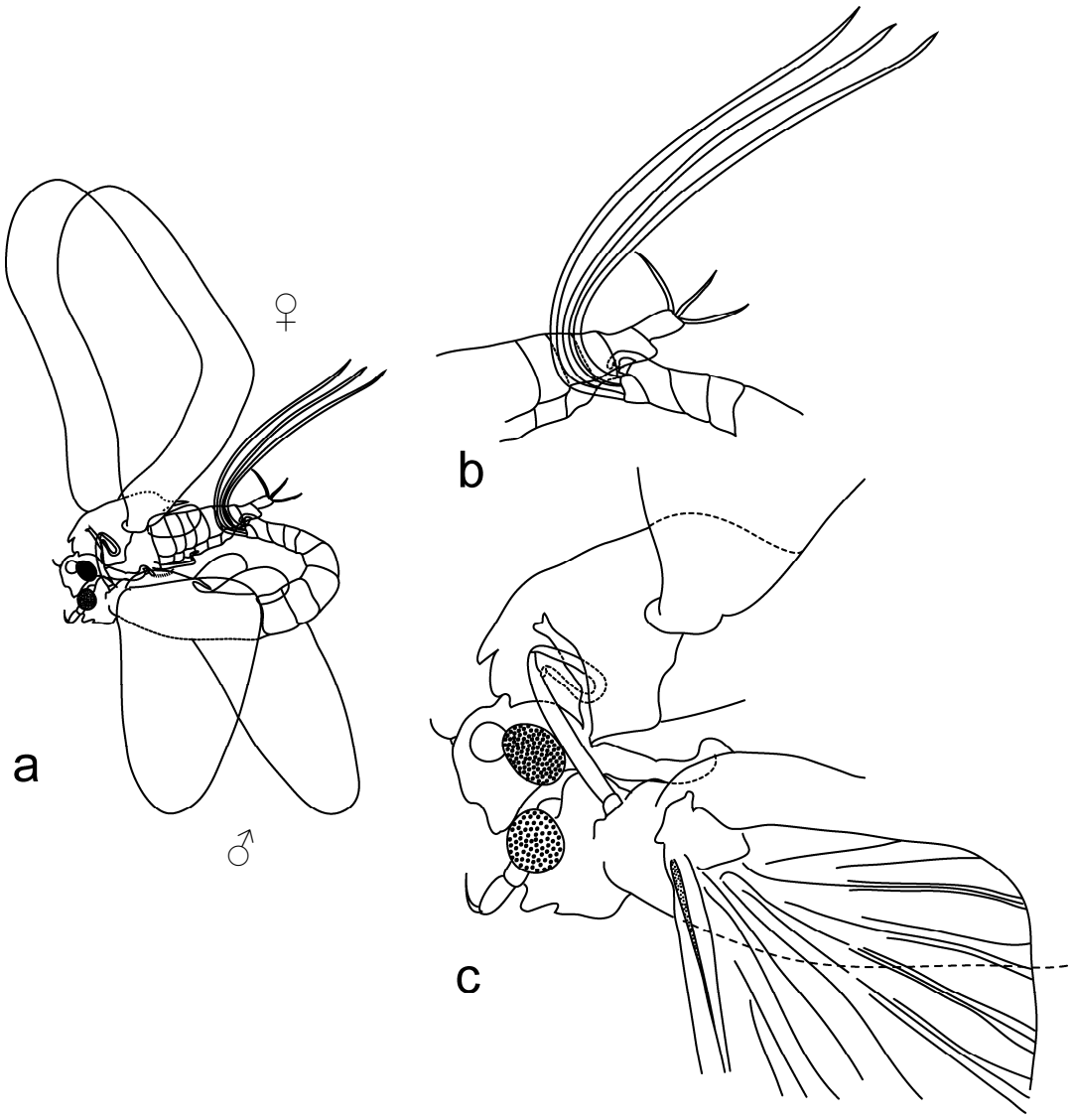
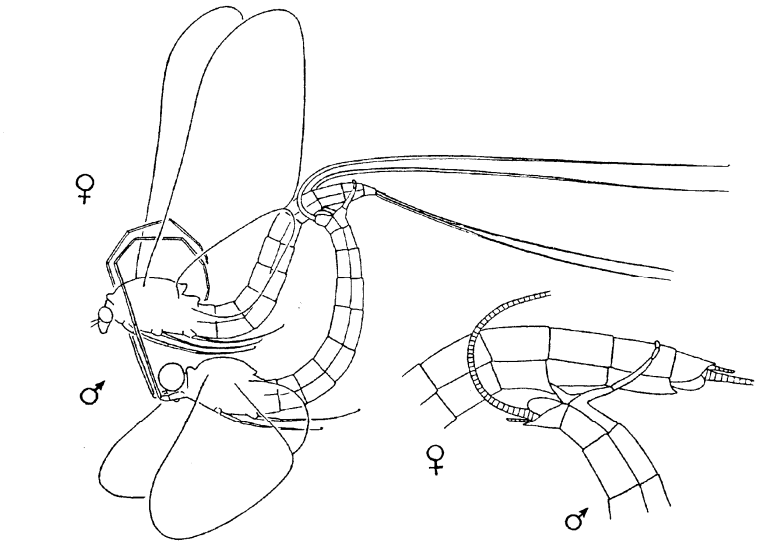
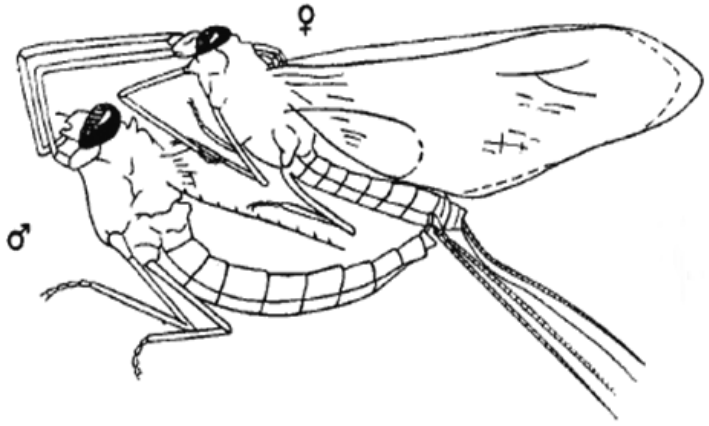


Figure 2.38. Proposed mating position of Prosopistomatidae in flight, (a) male grasps female from below using his legs twisted above his thorax, (b) copulation takes place with the clasper grasping the intersegmental spines, (c) the male leg and claw are within the groove and socket on the female thorax, holding the mating pair together.



a



b

Figure 2.39. Mating position of (a) *Parameletus chelifer* (Siphlonuridae) (from Kluge, 2004), (b) *Rhithrogena minus* (Heptageniidae) (after Brinck, 1957).

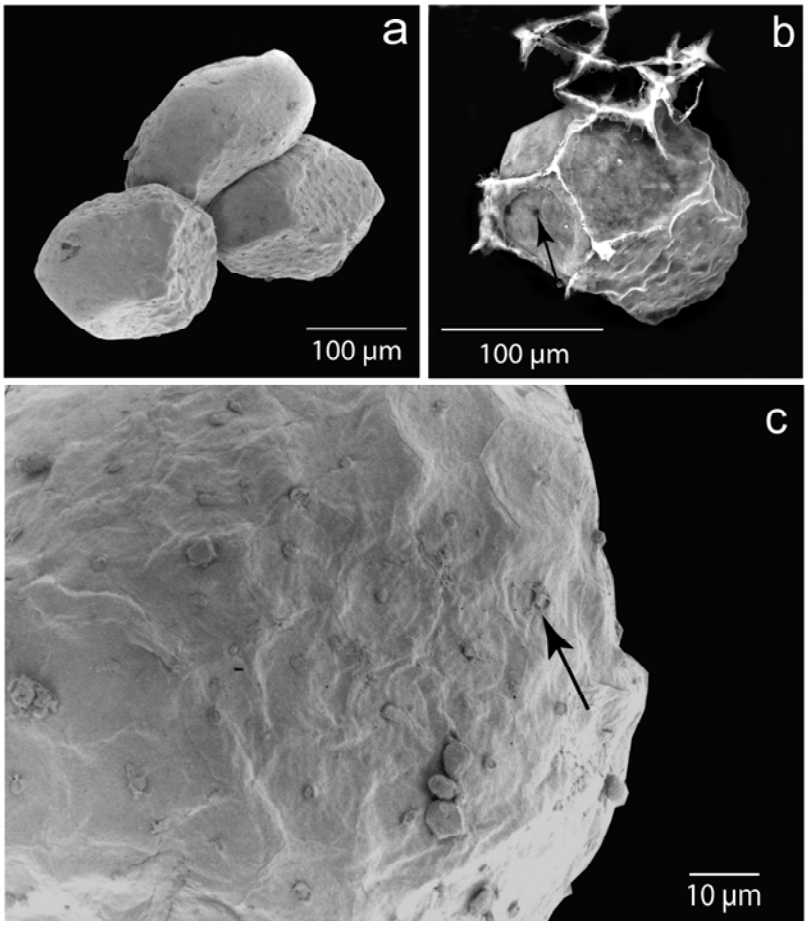


Figure 2.40. Scanning electron micrograph of eggs of *Prosopistoma variegatum* (a) cluster of eggs, (b) end on view, with funnelform micropyle arrowed, (c) close up of chorionic pattern, with one of the knob-terminated, coiled threads arrowed.

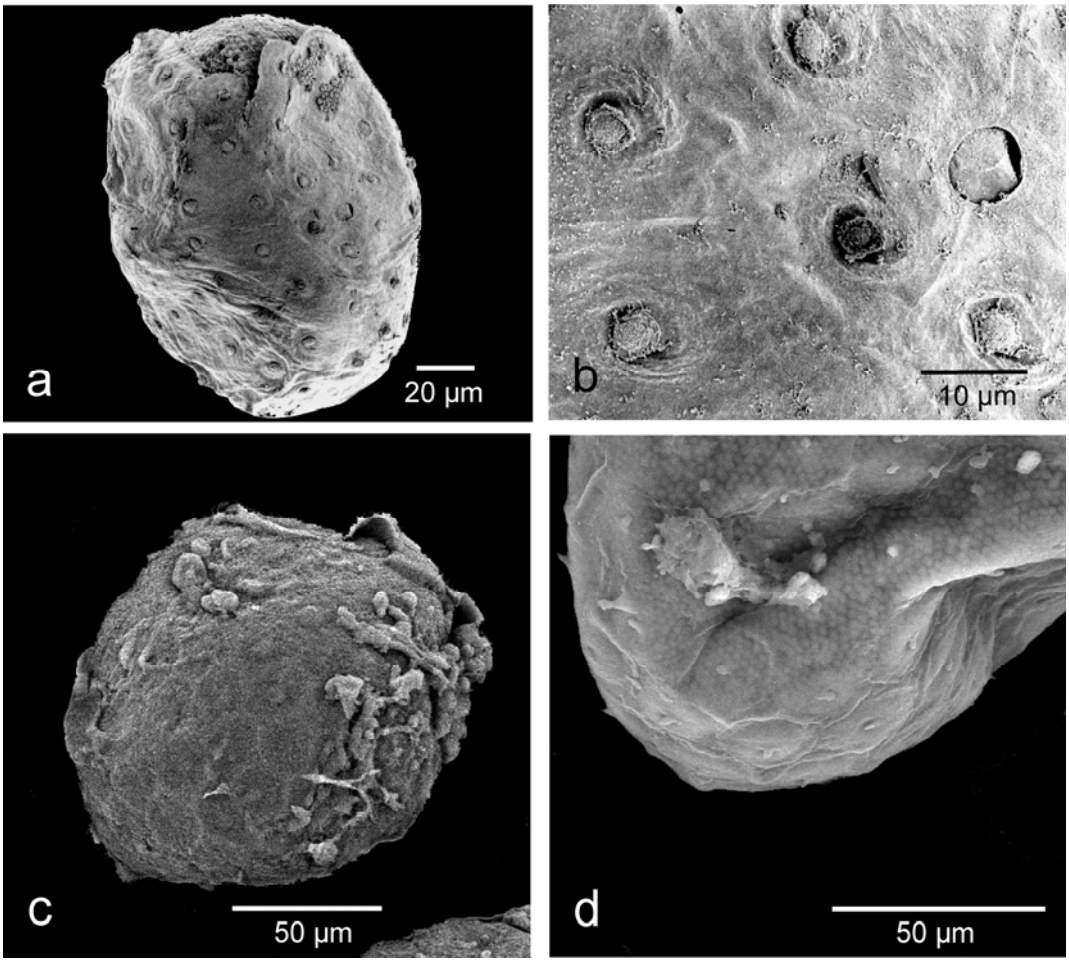


Figure 2.41. SEM images of prosopistomatid eggs. (a) Madagascan sp. 2, whole egg, (b) Madagascan sp. 2, closer view of knob-terminated coiled threads, (c) *P. africanum*, (d) *P. crassi*.

Chapter 3

Species delimitation using morphometric measurements

"Shape is a concrete representation of an otherwise nebulous concept"
wordnetweb.princeton.edu

"Shape is the functional form of a body, sculptured by the environment's contrasting forces" Desmond C. Weeks, 2010

Synopsis

In contrast to the detailed study of the structural morphology of the nymphs and winged stages of the Prosopistomatidae presented in Chapter 2, this chapter deals with measurements and shape. Multiple nymphal carapace measurements were taken to see if they characterise species. This exercise focussed on nymphs due to the low species representation as adults. However, truss measurements were also taken of adults' wings, despite the small sample size, to explore whether they could be used for species delimitation in the Prosopistomatidae. All available nymphal material, representing a wide selection of species, was measured. A relatively large collection of *P. crassi* and *P. variegatum* nymphs was available, enabling the use of these species to additionally investigate seasonal growth patterns and allometry. The allometry study questioned whether the ratios derived in younger instars would be similar to older instars; if the ratios were similar, this would allow extrapolation of ratios determined for mature nymphs to earlier instars, thus contributing to species identification of younger instars. The seasonal distribution of nymphal sizes provides another way of checking the delimitation of species, because if two or more species were living sympatrically, size differences could reflect not only seasonal differences within one species, but possibly identify the presence of more than one species based on ratios which give an indication of shape and therefore species. A simple scatter plot of Principal Component Analysis (PCA) components derived from the measurements shows trends, implied groupings and species delimitation. A plot of coordinates derived from Discriminant Function Analysis (DFA) of the same data indicated which of the measurements had the greatest influence on each species.

3.1 Introduction

The use of morphometric measurements to help with species recognition is not a new concept. Bookstein (1982, 1997) defined morphometrics as "the empirical fusion of geometry with biology". He introduced the idea of using "landmarks" on an object and repeating measurements between similar landmarks on related objects. This technique is widely used to help with species identification, both in animals (e.g. Loy *et al*., 2000; Ibañez *et al*., 2007), and plants (e.g. Henderson, 2006; van der Niet *et al.*, 2010). Instead of relying purely on simple one-dimensional measurements such as length, Strauss and Bookstein (1982) proposed the analysis of a truss of measurements to produce a two- or three-dimensional representation of shape which is repeatable between specimens and which accounts for changes in shape.

Measurements of a number of features on *Prosopistoma* nymphs were undertaken with three intended outcomes. The first was to measure a number of nymphs of one species looking at instars of different sizes to see if nymphal growth is allometric, and the implications of this for species delimitation of younger instars; this was done for two species, *P. crassi* and *P. variegatum*, as there was adequate material for both. The second was to see whether morphometric measurements of nymphs could be used as a means to distinguish individual species. The third aim was to use truss measurements of wings to identify adults, with homology of wing venation providing the landmarks for measurements to be repeatable between species.

3.2 Methods

3.2.1 Seasonal growth patterns and allometry of nymphal features

The first approach, investigating allometry between nymphs of different ages within one species, measured a larger number of individuals of *P. crassi* and *P. variegatum*, considering all sizes that were available (Appendix Table A3.1). In the case of *P. crassi*, a large sample of nymphs (n = 92) was available from the Vaal River and its tributaries due to surveys of these rivers from 1958-1961, thanks to the collecting efforts of Dr F.M. Chutter (Appendix Tables A2.1, A3.1). The mesh size of the nets used in Chutter's studies was 0.29 mm, far smaller than the net mesh size used by the current South African National River Health biomonitoring "SASS" sampling program, which has a 1 mm mesh size. As a consequence, current biomonitoring surveys may miss small *Prosopistoma* specimens. Chutter's collection provides by far the largest representation of nymphs of

one species collected over a number of years and seasons within South Africa, and offers an opportunity to examine nymphal growth in *P. crassi.* This material is housed at the Albany Museum. Nymphs of *P. variegatum* (n = 71), now housed at the Museum of Zoology, Lausanne, Switzerland, were collected by Dr Jean-Marc Elouard in Madagascar. Two other species for which more than 10 specimens were present (*P. mccaffertyi* (n= 12) and Madagascan sp. 2 (n = 30); Appendix Tables A2.1, A3.1) were also compared in this way to see if the results obtained from the first two species apply more generally.

While some of the younger instars were small (less than 0.5 mm in length), there is no way of being sure that they were first instar nymphs. Initially measurements were done using an eyepiece measuring graticule, but it was found to be easier and more accurate if the measurements were done from calibrated digital images using the freeware program 'ImageJ' and stored in an EXCEL spreadsheet. The specimens were adjusted so that they were, as nearly as possible, parallel to the plane of the objectives when photographed to eliminate any bias due to parallax.

Details of the source of all material investigated is listed in Appendix Table A2.1.

3.2.2 Morphometric analysis of nymphal features

Morphometric measurements of nymphs were undertaken using the measurements between landmarks shown in Figures 3.1-3.3, with particular focus on truss-type measurements of the carapace (Figure 3.1a, c) to see if this could give a measure of shape indicating species differences for the nymphs. The following measurements were taken: carapace width: width of carapace at its widest point (Figure 3.1a, c, line a-b); carapace length: length of carapace along the midline from anterior margin to posterior point above the orifice where water leaves the carapace (line c-d); diagonal measurements were also taken across the carapace (lines a-c, a-d, b-c and b-d) to estimate possible shape differences between species (Figure 3.1a,c), these, together with carapace length and width, form Strauss and Bookstein (1982) truss-type measurements. Head measurements included head width: maximum width measured across widest part of head (line e-f); distance between eyes: measurement between midpoints of each compound eye (line g-h); head length: from base of frons above clypeus to back of vertex (line i-j).

Ventrally (Figures 3.1b, d and Figures 3.2a, b), measurements were taken between the fore coxae (line k-l); between the bases of the hind coxae (line m-n); from the base of the prothoracic sternite to the anterior margin of the first visible abdominal segment (segment VII) (line o-p); from the base of the prothoracic sternite to the triangular point at

the end of the fused meso- and metasterum (line q-r); the length of the hind femur (line s-t). Finally, a measurement of carapace height over carapace length (Figure 3.3) gave an indication of the flatness of each species; this was termed "convexity". Although these are potentially useful for morphometric species delimitation, they were not used in these analyses for two reasons. Firstly, comparable data was not available for all species, and secondly too many variables were being considered, producing cluttered results; the analyses which follow therefore focussed only on the dorsal measurements. The ventral and convexity measurements are, however, useful for species descriptions. Total length, although useful for species description for size of the species, was found to be a less useful measurement as the tenth abdominal segment is retractile (an autapomorphy for this family) (Figure 2.3), therefore increasing variation in the absolute total length.

Measurements were made of over 350 nymphs representing different species (Appendix 3.1). In some instances, only one or two nymphs of a species have been collected, while others are represented by many specimens, making it difficult to compare the different species statistically.

3.2.3 Truss analysis of adults' wings

Truss measurements of imaginal wings (Figure 3.4a-c) were measured. For consistency, right hand wings were chosen. Measurement points represent landmark structures such as the costal brace and selected wing vein end points. Thus, the first part of the truss (truss 1) runs from the proximal margin of the costal brace to the wing margin where RA ends; truss 2 runs from the marginal end of RA to the margina end of MA, truss 3 runs from the marginal end of MA to the marginal end of CuP, truss 4 runs from the marginal end of CuP to the costal brace, truss 5 runs from the costal brace to the marginal end of MA, truss 6 runs from the marginal end of CuP to the marginal end RA. Very few specimens were available for measurement, either due to lack of material for certain specimens, or poor condition of wings in some of the material. Measurements for *P. pennigerum* were taken from an illustration by Fontaine (1955), for *P. pearsonorum* from an illustration supplied by Janice Peters (FAMU), and for *P. africanum*, from illustrations by Gillies (1954, 1956). A total of only 27 measurements were possible due to the shortage of adult material. While this sample size is really too small for statistical analysis, it was decided to use this data to see if any trends were visible, which would at least show whether this approach has potential for species delimitation in the adults.

3.2.4 Analysis of data

The comparison of seasonal size ranges for *P. crassi* and *P. variegatum* were plotted using Microsoft EXCEL, as were the investigations of the relationships between carapace length to width ratio with nymphal size. Data was analysed using a correlation matrix based Principle Component Analysis (PCA) in STATISTICA to see if species can be clearly distinguished using these morphometric criteria only. Nymphal data were log-transformed (done by taking the base-10 log of each observation, using the LOG function in Excel) to linearise allometric relationships so that the correlation coefficients perform optimally. The two principle factors from the PCA were plotted against each other in an overlaid scatter plot. The scree plots were checked to see whether Factor 3 was informative. A Discriminant Function Analysis (DFA) was performed in STATISTICA to see which of the variables had a stronger influence on which species.

3.3 Results

All measurements can be seen in Appendix Table A3.1.

3.3.1 Seasonal size range of nymphs

Figures 3.5a, b show the size variation of nymphs of *P. crassi* and *P. variegatum* over a period of twelve months. These data indicate that *P. crassi* emerges from December to January (summer), and that emergence is over by February, with only small nymphs present at that time. Interestingly, there are small nymphs present for most of the year, but the nymphs start to get larger from about July, reaching maximum size in January. Based on the time when nymphs are largest, *P. variegatum* seems to have its emergence in April-June, from late autumn to the onset of winter, with a possible second emergence in November. No *P. variegatum* specimens of this species were collected in March or December, although fieldwork was carried out. The data suggest that *P. crassi* is univoltine and *P. variegatum* is bivoltine. The continued presence in both species of smaller nymphal instars for much of the year occurring concurrently with a range of sizes, needs further investigation. It may indicate that some nymphs develop faster than others, or that egg laying is occurring for much of the year despite the females not always being at their peak size throughout the year. It could also indicate diapausing eggs. Sustained emergences and egg laying in spring for *P. crassi* could result in a faster growing summer population. There may be a slower growing overwintering population following on from recruitment throughout summer, with individuals developing slowly through winter and

into spring. For *P. variegatum* there may also be two or more co-occurring cohorts of nymphs.

Such differences in growth patterns may be useful for species delimitation with sympatric species. For example, *P. crassi* and *P. mccaffertyi* occur sympatrically in the Kruger National Park, South Africa; if one had enough nymphs of *P. mccaffertyi* over a period of time to do such measurements, life history and emergence times could be compared between species as these are an important part of understanding species development and behaviour. Knowing at what time of the year nymphs are mature, from data such as this, may help with adult association as one can deduce roughly when emergence is likely to occur, and plan a collecting trip to focus on catching adults at the appropriate time. At present, the adults of *P. crassi* remain unknown.

3.3.2 Allometric analysis of nymphal features

These data show the range of measurements which reflect details of nymphal development for *P. crassi* and *P. variegatum*, and have important implications to species delimitation procedures which follow. They show the growth of the nymphs of the species investigated to be allometric, implying that measurements and ratios can be applied to immature nymphs and are not limited to mature nymphs only.

Figures 3.6 – 3.8 show body length reflected against the ratio of carapace length-to-width. This ratio is often quoted as one of the metrics in descriptions of species of *Prosopistoma* species (e.g. Gillies, 1954; Peters, 1967; Dalkiran, 2009; Barber-James, 2010a). Gillies (1954) concluded that this ratio is useful even in small nymphs of *P. africanum*, and Peters (1967) also found this to be true for the Philippine species, *P. boreus*. However, Peters (1967) found that this ratio changes with nymphal size in certain other Asian species (*P. palawana*, *P. indicum* and *P. lieftincki*), so in those instances, a younger instar does not have the same ratio as an older nymph. The consistency of this ratio between different sized nymphs within a species was tested for several Afrotropical species (Figures 3.6 – 3.8). Measurements were compared for the carapace length-to-width ratio against total body length excluding caudal filaments, ignoring the retractile nature of the tenth segment, and the possible effect of this on a measure of total length. Figures 3.6a (*P. variegatum*) and 3.7a (*P. crassi*) show both the total length and the ratio plotted on a histogram, allowing a quick visual assessment to see that the carapace length-to-width ratio remains relatively constant while total nymphal length increases. The carapace length-to-width ratio remains the same for a particular species irrespective of the size of

the nymph. A plot of "Total length" against the "Carapace length-to-width ratio" produces the relationships shown in Figures 3.6b and 3.7b. This relationship was tested further and found to be true for two more species, *P. mccaffertyi* and Madagascan sp. 2 (Figures 3.8a,b). This makes the ratio a sound measurement for species identification even when nymphs are not mature, though, considering the observations by Peters (1967), may need to be tested for each species if dealing with immature nymphs.

The relationship between carapace width and head width was the next set of metrics investigated. Figure 3.9 shows a simple plot of nymphal head width against carapace width, showing an approximately linear relationship between different sized nymphs of *P. crassi*. This data was log transformed due to the relatively large sample size (n = 164), to reduce scatter of the data. This strongly linear relationship ($P < 0.0001$, $r = 0.98$, $r^2 = 0.97$) shows that growth in this species is allometric. Does this apply to other species, and can it be used to distinguish one species from another? Figure 3.10 shows a simple scatter plot of head width against carapace width for a broad selection of other *Prosopistoma* species. As smaller samples were being used due to fewer numbers of specimens for many of the species, the data was not log transformed. Distinct relationships can be seen for each species. The p-values (p), regression values (r) and coefficient of determination (r^2) values for each are given in Table 3.1.

3.3.3 PCA and DFA results

The number of measurements for *P. crassi* included in these plots was reduced as there were too many specimens relative to other species, and they dominated too strongly, thus only Vaal River specimens were used.

The first two principal components of the PCA analysis of the variables in Appendix Table A3.1, describe 97.25 % of the variation in the samples (Table 3.2). The remaining factors do not describe significant variation between them. A simple scatter plot of the first two factors (Figure 3.11) showed distinct species clusters, although several species overlap considerably, for example Madagascan species 2, *P. crassi* and *P. palawana*. Of particular interest is the scatter for *P. pennigerum*, where the data from the Russian population separates from that from the Spanish and French specimens.

The analysis was repeated using only the six carapace truss measurements (Figure 3.12). Factor 1 accounts for 98.30 % of total variation and Factor 2 for 0.59 %. Again the Russian and Franco-Spanish populations of *P. pennigerum* separate out, although not as clearly, and could alternatively be considered in an extension of the convex hull.

In a third plot (Figure 3.13), where the head measurements are considered without the carapace components, Factor 1 accounts for 94.43 % of total variation and Factor 2 for 4.55 %. *P. pennigerum* shows up in one cluster, showing that carapace shape is an important component in these analyses for this species. Apart from *P. variegatum*, there is considerable overlap between species, indicating that, head measurements alone are not useful for species delimitation in *Prosopistoma*.

In all three plots, *P. variegatum* stands out as the most distinctive species. This is not surprising, as visual comparison of *P. variegatum* with other Afrotropical species shows it to have a distinctive shape, although some of the Asia species also have a similar shape, e.g. *P. boreus*; however, using PCA factors, this species does not cluster near to *P. variegatum* (Figures 3.11-3.13). In the DFA plot (Figure 3.14), where the influence of each variable is mapped, *P. boreus* is placed adjacent to *P. variegatum*, with head width and distance between the eyes being the major components causing this. The DFA plot (Figure 3.14) provides a much better resolution of species than the PCA, and combined with the mapped variable influence, provides a clearer way of representing species differences.

3.3.4 Truss analysis of adults' wings

Measurements are shown in Appendix Table A3.2. This exercise using wing truss measurements was limited by the lack of suitable material, but nonetheless, patterns can be identified in the PCA analysis (Figure 3. 15). Furthermore, wings of each sex were quite distinctive in this plot. Open symbols represent female specimens and solid symbols represent male specimens. Females' wings tend to be larger than those of conspecific males. The first two principal components of the PCA of wing measurements (Figure 3.15) describe 99.33 % (98.26 % Factor 1 and 1.07 % Factor 2) of the variation in the matrix (Appendix Table A3.2), while the remaining factors did not significantly describe variation between the specimens (Table 3.3). The plot of the first two factors shows multiple distinct groupings based both on species and sex.

3.4 Discussion and conclusions

Morphometric measurements have been shown to be useful in Prosopistomatidae species delimitation. The size measurements in the species investigated shows that growth is allometric in these species, implying that ratios obtained from measurements of mature nymphs can be applied to immature nymphs of the same species. A very important

comparison should be drawn here with the Baetiscidae. A significant difference in the carapace development in the two families has been observed; Pescador and Peters (1974) reared nymphs of *Baetisca rogersi* through from the eggs stage to mature nymph, and demonstrated that the carapace is much less developed in younger instar nymphs, gradually developing in size and shape at each instar. In these studies, the carapace in *Prosopistoma* was found to be well developed in even the smallest nymph observed, and grew allometrically through to maturity. This has far reaching implications as to the homology of the carapace in these two families.

Seasonal differences in size can give an indication of emergence period, which is important in distinguishing species based on behaviour. A criticism of the data presented in Figure 3.5 is that the samples were not all taken from one site per species. Size of individuals may vary slightly between sites due to effects such as altitude (cooler temperature with higher altitude), and growth may respond to different localised climatic cues (e.g. individuals may grow faster and therefore be slightly smaller if they are from a warmer part of the country concerned). This effect has been shown for Simuliidae (de Moor, 1982, 1989). Despite such possible effects on the accuracy of the measurements, trends for each species are suggested.

Principal component analysis of a variety of measurements comparing length and shape of homologous structures in different species provides a visual way of delimiting species graphically. While such measurements may show overlap between different species, they can contribute to the overall definition and delimitation of a species. DFA was found to produce a clearer interpretation of which character variables influence the spatial representation of each species.

It would be interesting to compare wing truss measurements for a greater sample size, and also with species from other mayfly families to see if each family has a unique "wing truss signature". If this was the case, this approach could be used to develop a measurement-based means of species identification for non-expert taxonomists.

The development of the science of species delimitation based on morphometrics is taking on new dimensions in terms of numerical methods. Dujardin (2008) described the use of morphometric measurements in detecting cryptic members of medically important insect species. The measurements between morphological landmarks are seen not just as distance between these points, but the points themselves are given values as relative coordinates. This allows both size and shape to be visualized, and computer software has been developed to compare such coordinates in a system known as Procrustes

superimposition and visualization (e.g. Rohlf and Slice, 1990; Rohlf, 1999). This procedure was modified and used by Dujardin (2008) to compare coordinates from different individuals, which have all been brought to the same scale by superimposing them. These can then be compared statistically to identify cryptic species, which then allows further application of more expensive genetic methods to further determine whether the taxa being studied belong to the medically important group or not.

The use of computer-based algorithms to determine species identity is becoming an increasingly popular approach, as small differences which are less likely to be detected by human computation can be recognized using computers. Thus vast iterations, which were not possible until the age of faster computers, are now routine, and much of biology today is driven by computer programs which allow rapid calculations of complex algorithms. This aspect is explored further in the next chapter, where an artificial neural network is developed to delimit *Prosopistoma* species.

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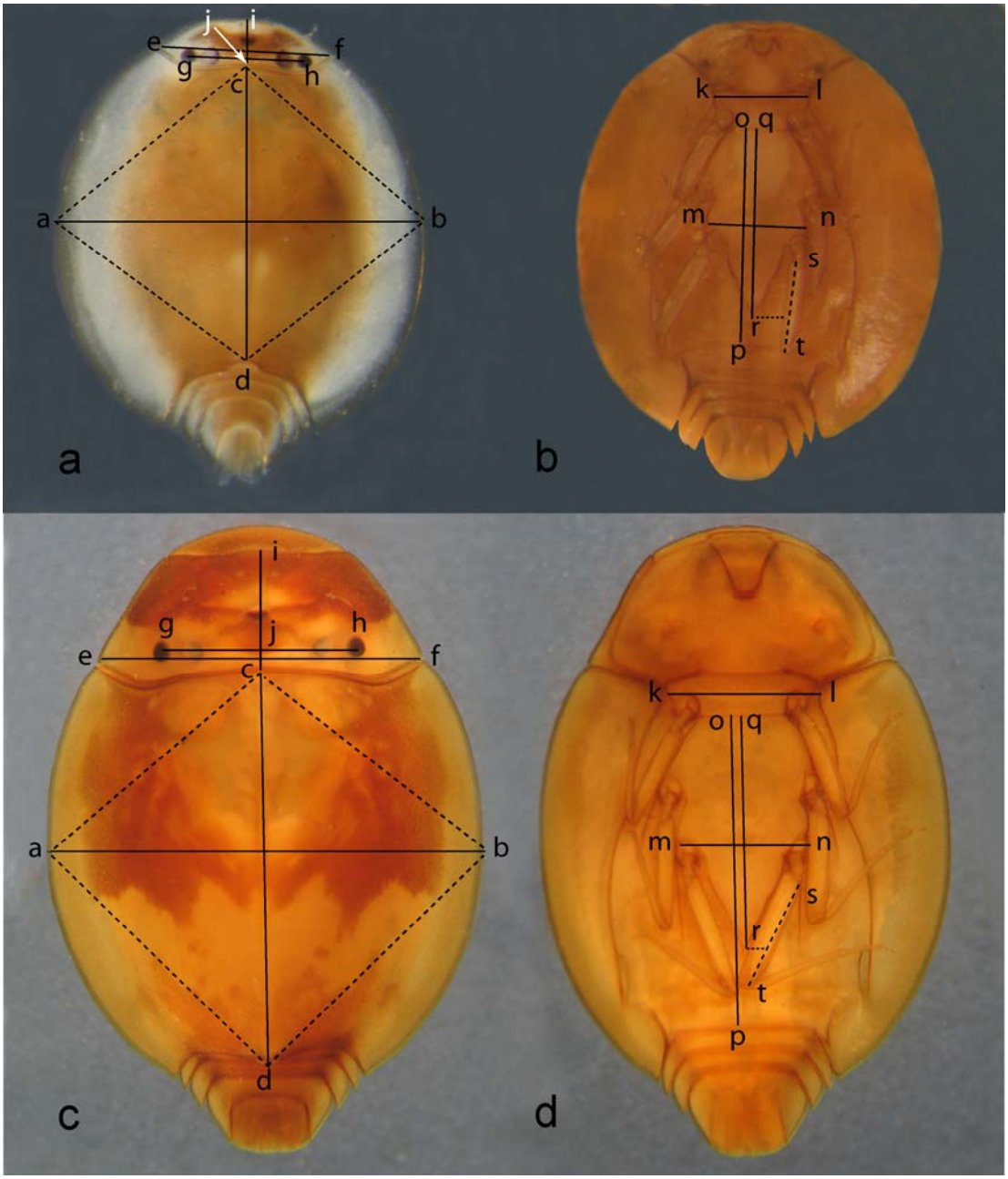


Figure 3.1. Measurements of external features of nymphs, using two species as examples; *P. crassi* (a) dorsal (b) ventral, *P. variegatum* (c) dorsal (d) ventral. Lettering explained in text.

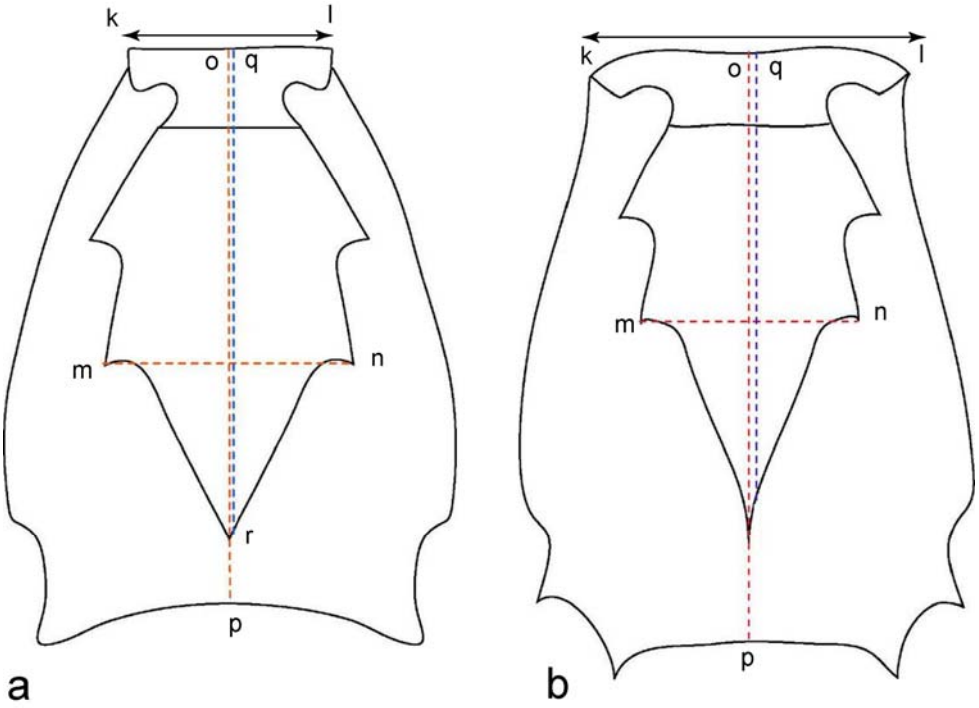


Figure 3.2. Ventral view of prothorax and fused meso and metothoracic slectites, showing details of measurements used for sternal plate ratios of (a) *P. crassi*, (b) *P. variegatum*.

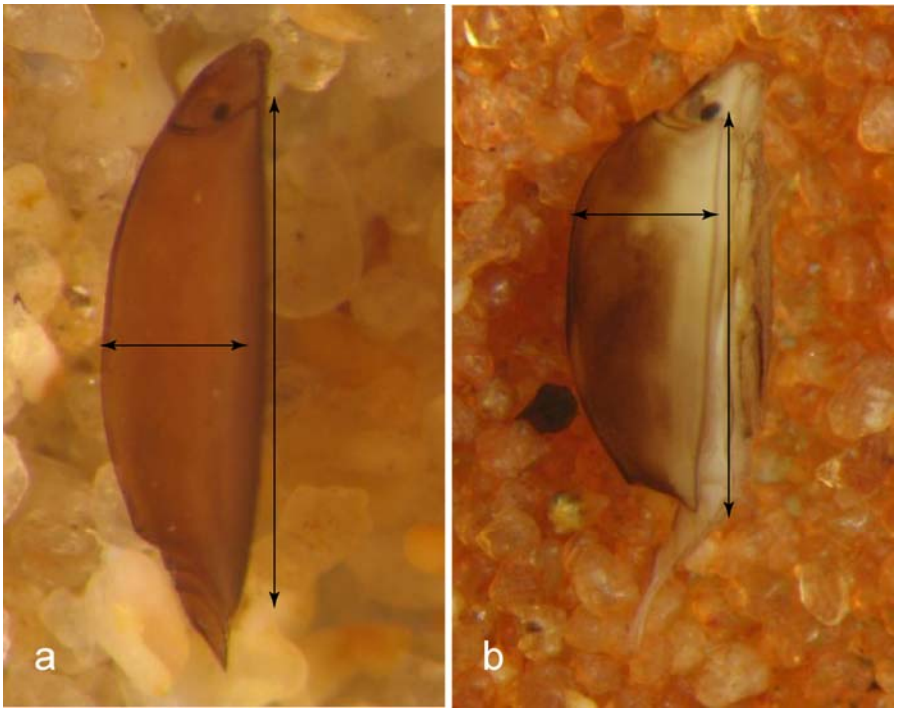


Figure 3.3. Lateral view of nymphs, showing "convexity", a ratio of maximum carapace height over carapace length, (a) *P. crassi* (b) *P. mccaffertyi.*

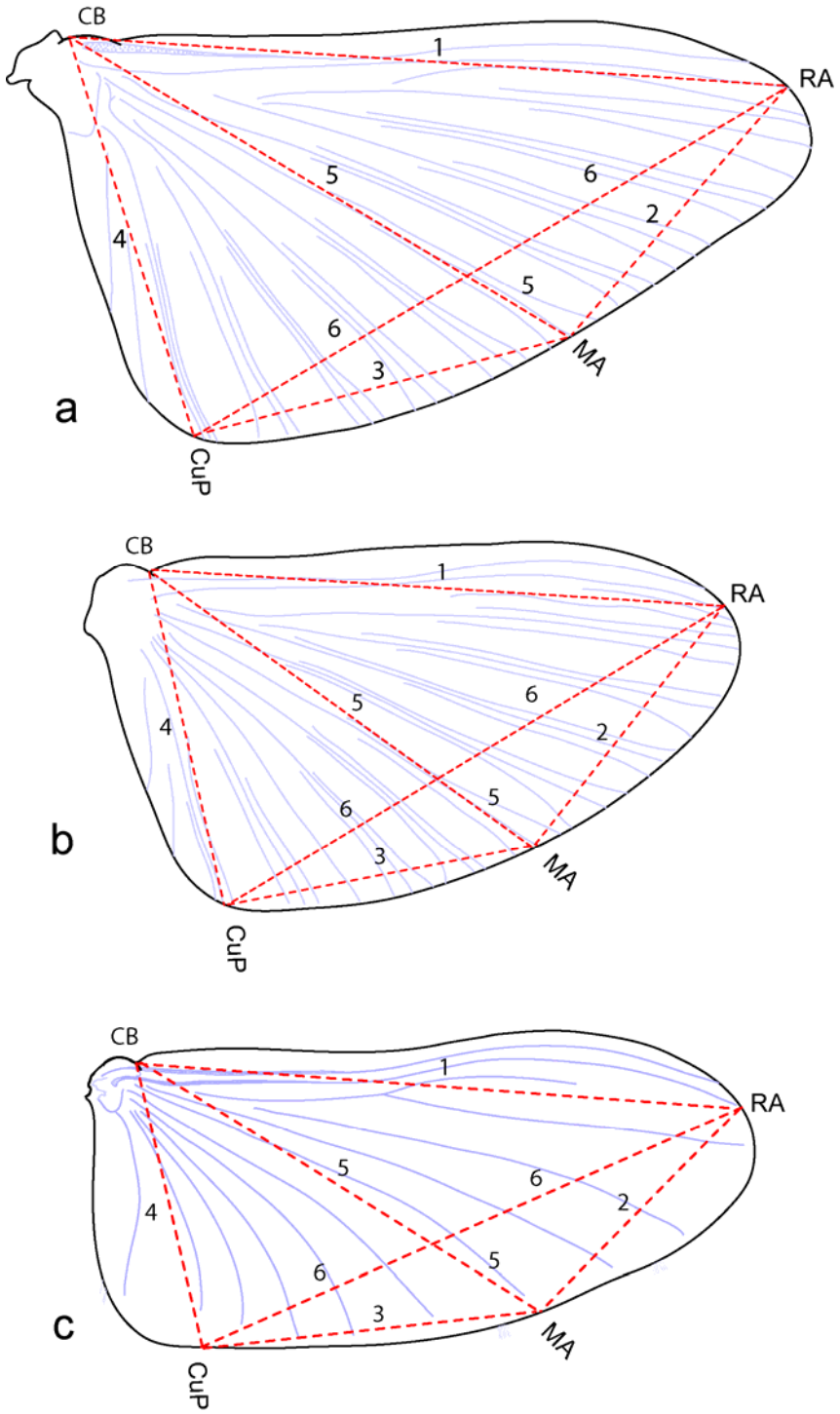
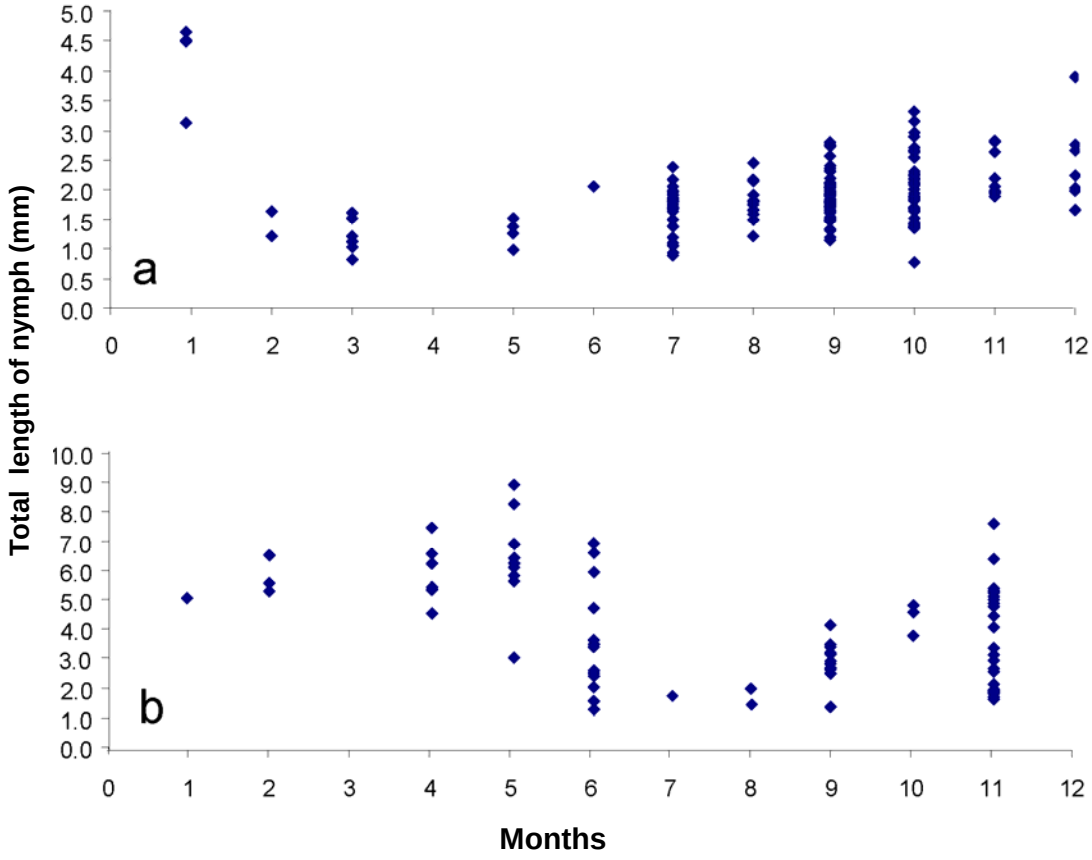


Figure 3.4. Examples of truss measurements in right forewing of (a) *P. variegatum* male (b) Madagascan sp. 1 male, (c) *P. mccaffertyi* female. Wing veins were used as landmarks. Numbering of truss distances for measurements discussed in text.



Figures 3.5. Seasonal size range of nymphs of (a) *P. crassi* from the Vaal River, South Africa (n = 92), and (b) *P. variegatum,* from several rivers in Madagascar (n = 71).

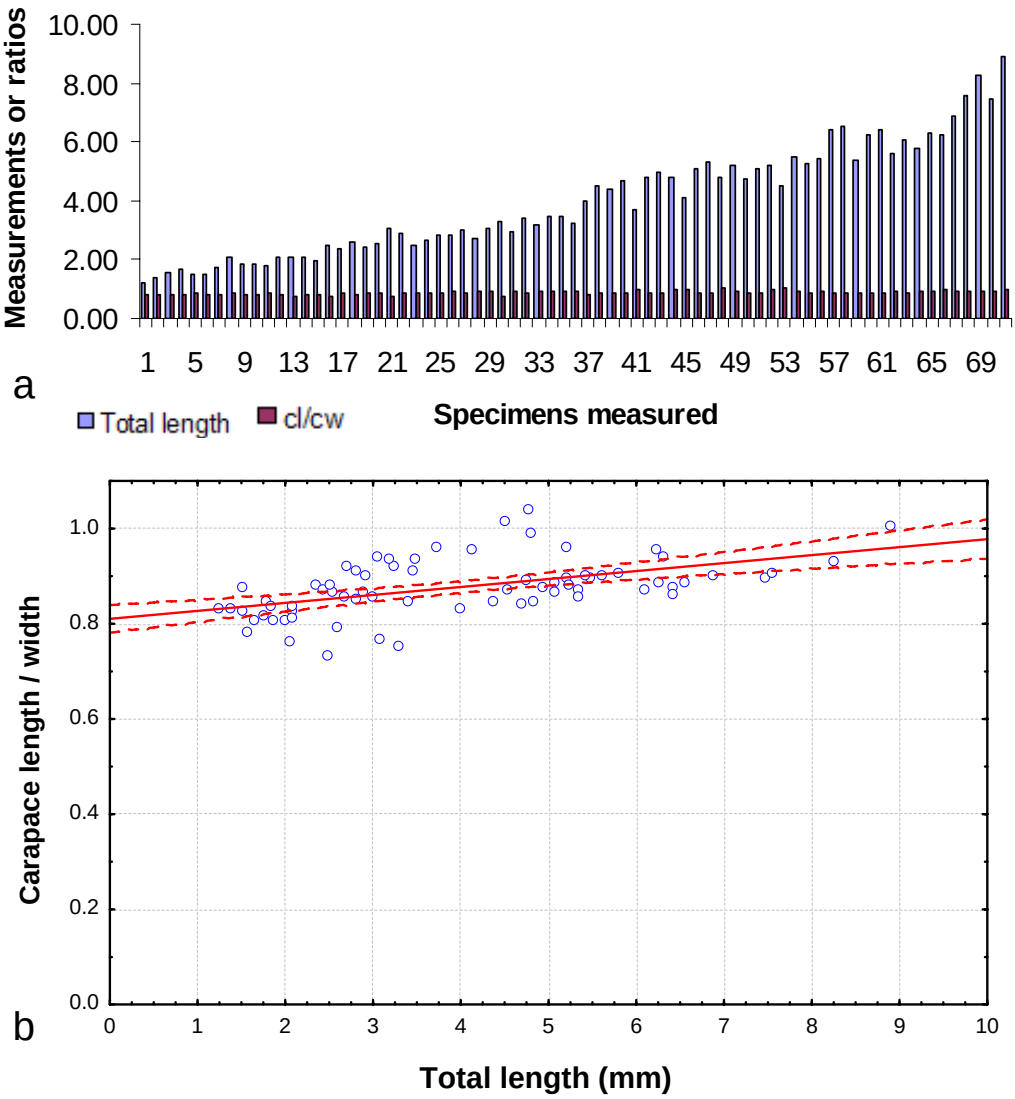


Figure 3.6. *P. variegatum* comparing ratio of carapace length to carapace width (cl/cw). (a) showing increasing total body length of nymph but relatively constant ratio (b) plot of total length against the carapace length to width ratio, $r^2 = 0.2701$, r = 0.5197,
$y = 0.8102 + 0.0168x$, n = 71; dotted red line indicates 95% confidence limits.

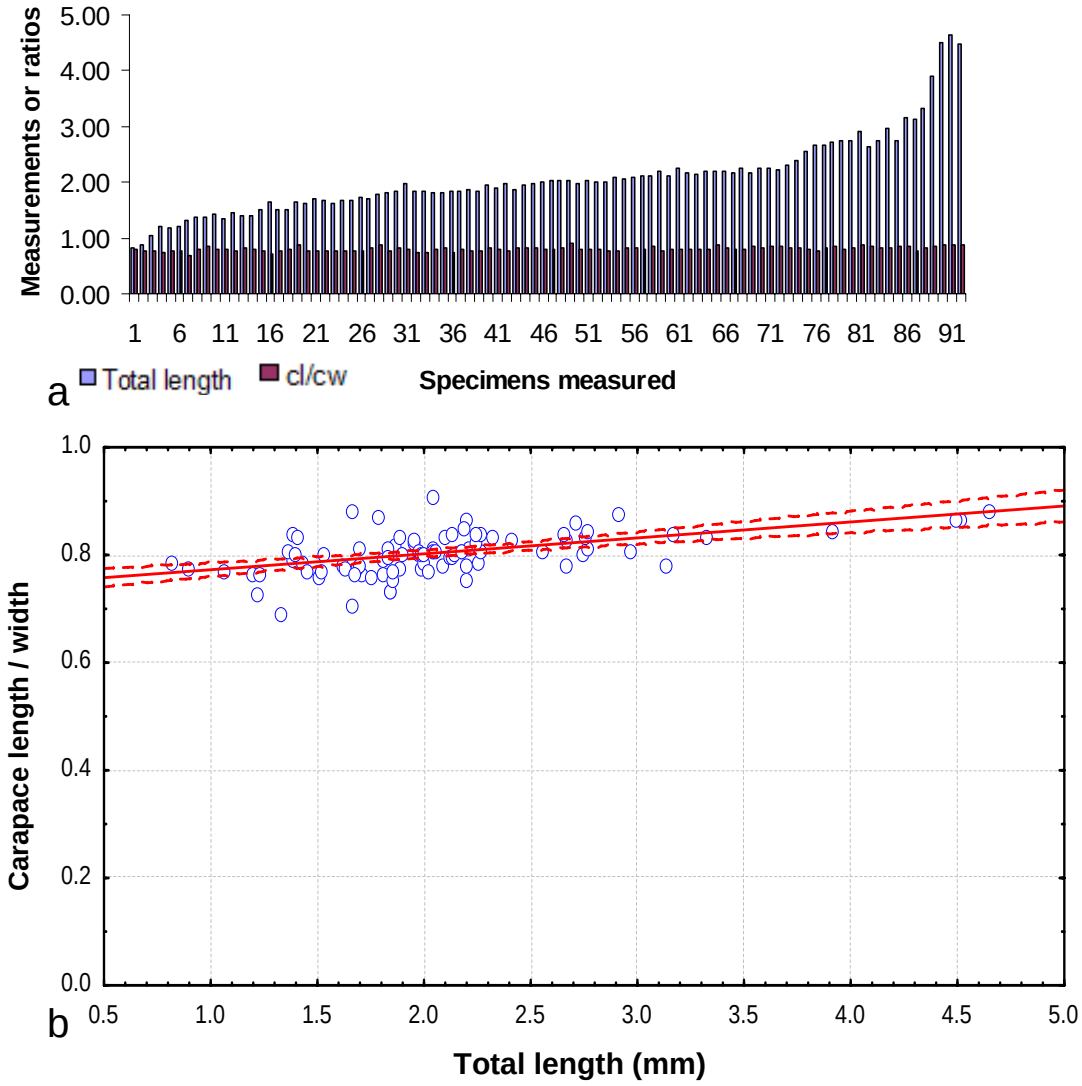


Figure 3.7. *P. crassi* comparing ratio of carapace length to carapace width (cl/cw) with (a) showing increasing total body length of nymph but relatively constant ratio (b) plot of total length against the carapace length to width ratio. Only specimens from Vaal River considered. $r^2 = 0.28$, r = 0.53, $y = 0.743+0.0296x$ n = 92, dotted red line indicates 95% confidence limits.

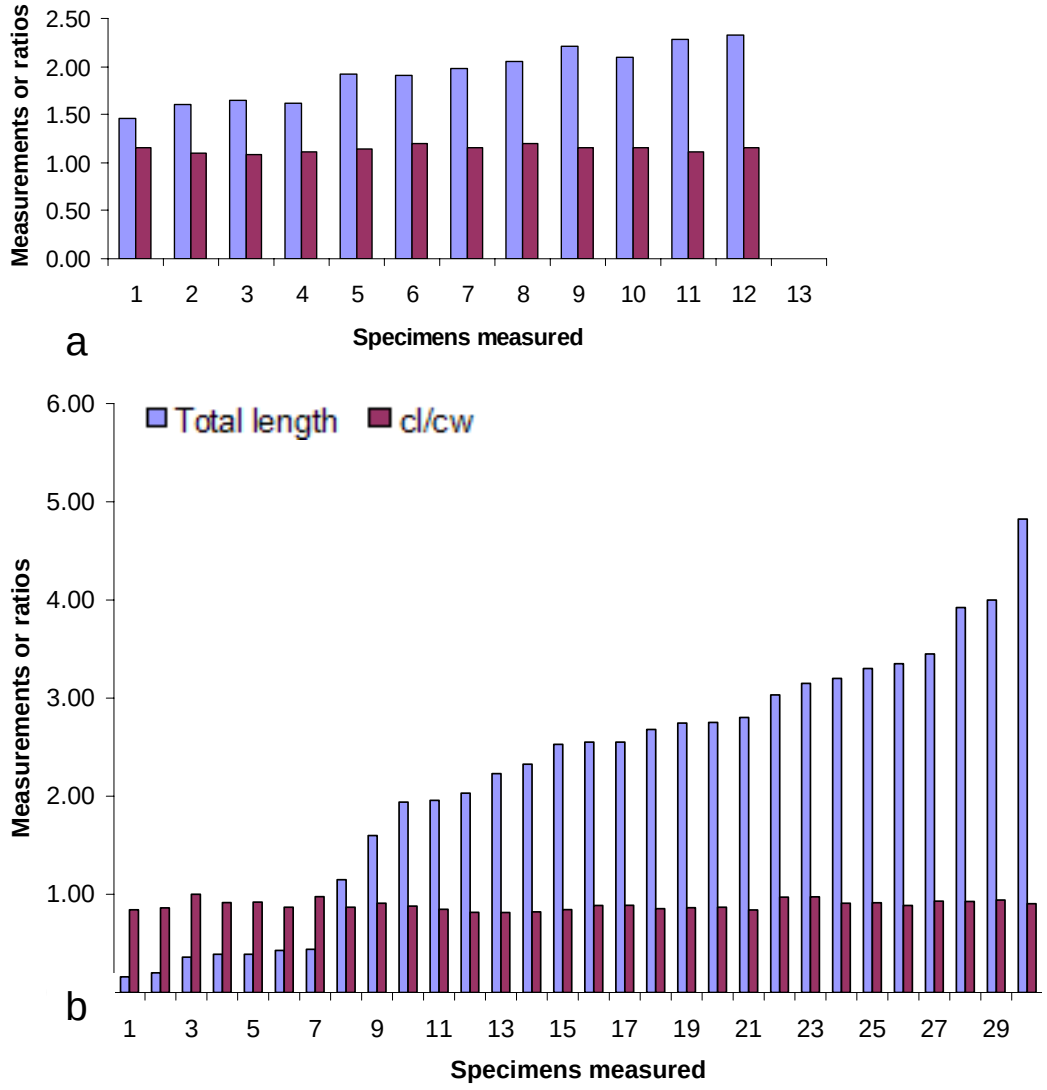


Figure 3.8. Two more examples showing comparison of ratio of carapace length to carapace width (cl/cw) with total body length of nymph.
(a) *P. mccaffertyi*, $y = 1.086 + 0.041x$; $r = 0.2955$, $p = 0.3269$; $r^2 = 0.0873$;
(b) Madagascan sp. 2, $y = 0.8255 + 0.0228x$; $r = 0.2950$, $p = 0.3786$; $r^2 = 0.0870$.

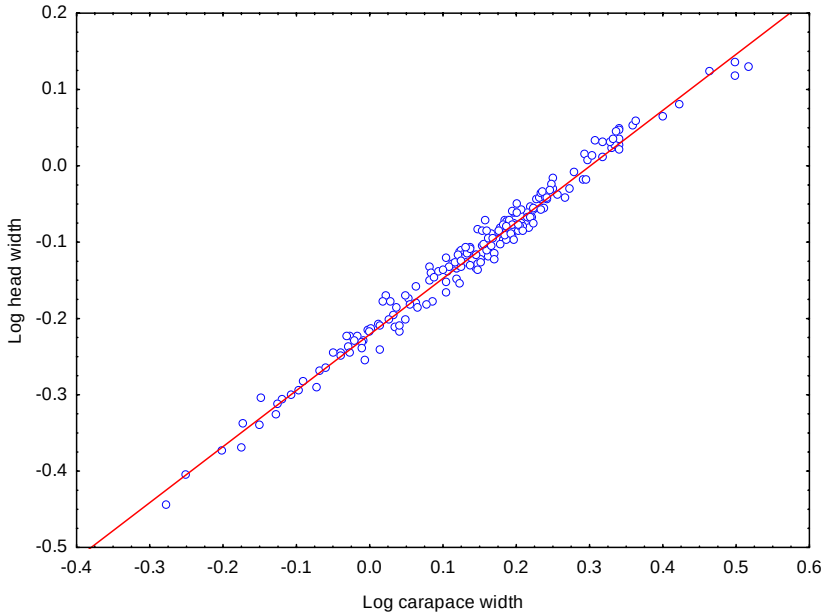


Figure 3.9. Plot of log transformed data of head width measurements against carapace width for *P. crassi*. r = 0.97; y = -0.1904+0.5938*x*, n = 164.

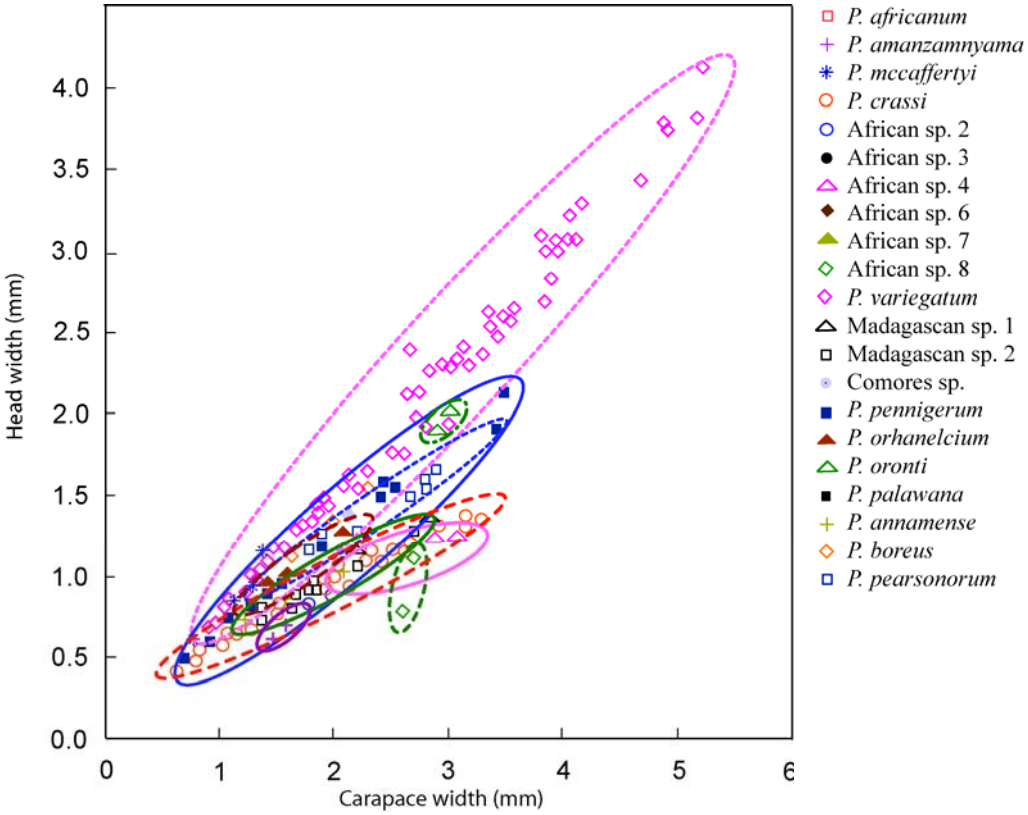


Figure 3.10. Relationships between nymphal head width and carapace width, showing trends between different species. Ellipses around species clusters have been visually estimated and drawn in. Statistics pertaining to the plot for each species are given in Table 3.1.

Table 3.1. Statistics pertaining to Figure 3.10.

Species	Equation	p	r	r^2	n
P. africanum	Too few samples for statistical analysis				1
P. amanzamnyama	y = 0.3255 + 0.3589*x	p = 0.0108	r = 0.6363	r^2 = 0.4049	15
P. mccaffertyi	y = 0.1806 + 0.4939*x	p = 0.0041	r = 0.7362	r^2 = 0.5421	12
P. crassi	y = 0.2582 + 0.3674*x	p < 0.0001	r = 0.9829	r^2 = 0.9660	92
African sp. 2	y = 0.5091 + 0.1774*x	p = 0.0157	r = 0.5598	r^2 = 0.3134	18
African sp. 3	y = 0.1797 + 0.4132*x	p = 0.0017	r = 1.0000	r^2 = 1.0000	4
African sp. 4	y = 0.2596 + 0.3449*x	p = 0.0003	r = 0.9680	r^2 = 0.2050	7
African sp. 6	Too few samples for statistical analysis				2
African sp. 7	Too few samples for statistical analysis				2
African sp. 8	y = 0.6358 + 0.1538*x	p = 0.3077	r = 0.4527	r^2 = 0.9371	7
P. variegatum	y = -0.0343 + 0.764*x	p = 0.0000	r = 0.9927	r^2 = 0.9855	71
Madagascan sp. 1	y = 0.3606 + 0.3531*x	p = 0.1464	r = 0.9737	r^2 = 0.9481	3
Madagascan sp. 2	y = 0.2783 + 0.3656*x	p = 0.00002	r = 0.9383	r^2 = 0.8804	11
Comores sp	Too few samples for statistical analysis				1
P. pennigerum	y = 0.1103 + 0.5613*x	p = < 0.0001	r = 0.9958	r^2 = 0.9917	14
P. oronti	Too few samples for statistical analysis				2
P. orhanelicum	y = 0.2537 + 0.4818*x	p = 0.0016	r = 0.9879	r^2 = 0.9759	5
P. lieftincki	Too few samples for statistical analysis				1
P. annamense	y = 0.2803 + 0.3534*x	p = 0.0195	r = 0.9995	r^2 = 0.9991	3
P. trispinum	Too few samples for statistical analysis				1
P. unicolor	Too few samples for statistical analysis				1
P. boreus	y = 0.1277 + 0.6126*x	p = 0.0212	r = 0.9994	r^2 = 0.9989	3
P. palawana	Too few samples for statistical analysis				2
P. sedlaceki	Too few samples for statistical analysis				1
P. pearsonorum	y = 0.3979 + 0.4196*x	p = < 0.0001	r = 0.9800	r^2 = 0.9603	13

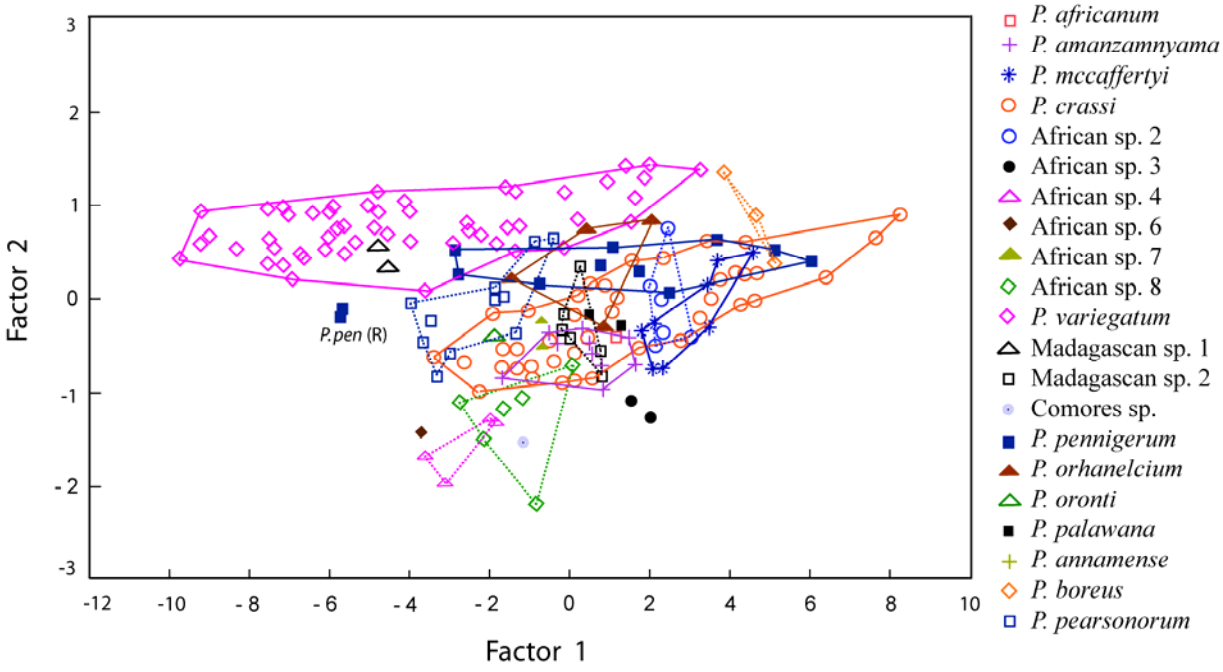


Figure 3.11. Scatterplot for first two components generated from the PCA analysis (Factor 1 and Factor 2) for all nymphal variables measured. Trends for species clusters estimated using convex hulls joining outer coordinates for each species. Eigenvalues of correlation matrix used for PCA indicate that Factor 1 accounts for 93.19 % of total variation and Factor 2 for 4.06 %. Specimens of *P. pennigerum* (*P.pen* R) from Russia were distinct from the specimens measured from France and Spain.

Table 3.2. Eigenvectors and Eigenvalues of PCA correlation matrix for logged data using all variables, showing first four factors, and cumulative variance (%).

Variable	Factors			
	1	2	3	4
Total length	-0.321	-0.078	0.037	0.677
Carapace l	-0.322	-0.213	0.087	-0.302
Carapace w	-0.322	-0.182	-0.280	0.099
Dist eyes	-0.310	0.317	0.692	-0.337
Head width	-0.314	0.350	0.295	0.438
Head length	-0.284	0.722	-0.564	-0.153
T1	-0.321	-0.243	-0.035	-0.040
T2	-0.323	-0.156	-0.093	-0.291
T3	-0.322	-0.170	-0.131	-0.157
T4	-0.321	-0.242	-0.043	0.0485
Eigenvalue	9.320	0.406	0.110	0.045
Cumulative variance (%)	93.19	97.25	98.35	98.80

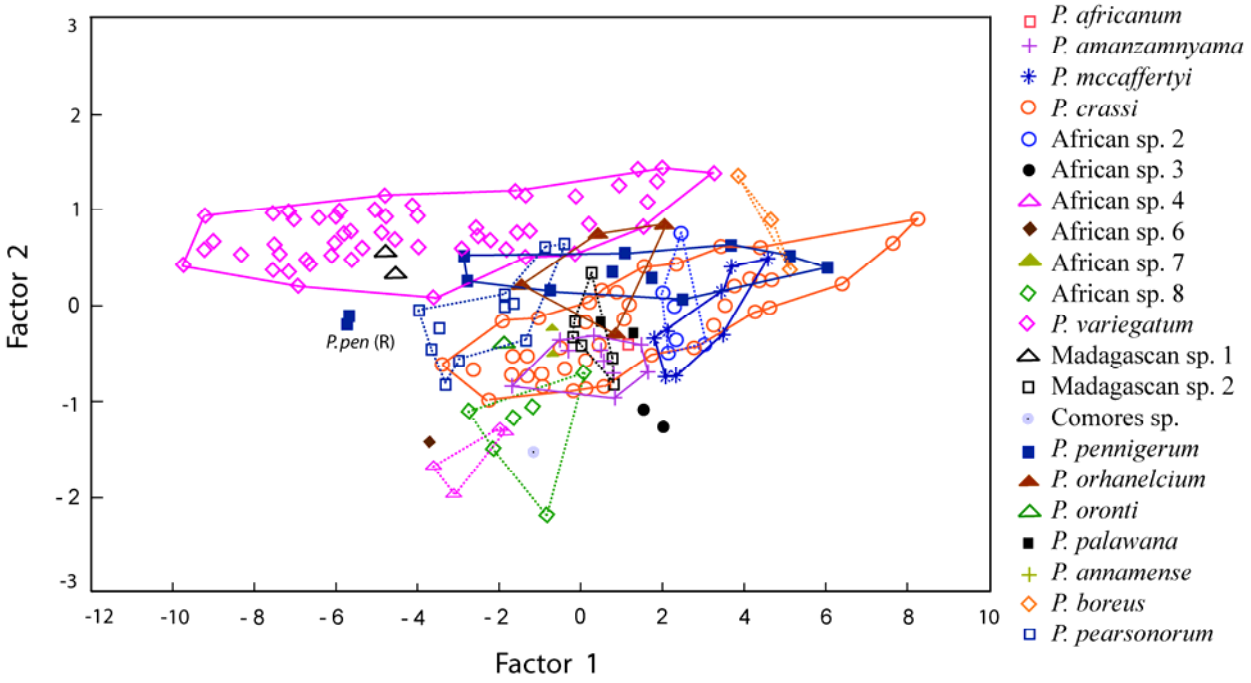


Figure 3.12. Scatterplot for first two components generated from the PCA analysis (Factor 1 and Factor 2) for carapace truss measurements only. Trends for species clusters estimated using convex hulls joining outer coordinates for each species. Eigenvalues of correlation matrix used for PCA indicate that Factor 1 accounts for 98.30 % of total variation and Factor 2 for 0.59 %.

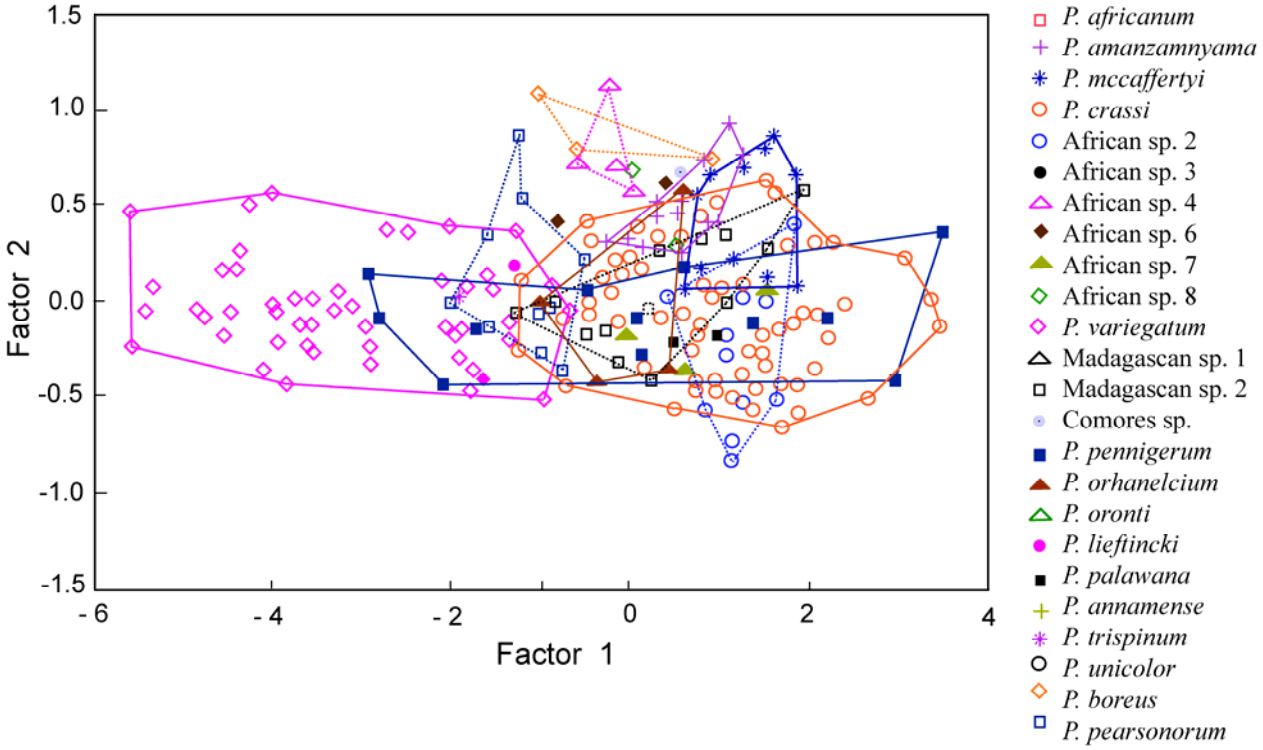


Figure 3.13. Scatterplot for first two components generated from the PCA analysis (Factor 1 and Factor 2) for nymphal head measurement variables only. Trends for species clusters estimated using convex hulls joining outer coordinates for each species. Eigenvalues of correlation matrix used for PCA indicate that Factor 1 accounts for 94.43 % of total variation and Factor 2 for 4.55 %.

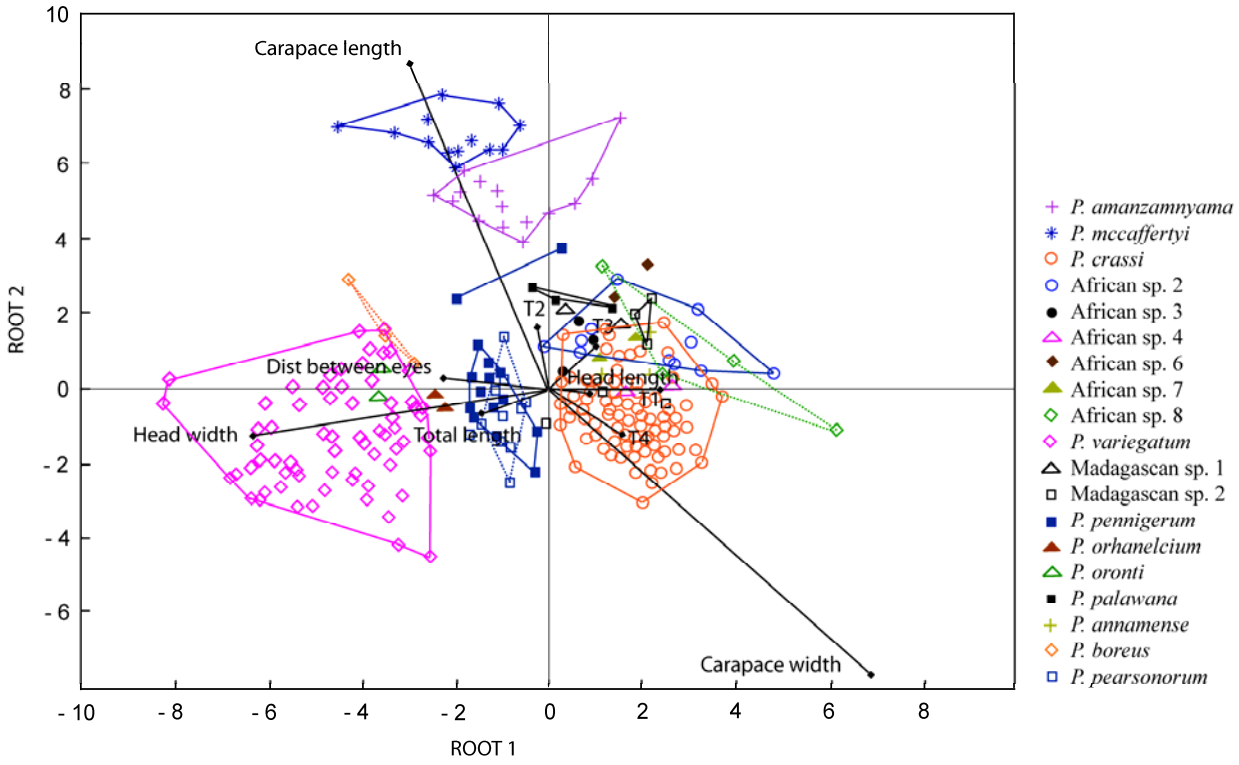


Figure 3.14. Scatterplot of DFA of all nymphal variables, showing root 1 against root 2; convex hulls used to demarcate boundaries of species as depicted by the morphometric measurements. Relative influence of each variable determining the spatial representation of each species is shown.

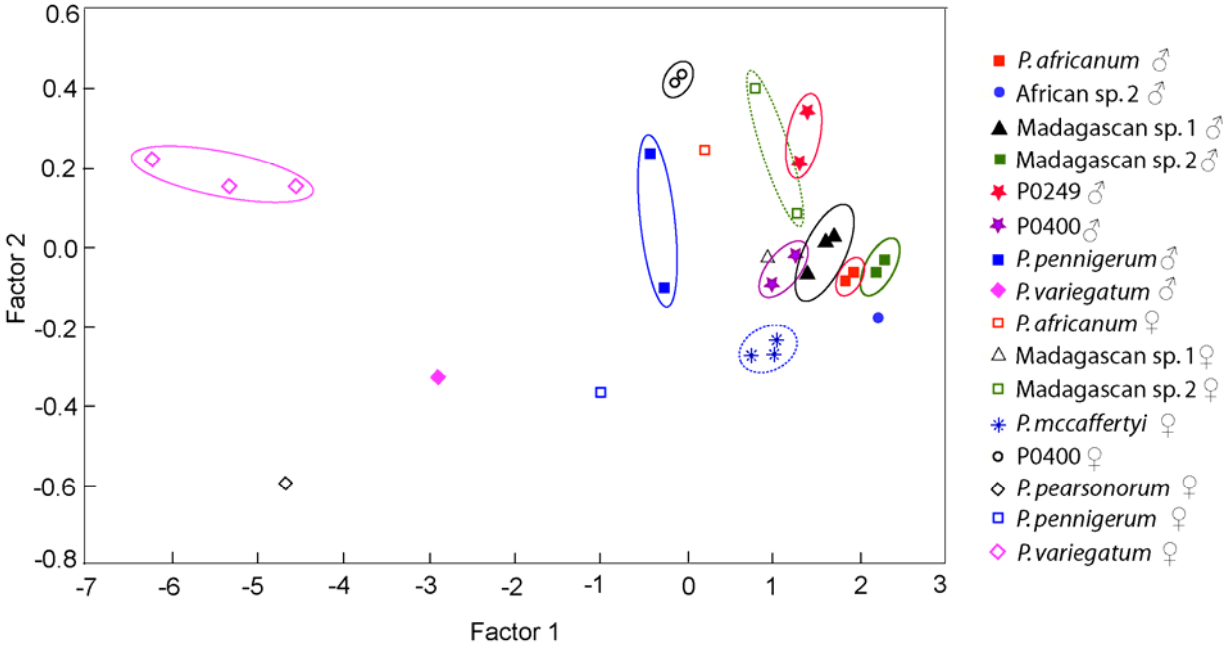


Figure 3.15. Scatterplot for first two components of the PCA analysis (Factor 1 and Factor 2) wing truss measurements. Trends for species clusters estimated using ellipses as too few species are represented to use convex hulls. Eigenvalues of correlation matrix used for PCA indicate that Factor 1 accounts for 98.26 % of total variation and Factor 2 for 1.07 %.

Table 3.3**.** Eigenvectors and Eigenvalues of PCA correlation matrix for adult wing truss variables showing first four factors, and cumulative variance (%).

Variable	Factors			
	1	2	3	4
T1	-0.410659	0.061725	-0.054011	0.694438
T2	-0.405669	0.625222	-0.377730	-0.086268
T3	-0.403804	-0.734470	-0.349477	-0.242235
T4	-0.408667	0.130671	0.696422	-0.462539
T5	-0.410080	-0.193619	0.388543	0.386680
T6	-0.410561	0.106195	-0.310320	-0.296937
Eigenvalue	5.90	0.06	0.02	0.009
Cumulative variance (%)	98.26	99.33	99.74	99.90

Chapter 4

Neural Network approach to species delimitation

"The concept of a species is a concession to our linguistic habits and neurological mechanisms" Haldane (1956)

"*The species problem is not primarily an empirical one, but it is rather fraught with philosophical questions that require but cannot be settled by empirical evidence"*
Pigliucci (2003)

Synopsis:

This chapter was carried out in collaboration with Professor Michael H. Burton (Mathematics Department, Rhodes University, Grahamstown), who wrote the scripts for the programming of MATLAB to produce these neural network analyses. He also provided instruction on how to do this, so that similar routines can be independently applied in future for other mayfly groups, or any other taxa. To make it directly useful to other taxonomists, a user interface will need to be developed with the help of an IT expert, to give the program a user-friendly front, but this is not tackled here. The principles are that the network can be used with 100 % accuracy to identify known species, and it can indicate whether a species is unknown or close to known species with a degree of certainty.

4.1 A Brief History of Neural Networks

The quest to get a computer to think intelligently as opposed to merely perform calculations is labelled the quest for "artificial intelligence" (AI). As will be shown in this section, "artificial neural networks" (ANNs) can be constructed to recognise patterns, classify objects, simulate processes and predict how the process will behave in the future. In a sense, a computer which runs an artificial neural network is exhibiting intelligence and we have taken a step towards realising the quest.

The first steps were taken by McCulloch and Pitts (1943) in a paper describing the properties of an artificial neuron. Hebb (1949) produced a paper which described a new learning process, which is known today as "Hebbian learning". These ideas were

implemented without the aid of a computer. The first computer simulations of artificial neural networks were made known at a conference in Dartmouth (Rochester *et al*., 1956). This is generally recognised as the start of AI. Shortly after this, the psychologist Rosenblatt published his work on perceptrons (Rosenblatt 1958, 1961). There was much enthusiasm about perceptrons in the 1960s until Minsky and Papert (1969) published a book which revealed that perceptrons were limited in their computational abilities and the AI quest entered the doldrums after this. Many research projects folded and mathematicians and computer scientists, fuelled by the "publish or perish" principle, turned their thoughts to other matters with short-term, achievable goals. However, during this time Widrow and Hoff (1960) developed some practical applications of a variation of the perceptron called ADALINE (ADAptive LInear NEuron). Layers of ADALINEs were called MADALINEs (Multiple ADALINEs). They developed a supervised learning procedure, known as "Widrow-Hoff learning", which was the forerunner of "backpropagation" (see below). Kohonen's work lead to the discovery of "self-organising maps"; these are networks which learn without targets and this process is called "unsupervised learning" (Kohonen, 1977, 1984a, 1984b, 1991). Hopfield (1982) discovered what is now known as "associative memory networks", and was awarded the Nobel Prize in physics, his work reviving interest in AI (Hopfield, 1982; Hopfield and Tank, 1985). A huge impetus to AI research was given by the invention of the backpropagation algorithm with initial results obtained by Werbos (1974, 1988). Further improvements were contributed by Parker (1985) and Rumelhart *et al*. (1986). More recently, the Fukushima developed networks called "cognitron" and "neocognitron" networks which are capable of recognizing handwriting (Fukushima and Wake, 1991).

Standard reference works on neural networks can be found in many books e.g. Churchland (1989), Beale and Jackson (1990), VerDuin (1994), Hagan *et al*. (1995), Skapura (1995), Patterson (1996), Haykin (1998), Jain and Vemuri (1999), Reed and Marks (1999), Zupan and Gasteiger (1999) and Wu and McLarty (2000). The book by Hagan *et al*. (1995) uses notation which is compatible with the Neural Network Toolbox for MATLAB, the program used in this chapter.

Hornik (1989, 1991) proved that an artificial neural network is capable of approximating a wide class of functions, including continuous functions, to an arbitrary degree of precision. If a tolerance is given (any tolerance, no matter how small –

arbitrarily small, so to speak), then a neural network will exist which is able to approximate the function to within the tolerance.

4.2 Artificial Neural Network (ANN) and species classification

The statistical approaches used to detect species from morphometrics (Chapter 3), and the phylogenetic approaches to relationship (Chapters 5 and 6) have applied known techniques to new data, but have not attempted to derive new methods. In this section, a new approach to the problem of identifying mayfly species, represented by the nymphs of the Prosopistomatidae, is undertaken, using the mathematical supervised artificial neural network (ANN) with backpropagation (Figure 4.1).

The product is a relatively simple tool which can aid in the identification of species, reducing the need for high-level taxonomic expertise, allowing accurate species identification amongst less well-trained para-taxonomists. Taxonomic expertise will always, however, be needed for any particular group of taxa to select characters to allow the creation of an effective ANN system such as this. Although this application produces a semi-automated identification system, it initially requires considerable human input to create, both in selection of characters and matrix construction, and in the later mathematics and programming. A limited amount of training would be necessary to familiarise the end user with both the organism to be identified and use of the program. It can therefore be seen as an expert system to help non-experts, who nevertheless need some training in the particular field, be it entomology, botany or another discipline (so that they can recognise the characters required to feed into the system to achieve identification of the species they have before them). However, they would not need the high level of specialised terminology and diagnostic skills needed by an expert. The identification is carried out using a limited but sufficient selection of characters to identify the organism and place it in relation to others to which it is related. If a new character was identified as being diagnostic for a group of taxa, the state of this character would have to be determined for each member of the group, and the neural network would have to be retrained to incorporate that character.

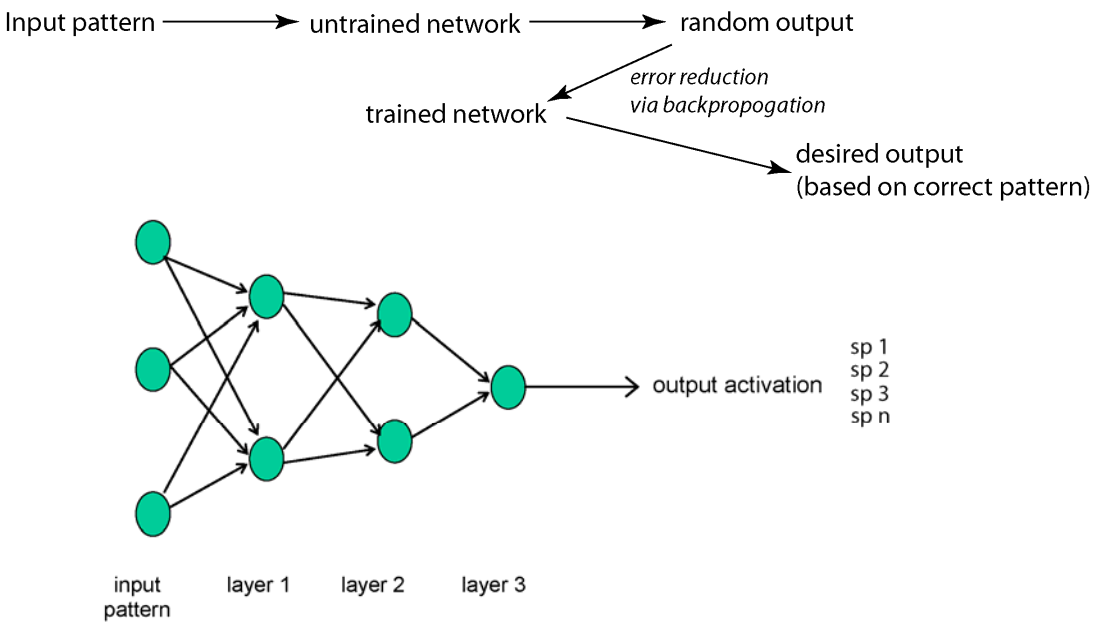


Figure 4.1. Schematic diagram of the principles behind supervised learning via a backpropagation three-layer artificial neural network, resulting in accurate species identification.

This exercise has been carried out using only morphological characters of nymphs of *Prosopistoma*, but could be done with other life cycle stages or even molecular data; for the latter, aligned sequences could be compared and classified in a similar way. Biological identifications are often clouded by the natural variations that occur within a species, and neural networks are a good way to model such multidimensional, non-linear variables. Some of the earlier numerical methods of species detection using computers were developed as far back as the 1970s, e.g. Doyen and Slobodchikoff (1974) produced a classification using phenetic, ecological and reproductive information to produce computer-derived "operational taxonomic units" (OTU's *sensu* Sokal and Sneath, 1963) which were seen as equivalent to species. The ideas leading to the program DELTA, used for generating automated species descriptions and identification keys, were first formulated by Dallwitz (1974, 1980), and lead to the first interactive computer-based identification keys: the software for this is being kept up to date with modern technology (e.g. Dallwitz *et al*., 1997, 2002 and at the DELTA web site - http://delta-intkey.com/). More recently, an interactive identification program, LUCID, has been developed by the University of Queensland, Australia. These are different to an ANN, and are more like keys or identification guides based on character recognition and set theory.

Although the application of the ANN approach to mayfly species delimitation in this chapter is novel, a literature search has shown that a number of other biologists in other disciplines have applied similar techniques. Some methods are character-based, using morphology or chemistry, while others use imaging or sound. Character-based models were used by Clark and Warwick (1998), who used a multilayer perceptron neural network to identify three species of iris, then 34 *Lithops* species, with 90 % confidence and only 52.9 % confidence, respectively. Clark (2003) developed this further with *Lithops*, finding that some species were always identified accurately and others never were; he deduced that this was pointing out errors in the taxonomy of the group, implying that the initial perception of the species was flawed. This may be another application of the ANN technique (i.e. is the "species" under investigation really a distinct species?). This begs the question of just how closely neural networks can reflect species characters. If two species are closely related and the feature list is inaccurate, then it may not be able to classify correctly, but this is an error at the data entry level, not of the network. The neural network will be able to associate a list of features obtained from a specimen chosen from the species that it has been trained to know. However if the feature list is incomplete or the measurements are faulty it will output a list of possible species, indicating that there is insufficient or inaccurate data to discriminate. If the feature list is indeed from a new species, unknown to the network, it will not be able to classify it and will indicate that is belongs to none of the characterised species.

Giacomini *et al.* (2000) used biochemical and protein extracts from bacteria as characters for species identification, using both a supervised backpropagation ANN and an unsupervised competitive Kohonen ANN, using MATLAB. They found that both methods gave a successful (though with less than 100 % accurate) identification of the bacterial strains investigated. Further work on bacterial identifications, which were characterized using infrared spectroscopy, resulted in 99.16 % successful classification using a backpropagation ANN (Mouwen *et al.*, 2006). Hernández-Borges *et al.* (2004), using aliphatic hydrocarbons (pollutants) extracted from intertidal limpets, classified the limpet species using a backpropagation supervised ANN, showing that different species metabolise pollutants in different ways, and that these differences can be used to categorize the species. A possible flaw in this approach is that species are adaptable from within their gene pool and isolated populations of a species can then appear to be different species.

Do *et al*. (1999) classified wolf spiders using imaging of one particular selected feature from several different species. The characters were based on greyscale values of pixels in the images, and, using an ANN, they successfully classified the spiders based on this one feature. Chesmore and Ohya (2004) used a backpropagation ANN technique for discriminating between the calls of grasshoppers and hence distinguish species according to their sounds. Du *et al*. (2007) developed a new approach to evaluate and analyze leaf shape using a generalized differential evolution training algorithm (one of the many kinds of algorithms developed to reduce errors by producing weights and biases in the network which result in zero error) to train multilayer perceptron neural networks and a multiscale Fourier descriptor method to recognize shape. Messina *et al*. (2009) have applied backpropagation ANNs to classify leaves of *Banksia* species using phyllometric parameters (a standard technique for measuring leaf morphology).

These few examples serve to illustrate the wide variety of approaches to delimiting species using ANN. The use of this approach for distinguishing species of prosopistomatid nymphs could not have been done without the help of a mathematician to write a program to train a network using the mathematical software (MATLAB).

4.3 Methods

A matrix of nymphal characters (character, or pattern matrix) was created and coded (Appendix Table A4.1). These are all morphology-based, and further details of the characters can be seen in Chapters 2 and 5. The coded characters are then fed through the neural network (designed in this instance using the program MATLAB). This produces a mathematical classification function for each known species. It produces a single function (the ANN) that embodies all species simultaneously (i.e. which acts on each feature in the list.

One consideration is that the characters states (or “features” to use neural network terminology) do not have to be synapomorphies. This exercise is not looking for relationships, but simply aims to distinguish species, and as identification relies on unique combinations of character states, these need not be autapomorphies (or even necessarily derived states). Hence the character matrix for this exercise need not be the same as a matrix used for phylogenetic analysis. There, approaches require categorical variables, while phenetics also draws on continuous variables. Four different approaches were used

to train the neural network, which are described in Appendix 4. There were three layers (two hidden and one output layer) with 66, 33, 33 neurons respectively. It was trained by the scaled conjugate gradient backpropagation method.

4.4. Results

The mathematics behind this Artificial Neural Network is given in Appendix 4. Details can be seen in the script "mayflyutrain". In order to avoid overfitting, the script was initially run over many epochs and the performance function was observed. When it finally settled to a nearly flat line (very small gradient) the number of epochs could be estimated. The ANN was then trained over 3000 epochs, estimate by the experience of the programmer. The architecture (number of neurons and the transfer functions in each layer) can also cause overfitting. A supervising script which varies the number of neurons in the layers, and records the sum of the squares of the errors, could be used but instead, simple experimentation was used, again based on experience. The structure is not critical and ANNs with different architectures can do just as well. This is standard for constructing ANNs. Finally, a validation set was defined. While training, the performance (SSE or MSE) on this set is measured and, if it increases for more than a specified number of epochs (300 in this case), training is terminated. This also helps to avoid overfitting and improves generalisation.

The result of this exercise is a function which can be run in MATLAB, which can accurately identify any of the species it has been trained to recognise. When the trained neural network was presented with characters of any of the species, it could correctly identify each species with 100% success (Figure 4.2). When presented with characters which had been slightly perturbed, representing an unknown species, it could give a likelihood of this being close to a known species, or alternatively indicate that the species could not be identified (see Appendix 4 "The Test Script" for a worked example of this). A measure of the importance of each character in defining a species was obtained by removing each of the characters in turn and seeing which caused the biggest deviation from the correct identification when all characters were used. The deviation from the correct classification was used as a measure of the significance of the feature.

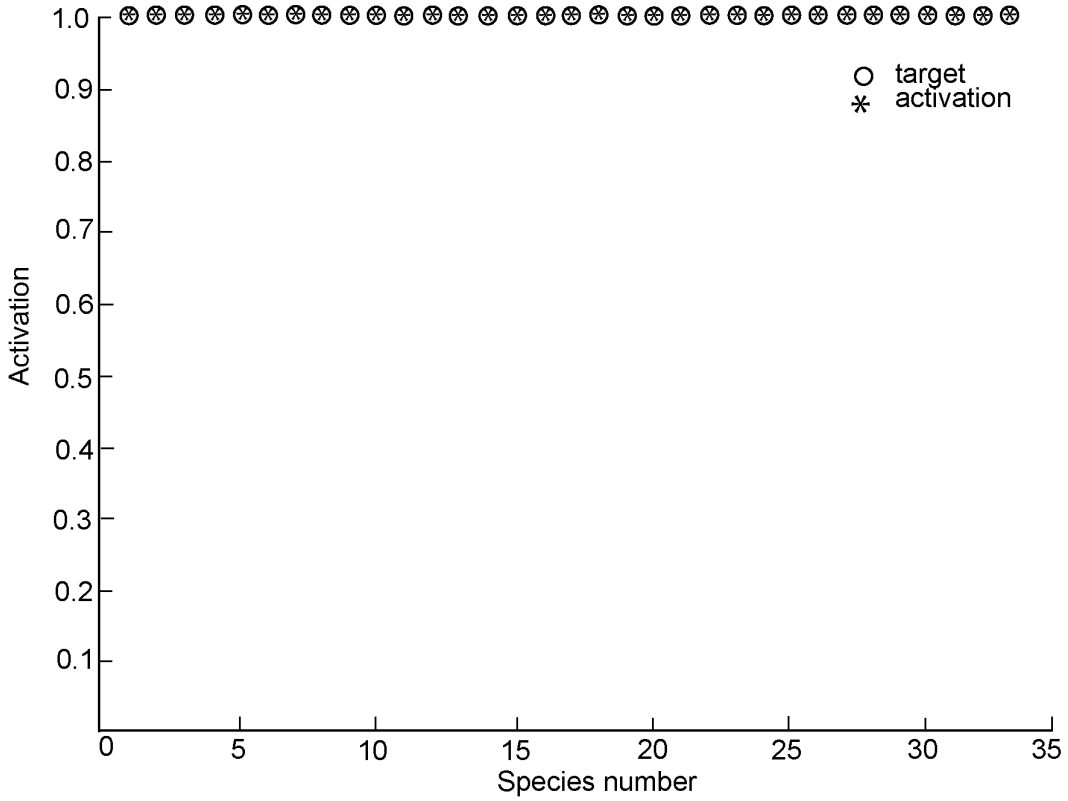


Figure 4.2. Species (given as a number from the species list) showing that the activation for each species, produced from the input patterns, match the target. O indicates the targets and * the activation of the neural network. If they coincide, the neural network has correctly matched the input pattern and target. This is the case for all 33 species, as shown here.

4.5 Dicussion and Conclusions

Once the neural network is classifying (i.e. recognising and identifying) known species accurately, characters from unknown specimens of that taxon can be fed in, and will be placed either as a known species, close to known (with probability of accuracy), or unknown species. The questionable species can then be sent to an expert for confirmation or description as a new species, useful when there is a shortage of trained taxonomists. It is possible to produce a java script website, where the animal can be identified using this program embedded in a user-friendly front. A stand-alone executable file can be produced, so that the user can take a disc and use the program to identify species in the field (provided they have a microscope, as this does not give them the excuse not to examine the animal in detail). A neural network can be produced for any well-defined group of organisms. An expert on that group would have to compile the characters, and design the neural network in a program like MATLAB, STATISTICA, or other such computational

environments. The steps used for this analysis can be repeated for any taxa, just replacing the characters of the required taxa and the number of entities being classified. Once this is done, it can be used by non-experts, whose only training will to identify the characters and run the program. Thus the system could be used by a para-taxonomist who does not necessarily have to know the group.

Gaston and O'Neill (2004) discuss the use of automated species identification, questioning why it has not become more widely used. They identify the accuracy of identification of morphologically similar species as being one of the stumbling blocks, as the networks in many cases have not produced reliable results, not being able to account for natural variation, which a well-trained human eye can do. The ANN developed with the *Prosopistoma* nymphs allows for some flexibility, giving a probability of a particular identification, thus allowing for some of the natural variation seen within and between populations of a species. While imaging approaches may still be limiting, character-based approaches such as the one demonstrated here can be implemented relatively easily. It can also classify with relative accuracy when features are missing, though this depends on the number of features and their importance. Thus this is a useful and reliable tool which has great potential for further development in artificial species recognition.

The use of such systems is necessary in an era when specialist taxonomists are too few to cope with the needs of biodiversity studies. Before biodiversity assessments can be carried out, it is necessary to know the fauna and flora. This is needed to implement management plans to reduce the rate at which species are going extinct due to anthropogenic activities. Even if a system can be introduced where species do not immediately get a formal name, it is important that they can be recognized as distinct entities, both ecologically and from an evolutionary perspective. Thus this can provide a tool for assessing biodiversity and conservation thereof, in the light of the sadly declining number of specialist taxonomists globally. The knowledge provided by one expert taxonomist can facilitate the identification of the required organisms by many less-skilled people.

Chapter 5

Phylogeny of Prosopistomatidae based on nymphal and adult morphology

"Life is a copiously branching bush, continually pruned by the grim reaper of extinction, not a predictable ladder of progress" Stephen J. Gould, 1991 (p. 35)

"If you don't know the names, the knowledge of things is wasted"

Isodorus (Greek botanist)

Synopsis

The previous two chapters looked at ways to empirically categorise species, using measured differences to produced clearly delimited species units. This chapter examines relationships between these species using shared, derived morphological characters to reflect phylogeny. Having identified and described characters in Chapter 2, this chapter deals with choosing characters which are useful for determining phylogeny, recognising homology and different character states. Relationships are investigated using parsimony analyses to assess matrices of shared derived nymphal and adult characters and character states. Although adult material is not available for many of the species, comparison of representatives of each clade defined by nymphal morphology was possible using available species. Maximum parsimony is used to produce phylogenies. A "supertree" is constructed using the program "Clann" to compensate for having many taxa missing as adults. The results indicate the possibility of two genera.

5.1 Introduction

With attempting to understand relationships between species comes the need to have a clear definition of what constitutes a species. Clearly a species in the sense of phylogeny must be seen in the light of the evolutionary processes that have shaped it, rather than as a product of an arbitrary assignation of phenetic characters which may have no bearing on its relationships with other species. Wilkins (2002, 2003) and Mallet (2004, 2007, 2008a) provide comprehensive reviews on species-concepts. de Queiroz (2007) also provides a

useful summary of the types and applicability of the different concepts, suggesting a unified approach where species only need to be seen as evolving separately from other lineages. His unified species concept continues to accept the properties that have been considered important in defining species by previous authors. However, it avoids treating any of those properties as necessary to a species. In this way, de Queiroz provides a way of separating theoretical species concepts from practical concepts required for species delimitation. This is similar to the approach taken in this thesis, with species delimitation (Chapter 3 and 4) being distinguished from the determination of species relationships in this and the following chapter.

In this chapter, species are considered from an evolutionary perspective, using phylogeny to reflect relationships. The first step in a morphology-based phylogenetic analysis is to assess whether characters and the states within a selected character are homologous. This is done by seeking categorical variation between taxa that experience has suggested will be homologous and informative, and these choices are tested by the analysis by seeing how the character states group together within the different taxa or how they influence the branching on the trees derived from these characters. The second hurdle is whether suites of characters are correlated, and therefore lack independence because they are all expressions of one unifying adaptive theme (for example, when considering structures on mandibles, whether different mandibular characters are independent, or linked together to perform one function). This question encompasses the cross-validation test of using independent data sets, e.g. by adding more morphological characters, which are parsimony-informative, from the same life history stage; by adding characters from a different life history stage (such as the adult) which is under different evolutionary pressures and therefore may give an independent picture; and by using evidence from molecular analysis, which is independent of the first two data sets (although the genes determine the morphology to some extent). Unfortunately the comparisons here are data-deficient due to lack of comparable material for all life history stages for each species. For example, no male adult material is known for any of the Oriental or Australasian species, so the focus is on only eight males (Afrotropical and the European species, from France) and eight female subimagos (Afrotropical, European and Australian, not all the same eight species), giving a total of ten species which have at least one sex of the winged stage known.

Once clades which are supported by a number of different datasets are identified, the next set of questions arises. Are the terminal points shown on the trees really representative of species? What do the clades show? Are the clades sufficiently different from each other to support the creation of a separate genus? These questions are addressed as the results of this research unfold below.

A further approach to test relationships between species is to relate them to habitat requirements. What ecological conditions support each species now defined? Factors considered in this study include size of river, flow conditions, water quality (e.g. pH, electrical conductivity, total dissolved solids), and type of biome through which they flow (e.g. forest or grassland). This is dealt with in Chapter 8, where these parameters are investigated in terms of how they may affect biogeographic relationships between the species. Seasonality of emergence of adults can also be taken into account; this can be of particular importance when two species occur sympatrically, as different emergence times could keep them from interbreeding. As life history is not a focus of this thesis, field investigations looking at such behaviour was not carried out.

5.2 Methods

Material examined was obtained from a number of sources, listed in Appendix Table A2.1. Figures 5.1-5.3 give a visual representation of the dorsal aspect of some of the species as nymphs; visual assessment provides the first step in recognizing different species based on patterns, shape and size. It is not reliable beyond being a starting point for species recognition, as some of the species are very variable in appearance from different localities, e.g. *P. variegatum* (Barber-James, 2010b), and others that are different species may look superficially similar (e.g. African sp. 2 and sp. 7). The choice of characters is important for phylogenetic studies, as they have to be deemed homologous in order to reflect relationships. For an identification system such as the artificial neural network approach (Chapter 4) or for identification keys, this is not important, and there the characters can be phenetic, so long as the character matrix can provide the species with a unique identity. Illustrations of the major nymphal characters and characters states used in determining phylogeny are given in Figures 5.4 - 5.7.

Adult characters, observed as described in Chapter 2, and character states are recorded in Appendix Tables 5.2 and 5.3. Figure 5.8 gives an example of two character

states for the male forewing, where the leading edge is either gradually tapered (Figure 5.8a) or the wing is abruptly tapered to give a more pointed distal wing margin (Figure 5.8b); in the material examined there was not an intermediate state. Unfortunately, no adult male material is available for any of the Oriental species (except for an undescribed male from Malaysia, which may be *Prosopistoma wouterae* (Peters, 1967), which was not examined for this thesis), or Australasian species, where only females have been collected. Therefore only five Afrotropical species and the only known European species, and the female of the only known Australian species have been studied. Only ten species are known in the adult stage (Tables 1.1, 2.1), eight male and eight female, with some known as either male or female, few being known in both sexes. While it would be preferable to have more species represented, this is enough to test whether the phylogeny obtained from nymphal morphology alone is a true reflection of the relationships within the family based on morphology alone.

The initial thrust of the work reported here focussed on nymphal material of Afrotropical *Prosopistoma* species only. Nymphs of fifteen previously undescribed species from a number of localities in the Afrotropical region (Figures 5.1, 5.2; Appendix Table A2.1) have been recognised (Barber-James, 2010a and unpublished data), and a further two species from Madagascar are known as adults only. Although the defining characters of most of the undescribed species are tabularised (unpublished), the prospective names of these taxa cannot be given until these are formally published elsewhere. For this reason, numbers have been allocated to each species. Thirty three species of the 37 currently recognised (but not all formally described) species were used in the phylogenetic analysis based on nymphal characters (Table 1.1), excluding *P. deguernei* because not enough is known about this species, described from an immature nymph, and material is not available for closer examination.

A number of morphological and morphometric characters were found to be useful for deriving a phylogeny (Appendix Table A5.1). Some of these, such as number of antennal segments (Figure 5.4e,), antennal length relative to head (Figure 5.4d), ratio of antennal segment three to rest of antenna (Figure 5.4f), notching of carapace (Figure 5.4b), mandibular features such as number of setae beneath the canines (Figure 5.6a-c), length of maxillary palp (Figure 5.6d,e), shape of postmentum (Figure 5.6f,g), or gill features (e,g. Figure 5.7), are more phylogenetically informative than others. Other structures which provide good characters for identification purposes and show differences between species,

are adaptive and therefore do not reflect relationship. For example, the number of serrations on a mandibular incisor could be useful in a species description, but produces "noise" in a phylogeny.

Baetisca rogersi Berner was chosen as outgroup in this analysis, as Baetiscidae and Prosopistomatidae are currently widely believed to be sister taxa (e.g. Wang *et al*., 1997; Kluge, 1998; Ogden and Whiting, 2005; Sun *et al*., 2006). Analysis of coded characters was done using the program "Tree analysis using New Technology" (TNT) (Goloboff *et al*. 2008) which was run using the tree bisection reconnection (TBR) algorithm to search for the most parsimonious trees. Majority Rule consensus was chosen, as the strict Nelsen consensus method was found to collapse the nodes giving too little visual resolution.

The emphasis of recent research was on the nymphs of the Afrotropical species, and over 60 characters were quantified for these species. Of these, only 17 characters were selected for the nymphal phylogeny (Appendix Table A5.1). Some characters which were initially considered were not phylogenetically informative, and therefore not used, e.g. whether the integument of the carapace had scales; whether strange slits seen in the carapace margin were present or absent (found to be an autapomorphy for African sp. 6); the relative size and number of serrations on the outer canine of a mandible; the number of small subapical serrations along the inner margin of the inner canine of the mandible. Additionally, several characters seen in Afrotropical species have not been determined for all of the remaining world species. For the non-Afrotropical species, some of the character states were extracted from the literature, as material was not at hand for examination for each species. Thus some potentially useful characters, such as sternal plate ratios and convexity of carapace (length divided by height), were omitted in this analysis due to having too few instances of this character recorded for phylogenetic analysis.

Parsimony analysis was carried out using a number of different combinations of data. The nymphal characters were assessed on their own, and characters for male imagos and female subimagos were also analysed separately, producing a separate phylogeny for each. The data were then all combined into one supermatrix and analysed together, and the synapomorphies responsible for defining different clades were listed at the relevant nodes of the combined-data tree. The problem with this analysis is that there is a lot of missing data because of the few species known in the winged stages. Nixon and Wheeler (1992) have shown that missing characters result in nearly random placements of the affected taxa in phylogeny reconstruction using maximum parsimony, resulting in reduced resolution of

consensus trees (Huelsenbeck, 1991). Supertree construction has been developed as a way to minimize this. A phylogenetic supertree allows a source tree to be combined with other sets of trees which have no taxa in common, so long as there are additional trees that overlap both (Creevey and McInerney, 2005). Bininda-Emonds (2004) offers a useful review of the development of supertrees as an approach to resolve difficulties in combining data for phylogenies when there are large sets of missing data. He distinguishes between the supermatix approach (used above) and the supertree approach in the following definitions:

Supermatrix approach: a phylogenetic approach in which separate character data sets are concatenated and analyzed simultaneously to yield a phylogenetic tree.

Supertree approach: a phylogenetic approach in which phylogenetic trees are combined to yield another phylogenetic tree. Distinguished from classic consensus techniques in that the source trees need only have overlapping rather than identical taxon sets.

The matrix representation with parsimony (MRP) supertree consensus method (Baum, 1992; Ragan, 1992), implemented in the program Clann (Creevey and McInerney, 2005) was used to combine the most parsimonious trees (MPTs) obtained for each of the three life history stages (i.e. nymphs, males and females). Nodes of the input trees were weighted according to the proportion of the total input trees that were incorporated.

5.3 Results

The characters used for estimating the phylogenies are listed in Appendix Tables 5.1-5.3. The Majority Rule consensus tree from nymphal characters only, produced using TNT, indicates two distinct clades (Figure 5.9). One clade consists of some of the Madagascan and Oriental species, and all of the Palaearctic and Australasian species, which share many synapomorphies with *P. variegatum*; the other clade links all of the sub-Saharan African species, the Comores species, some Madagascan species and some of the Oriental species. For the purpose of the biogeographical discussions based on this phylogeny (Chapter 8), the species sharing synapomorphies with *P. variegatum* are referred to as the "*P. variegatum*" clade, while the clade dominated by all of the sub-Saharan African species, and including a few species from Madagascar and Asia, is referred to as the "*African*" clade.

Phylogenies based on adult characters are shown in Figures 5.10. The combined matrix of all adult characters (Figure 5.10a) shows two clades, one closely approximating the "*P. variegatum*" clade seen in the nymphs, and the other with species which fall into the nymphal "*African*" clade. The only species which differs in placement from the nymphal phylogeny is *P. pearsonorum*, the Australian species. This is included in the "*African*" clade in the adult cladogram, but in the "*P. variegatum*" clade in the nymphal cladogram. Due to the problem of missing data possibly obscuring relationships, *P. mccaffertyi* and *P. pearsonorum*, known only as females, were omitted from a second analysis (Figure 5.10b). This again gave a cladogram showing the *P. variegatum*" clade, represented by three species, as distinct from the "*African*" clade. Relationships were then tested using characters for each sex separately. Figure 5.11a shows the Majority Rule consensus cladogram from three trees based on female characters only. Here, the "*African*" clade is well recovered, but *P. variegatum* is isolated from the other two members of its clade. Using male characters only (Figure 5.11b), a single tree was produced. In this cladogram, the traditionally "*P. variegatum*" clade groups together, but two members of the "*African*" clade group more closely with this than the rest of the "*African*" clade.

The Majority Rule consensus tree of all the characters combined into one supermatrix, based on 20 trees produced by maximum parsimony analysis in TNT, is not well resolved (Figure 5.12). It, however, fully recovered the two clades derived from the nymphal morphological phylogeny analysis. Due to many missing data entries for this tree because of lack of adult representatives in many species, the quality of the analysis is reduced. It is better to view the nymphal-derived and adult male and female trees independently as they give a truer reflection of relationships. Synapomorphies responsible for defining the major clades are listed at nodes in Figure 5.12.

The Supertree produced in Clann used a combination of 20 nymphal trees (given weight 0.03), 3 female trees (given weight 0.33) and 1 male tree (weight 1). Nine supertrees were produced to give the consensus Majority Rule supertree (Figure 5.13), synthesized using maximum parsimony analysis in TNT. While the "*P. variegatum*" clade could still be distinguished as an entity (indicated by parentheses in Figure 5.13), the relationships between the species from the "*African*" clade were fragmented into a number of smaller clades. This construction is generally weakly supported except for the "*P. variegatum*" clade.

5.4 Discussion

The cladograms produced here show strong support for the division of the Prosopistomatidae species into two clades. Synapomorphies causing this division are listed (Figure 5.12). Thus, character 5, (length of third antennal segment), 8 (number of antennal segments), 9 (antennal length from base to anterior margin of head), and 15 (shape of anterior margin of carapace) are the strongest characters linking the "*P. variegatum*" clade, with character 10 (length of inner canine of mandible relative to outer canine), 16 (prominence of labrum when nymph viewed dorsally), 17 (number of major filaments branching off gill 1), 19 (forewing length of male), 36 **(**shape of female forewing) and 37 (length of female forewing) grouping members of the "*African*" clade.

It is tempting at this point to suggest erection of a second genus, based on morphological characters, to accommodate the species which group in the "*African*" clade. However, considering the results obtained in the following chapter, this would be premature.

5.5. Conclusions

The morphologically-based phylogenies indicate two main lineages within the Prosopistomatidae. The "*P. variegatum*" clade is currently clearly represented by three Madagascan species, two Australasian species, three Palaearctic species, and four Oriental species. The other sixteen Afrotropical species and seven Oriental species fall into the "*African*" clade. Since it is unlikely that similar selection pressures operate on more than one life history stage, the use of more than one stage in phylogenetic analysis increases the probability that character states are not correlated with adaptive features. Different sets of genes are turned on during different life stages, and hence nymphs and adults provide characters which are not related to each other functionally. Thus the independent adult characters corroborate the relationships seen in the nymphs-only derived phylogeny. The use of different life cycle stages which both produce evidence for two clades indicates a sound and well supported morphological phylogeny. A number of populations of the European species need to be studied further to see whether morphological indicates that they are actually represented by more than one species.

Detailed ecological and behavioural knowledge, such as river size, flow rate, depth, substrate type and season of maturity of the nymphs, and flight period of the winged stages may also add to the understanding of relationships within this enigmatic group.

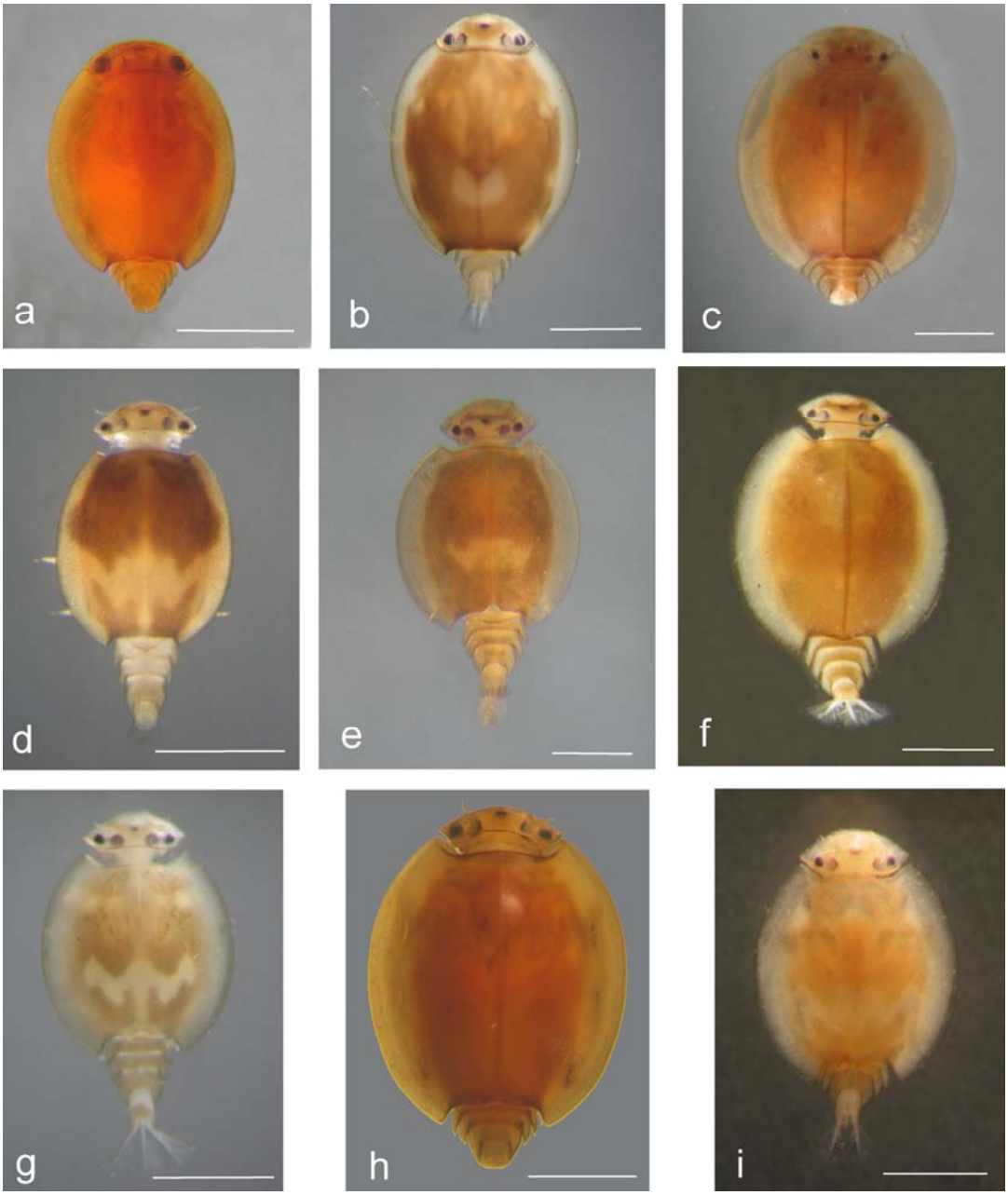


Figure 5.1. Dorsal aspect of nymphs of a selection of the nymphs of African species of *Prosopistoma*, (a) *P. africanum*, (b) *P. amanzamnyama*, (c) *P. crassi*, (d) *P. mccaffertyi*, (e) African sp. 2, (f) African sp. 8, (g) African sp. 3, (h) African sp. 6, (i) African sp. 7.

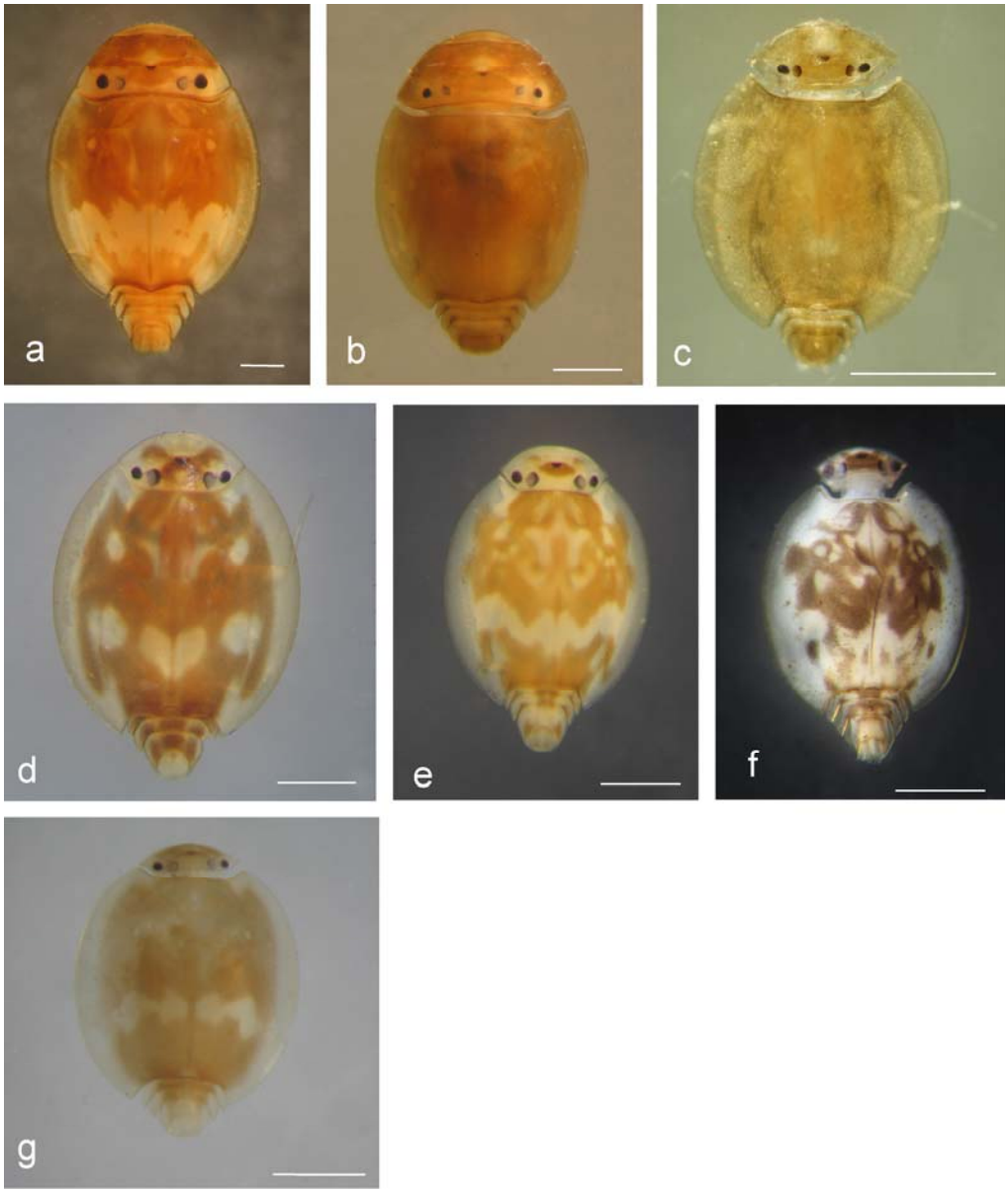


Figure 5.2. Dorsal aspect of nymphs of Madagascan and Comores species of *Prosopistoma*, (a) *P. variegatum*, (b) Madagascan sp. 5, (c) Madagascan sp. 4, (d) Madagascan sp. 1, (e) Madagascan sp. 2, (f) Madagascan sp. 6 (uncharacterised), (g) Comores sp. 1.

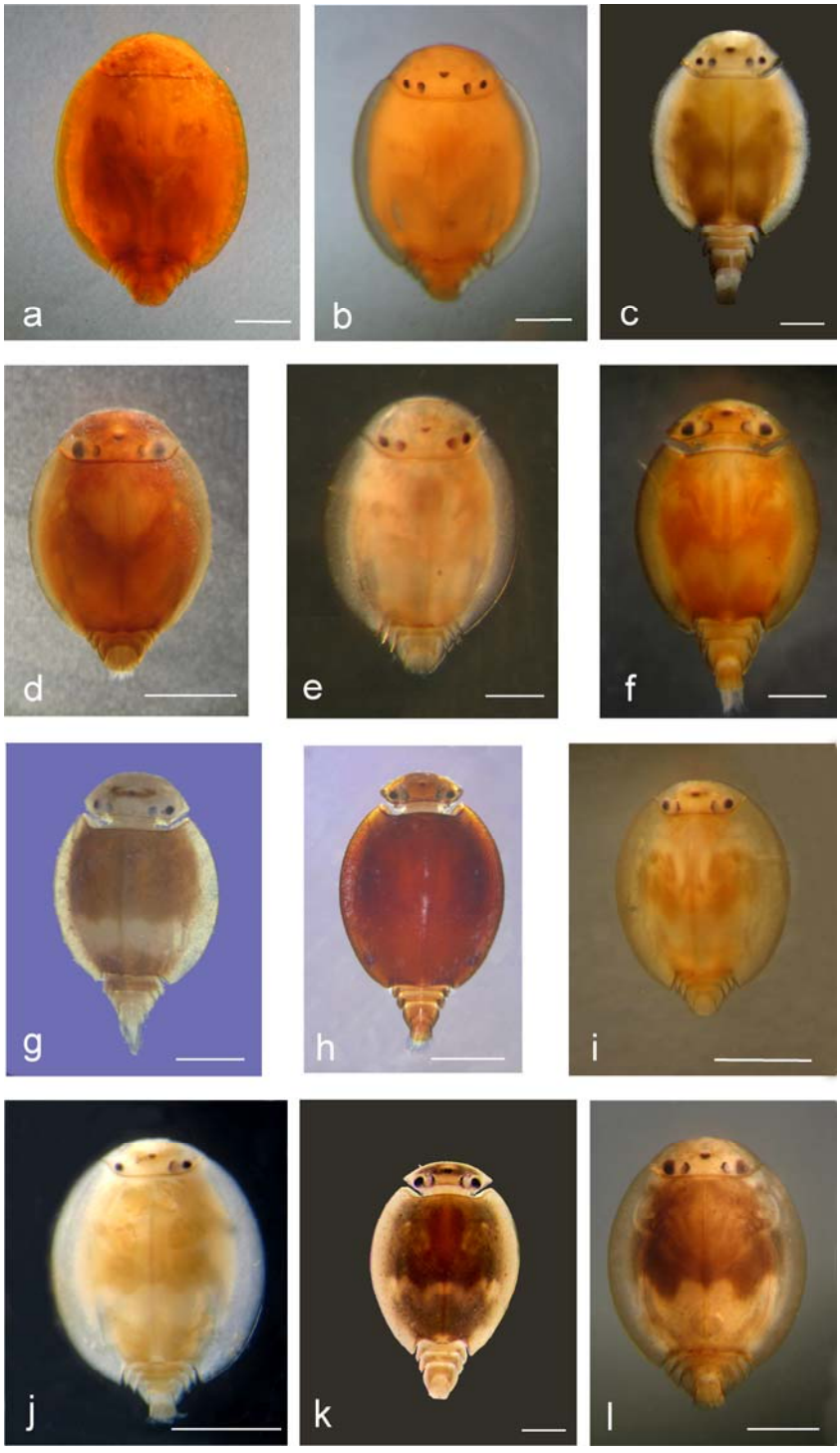


Figure 5.3. Dorsal aspect of nymphs of a selection of *Prosopistoma* nymphs representing European, Oriental and Australasian species. (a) *P. pennigerum* (France), (b) *P. pennigerum* (Spain), (c) *P. pennigerum* (Russia), (d) *P. oronti*, (e) *P. orhanelicum* (Turkey), (f) *P. lieftincki*, (g) *P. trispinum*, (h) *P. unicolor*, (i) *P. wouterae*, (j) *Prosopistoma* sp (Vietnam), (k) *Prosopistoma* sp (Thailand), (l) *P. pearsonorum*.

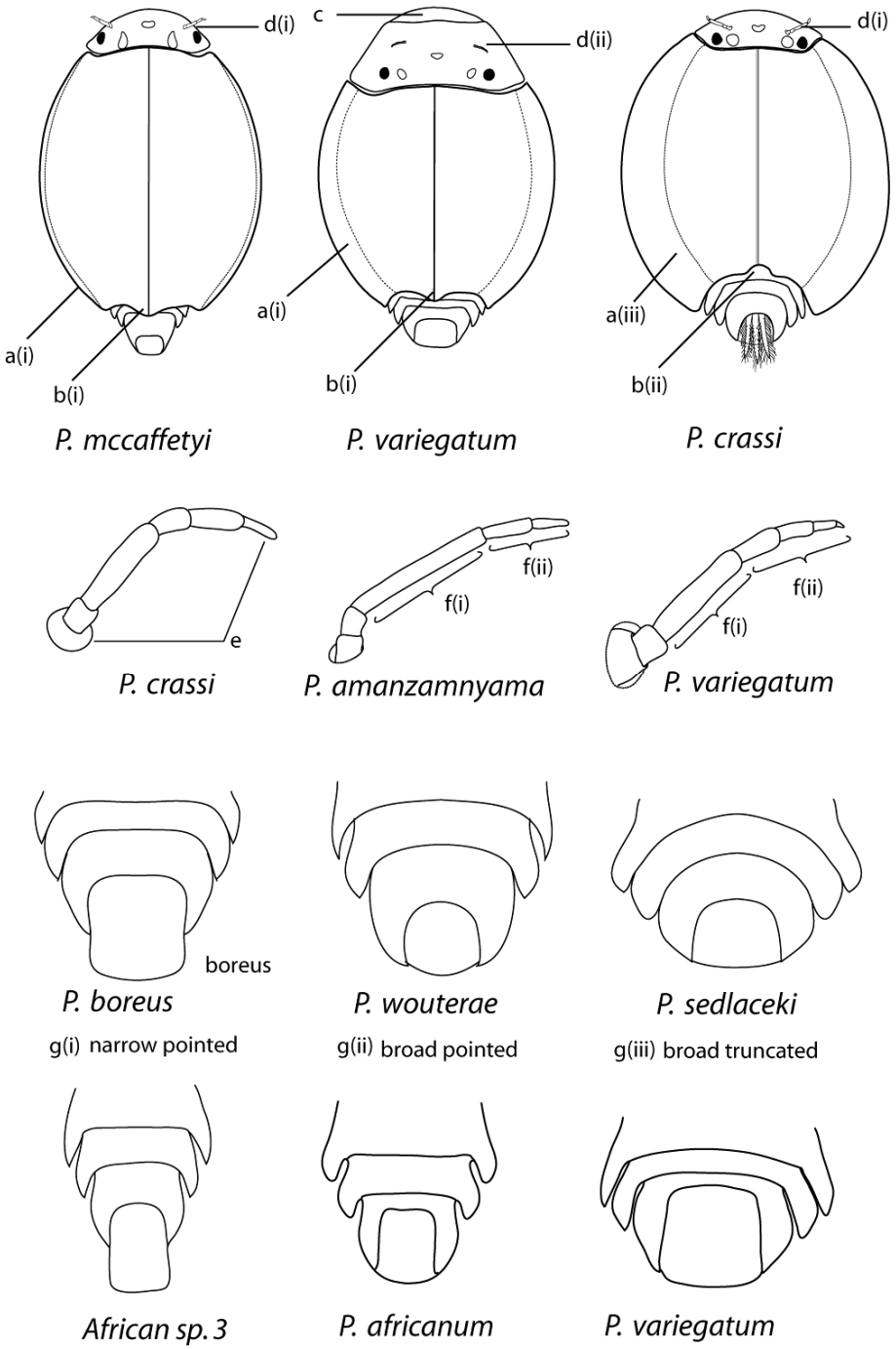


Figure 5.4. Illustration of nymphal characters considered for phylogeny, with examples; (a) flange width (ai) very narrow to narrow or (aii) wide, (b) carapace posteromedially (bi) notched or (bii) protruding, (c) labrum visible in dorsal view (d) relative length of antenna to anterior head margin (di) equal to or longer (dii) shorter, (e) number of antennal segments, (f) length of antennal segment III (fi) in relation to remaining antennal segments (fii), (g) shape of posterolateral projections of abdominal segments 7-9 (gi) narrow-pointed, (gii) broad-pointed, (giii) broad-truncated.

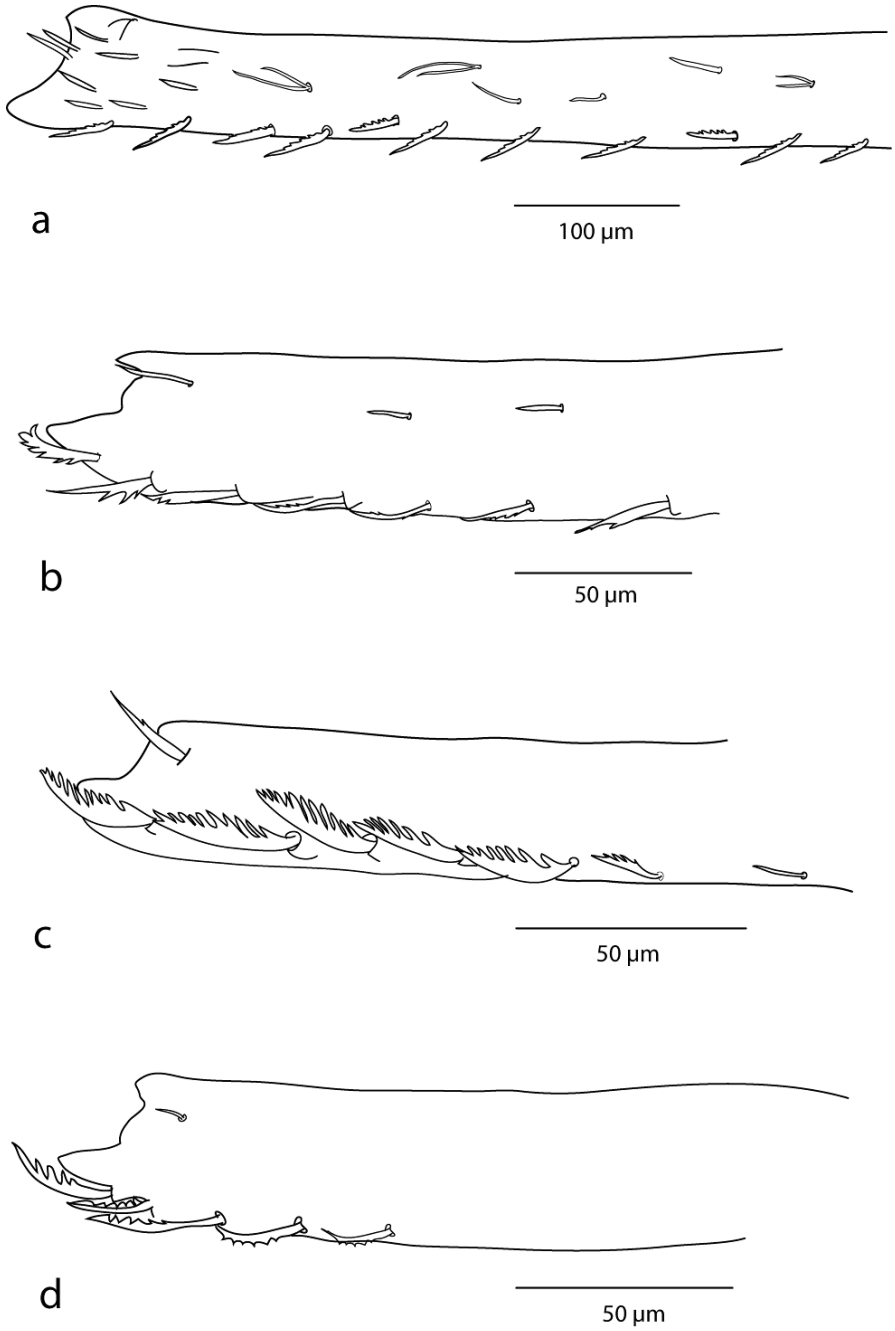


Figure 5.5. Examples of pectinate setae on nymphal foretibiae, (a) *P. variegatum*, (b) *P. crassi*, (c) Madagascan sp. 2, (d) African sp. 2.

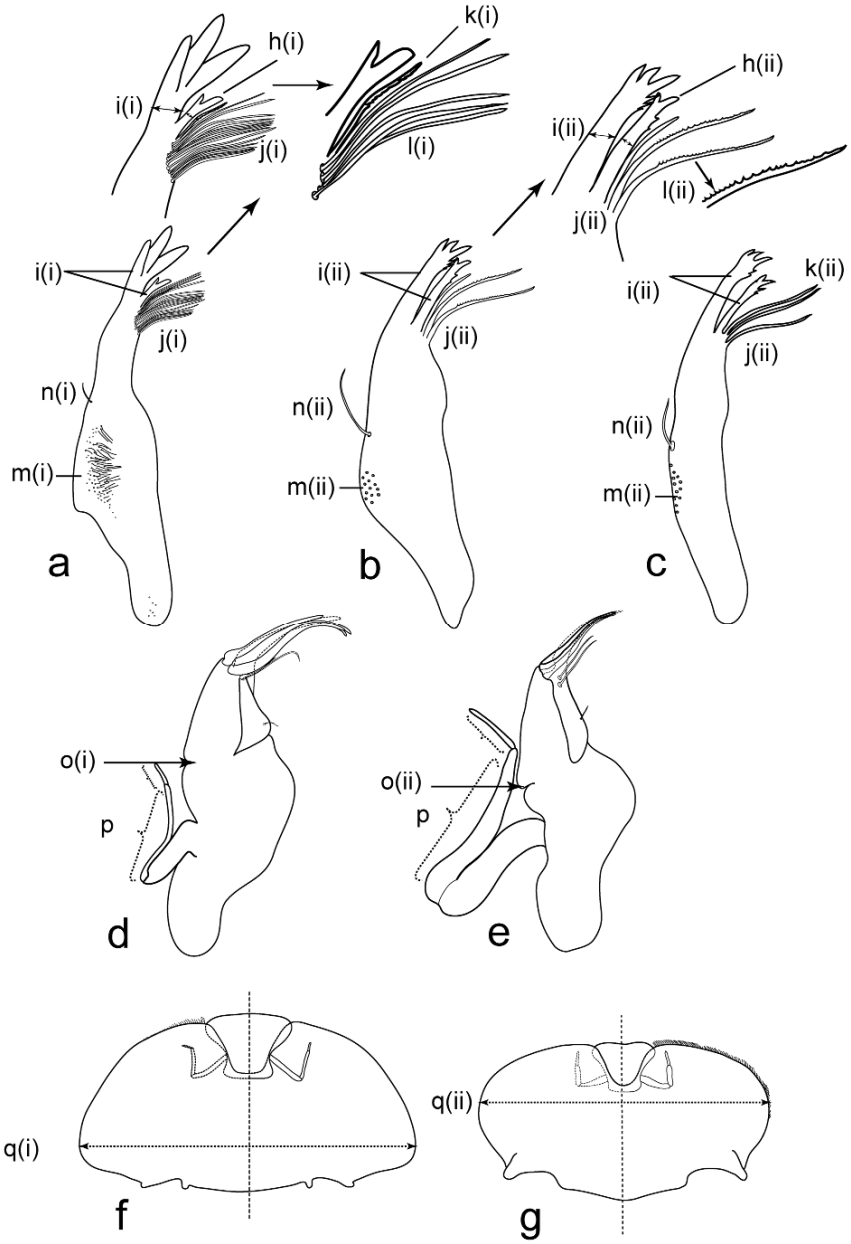


Figure 5.6. Further characters used for neural network and phylogeny, (h) length of inner canine of mandible compared to outer canine, (i) width of outer canine of mandible compared to width of inner canine, (j) number of setae beneath canines of mandible, (k) Mandible with or without short, thicker serrated seta preceding rest of setae below canine, (l) Setae beneath canine of mandible smooth or serrated, (m) number of setae or setal sockets at rear end of mandible, (n) relative length of seta on lateral margin of mandible (o) seta maxillary palp extending beyond the notch which marks the separation between the stipes and galea-lacinia or not reaching the notch, (p) relative length of maxillary palp segments, (q) position of widest part of postmentum widest near central axis or widest near base (proximally). Species represented: (a) *P. variegatum*, (b) African sp. 3, (c) *P. crassi*, (d) *P. variegatum*, (e) *P. amanzamnyama*, (f) *P. variegatum*, (g) *P. amanzamnyama*.

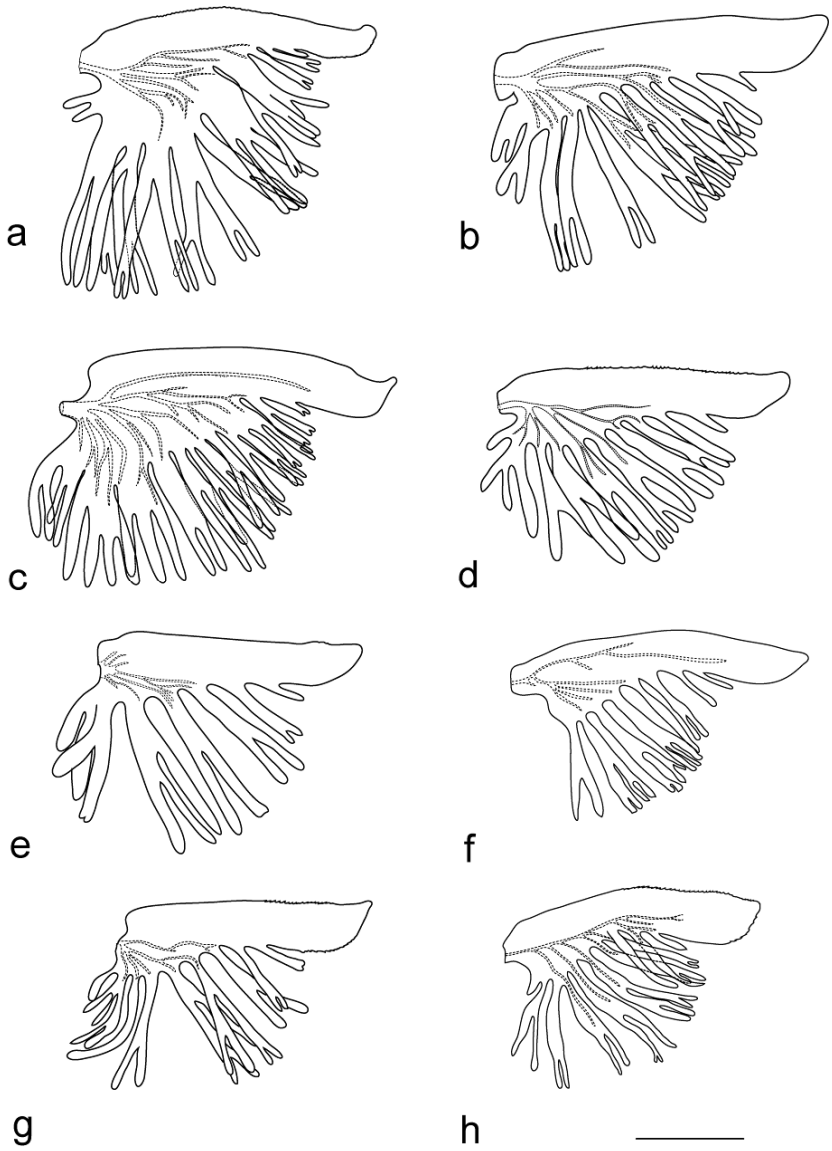


Figure 5.7. A comparison of structure of gill 1 in: (a) Madagascan species 5, (b) *P. pearsonorum*, (c) *P. variegatum*, (d) African species 4, (e) Comores species, (f) *P. crassi*, (g) *P, mccaffertyi*, (h) *P. amanzamnyama.*

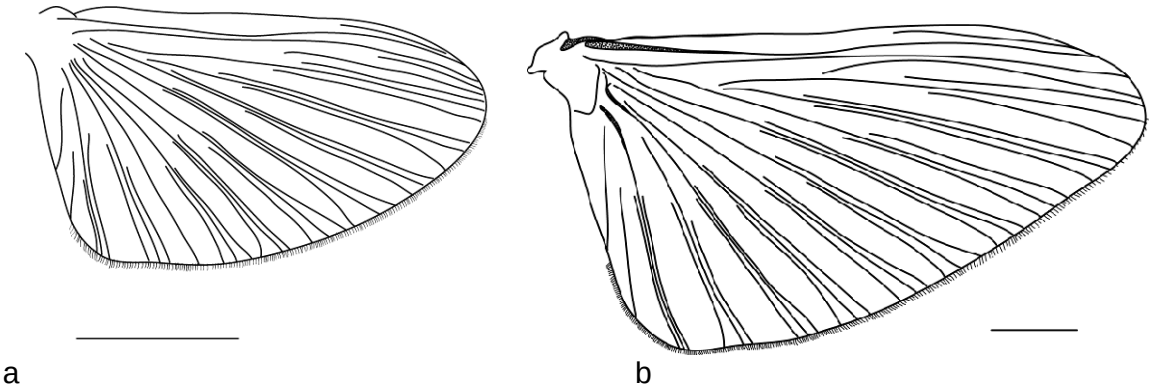


Figure 5.8. Comparison of male forewing showing (a) a more rounded apex, as seen, for example, in Madagascan sp. 1, and (b) tapering effect, where the leading edge tapers off sooner basally to give more pointed appearance (as seen for example in *P. variegatum*.

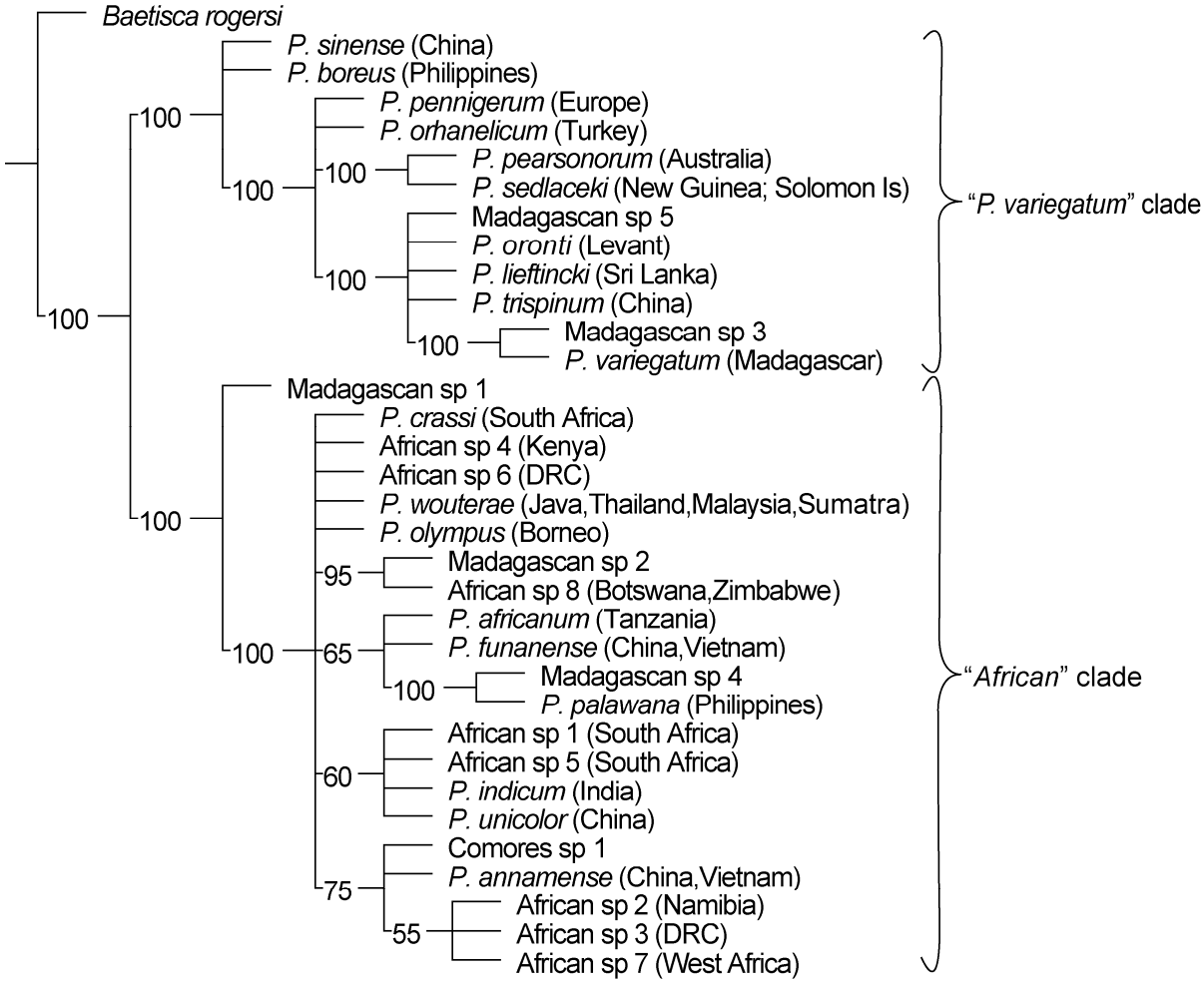


Figure 5.9. Majority rule consensus tree for world *Prosopistoma* species calculated from 20 trees derived from nymphal characters recorded in Appendix Table A5.1. Nodes with over 50% support are indicated; tree length = 51.

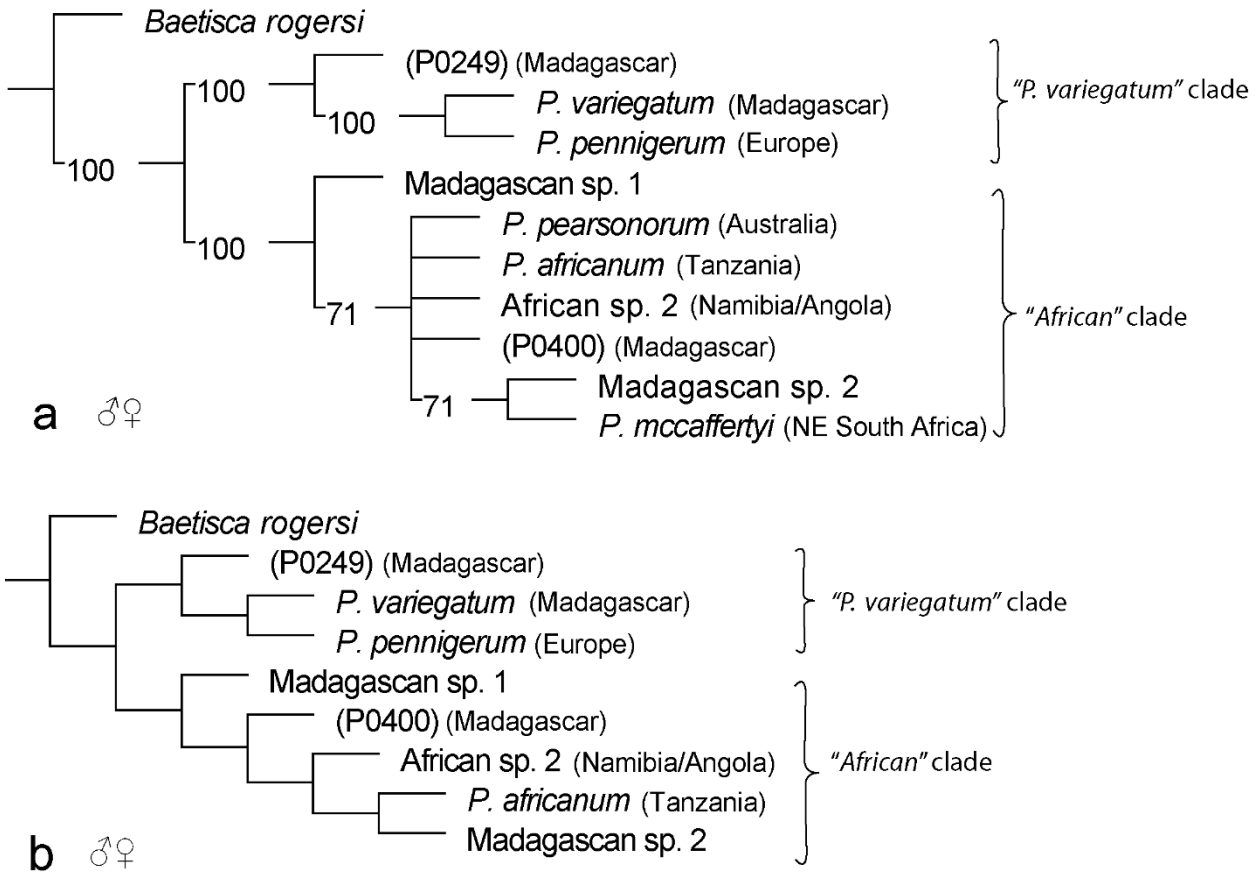


Figure 5.10. (a) Majority rule consensus tree from seven trees produced from combined male and female adult characters (note: *P. mccaffertyi* and *P. pearsonorum* are only known as females, so many characters are missing for these two branches). Nodes with over 50% support are indicated; tree length = 23, (b) single tree produced when analysis was rerun without *P. mccaffertyi* and *P. pearsonorum*; tree length = 23. Adults of two unknown Madagascan species are included, these are not correlated with any of the recognized nymphal species, and are therefore not in the nymphal analysis

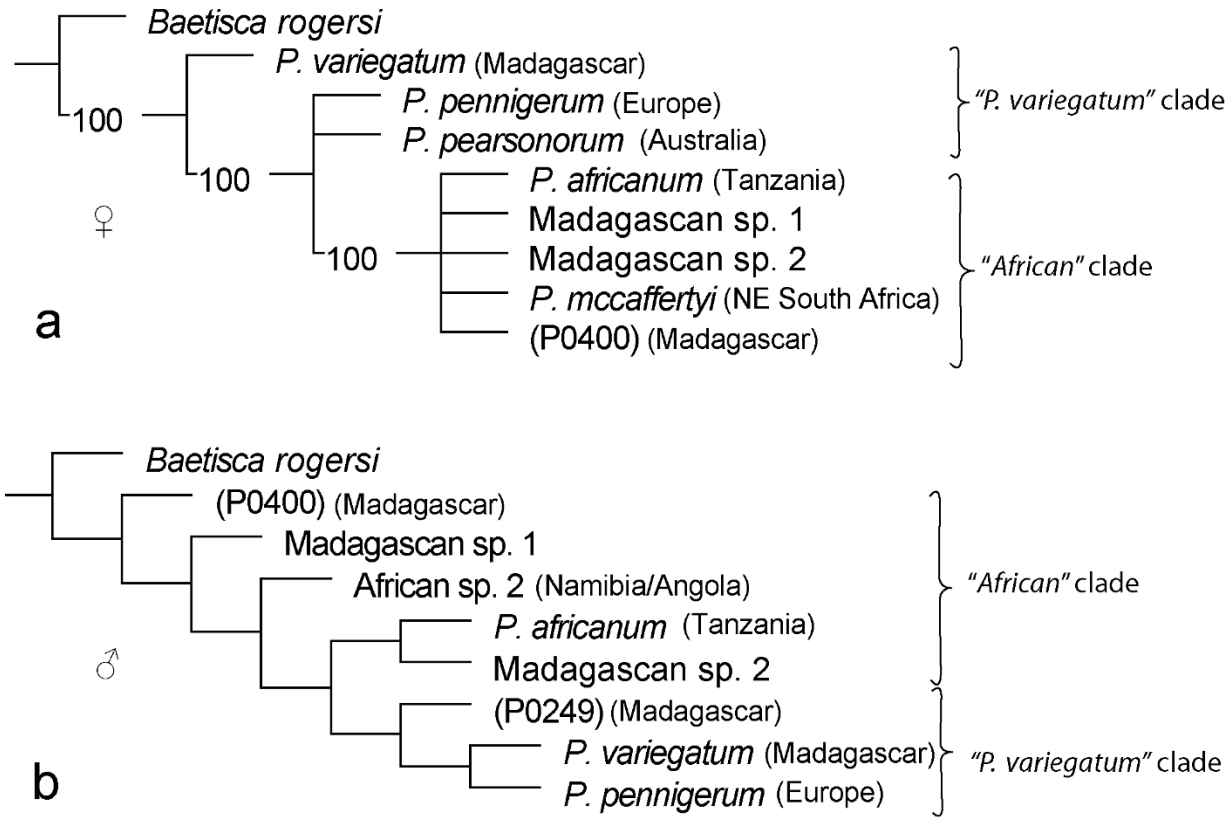


Figure 5.11. (a) Majority rule consensus tree from three trees produced from characters from female subimagos only. Nodes with over 50% support are indicated; tree length = 11, (b) single tree from characters from males imagos only; tree length = 39.

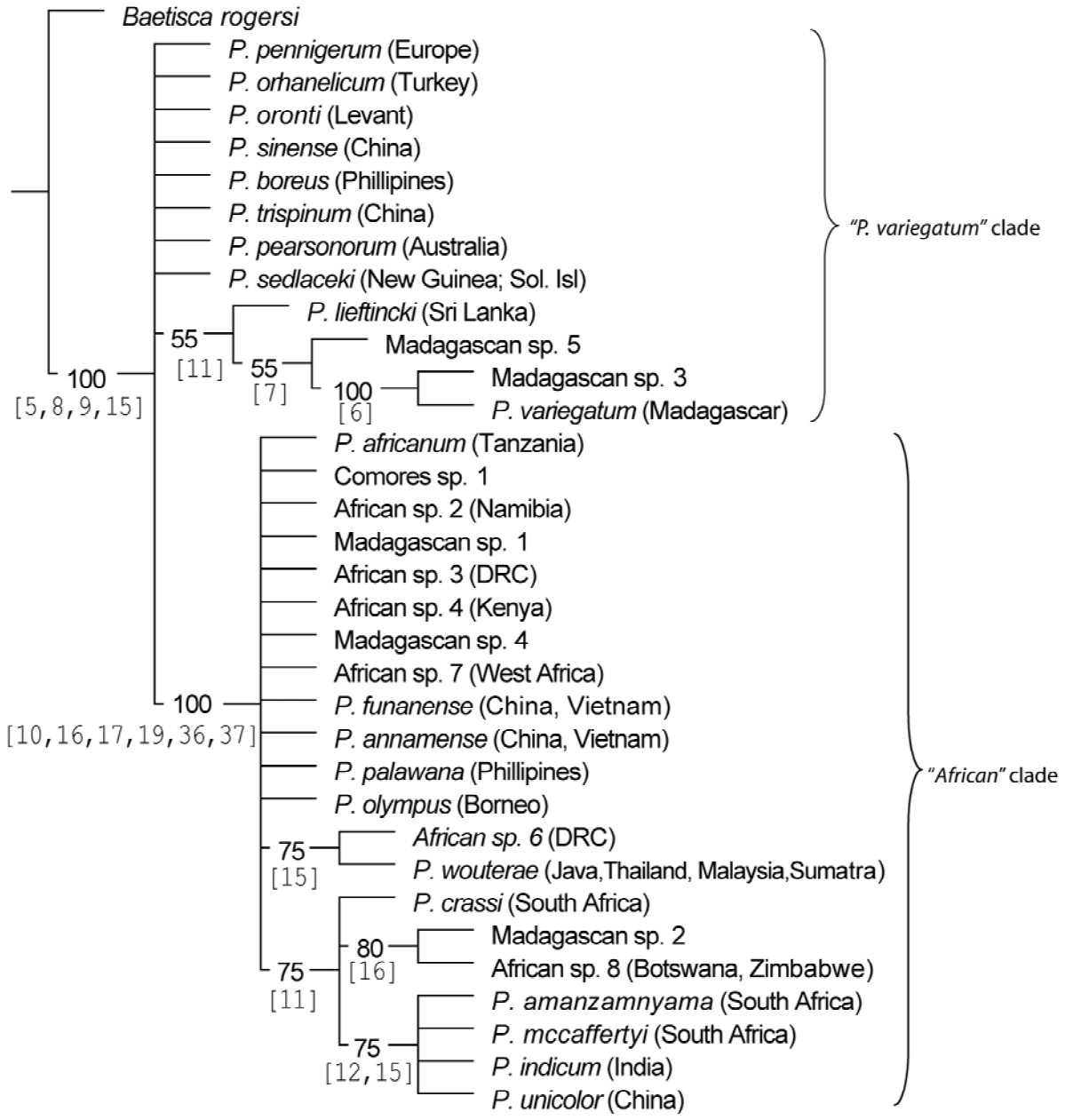


Figure 5.12. Majority rule consensus of 20 trees produced from a supermatrix of nymphal and adult characters. Nodes with over 50% support are indicated; tree length = 170. Due to a large number of unknown characters in the adults, this tree has poor resolution, and it is more appropriate to refer to the resolution in the smaller trees considering each life history stage separately. Common synapomorphies are indicated in square brackets.

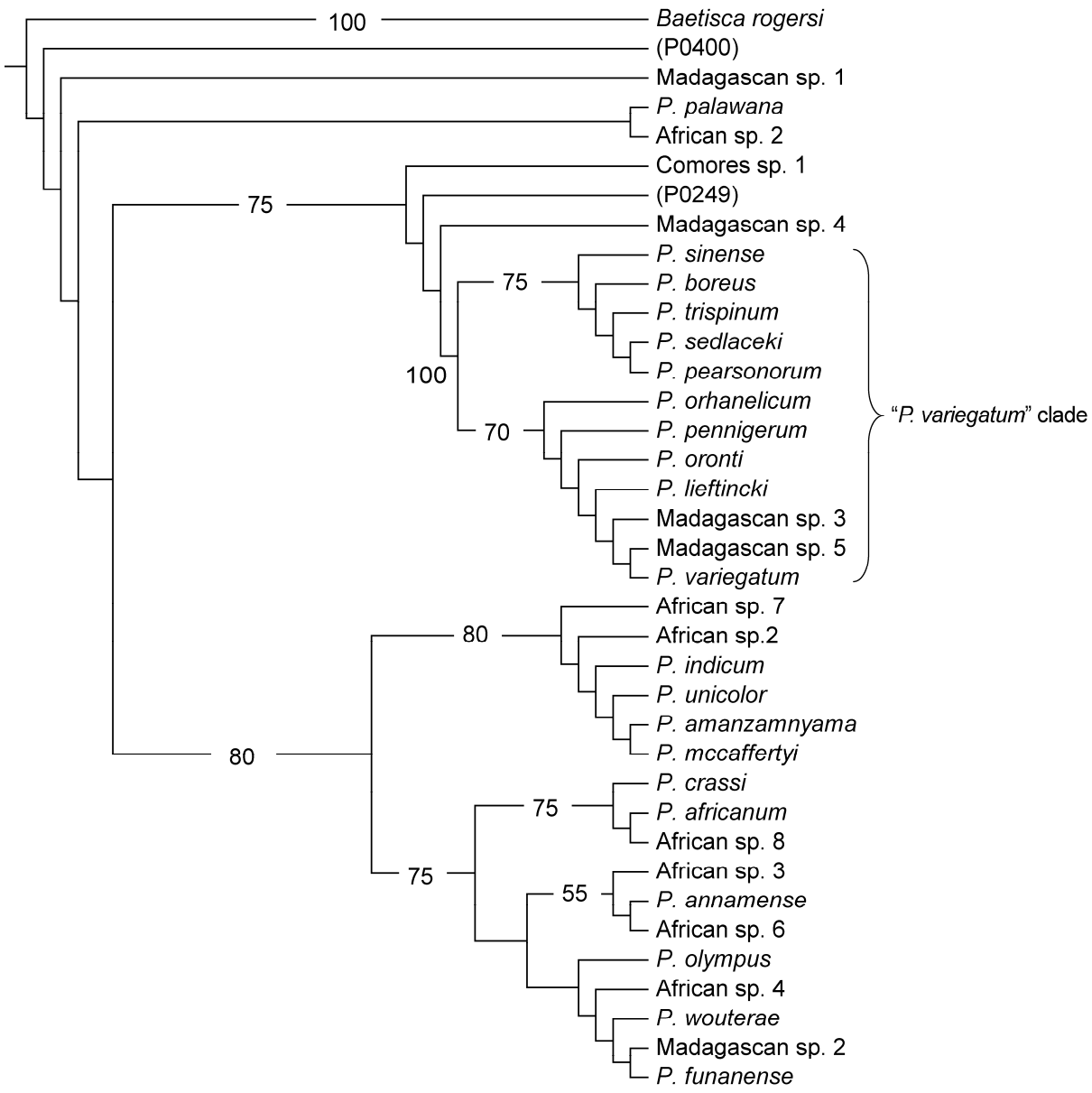


Figure 5.13. Supertree produced in Clann using combination of 20 nymphal trees (given weight 0.03), 3 female trees (given weight 0.33) and 1 male tree (weight 1). Nine supertrees were produced, giving the consensus Majority Rule supertree, above. Nodes with over 50% support are indicated.

Chapter 6

Molecular phylogeny of the Prosopistomatidae

"Science seldom proceeds in the straightforward logical manner imagined by outsiders. Instead, its steps forward (and sometimes backward) are often very human events in which personalities and cultural traditions play major roles."

James Watson: The Double Helix" 1968

Synopsis

This chapter continues the quest to understand the relationships between the extant species of *Prosopistoma*, and uses standard Bayesian and Parsimony methods applied to molecular sequences to achieve this. These phylogenetic analyses show a close relationship between the European species (*P. pennigerum*) and one of the African species (*P. crassi*). Other African and Madagascan species and the Australian species are closely related. The only Asian species investigated (*P. wouterae*), does not group as closely with the other species as may be expected. An estimation of the relative ages of these species, and the age of their lineage, contributes an important part to understanding these relationships. A relaxed molecular clock method was applied to the data, which were expanded with additional Ephemeroptera sequences and an odonate sequence from GenBank. Several fossil dates were used to constrain nodes to estimate both the time of origin of the lineage, the time of origin of the current species and the relationships between these and representatives of other mayfly lineages. Results based on Prosopistomatidae, Baetiscidae and Oligoneuriidae sequences only, indicate that Prosopistomatidae and Baetiscidae diverged 387.96 Ma, with the oldest crown-group species (*P. wouterae*) 131.49 Ma. According to the same model, crown-group Baetiscidae appeared around 278.67 Ma. Using a broader set of taxa, the Prosopistomatidae stem-group diverged from other mayfly lineages 319.17-168.31 Ma, while the age for the youngest crown-group species, *P. crassi* ranges from 3.49-1.21 Ma.

6.1 Introduction

The main purpose of the molecular phylogeny was to confirm or refute the morphology-based phylogeny and conclusions derived from this. The questions addressed by this section are as follows:

(1) Whether there are really two clearly defined clades as shown by the nymphal morphology-based phylogeny.

(2) If there are two clades, are they distinct enough to deserve placement in separate genera?

(3) Is there really only one species represented across Europe?

(4) Is it possible to work out the ages of the ancestral stem-groups and the crown-group species using the molecular data gathered here, in conjunction with fossil data and molecular clock methods?

(5) Do Asian or African species represent the youngest clade?

(6) Are West African species younger than East African species?

(7) Is it possible to link adult and nymphal material in Madagascan species, where both adult and nymphal material is available for some species, but not associated?

(8) Is *P. crassi* a morphologically variable species, or does it constitute a species complex?

As part of a global analysis of mayfly phylogeny based on molecular evidence, genes of two prosopistomatid species have already been sequenced (Ogden and Whiting, 2005). One is *Prosopistoma wouterae* from Asia; the other, *Prosopistoma* sp. EP166, is recognised as the species currently designated as Madagascan sp. 2 in this thesis. Material of both species has been examined morphologically and it is also included in the morphological phylogeny. Ogden and Whiting (2005) analysed these two specimens using mitochondrial 16S ribosomal RNA and the nuclear 18S ribosomal RNA, 28S ribosomal RNA, and Histone 3 protein coding genes. The ribosomal 16S region of mitochondrial DNA gene is quite informative at the species level and used regularly in other mayflies (eg. Tojo and Matsukawa, 2003; Monaghan *et al*., 2005). Another widely used gene is the cytochrome oxidase subunit I (COI) region of mtDNA (eg. Williams *et al*., 2006), which failed to produce sequences in this study on *Prosopistoma*, and was not used by Ogden and Whiting (2005). The nuclear genes used by Ogden and Whiting (2005) are also fairly routinely used, although more at the supraspecific level as they tend to evolve more slowly and be conserved, and are therefore more useful in old lineage delineation and at higher taxonomic levels (eg. Ogden and Whiting, 2003).

Unfortunately not all species were available for analysis as fresh material is scarce, especially from Asia. Despite numerous requests to Asian colleagues for material, only material from Vietnam was obtained, and the DNA from this did not amplify. A selection of species representing as many major biogeographical regions as possible was tried for

DNA extraction, amplification and sequencing, even when the material was potentially too old. Sequences for two additional Prosopistomatidae species were available from GenBank.

6.2 Methods

6.2.1 Laboratory procedures

Laboratory work was carried out at the Leibniz Institute for Freshwater Ecology, Berlin, Germany. *Prosopistoma* species (Appendix Table A6.1) were selected for molecular analysis, based on age and state of preservation of material available. Genomic DNA extraction was attempted on some specimens even though they were collected over ten years ago, as available fresh material was limited. Seventeen species out of the total 37 species recognised in this thesis (Table 1.1) were analysed, representing as many major biogeographical regions as possible. DNA was extracted from whole nymphs or adults using a Qiagen Dneasy Tissue Kit. A volume of 180 µl of ATL tissue lysis buffer was added to each specimen, with 20 µl proteinase-K solution. These were placed in a water bath at 56°C and left for approximately 12 hours. The remaining intact chitinous exoskeleton of each species was removed and preserved in 80% ethanol as voucher material (museum catalogue numbers are given in Appendix Table A6.1). Remaining genomic DNA extract is stored at -80°C at the Leibniz Institute. The standard Qiagen extraction protocol was followed on the preliminary extraction product, with a few modifications, namely the use of 100 µl of AE buffer rather than the stated 200 µl as the *Prosopistoma* specimens tend to be very small (often only just over 1 mm in total length). Two elutions were retained.

PCR amplification was performed using a VWR DuoCycler thermocycler. The following procedures were carried out with a selection of relevant primers which had previously been tested on mayflies. Six genes were targeted for amplification and sequencing (Table 6.1).

CO1: A total volume of 25 µl of buffer mixture containing the forward and reverse primers (Table 6.1), nucleotides (dNTP) and Taq enzyme and 1 µl of extract were subjected to 94˚C preheating for 2 minutes; 35 cycles at 94˚C for 30 seconds, 52˚C for 30 seconds, 72˚C for 60 seconds, with elongation at 72˚C for 10 minutes. This was repeated at 48˚C annealing temperature using 2 µl of extract, after a poor yield of DNA in the PCR product, using 38 cycles.

16S and 18S: As above, using the primers indicated in Table 6.1, and using 94˚C for 5 minutes, 35 cycles at 94˚C for 30 seconds, 48˚C for 30 seconds (lower annealing temperature), 72˚C for 60 seconds with elongation at 72˚C for 10 minutes.

Once these more standard genes had been attempted, Wingless and Pepck nuclear genes were also attempted. A gradient PCR for these genes was run on selected samples which had been shown to have useable DNA to establish optimal annealing temperatures and sample volumes. The annealing temperature gradient ran from 46˚C to 60˚C.

Pepck: A standard cycle using an annealing temperature of 48˚C was found to be optimal. Primers as in Table 6.1.

Wingless: A standard cycle using an annealing temperature of 44˚C was found to be optimal. Primers as in Table 6.1.

Further gradient PCR's were run on selected samples with low DNA yield to establish optimal annealing temperatures, PCR cycle length and sample volume to maximize the chance of getting DNA from these specimens. This was done for CO1, 16S RNA, 18S rRNA and Histone 3.

Table 6.1 Genes selected for analysis, and primers used.

Gene	Type	Forward primer	Reverse primer
CO1	Mitochondrial	HCO (Folmer *et al*., 1994)	LCO (Folmer *et al*., 1994)
16S rRNA	Mitochondrial	16Sar (Simon *et al*. 1994)	16Sb2 (Palumbi *et al*.,1991)
18S rRNA	Nuclear	18S 5' (Whiting *et al*. 1997)	18Sb5.0 (Whiting *et al*. 1997)
Histone 3 (protein coding for the nucleosome (H3))	Nuclear	H3aF (Colgan *et al*. 1998)	H3aR (Colgan *et al*. 1998)
Pepck (Phosphoenolpyruvate carboxykinase)	Nuclear	Pep FB12 (Vuataz, unpublished)	Pep RB45 (Vuataz, unpublished)
Wingless	Nuclear	WgF2 CT(AGT) CCA TTA TTC CGT GTA GTT GG (Vuataz, unpublished)	WgR498 GTA CAT TCA CAT CTT TCT CTT AC (Vuataz, unpublished)

Availability of adequate DNA for sequencing was tested by running the PCR products on Agrose gel electrophoresis. The Agrose gel was prepared using 1.8 mg Agrose powder in 90 ml TAE (tris EDTA acetate) buffer, with 0.6 ml Ethidium Bromide. Later runs were done with Gel Red, which is less toxic than Ethidium Bromide. Aliquots of 0.5 µl of loading dye were mixed with 4 µl of PCR product and placed into individual wells in

the gel, which was run at 80 V for 20 minutes. PCR product size (bp) was measured using a single lane of Hyperladder IV (BioLine) to verify whether the target fragment was amplified. Once the electrophoresis had taken place, the gel was viewed under ultraviolet light using a Bio-Rad Quantity-One trans-illuminator and photographed digitally. Clean-up of PCR products which had adequate suitable DNA was done following the Promega PCR clean-up protocol. Further testing of genomic DNA concentration in the samples was done using a Thermo Scientific (Wilmington DE, USA) NanoDrop 2000 to measure DNA concentration (ng / uL), to test whether samples were likely to have enough DNA to produce sequences or not. Cleaned DNA strands were sequenced in both directions using the PCR primers, with a Beckman Coulter CEQ 8000 Genetic Analysis System automated sequencer.

Table 6.2. Fossil age estimates, originally derived from geological evidence, used for constraining nodes in BEAST molecular clock analysis.

Family	**Genus, species**	**Era**	**Period**	**Age (Ma)**	**Site (modern)**	**Source**
Aeshnidae	*Aeshna hageni*	Mesozoic	Upper Jurassic	205.7 - 197	Switzerland	EDNA database
Siphlonuridae	*Albisca trachaeta*	Mesozoic	Upper Cretaceous	142 - 98.9	Mongolia	Sinitshenkova, 1989
Siphlonuridae	*Huizgougenia orbicularis*	Mesozoic	Lower Jurassic	159.4 - 142	China	Lin, 1980
Ephemeridae	*Australiphemera revelata*	Mesozoic	Upper Cretaceous	142 - 98.9	N.E. Brazil	McCafferty, 1990
Leptophlebiidae	*Clavineta cantabilis*	Mesozoic	Jurassic	205.7 - 142	Mongolia	Sinitshenkova, 1991
Oligoneuriidae	*Colocrus indivicum*	Mesozoic	Upper Cretaceous	142 - 98.9	N.E. Brazil	McCafferty, 1990
Oligoneuriidae	*Colocrus magnum*	Mesozoic	Lower Cretaceous	114	N.E. Brazil	Staniczek, 2007
Baetiscoidea	*Balticobaetisca velteni*	Cenozoic	Paleogene	54.8 - 33.7	Baltic	Staniczek and Bechly, 2002
Baetiscoidea	*Balticobaetisca stuttgardia*	Cenozoic	Paleogene (Eocene)	39.9 - 59.8	Baltic	Godunko and Krzemiński, 2009.
Baetiscoidea	*Protobaetisca bechlyi*	Mesozoic	Lower Cretaceous	125 - 112	N.E. Brazil	Staniczek, 2007
Baetiscoidea	Unnamed ?Siphloneuridae Jell & Duncan; Stanickzek to Baetiscoidea	Mesozoic	Lower Cretaceous	118 - 115	Victoria, Australia	Jell and Duncan 1986, Staniczek, 2007
Baetiscoidea (†Cretomitarcyidae)	*Cretomitarsys luzzii*	Mesozoic	Upper Cretaceous	93.5 - 89.3	New Jersey, North America	Sinitshenkova, 2000b, McCafferty, 2004

6.2.2 Phylogenetic analysis

For the choice of outgroup taxa, molecular (Ogden and Whiting, 2005, Ogden *et al*., 2009) and morphological (Fontaine, 1958; Edmunds 1972; Kluge, 1998; Wang, McCafferty and Bae, 1997) evidence to date indicates that Baetiscidae are the sister taxon to Prosopistomatidae. *Baetisca rogersi* Berner was selected as the outgroup, as was the case in the morphological phylogeny. It was decided to expand the outgroup comparison to include a second species, *Baetisca lacustris* McDunnough, using sequences from Genbank. As Ogden *et al*. (2009) showed a sister relationship between the Oligoneuriidae and the Posteritorna, three Oligoneuriidae species were also included, *Lachlania saskatchewanensis* Ide and *Oligoneuriella rhenana* (Imhoff) from Genbank, and *Elassoneuria trimeniana* (McLachlan) which was sequenced at the same time as the Prosopistomatidae specimens. These are used as the outgroup for the Posteritorna in these analyses. For the 16S gene, only *Oligoneuriella* sequences were available for outgroup comparison (Table 6.2), no 16S sequences were available for *Baetisca* species.

Sequence trace files were checked and edited using a trial version of Codon Code Aligner. These consensus sequences were imported into Mesquite (Maddison and Maddison, 2010), with preliminary alignment using the sequence alignment program ClustalW (Chenna *et al.* 2003). Manual alignment followed to complete alignment. BLAST (Basic Local Alignment Search Tool) analysis was done on Genbank to see if the sequences were close to any other taxa on Genbank and to check for contamination.

Once the additional sequences from Genbank were incorporated and all were aligned, each gene dataset was tested for the most appropriate model of sequence evolution as determined by MrModeltest v.2.2 (Nylander, 2004) using MrMTgui (http://www.genedrift.org/mtgui.php) to determine a separate model for each gene. The combined molecular dataset consisted of 15 terminals (including five outgroup samples). For all analyses, all characters were treated as unordered and equally weighted. The aligned sequences were saved in Nexus format for maximum parsimony analysis in PAUP*, submitted to the University of Oslo Bioportal: https://www.bioportal.uio.no for analysis, and Bayesian Inference (BI) in MrBayes v.3.1 (Huelsenbeck and Ronquist, 2001). Analyses were conducted for each dataset independently.

Each BI analysis comprised two independent runs each of ten million generations. Random starting trees with four Markov chains (one cold, three hot) were used with trees sampled every 1000 generations. All model parameters except branch length and topology were unlinked. Stationarity was determined when the log likelihood (-ln *L*) scores plotted

against generation time reached a stationary value, and when the potential scale reduction factor (PSRF) was equal to 1. The first 1000 trees (10%) sampled were discarded from each run as burn-in. The remaining trees were then used to compute the majority rule consensus Bayesian topology and posterior probability values. Bayesian analysis was conducted at the Computational Biology Service Unit, Cornell University BioHPC: http://cbsuapps.tc.cornell.edu/mrbayes.aspx. Posterior probability (Bernardo, 1979; Berger and Bernardo, 1989) was used as a measure of node support; a posterior probability of > 0.95 is considered well supported.

Parsimony analyses were performed using PAUP* version 4.0b10 (Swofford, 2002). A simple heuristic search with tree bisection-reconnection (TBR) branch swapping was used to find the approximate length of the shortest tree. This was followed by a random input analysis using 1000 repetitions and TBR branch swapping, where trees shorter or equal to the shortest heuristic tree were retained. This process was repeated until no shorter trees were found. All trees equal to or shorter than the shortest tree found in the simple search were used to compute the strict consensus tree. Nodal support was obtained using 100 bootstrap pseudoreplicates (Felsenstein, 1985) in PAUP*.

6.2.3 Molecular Dating

Further analyses were performed incorporating selected additional sequences from Genbank (Table A6.2), using the 16S, 18S and Histone 3 genes as these were the only genes available from the *Prosopistoma* analyses. The first attempt at molecular dating included the gene sequences obtained in this study for the *Prosopistoma* species, ***B. rogersi*** and *E. trimeniana*, and GenBank sequences for Ogden and Whiting's (2005) *Prosopistoma* species, as well as additionally ***B. lacustris*** (the only species on GenBank with comparable genes) and two additional Oligoneuriidae species (*L. saskatchewanensis* and *O. rhenana*. Nodes were constrained to a minimum age using a Baetiscoidea fossil estimated to be 112-125 Ma old.

In an attempt to do a deep-level dating, sequences were sought for Odonata as outgroup, and representatives of a selection of other mayfly families to provide a more broad-based comparison. An uncorrelated lognormal (UCLN) relaxed clock in BEAST v.1.5.2 (Drummond and Rambaut, 2007) was used to estimate node ages, constraining relevant nodes where fossil dates were known. A Yule speciation prior and random starting trees were used. Analyses were run using the Computational Biology Service Unit, Cornell University BioHPC, with one run of 50 million generations and a discard burn-in of 0.5

million generations to achieve stationarity. TreeAnnotator v.1.5.2 was used to summarise trees and to produce a consensus, which was then viewed using FigTree v.1.2.3. Both of these programs are part of the BEAST package (Drummond and Rambaut, 2007).

Fossil ages were obtained from the EDNA database (http://edna.palass-hosting.org) or from the publications indicated in Table 6.2. Different fossil-constrained nodes were used in an attempt to date the lineages, to see whether the dates obtained would vary depending on the fossils selected. A predicted model of relationships based on fossil dates and the phylogeny derived by Ogden *et al*. (2009) is given in Figure 6.5.

6.3 Results

6.3.1. DNA extraction and sequencing

DNA extraction and amplification was attempted on the species given in Table A6.1. It was decided that if this was successful for older samples (greater than ten years), further specimens available only as old material would be included. However, as little or no high molecular weight DNA could be extracted and no PCR products could be amplified from there, older samples were not attempted further. It was found that, unfortunately, several important species needed to cover the overall distribution pattern did not produce DNA. Obtaining fresh or adequately preserved *Prosopistoma* material is a drawback for molecular work on this group as they are difficult to collect and not often found. The nymphs are also not often collected in large numbers at any site, although adults are occasionally attracted to light in large numbers (M. Sartori, Musée de zoologie, Lausanne, Switzerland, pers. comm., 2010).

BLAST analysis put the sequences obtained from these analyses with the other Prosopistomatidae Genbank sequences, as expected, showing that the DNA was from the specimens and not a contamination from another source. Sequences were obtained for several species (Table 6.3), but often different genes were successful for different species. 18S genes provided the most consistent sequences, possibly because 18S is highly conserved and primers are universal. In several instances, although DNA banding was apparent in the Agrose gel electrophoresis, and was measurable, sequences could not be obtained. The NanoDrop measurements for DNA concentration gave an indication of which species were likely to produce successful sequences. Ideal values should range from 30-50 ng / uL but some of the older samples produced values of 3-5 ng/ uL i.e. an order of magnitude lower.

6.3.2. Phylogenetic analysis

The final molecular dataset for the simple phylogenetic analyses (baetiscoid species and outgroups) consisted of 2394 bp for the combined analysis, comprising 1743 bp (18S), 305 bp (16S) and 344 bp (H3) (Table 6.4). The initial alignment of 2528 bp was pruned to remove areas where one sequence tailed off with more base pairs than the adjacent sequences, for example between 18S and 16S sequences. The models used for each gene in the Bayesian analysis are given in Table 6.4. Bayesian inference resulted in the consensus trees shown in Figure 6.1, (18S gene only), Figure 6.2 (16S gene only) and Figure 6.3 which combines 16S, 18S and Histone 3. A tree for Histone 3 alone was considered pointless as too few species were represented. Parsimony analysis yielded the trees given in Figure 6.1-6.3, with the support statistics given in Table 6.3. Although some of the nodes lack support, the congruent topologies of the Bayesian and parsimony trees indicate that the estimate of relationship between the *Prosopistoma* species is relatively reliable using the given genes.

Table 6.3. Gene sequences used for phylogenetic analysis (X). GenBank accession numbers are given for additional sequences. Blanks indicate that sequences were not obtained, or were not available on Genbank. No results were obtained with CO1, Pepck or Wingless.

Species	Genes used in alignment		
	16S	18S	Histone 3
Elassoneuria		X	X
Lachlania	AY749801	AY338701.1	AY338623.1
Oligoneuriella	AY749823.1	AY749897.1	AY749745.1
B. rogersi		X	
B. lacustris		AY749865.1	AY338627.1
P. wouterae	AY749810.1	AY749882.1	AY749734.1
Madagascan sp. 2	AY749827.1	AY749904.1	
African sp. 2	X	X	
P. pearsonorum	X	X	
P. pennigerum (Russia)	X	X	
*P. pennigerum (*Spain)	X	X	
P. variegatum		X	
African sp. 8		X	
P. crassi	X		
P. crassi-like	X		

Table 6.4 Data characteristics and analysis summaries.

		Characters			Parsimony analysis				Bayesian analysis	
Genes	No of taxa	Base-pairs (bp)	Parsimony informative	% pars informative	No of trees	Score (tree length)	CI	RI	Model (AIC)	lnL (Harmonic Mean)
18S	13	1743	70	4.02	10000	84	0.905	0.925	HKY+G	-3139.90
16S	8	305	47	15.41	2	100	0.660	0.547	GTR+I	-1180.38
H3	5	344	-	-	-	-	-	-	Did not test	N/A
ALL DATA	15	2394	152	6.35	3655	274	0.737	0.699	GTR+I+G	-5583.14

6.3.3. Molecular dating

The Majority rule Bayesian chronogram obtained for the first analysis (Figure 6.4), which used Baetiscoidea representatives and Oligoneuriidae only, indicates that the only Asian species represented, *P. wouterae*, is the oldest of the species investigated (lineage originating 131.49 Ma, with posterior probability support of 0.98). The next oldest branch groups an African and Madagascan species of similar age (111.22 Ma, although the support for this is not strong). A second African species and the Australian species *P. pearsonorum* have minimum time to most recent common ancestor at 71.99 Ma and 42.55 Ma respectively. These results indicate that both *P. pennigerum* and *P. crassi* are relatively young species, no more than about 2.5 million years old.

Prior to the onset of the broader analyses based on a wider selection of taxa, a model chronogram was hypothesized (Figure 6.5) based on fossil ages and Ogden *et al*'s (2009) results. The second set of analyses, using a dragonfly as the outgroup, and constraining the nodes with various different fossils, produced the chronograms seen in Figures 6.6-6.9. Figure 6.10 summarises the relationships and range of ages resulting from the BEAST chronograms (Figures 6.6 -6.9), at family level, while Figure 6.11 summarises these relationships at species level.

6.4. Discussion

There was no resolution between *Prosopistoma* species and *Baetisca* species with the 18S alignment (Figure 6.1), showing that this is not a suitable gene for species-level differentiation, which was expected due to the conserved nature of the 18S gene. With the 16S alignment (Figure 6.2), no distinction was shown between *P. pennigerum* from Europe

collected in different geographic regions (Russia and Spain). Furthermore, in the consensus *P. pennigerum* could not be distinguished from *P. crassi* or a second species from southern Africa which is morphologically similar to *P. crassi,* but was questionably a second species based on morphology. As *P. pennigerum* and *P. crassi* are clearly morphologically distinctive and are widely geographically separated, this seems to be a poor reflection of relationship between these species. The lack of differentiation in 16S rRNA would suggest recent gene flow but a second mtDNA marker would be needed to confirm this. The low mtDNA divergence indicates that these "species" can interbreed and shows widespread geneflow, but this is impossible due to geographic distance. Perhaps there is something in the 16S gene that needs to be functionally conserved for *Prosopistoma* species.

The consensus tree from the combined genes (Figure 6.3) reflects further problems as the genus *Baetisca* is paraphyletic in this phylogeny. *Baetisca* is widely accepted as a monophyletic group based on morphology (e.g. Pescador *et al*., 2009). This analysis shows that the genes used are less suitable for species differentiation. This should be used as a warning on the importance of choosing suitable genes for molecular phylogeny, using multiple genes, and preferably to choosing more rapidly evolving genes for species-level analysis. If CO1 genes had successfully been sequenced, a fundamentally different phylogeny may have resulted. Due to the shortage of suitable material, this could not be further pursued at this moment.

When trying to constrain all the possible nodes simultaneously with the relevant fossil date for each node, it was found that ages became unrealistic (for example, in one analysis, the outer node gave a date of over 800 Ma). It was found best if dating was restricted to one lineage per analysis, as confidence intervals were too large when using multiple node constraints, possibly due to an accumulation of errors due to any given fossil date having its own error margin, or with one or more of the fossil dates conflicting and thereby biasing the analysis.

The relative ages of the *Prosopistoma* species gives a different biogeographical scenario to that envisaged from the purely morphological studies (Barber-James, 2009). Instead of an African-centred Gondwanan vicariant dispersal, it would seem that the modern lineages may have dispersed from Asia in two directions, reaching Australia and Africa. The relatively young age of the European species confounds this argument; perhaps this area was recolonised from Africa following the Pleistocene glaciations; this may provide a way to explain the genetic similarity between *P. crassi* and *P. pennigerum*. This would have to assume though that the *P. crassi* - *P. pennigerum* ancestor was much more

widely spread across Africa than P. *crassi* is currently known to be. This is discussed further in Chapter 8.

Another outcome of this research was that the Oligoneuriidae were not a sister group to Baetiscidae and Prosopistomatidae (as suggested in one of the constructions shown by Ogden *et al*. (2009). Surprisingly, Baetiscidae and Prosopistomatidae also do not show sister group relationships. Thus the relationships between groups are still far from resolved, and further research is needed to improve these concepts. The results of this research must be seen as preliminary, with further sequencing being carried out if fresh material can be acquired. Suitable primers for CO1 in Prosopistomatidae mayflies also need to be designed.

6.5. Conclusions

The results are informative despite the inherent limitations of studying such an elusive and rare taxonomic group. In answering the questions set out at the onset of this exercise, the following conclusions can be drawn.

(1) The genes used in this study do not show two clearly defined clades, as concluded in the nymphal and adult morphology-based phylogeny.

(2) There is no evidence for erecting a second genus, which the morphological evidence would strongly suggest.

(3) It seems that there is only one species represented across Europe.

(4) The ancestral stem-group of the Prosopistomatidae seems to be some 243 -159 million years old, and the crown-group *Prosopistoma* species some 89 to 57 Ma.

(5) Asian species are older than the African taxa.

(6) The age of West African species could not be determined as genes from those species did not amplify.

(7) It was not possible to link adult and nymphal material in Madagascan species, due to age the preserved adult material.

(8) Nymphs which looked outwardly different to *P. crassi* were shown to have similar genetic makeup, indicating that there is only one species, but with morphological variation. This was not one of the proposed new species.

This preliminary investigation of Prosopistomatidae genetic markers needs to be followed up using additional quickly evolving genes for more species, which would give

clearer distinction between recent species (if not mutationally saturated), and a larger sample size for each. 16S rRNA was the only relatively quickly evolving gene used successfully in these analyses. CO1 may have produced a clearer indication of crown-group species relationships than the genes for which results were obtained. It is a notoriously difficult gene to amplify consistently in mayflies, particularly if DNA quality is low (Michael Monaghan, Leibniz Institute for Freshwater Ecology, Berlin, Germany, pers. comm., 2010) and better primers need to be designed for this, which was beyond the scope of this thesis. Additional fresh material is needed, and further work needs to be done to find a way to amplify and sequence this gene for this mayfly family. The 18S gene is a more slowly evolving gene and is therefore a preferred gene for studies investigating higher level taxonomic resolution and for ancient lineages (Hoy, 2003). With the *Prosopistoma* species, the level of mutational saturation was too high to give useful species-level differentiation. The 16S genes gave a better level of resolution, but sequences were available from too few species for a comprehensive comparison. There are a number of discrepancies in the results which indicate caution before making hard and fast conclusions about the origin and dispersal of the extant *Prosopistoma* species. Certainly the molecular evidence as it is does not support the splitting of the family into two genera, which the morphological evidence strongly suggests. Before these conclusions are taken as definitive, it would be wise to investigate several other genes as was intended at the beginning of this study. While it would be ideal to collect more material and rerun all species with a variety of primers to find which produce the best results, it was deemed that this should be seen as future research and was beyond the scope of this thesis. A further unexpected outcome of these results is that they do not support the widely accepted sister relationship between the Prosopistomatidae and Baetiscidae. This also needs further investigation.

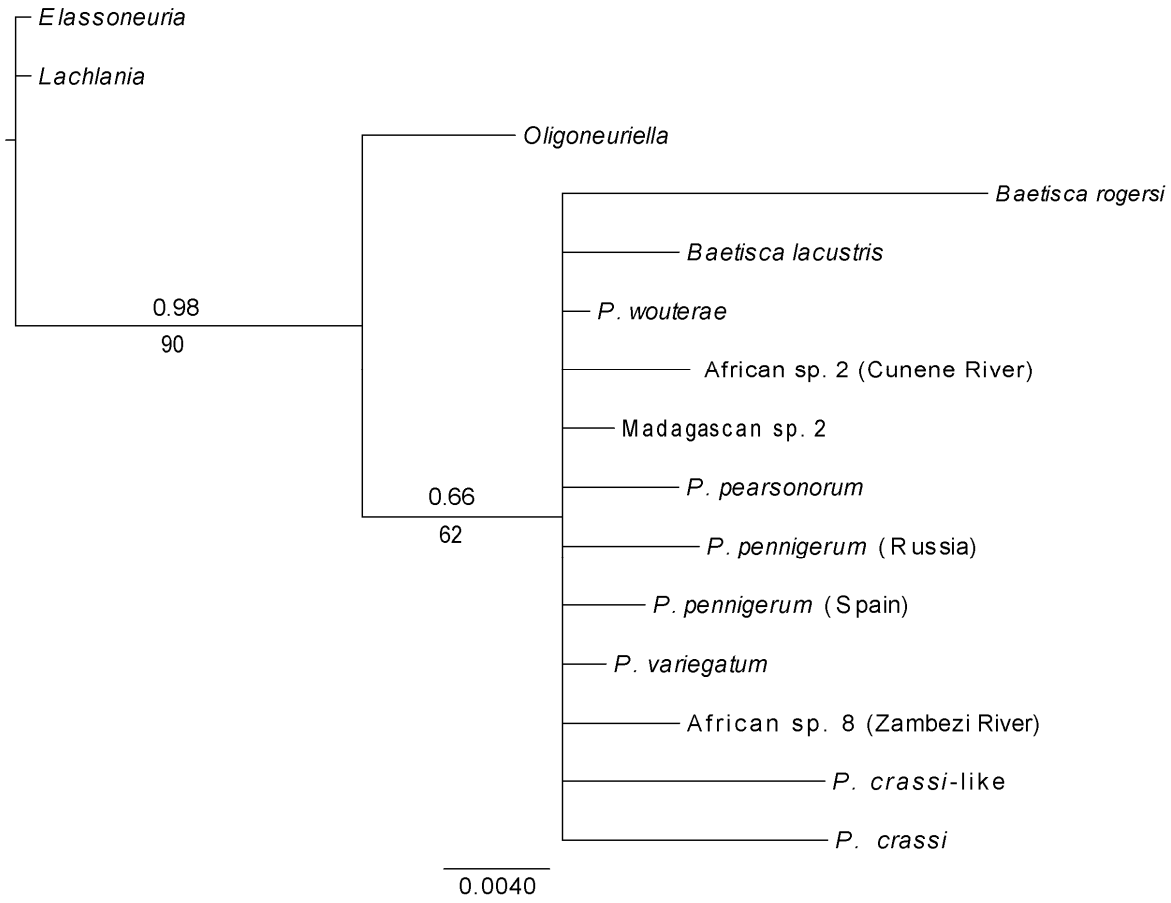


Figure 6.1. Majority rule Bayesian phylogram for 18S gene sequences, with posterior probability (above line) and Parsimony bootstrap value (below line). Using this gene, differences between the *Prosopistoma* species could not be distinguished. Scale bar represents substitutions per site.

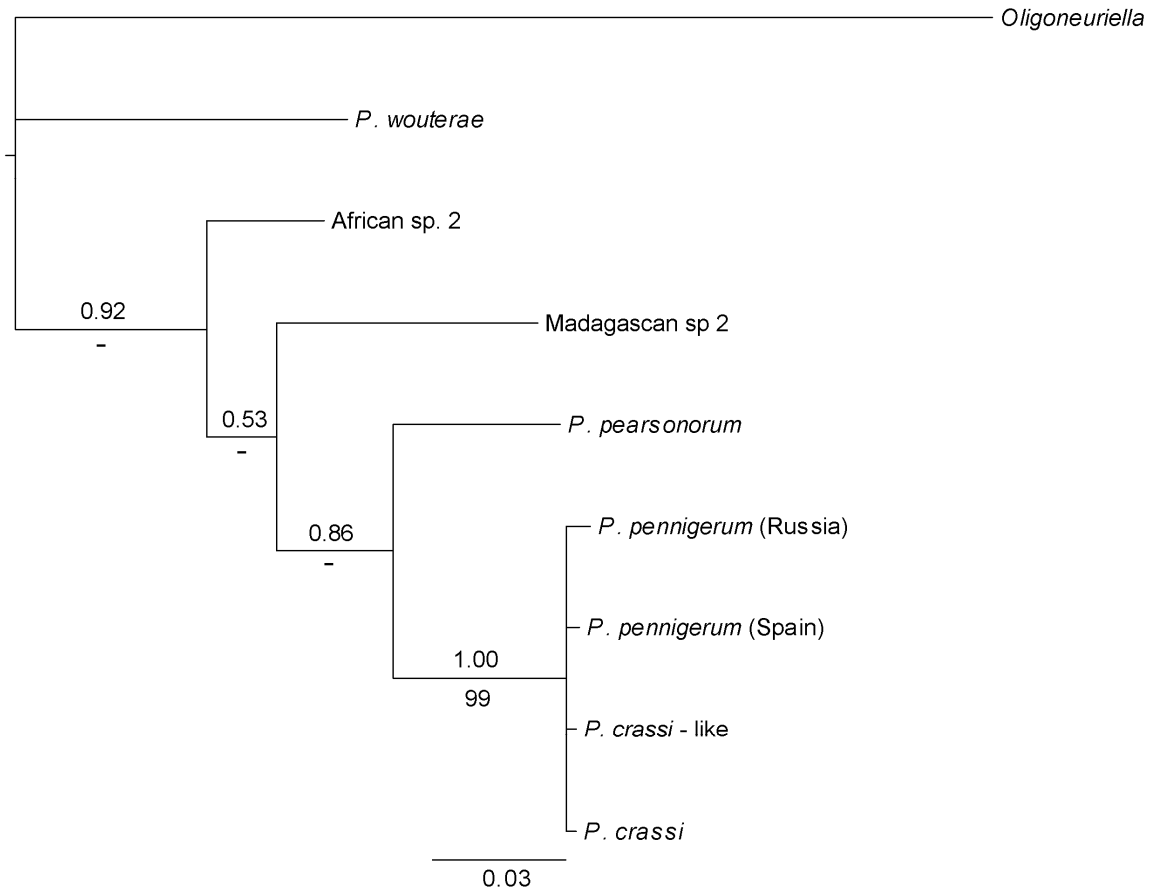


Figure 6.2. Majority rule Bayesian phylogram for 16S gene sequences, with posterior probability indicated above the line and parsimony bootstrap value below the line (support below 50% indicated by –). Scale bar represents substitutions per site.

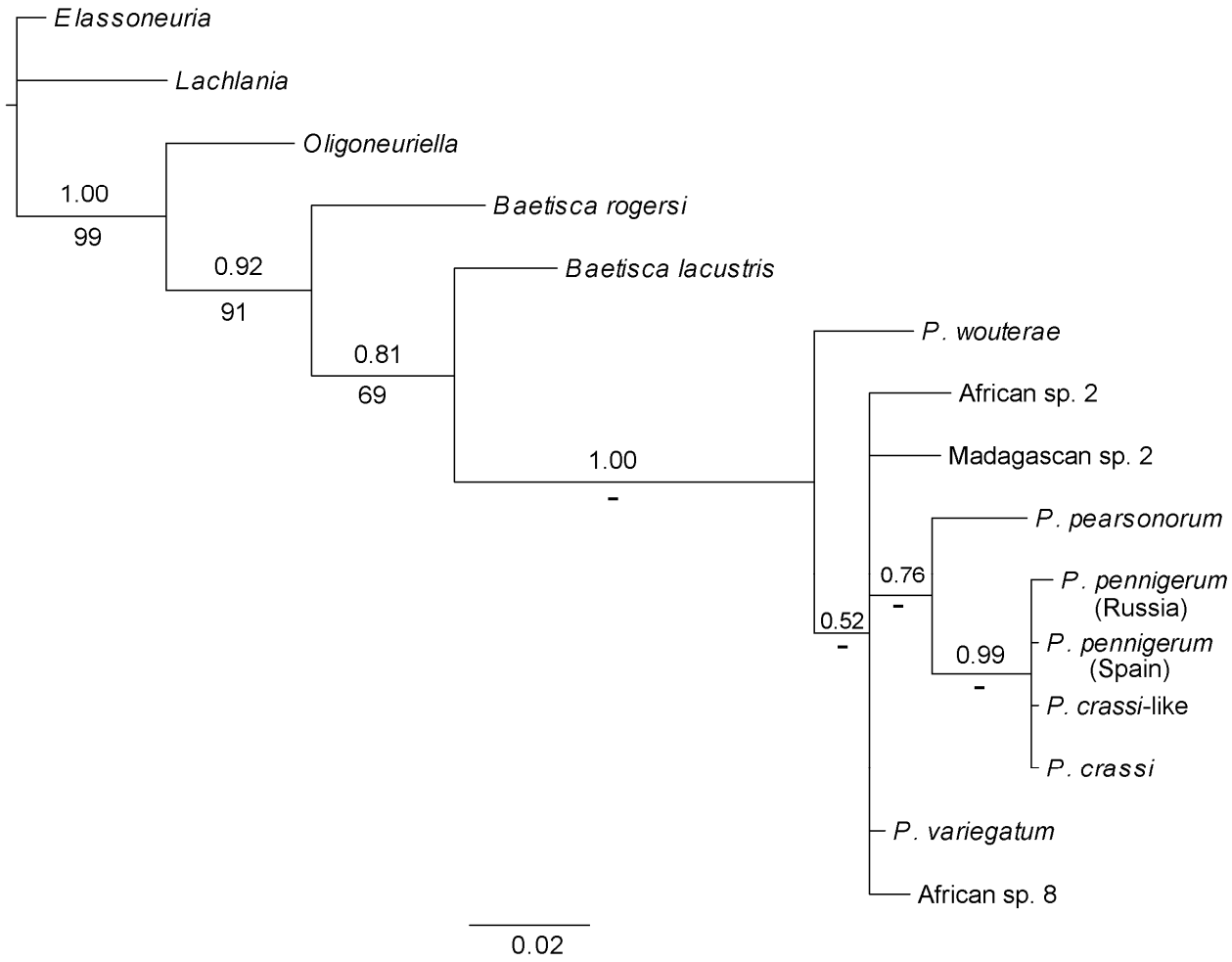


Figure 6.3. Majority rule Bayesian phylogram for combined 16S, 18S and histone 3 gene data, with Bayesian posterior probability indicated above the line and Parsimony bootstrap values below the line (support below 50% indicated by –). Scale bar represents substitutions per site.

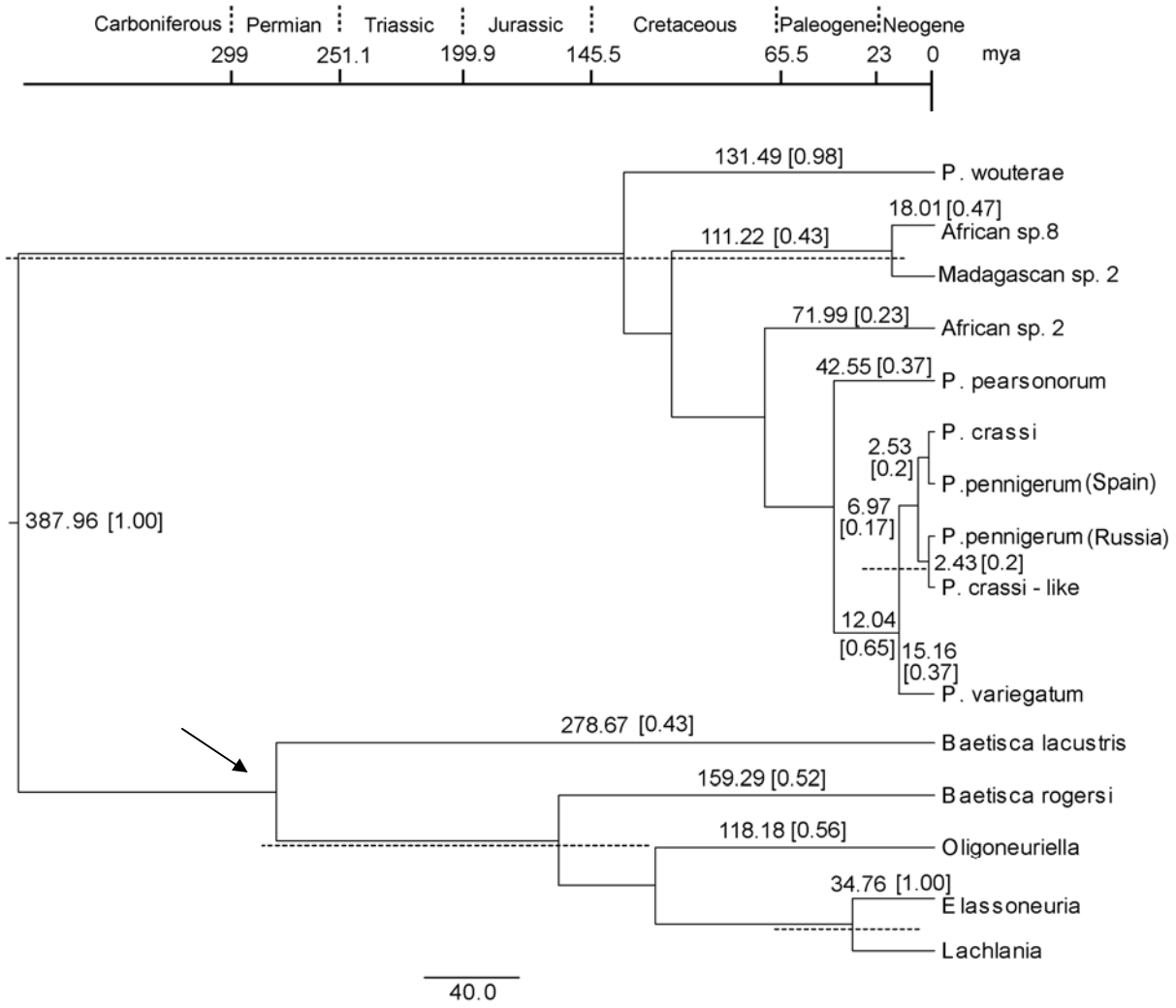


Figure 6.4. Majority rule Bayesian chronogram of Prosopistomatidae species, with selected Baetiscidae and Oligoneuriidae as the outgroup. Numbers on branches indicate estimated ages in Ma, with posterior probability support [ ]. 95 % HDP (highest posterior density) are indicated by dotted lines. Node constrained (arrowed) using 112-125 Ma Baetiscoid fossil dates.

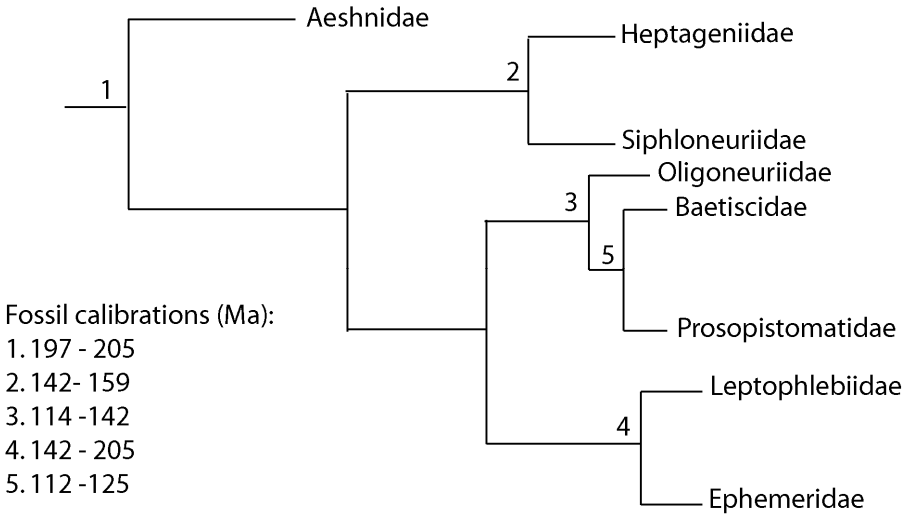


Figure 6.5. Hypothetical relationships and dates between major lineages as predicted based on Ogden *et al.*'s 2009 phylogeny, showing fossil contraints, used in the BEAST analyses, as a minimum age for each node. The odonate family Aeshnidae was chosen as the outgroup.

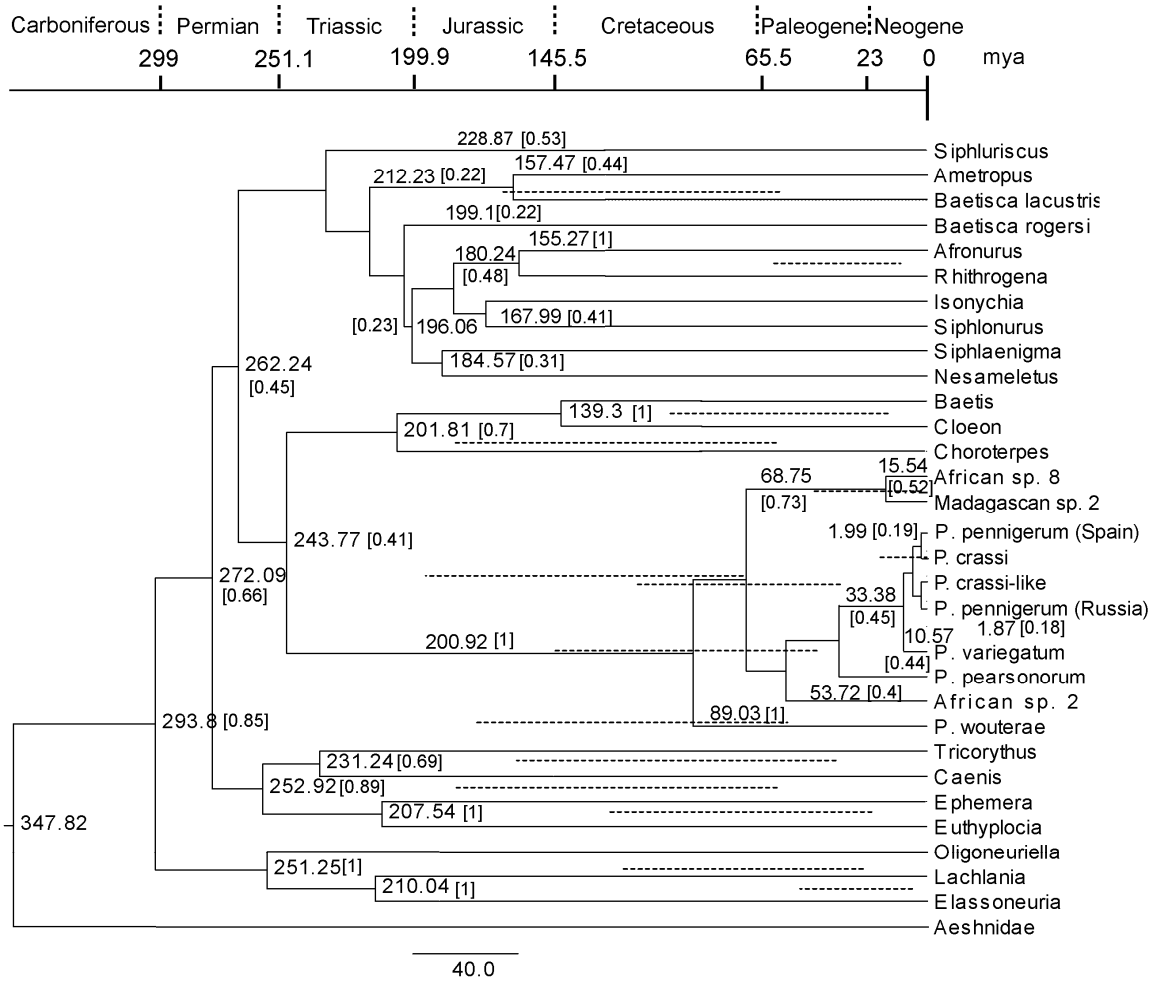


Figure 6.6. Majority rule Bayesian chronogram, with dragonfly outgroup (*Oplonaeschna* sp.). Numbers on branches indicate estimated ages in Ma, with posterior probability support [ ]. 95 % HDP (highest posterior density) are indicated by dotted lines. Nodes calibrated with 197-205 Ma Aeshnidae fossil.

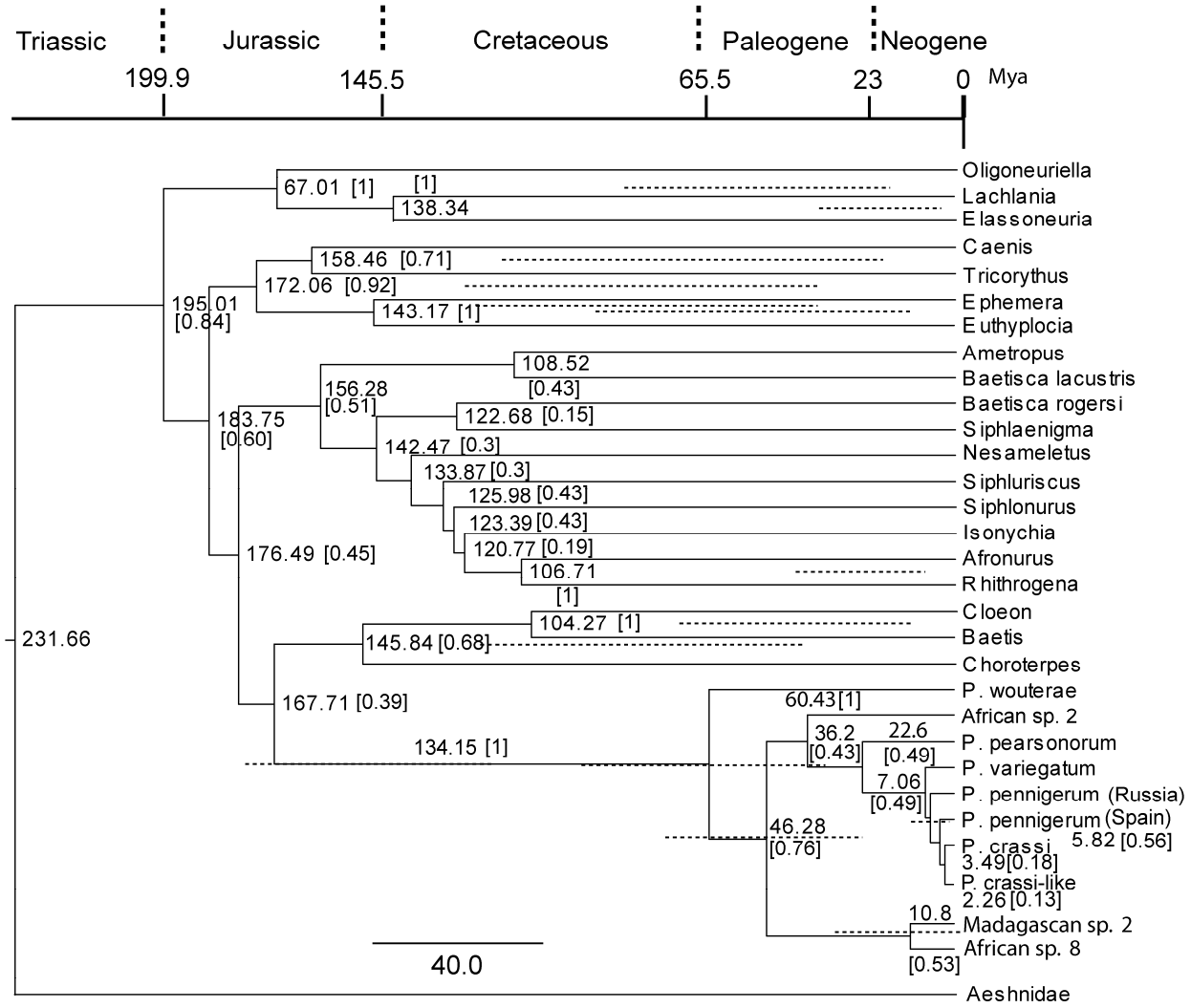


Figure 6.7. Majority rule Bayesian chronogram, with dragonfly outgroup (*Oplonaeschna* sp.). Numbers on branches indicate estimated ages in Ma, with posterior probability support [ ]. 95 % HDP (highest posterior density) are indicated by dotted lines. Nodes calibrated with 114-142 Ma Oligoneuriidae fossil.

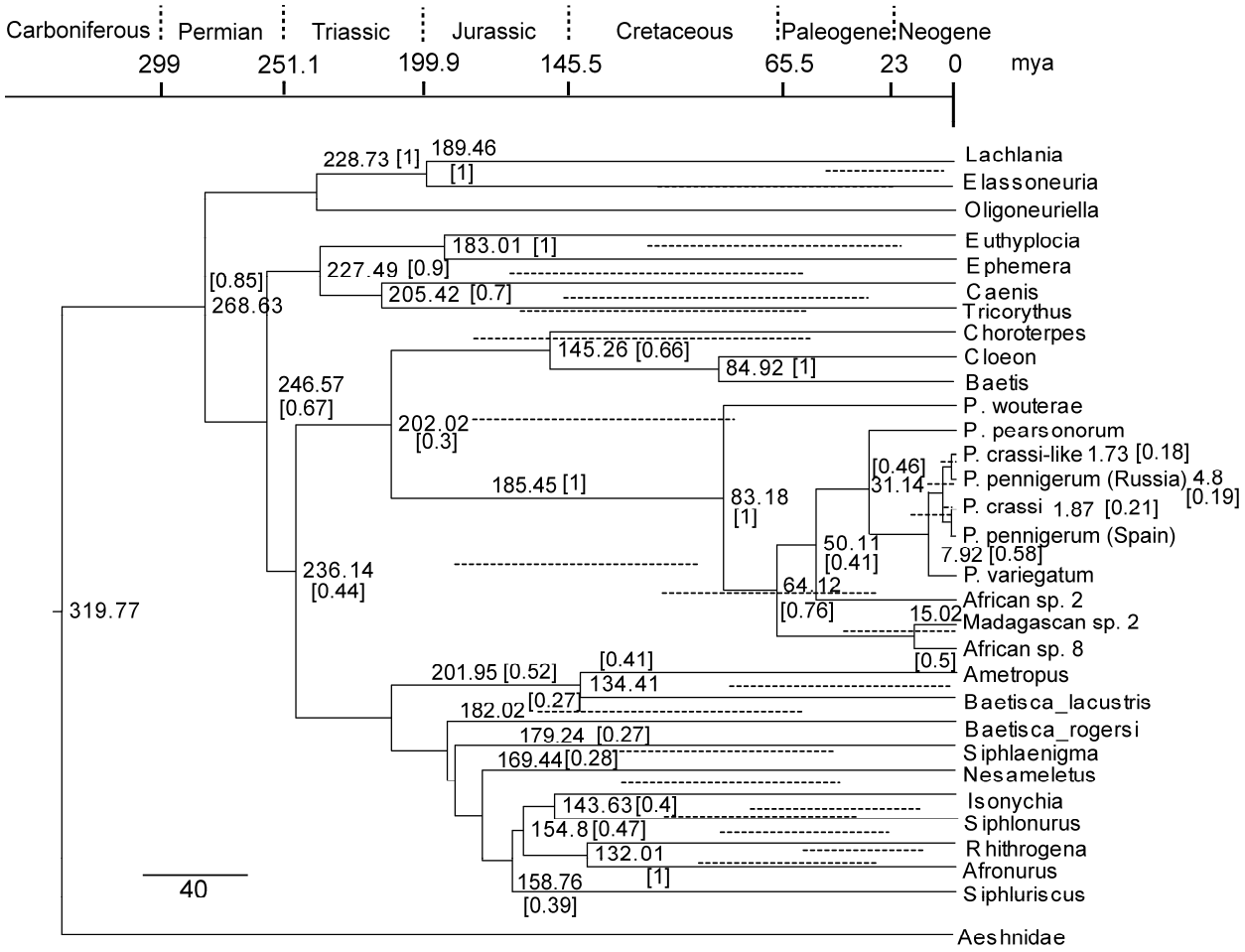


Figure 6.8. Majority rule Bayesian chronogram, with dragonfly outgroup (*Oplonaeschna* sp.). Numbers on branches indicate estimated ages in Ma, with posterior probability support [ ]. 95 % HDP (highest posterior density) are indicated by dotted lines. Nodes calibrated with 142-205 Ma Leptophlebiidae fossil.

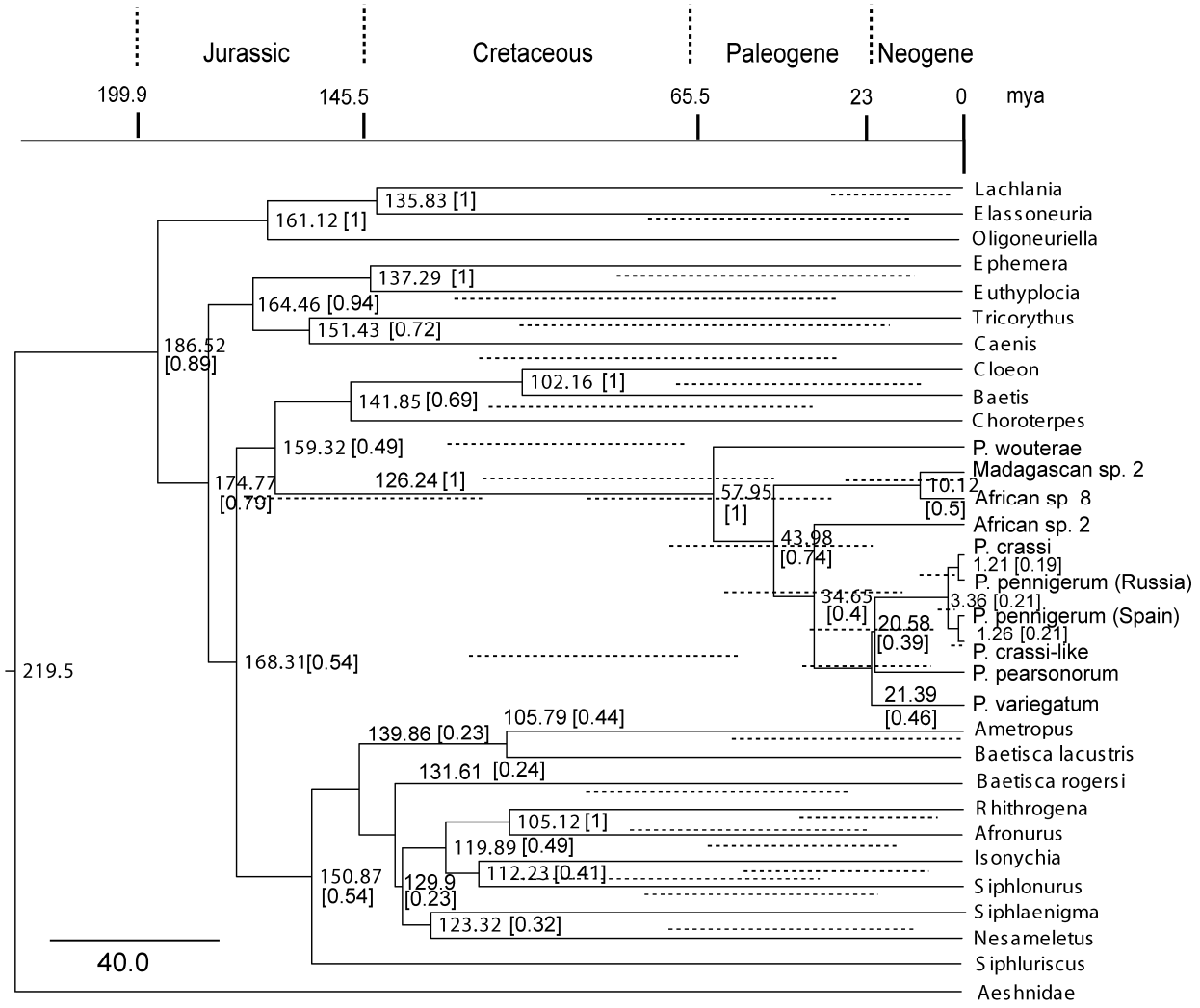


Figure 6.9. Majority rule Bayesian chronogram, with dragonfly outgroup (*Oplonaeschna* sp.). Numbers on branches indicate estimated ages in Ma, with posterior probability support [ ]. 95 % HDP (highest posterior density) are indicated by dotted lines. Nodes calibrated with 112-125 Ma Baetiscidae fossil.

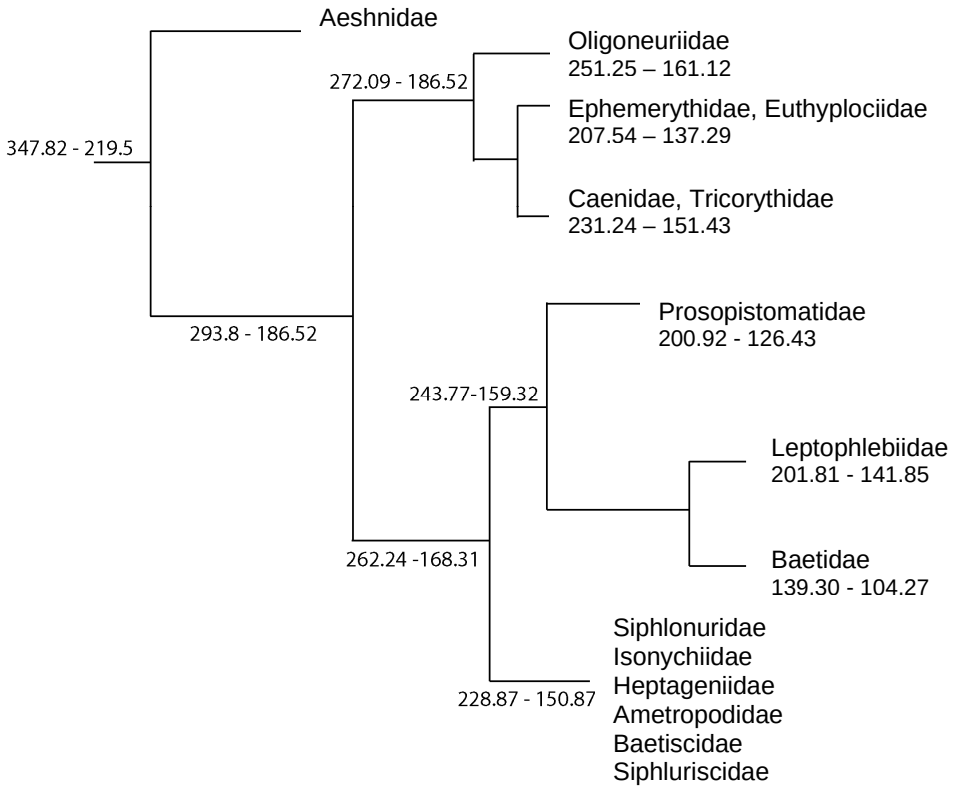


Figure 6.10. Summary of observed family relationships, showing range of dates obtained for stem group branching (compare with hypothesized scenario in Figure 6.5).

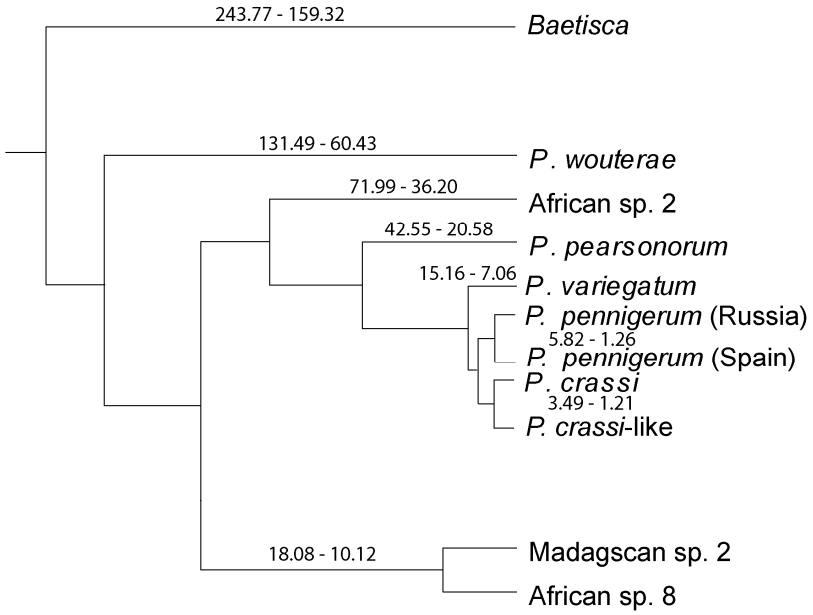


Figure 6.11. Summary of observed species level relationships, showing range of dates obtained from BEAST analyses based on different fossils node constraints.

Chapter 7

The palaeo-history of the Ephemeroptera, and proposed origin of stem-group Prosopistomatidae based on fossil evidence

"Present distributions, even though complex, are but the surface of a pattern that extends millions of years into the past, and there is no justification for assuming a uniformitarian principle concerning it." Ball, 1975

Synopsis

This chapter investigates the Ephemeroptera, and the Baetiscoidea in particular (Prosopistomatidae Lameer 1917 and their Nearctic relative, the Baetiscidae Ulmer 1933), in the context of other primitive insects known from the fossil record, many of which became extinct at the end of the Paleozoic and during the Mesozoic. Although this chapter is largely a synthesis of fossil literature, the analysis of this information implies that the Baetiscoidea may have separated from the main ephemeropteran stem-group lineage much further back in evolutionary history than currently thought. A faint trace of primitive archedicyton, evident in the wings of Prosopistomatidae, (Chapter 2, Figure 2.19) is recorded in this family for the first time. This is one of several plesiomorphic characters retained in the group, supporting their primitive origins. Comparisons are drawn between wing venation characters in selected examples of recent mayfly families and some of the extinct stem-group lineages. Parsimony analysis of these characters supports the hypothesis of earlier branching of the Posteritorna (Baetiscoidea) lineage from the main lineage leading to other extant mayfly families. Ages of lineages are given as millions of years ago (Ma).

7.1 Introduction

Before further investigating the biogeography of the extant Prosopistomatidae (i.e. the crown-group species – Chapter 8), it is useful to see this lineage in context relative to other extant mayfly lineages and their ancestors, thereby providing an understanding of the order and its roots as a whole (i.e. investigate the relationships with stem-group

taxa). The Order Ephemeroptera is an extant ancient insect lineage, the fossil record showing that it dates back to the Carboniferous and Early Permian periods circa 290 Ma (Tshernova, 1965; Kukalová, 1968; Sinitchenkova, 1984; Kukalová-Peck, 1985; Grimaldi and Engel, 2005). This estimated time of origin is well supported by the dated molecular phylogeny (Chapter 6, Figure 6.10), where several different fossils, including a dragonfly fossil from the Upper Triassic, were used to estimate the ages of lineage diversion. The Ephemeroptera is one of about 12 orders of insects that appear in the fossil record during the mid-Carboniferous around the Mississipian-Pennsylvanian Epoch boundary some 320 Ma (Ogg *et al*., 2008). Some of the other insect orders that appear include the Protodonata, Diaphanopterodea, Palaeodictyoptera, Megasecoptera (all palaeopterous), as well as neopterous representatives Archaeorthoptera, and Blattodea. Recent discoveries at Hagen-Vorhalle in western Germany (Brauckmann and Herd, 2007) indicate that this diversification was sudden, starting in the Early Carboniferous.

It is important at this point to mention that "Palaeoptera" in this chapter refers to the group of insects that cannot fold their wings, recognizing that many authors consider it to be paraphyletic (see Appendix Table A7.1). The term Palaeoptera was introduced by Martynov (1923) to include the Ephemeroptera, Odonata and extinct relatives known from the fossil record which hold their wings vertically above the thorax (Figure 7.1a), or outstretched on either side of their body. They lack a flexor muscle, which, in the Neoptera, pulls the third axillary sclerite causing the posterior part of the wing to swing rearwards and fold to lie flat over the body (Martynov, 1925). The Palaeoptera (literally, primitive wings) were considered the sister group of all other extant primarily winged orders, the Neoptera (new wings). This concept continues to be supported by some authors, but the monophyly of this group has been contested by many others (Appendix Table A7.1). More recently, the Ephemeroptera *per se* have been considered to be the sister group of Odonata and Neoptera (e.g. Kristensen, 1991; Soldán, 1997; Whiting *et al.*, 1997; Fürst von Lieven, 2000; Staniczek, 2000; Wheeler *et al.*, 2001; Ogden and Whiting, 2003; Grimaldi and Engel, 2005). This is based on a number of morphological features unique to mayflies e.g. the costal brace, structure of the nymphal hypopharynx, presence of the subimaginal stage (e.g. Kristensen, 1975; Soldán, 1997), as well as evidence based on recent, DNA-based phylogeny (Whiting *et al*., 1997; Wheeler *et al*., 2001; Hovmöller *et al*., 2002; Zhang *et al*., 2008). Essentially this is a three body problem - Ephemeroptera, Odonata, and Neoptera - and every possible relationship has been proposed.

Ephemeroptera exhibit many primitive ancestral characteristics shared with several other extinct orders. They are considered the most ancient of extant pterygote insect lineages (e.g. Hamilton, 1972a; Edmunds and McCafferty, 1988; Brittain and Sartori, 2003; Barber-James *et al.*, 2008). These characters include the presence of two cerci and sometimes a median caudal filament at the posterior end of their abdomens in both the nymphal and adult stages (Figures 7.1a, b). Such caudal appendages are also found in the †Monura, Archaeognatha and Zygentoma, wingless relatives which are phylogenetically basal to the Ephemeroptera and Odonata (e.g. Terry, 2003; Gullan and Cranston, 2005). Further ancestral characters seen in Ephemeroptera include the palaeopteran feature of inability of the winged stages to fold their wings back horizontally over the abdomen (Figure 7.1a), and the presence of two winged stages (the subimago and sexually mature imago). This is thought to be a remnant of a life history pattern that was common in Paleozoic, now extinct insect lineages (Edmunds and McCafferty, 1988; Kukalová-Peck, 1978). Amongst extant insect orders, the subimago is unique to Ephemeroptera, and phylogenetically important as it is an autapomorphy that defines the Ephemeroptera as a clade distinct from other extant insect orders, grouping them with fossil orders. The presence of a costal brace at the base of the forewing (Figure 7.2a) is another key character linking mayflies and their stem groups, i.e. other extinct Paleozoic insects. Other defining characteristics of the Ephemeroptera include paired penes in the male imago, extended forelegs in the male imago occurring in all families except Prosopistomatidae (elaborated upon in the discussion of morphology, Chapter 2), and vestigial to absent mouthparts in the imagos.

Fossil evidence points to mayflies having attained their highest diversity during the Mesozoic, mainly during the Jurassic and Cretaceous Periods (e.g. Sinitshenkova, 1984, 1985, 1986; McCafferty, 1990; Brittain and Sartori, 2003), which covers the time span 199.6 – 65.5 Ma (Ogg, 2009), a period covering some 131.1 million years. Dramatic extinction events such as the Cretaceous-Paleogene (K-P) mass extinction (65.5 Ma) had a profound effect on modern mayfly diversity and distribution; McCafferty (1990, 1991) noted that the Mesozoic mayfly fauna was considerably different compared to the Cenozoic fauna, following the K-P extinctions. The taxa present today represent the surviving branches of continually evolving mayflies, and many of the stem group taxa, known from fossils and representing older lineages or ancestral stock, have disappeared. Although background extinction takes place more or

less constantly (Raup, 1994; Labandeira, 2005), past mass extinction events have had notable effects on the composition of the mayfly families and genera known today.

Looking further back in time, an earlier and much more extreme extinction event, the Permian-Triassic (P-T) event occurred some 251 Ma (e.g. Kaiho *et al*., 2001). Based on fossil evidence of selected insect groups, some authors have noted that Permian (Paleozoic) and Triassic (Mesozoic) entomofaunas are considerably different on either side of the P-T boundary (e.g. Labandeira and Sepkoski, 1993; Béthoux *et al.*, 2005; Labandiera, 2005). The existence of mayfly-like fossils in Permian rocks has been well documented e.g. Kukalová (1968), Carpenter (1933, 1939, 1963, 1980), Demoulin (1970), Handlirsch (1919, 1922), Sellards (1907), Martynov (1928, 1932, 1938). Even earlier stem-group fossils are recorded from the Carboniferous (Brongniart, 1893; Lameere, 1917a,b; Martynov, 1923; Tillyard, 1932; Carpenter, 1963). These stem-group relatives of the mayflies are considerably different from modern mayflies in that the developing wing pads of the nymphs are not sheathed as in modern mayflies, and do not lie flat against the body as seen today (Kukalová, 1968), and the fore and hind wings of the adults are equal in size (homonomous). The Syntonopteroidea, including Syntonopteridae (e.g. *Lithoneuria*) and Bojophlebiidae were Carboniferous relatives of the Ephemeroptera, probably extinct before the end of the Permian (Handlirsch, 1911, Kukalová-Peck, 1985), while families in the Protereismatoidea e.g. Protereismatidae (including Jarmilidae and Oborophlebiidae), Misthodotidae (including Eudoteridae) and Palingeniopsidae (*sensu* Carpenter, 1980) were extinct by the end of the Permian.

7.2 Methods

Insect wing venation homology and articulation has been the subject of much debate for nearly a century (e.g. Lameere, 1917; Martynov, 1931, 1938; Morgan, 1912; Needham, 1935a,b; Tillyard, 1919, 1923, 1932; Kukalová-Peck, 1983, 1985; Kukalová-Peck *et al.*, 2009; Willmann, 1999). Certain wing veins may provide phylogenetic evidence of relationship between extant and fossil stem-groups, e.g. the relative positions of CuA and CuP, triadic arrangement of MA, or the origin of RA relative to RP (Edmunds and Traver, 1954b; Kukalová-Peck, 1985).

To investigate the relationships between the fossil insect forms and their recent successors, a literature survey was undertaken to assess which salient characters would be useful to estimate phylogenetic relationships. As fossil insects are most frequently

represented by wing imprints, wing characters, especially venation, comprised most of the characters selected from representatives of different groups. Characters were coded for parsimony analysis performed in TNT (Goloboff *et al*. 2008). The following characters were deemed to be of particular significance: presence or absence of costal brace; wings homononous or heteronomous; position of veins CuA and CuP relative to the tornus; triadic branching of RP, M and Cu; where bases of RA and RP fuse; presence or absence of an archedictyon. These are discussed further in section 7.4 which provides background to each character.

Taxa investigated included the enigmatic †*Triplosoba* Brongniart and †*Lithoneura* Carpenter, whose taxonomic placement is not readily agreed upon; a member of the Palaeodictyoptera (*Stenodictya* Brongniart); two mayfly like-creatures which had costal braces but with homonomous wings, the Permian †*Protereisma* Sellards and †*Misthodotes* Sellards, the Triassic †*Litophlebia* (Riek), which existed some 60 Ma later (Figure 7.6), and the †Hexagenitidae Lameer, a Jurassic fossil lineage with relatively large hindwings (Tshernova, 1962; Demoulin, 1971), but characters otherwise typical of modern Anteritorna.

Two approaches were taken in the choice of outgroup. Firstly, a hypothetical outgroup was used, where each character was considered to be plesiomorphic, and secondly one of the ancient taxa, †*Triplosoba*, was used as it has many underived characters. †*Lithoneura* could also have been designated as outgroup for the same reason.

7.3 Results

Appendix Table 7.2 summarises the characters used for the parsimony analysis, and their states, which are ordred as shown in the table. Four shortest trees, using an all-primitive character hypothetical outgroup (tree length=13, CI=62; RI=70); the majority rule consensus of these trees is shown in Figure 7.4a. Using †*Triplosoba* as outgroup, three shortest trees were produced (tree length=13, CI=62, RI=68). The majority rule consensus tree is shown in Figure 7.4b.

The Posteritorna consistently came out as sister to the Anteritorna plus †Litophlebiidae and †Hexagenitidae. This clearly shows that both the South African †Litophleiidae and Asian-European †Hexagenitidae are members of the Anteritorna clade, and supports the early splitting of Posteritorna and Anteritorna. The clade

comprising Posteritorna, Anteritorna plus †Litophlebiidae can be seen as the "true" Ephemeroptera.

7.4 Discussion of primitive characters

All of the groups considered in this analysis were paleopterous, and share some characters with extant Ephemeroptera. The †Palaeodictyopteroidea (including †Palaeodictyoptera, †Permothemistida, †Megasecoptera, †Diaphanopteroidea and (perhaps) the near dipterous †Permothemistida) are an extinct lineage of insects present during the Permian, now thought to have no modern descendants (Grimaldi and Engel, 2005). The paleopterous condition of their wings was the principal reason for the placement of the †Palaeodictyopteroidea along with Ephemeroptera and Odonata in the Palaeoptera by many authors (e.g. Riek, 1970). They became extinct by the end of the Permian, and Carpenter (1980) suggests that they were already receding even by the early Permian, as the members of the order are only sparsely represented in Permian deposits. The †Diaphanopteroidea were an exception in the †Paleodictyoptera, having independently acquired the ability to fold their wings flat over their backs (Hamilton, 1972c). The †Palaeodictyopteroidea had little else in common with Ephemeroptera; there was no costal brace or triadic branching of wing veins (Carpenter 1980) (characters autapomorphic for the Ephemeroptera); unlike the mayflies, the palaedictyopteran meso- and metathoracic wings were similar (homonomous), often patterned (e.g. Carpenter 1964), the prothorax had wing-like, non-articulating paranotal lobes (e.g. Carpenter, 1971; Kukalová-Peck, 1978). They were also characterized by beak-like sucking mouthparts (Kukalová, 1970). Despite these apomorphies, the Paleodictyoptera shared certain symplesiomorphies with the Ephemeroptera, notably the three frontal ocelli (Jeannel, 1960), and filamentous caudal filaments, which were often covered with dense setae. They also had the archedictyon-like mesh between the main veins.

Further details of the characters used for the phylogenetic analysis follow.

7.4.1 Costal brace

This is a thickened veinlet connecting C to RA at the base of the wings of modern Ephemeroptera and their stem-group ancestors, and is autapomorphic for the order (Kukalova-Peck 1974; Gullan and Cranston, 2005; Grimaldi and Engel, 2005).

7.4.2 Homonomous or heternonomous wings, and position of the tornus

Stem-group mayflies and their sister lineages had homonomous wings (Figures 7.5a, 7.6a-c), while recent mayflies tend to have their hindwings considerably reduced in size in comparison to the forewing. Hindwings may even be absent in some families, e.g. the Caenidae (Figure 7.5c). With this reduction in hindwing size, the shape of the forewing has changed to accommodate the hindwing, with the formation of an acute angle along the basal margin of the forewing, the tornus (Figure 7.5b). The two wings fit together to provide an almost uniform surface area, which Riek (1976) suggested has developed as a mechanism to maximize surface area for flight. In some cases, when the hindwing is completely lost, the tornus is lost and the basal hind-margin of the forewing is expanded, increasing the cubital-anal area as a replacement for the hind wing (e.g. Figure 7.5c). In all recent mayflies, with the exception of the Prosopistomatidae and Baetiscidae, the tornus is situated between CuA and CuP, which are widely separated. In these two exceptional families, the tornus is situated behind the apex of CuP (hence the introduction of the terms Posteritorna and Anteritorna by Kluge (1998) (see Chapter 1, pp. 7-8). In the examples given in Figure 7.6, many of the older lineages (Carboniferous and Permian) have the veins CuA and CuP lying more or less parallel to each other (Figures 7.6a, b, d), similar to the condition seen in *Prosopistoma* and *Baetisca* (Posteritorna) (Figures 7.6 f, g). However, many fossils which occurred between the Triassic and Jurassic boundaries have the same placement of tornus relative to CuA and CuP as seen in most other extant mayfly families. These include the †Mesephemeridae Lameere 1917c, †Mesoplectopteridae Demoulin 1955, †Mesonetidae Tshernova 1969, and †Hexagenitidae Lameere 1917, (e.g. Demoulin, 1955; Tshernova, 1969; Riek, 1976; Kukalová-Peck, 1985; McCafferty, 1990; Grimaldi and Engel, 2005; Sinitshenkova, 2002; Zhang, 2005), probably representing a lineage which developed after the Permian-Triassic (P-T) extinction event, sharing common ancestry with the modern Ephemeroptera (Figure 7.3). This point is important in the development of the argument pertaining to the evolution of the Baetiscoidea.

The oldest known stem-group baetiscoid fossil is known from the Crato fossil bed in Brazil (Cretaceous, late Aptian, 117-94 Ma) (Staniczek, 2007), but based only on the relative positions of CuA and CuP to the tornus, the ancestry of the group is seen to likely be older.

7.4.3 Triadic branching of veins and relative stem positions of RA and RP

Prosopistomatid wing venation, with its great reduction of veins, appears to share several of the primitive conditions noted in some of the stem-group ephemeropteran taxa and related lineages, e.g. RA is independent from RP (as seen in †*Lithoneura* (†Syntonopteridae) (Carpenter, 1938; Willman, 1999). Prosopistomatidae also do not have triadic branching of MA (although it cannot be excluded that this may be a result of secondary reduction); all other recent mayflies have triadic branching of MA. This condition is absent in some of the early insects, e.g. in the Carboniferous †*Triplosoba* (Figure 7.5a). †*Triplosoba* also has RP fused near the wingbase (Willmann, 1999), a condition also seen in *Prosopistoma*. The question arises of whether the venation seen in the Prosopistomatidae is a secondary reduction, or represents a reversion to an ancestral state. *Baetisca* does have triadic branching (Figure 7.6g), so, based on their putative sister relationship, this condition is taken for the phylogenetic analysis as representing the group, and *Prosopistoma'*s venation is taken as representing reduction This also follows Dollo's rule that evolution rarely reverts to an earlier specialized form (Farris, 1977).

7.4.4 Archedictyon

Archedictyon (a term apparently coined by Brongniart, 1854), is considered a primitive condition (e.g. Martynov, 1924b; Kukalová, 1970), a shared synapomorphic character between several ancient lineages (Appendix Table A7.2), and therefore phylogenetically important. How does this relate to the Prosopistomatidae, a family with considerably derived wing venation (see Chapter 2)? An archedictyon forms a reticulate, irregular network of polygonal areas between the veins, superseded by proper cross veins in more derived families. Several extant mayfly families (e.g. Ephemeridae, Polymitarcyidae (Figure 7.7c,d), and Leptophlebiidae) have a similar looking archedictyon-like mesh between their main veins. Needham (1935) described the archedictyon as consisting of ridges of chitin of hypodermal origin, which formed between the trachea (source of main veins) as the wings expand. These are not true cross veins, which are formed as chitin thickens around what were originally tracheoles. Hamilton (1972b,c) described six basic types of cross venation, with archedictyon considered the ancestral condition, due its prevalence in wings and notal lobes of early fossil insects. This consists of "an irregular meshwork of generally pentagonal cells between the much heavier veins and intercalaries". He did not mention its presence in modern mayflies, though noted that the condition is seen in Odonata. Although this condition is apparently seen in some

Ephemeroptera, closer examinations (Figure 2.7c,d) shows that the multiple cross veins seen in some ephemeropteran families is more like Hamilton's polyneurous condition, where the main veins are only slightly heavier than the crossveins, which are more widely spaced than the crossveins of the true archedictyon, and are usually parallel (Figure 7.7 c,d). This indicates that a true archedictyon *per se* is not present in modern mayflies. An archedictyon was common in primitive insects, but became simplified over time in more recently derived insects into a series of cross veins, although both tracheated crossveins and archedictyal crossveins can occur together (e.g. as seen in Odonata). In many groups, crossveins have become further reduced. The Caenidae are a recent mayfly group providing an example of a lineage with extremely reduced crossveins, and the Prosopistomatidae even more so. However, on examination of Prosopistomatidae wings during these studies, a faint background reticulation has frequently been noticed, visible under phase contrast lighting conditions (Chapter 2, Figure 2.19a,b) (not seen in Caenidae). This is thought to be a trace of the old archedictyon, which is no longer generally expressed in the Ephemeroptera, but a character which has been preserved in ontogenetic memory over tens of millions of years and somehow switched on again. The power of ontogenetic memory and wing development is exemplified by recent research on other insects. Whiting *et al.* (2003) showed that in some stick insect species, wings derived secondarily from non-winged forms. Fig wasps are known to be able to alternate between winged males or non-winged males within one species (Cook *et al*., 1997; Wang *et al*., 2010)

The fact that archedictyon-type mesh venation has been preserved in some non-palaeopterous orders, e.g. the Neuroptera (Figure 7.7 e-h), and Hemiptera, e.g. some Cicadidae, indicates that this condition has persisted and been expressed in some lineages for millions of years. As mentioned earlier, the main veins in insect wings are considered to be the remnant of a tracheal system (e.g. Needham, 1935), with veins developing around the trachea. Thus, the original function was for oxygen transportation, not wing strengthening. The wing venation seen in each insect lineage has evolved over time due to vein reductions, but the original venation was co-opted for a function different to the original; this is an example of exaptation *sensu* Gould and Vrba (1982). Crossveins are considered to have progressively arisen from the mesh-like primitive archedictyon. The presence of this trace archedictyon in prosopistomatid wings may be of great importance in understanding the evolution of wing venation in the Prosopistomatidae, and the branching of this lineage from other mayflies. Have the

genes for this trait in Ephemeroptera been turned off for tens of millions of years, with the possibility of atavistically returning in a group with no cross veins? This is known in some Cicadidae (the Polyneurini) (Martin Villet, Rhodes University, pers. comm., 2010). Alternatively, perhaps it adds more evidence that the Prosopistomatidae lineage broke off from the stem-group mayfly early in the evolution of the Ephemeroptera. This is not shared with the Baetiscidae, which have abundant cross veins of the ordinary type.

7.5 Discussion of results, and conclusions

Edmunds and Traver (1954b) suggested that †Syntonopteridae may have been true mayflies, and that the †Paleodictyoptera were an off-shoot from the ancestral Ephemeroptera stem-group rather than ancestral themselves. The phylogenies (Figure 7.4a, b) do not place †Syntonopteridae with Ephemeroptera, but agree with both them and the †Paleodictyoptera being sister lineages rather than ancestral. Further, Edmunds and Traver (1954b) concluded that mayflies did not arise from the so-called Protephemeridea, represented by †*Triplosoba*. Rather, they proposed common ancestry for †*Triplosoba* and †Syntonopteridae, a lineage which became extinct. This is possible (Figure 7.4a), although Figure 7.4b does not support this.

The fundamental similarities in wing venation between the Permian †Misthodotidae to that of Baetiscidae gave Edmunds and Traver (1954b) cause to suggest that the Baetiscoidea arose from a †Misthodotidae-like ancestor, while most other mayfly lineages arose from a †Protereismatidae-like ancestor, and the burrowing mayflies arose from a †Palingeniopsidae-like ancestor. Riek (1973) also drew parallels between the wing venation of modern Baetiscidae with the Permian †Misthoditidae, implying, correctly, that the Baetiscidae have shared features with this primitive family. Riek further inferred that the †Misthoditidae may be been part of the stem group lineage from which Baetiscidae arose, and thus agreeing with Edmunds and Traver (1954b). The results shown in Figure 7.4 indicate that †Misthodotidae and Baetiscidae are more likely to have shared ancestry rather than direct descent from †Misthoditdae-like ancestors. The †Protereismatidae are also more likely to be a sister lineage rather than ancestral to modern mayfly lineages. The placement here of †*Lithophlebia* within the Ephemeroptera *s.s.* concurs with Rasnitsyn and Quicke (2002) and does not support Hubbard and Kukalová-Peck's (1980) placement of it with the Palaodictyoptera. However, the position of †Litophlebiidae is surprising in that, having CuA and CuP closely aligned and not separated by a tornus, it may have been expected that they would align with the

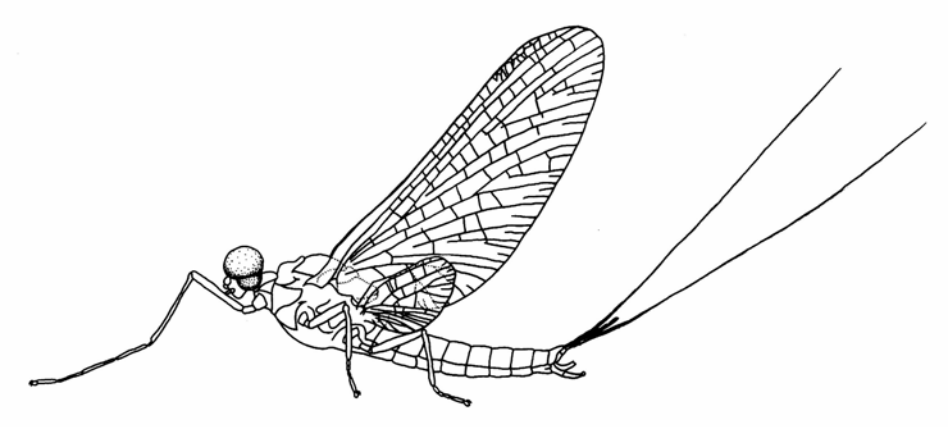
Baetoscoidea (Posteritorna) group. It could also have been expected for them to group with other groups that have no costal brace. This needs further investigation, but is beyond the scope of the aims set out here.

Another aspect of wing structure to consider is the arrangement of the sclerites at the base of the forewing (Figure 2.20). The three axillary sclerites at the wing base of Ephemeroptera are considered homologous to the first axillary, the second axillary and the third axillary of Neoptera (Willkommen and Hörnschemeyer, 2007). While the evolutionary significance of the placement of the sclerites in the different groups would undoubtedly shed further light in these relationships', it is very difficult to determine sclerite structure in fossil wings, and this is also beyond the scope of the present study.

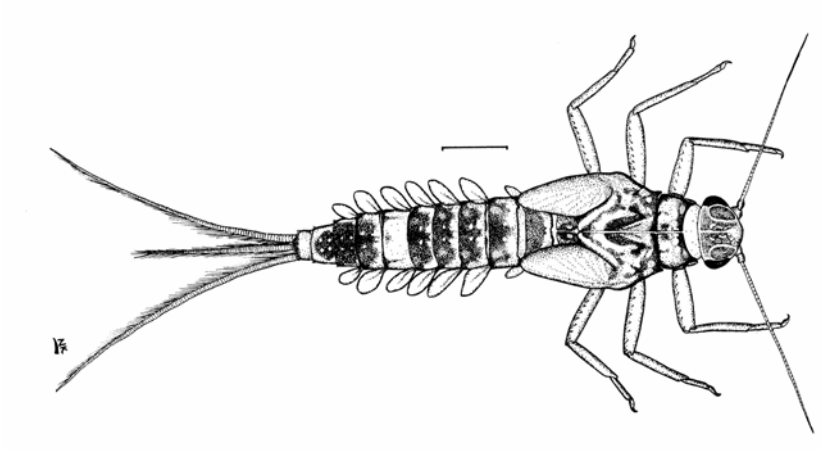
It is important to realize that because a lineage is geochronologically ancient, this does not necessarily mean that it has changed substantially from its ancestral state. Evolution does not work in a gradual progression of development, but typically, as stated in the punctuated equilibrium hypothesis of Eldredge and Gould (1972), Gould and Eldredge (1977), occurs in random bursts, some changes resulting in establishment of a successful lineage, others not. Some lineages, even if successful, may be eliminated by chance events such as global mass extinction, or regional extirpation of a remaining last population. Thus, the pathway of evolution of any group of living organisms is not a linear progression. Some of the fossils may be misplaced as a close relative when they are not part of a direct lineage. The placement of fossil insects within a higher classification is often difficult as only a few structures, usually only wings, are preserved, resulting in temporal snap-shots. Thus, for example, the contentious debate over whether *Triplosoba* Handlirsch 1906 was a member of the Palaeodictyoptera, Protoephemeroptera or Ephemeroptera is a matter of interpretation of the characters evident in the fossils. Carpenter (1963, 1992) accordingly placed this taxon within the Ephemeroptera, while Forbes (1943) placed it in the Palaeodictyopteroidea; Willmann (1999) maintained that it is neither a member of the Paleodictyopteroidea nor the Ephemeroptera, but closer to the latter. Prokov and Nel (2009) reinterpretated the wing venation, and suggested the placement of *Triplosoba* in the Paleodictyopteriodea once more. A second example is *Lithoneura* Carpenter 1938. Carpenter (1938, 1987, 1992) assigned it to Paleodictyoptera (Syntonopteridae), whereas Edmunds and Traver (1954b), Edmunds (1972), Wootton (1981) and Willmann (1999) placed it in Ephemeroptera, and others (eg. Laurentiaux, 1953) placed it in an order of its own, the Syntonopteroidea. It is important to understand which synapomorphies bind the

Ephemeroptera into one group, both in order to see them in the context of their extinct relatives and in terms of their placement with respect to other extant insect lineages. Hamilton (1972c) maintained that the ephemeropteroid lineage was already highly specialized when it appeared in the Upper Carboniferous fossil record, based on features such as the retention of a free radius, and the molecular clock evidence presented in this thesis supports this.

As Willmann (2007) points out, some lineages have changed very little in tens of millions of years. Ephemeropteriod insects are seen to be a "persisting type" (*sensu* Huxley, in Willmann (2007)), since these have been recorded as fossils as far back as the lower Cretaceous. However, no "crown group" (modern) mayflies have been found in the Paleozoic, while most of the stem groups seem to have been extinct by the end of the Permian. The biogeography of the crown group species of Prosopistomatidae is discussed in Chapter 8.



a



b

Figure 7.1. (a) Typical adult male Ephemeropteran, showing non-folding palaeopterous wing condition, (b) typical nymph (Baetidae). (Scale equals 1 mm). (Drawing of nymph by Nikki Köhly)

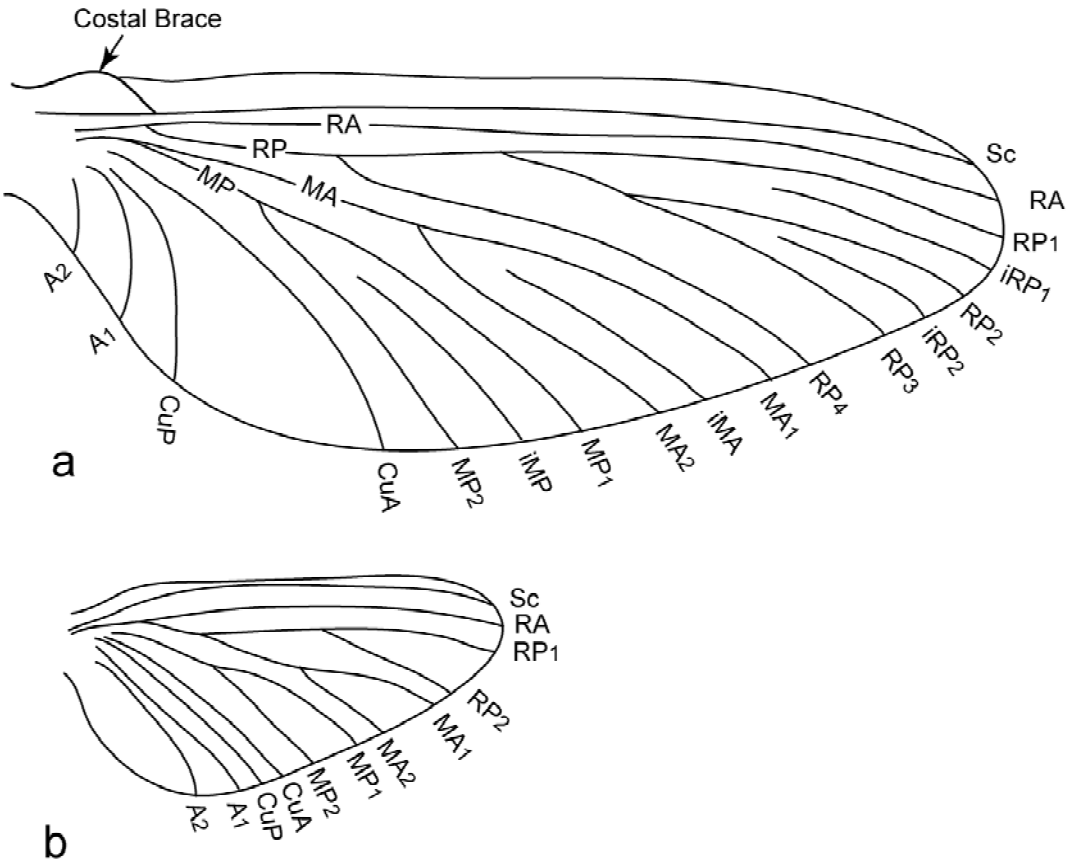


Figure 7.2. Generalised Ephemeroptera wings, (a) forewing, showing costal brace and typical venation, and (b) hindwing. Venation notation follows Kukalová-Peck (1983).

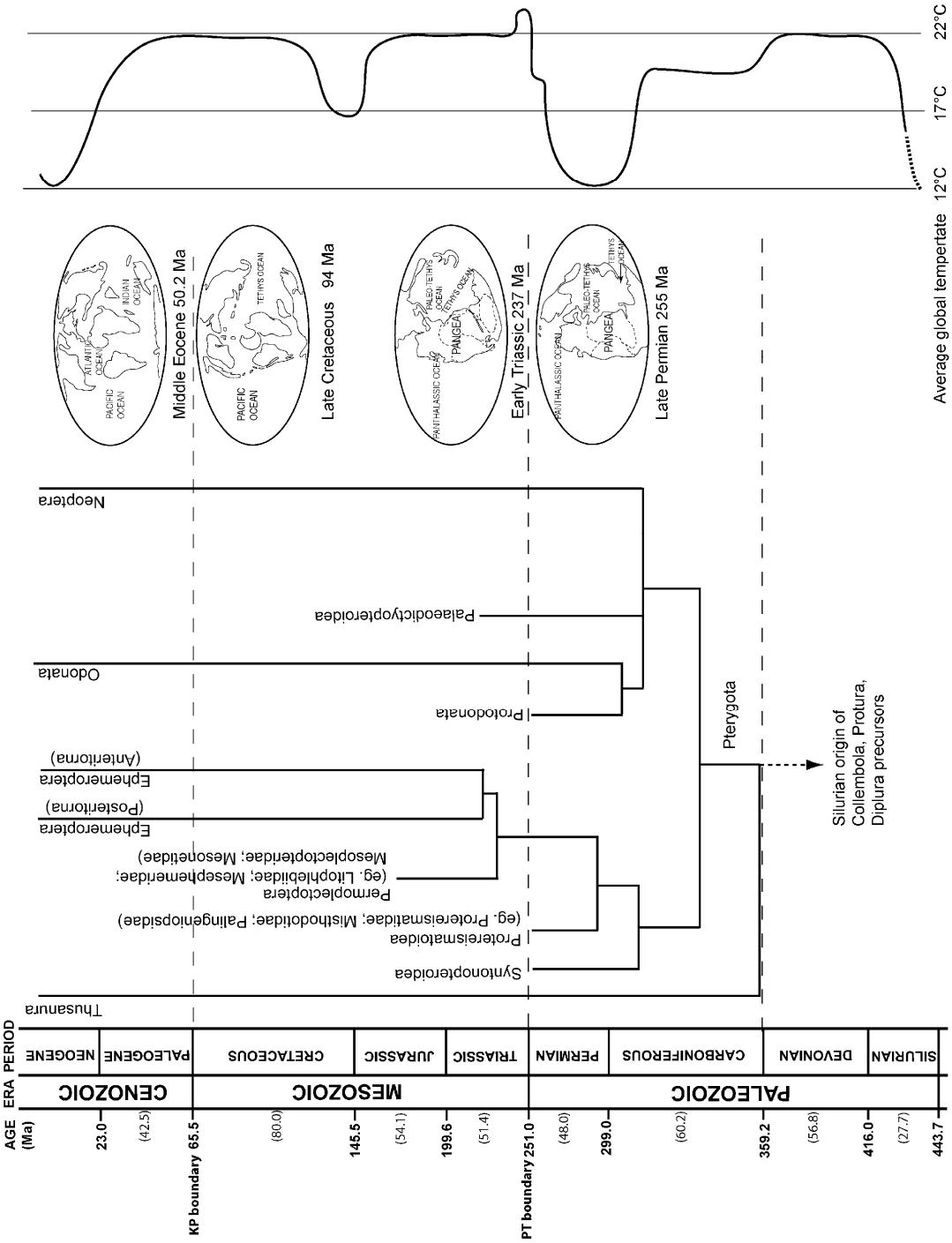


Figure 7.3. Postulated relationships between fossil insects precursors and modern insect lineages, focussing on the Ephemeroptera, showing possible early separation of Posternitorna and Anternitorna (sensu Kluge, 1998). Synthesized from Edmunds and Traver (1954), Riek (1973), Grimaldi (2001), Rasnitsyn (2002), Grimaldi and Engel (2005), Prokop and Nel (2009). Note that "Permoplectoptera" as a rank is almost certainly paraphylectic. Stratigraphy time line and temperature from Scotese. Time interval between each period indicated by numbers in parenthesise. *Triplosoba* and *Lithoneuria* unplaced due to lack of agreement in literature.

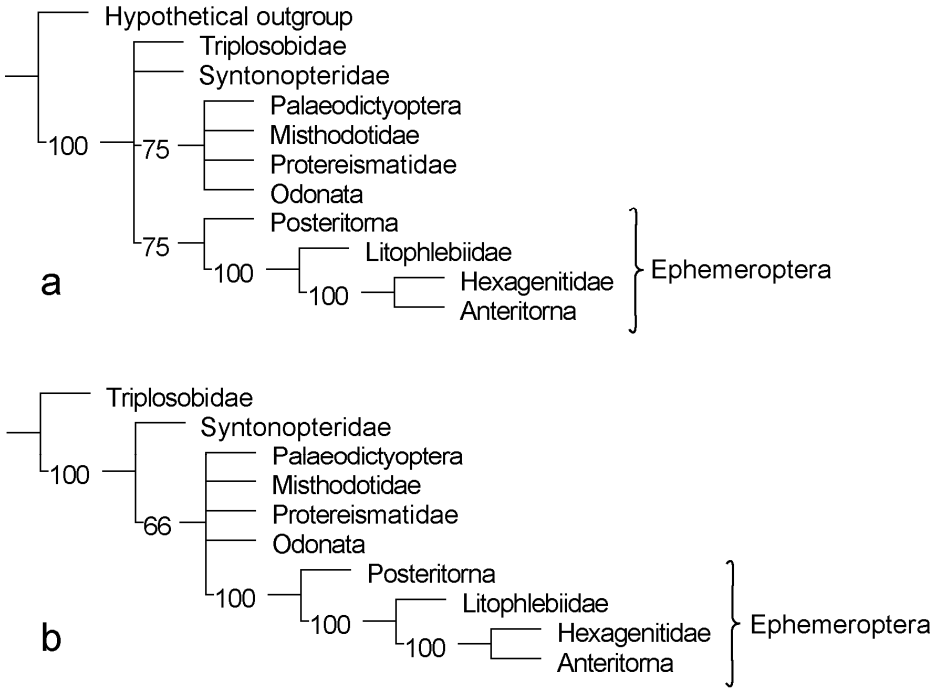


Figure 7.4. Parsimony analysis showing possible relationships between stem-group fossil lineages, the Posteritorna (i.e. Baetiscoidea) and Anteritorna (all other extant mayflies). (a) majority rule consensus from four trees, using an all-primitive character hypothetical outgroup (tree length=13, CI=62; RI=70); (b) majority rule consensus of three trees, using †*Triplosoba* as outgroup (tree length=13, CI=62, RI=68). Posteritorna is placed as a sister clade to the Anteritorna (which includes fossil example †Hexagenitidae), with †Litophlebiidae an intermediate lineage, and although taxa classify as "true" mayflies, distinct from the stem-group lineages.

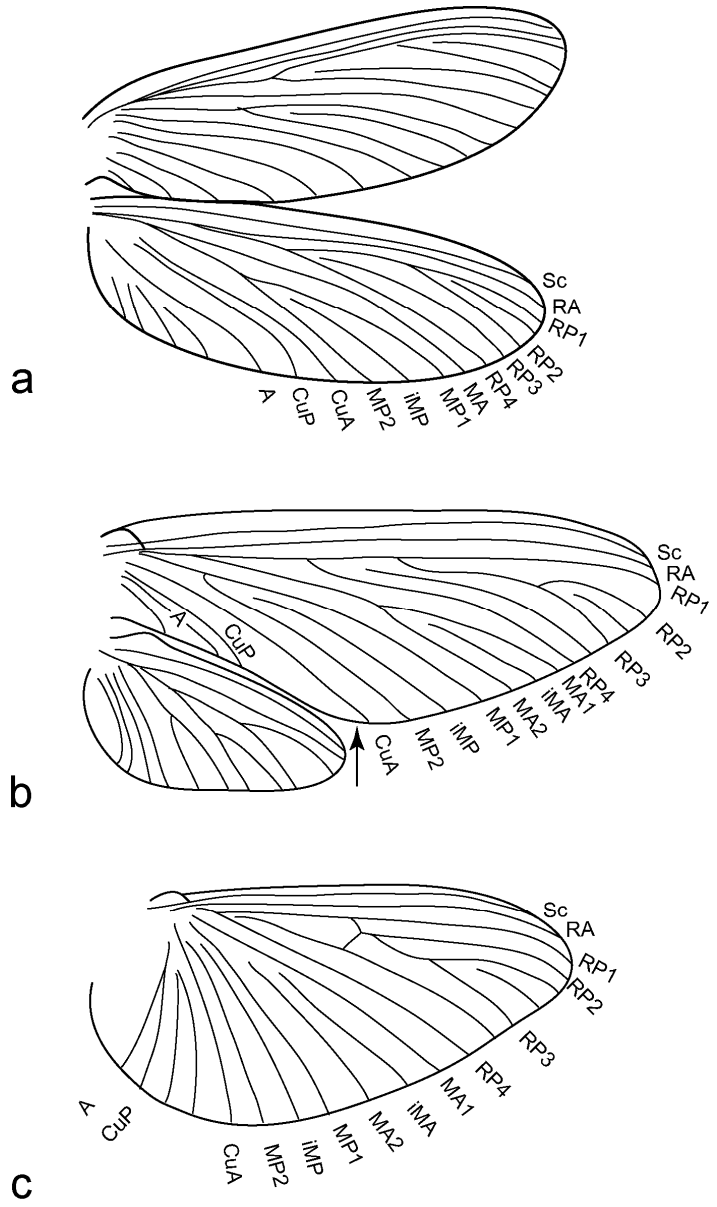


Figure 7.5. Change in position of the veins CuA and CuP in relation to the reduction of hindwing size. (a) †*Triplosoba* (homonomous wings – note the absence of a costal brace, so this is not a true mayfly), (b) *Siphlonurus* (reduced hindwing), (c) *Caenis* (no hind wing, broadened cubital-anal region). Position of tornus arrowed.

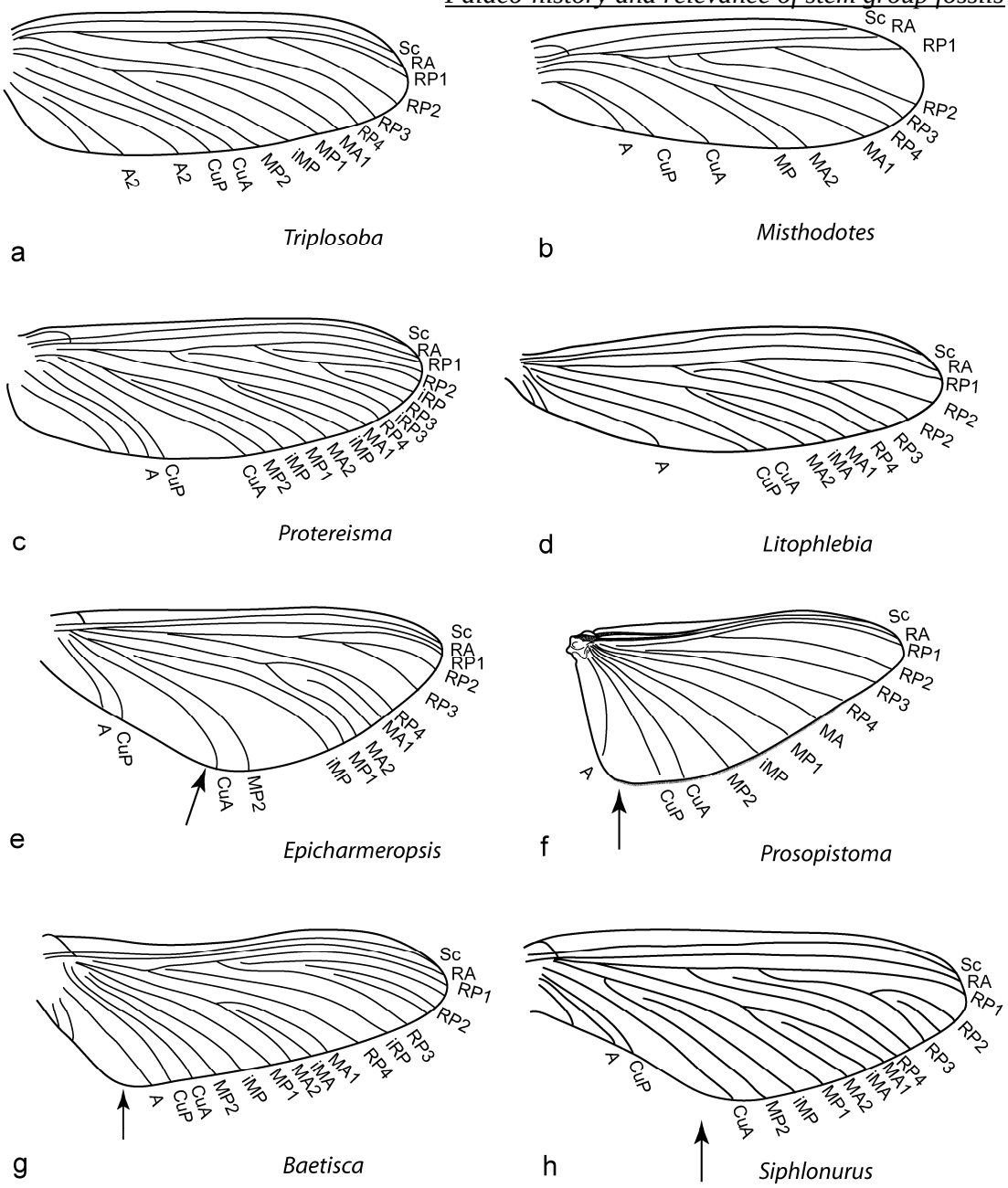


Figure 7.6. Comparison of the relative position of the cubital veins in the forewings of early Ephemeroptera stem-groups, and extant families, with position of tornus, when present, indicated by an arrow. Only main veins are shown. (a) †Triplosobidae (Carboniferous) (after Carpenter, 1992), (b) †Misthodotidae (Permian) (after Carpenter, 1992), (c) †Protereismatidae (Permian) (after Carpenter, 1992), (d) †Litophlebiidae (Triassic) (after Riek, 1976), (e) †Hexagenitidae (Jurassic) (after Huang *et al*., 2007), (f) Prosopistomatidae (extant), (g) Baetiscidae (extant) (after Pescador and Peters, 1974), (h) Siphlonuridae (extant) (after Kluge, 2004).

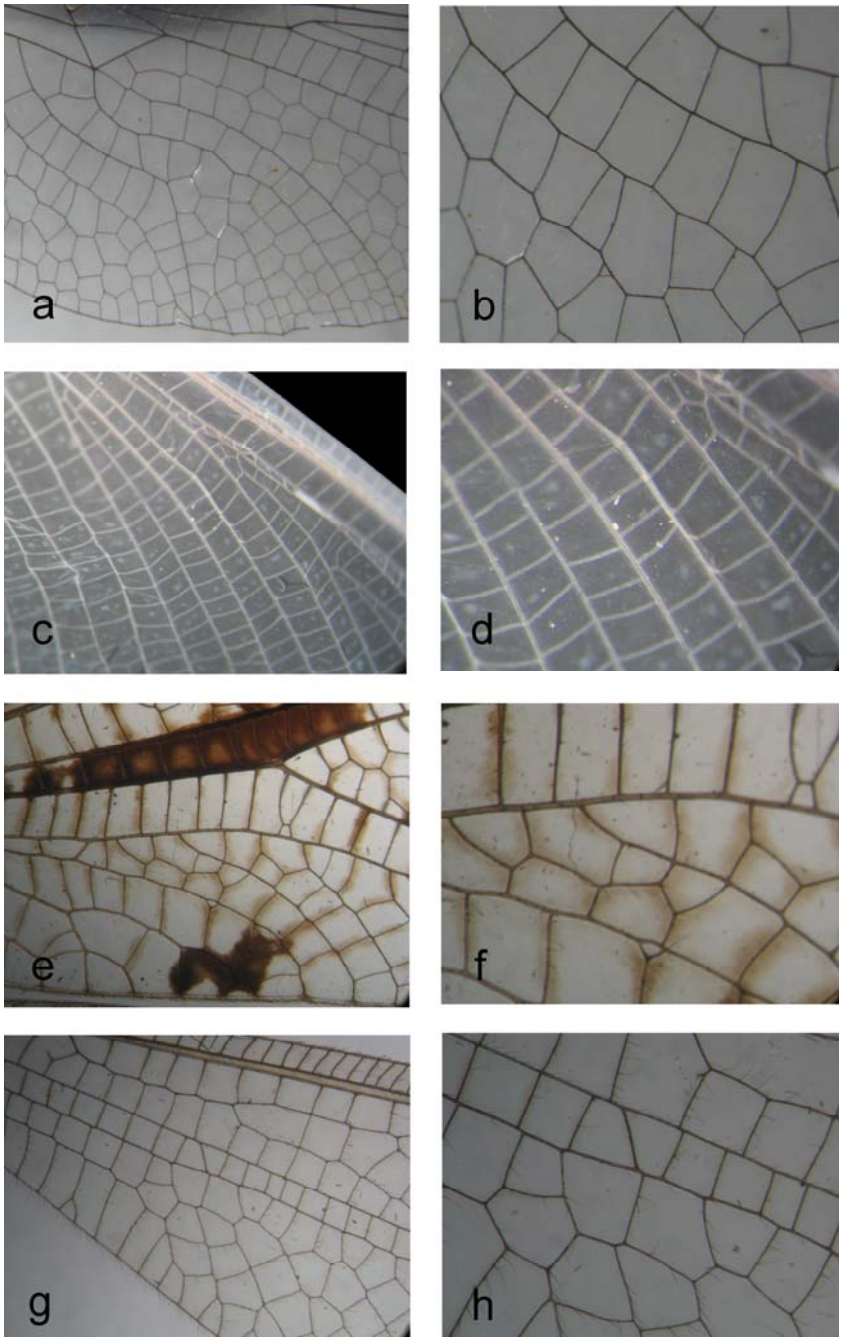


Figure 7.7. Cross veins as seen in (a,b) Odonata (Libellulidae, *Trithemis* sp.) showing a typical archedictyon structure, (c,d) Ephemeroptera (Polymitarcyidae, *Povilla* sp.), showing a polyneurous structure, (e-h) Neuroptera (Myrmeleontidae) - (e,f) *Maula* sp. and (g,h) *Lachlathetes* sp. showing an archedictyon structure, retained in a non-palaeopterous group. For *Prosopistoma* archedictyon, see Figure 2.19.

Chapter 8

Historical Biogeography of the Prosopistomatidae

"The chameleon changes colour to match the earth, the earth doesn't change to match the chameleon" African proverb

"It was 1946 ... the University of Cambridge ... and we were not allowed to dream about Gondwana" Sir David Attenborough (In: Andersen, 2001)

"A reasonable biogeographer is neither a vicarianist nor a dispersalist but an eclecticist" Simpson (1980)

Synopsis

This chapter examines the global distribution of recent prosopistomatid species and their extinct relatives, both in terms of locality, and by considering factors which may have influenced these patterns. Relationships shown by phylogenies derived in Chapters 5 and 6 are the key to this chapter, which investigates the "why" of these relationships. Both historical and present day ecological conditions are investigated, including a synthesis of changes in global palaeo-climates relating to fossil distribution and the implications of this for crown-group distribution. Vicariance and dispersal have both played a role in forming these distributions. Phylogenies based on molecular genetics (Chapter 6) suggest dates of more recent lineage divergence of the crown-group species, and are used further in this chapter to discuss dispersal from Asia southwards to Australia and westwards to Europe. The disjunct distribution of closely-related species shown by the molecular phylogeny is put into the context of Cenozoic climate changes within Africa. Relationships between different regions, chosen because of congruent distributions of other freshwater-dependent organisms, are compared using track analysis methods, to see if there is a common trend between unrelated taxa, which would have biogeographical implications. These distributions and the relationships between them are further investigated using Sørensen's coefficient of similarity and multi-dimensional scaling. Abiotic parameters of the rivers in which extant species of Prosopistomatidae have been collected are collated, and ordination analysis is applied to these results to see how each parameter may relate to species distribution. For example, do certain species have a preference for deeper or faster flowing

rivers, and do these group together even when the species are from different regions; does this show a common origin, or is it a secondary adaption?

8.1 Introduction

Why are species found today distributed as they are, and do phylogenies help to interpret this? Chapter 7 placed the lineage in relation to its stem-group relatives by reviewing the deeper phylogenetic history of the stem-groups from a palaeontological perspective. The next step in unraveling more recent biogeography looks at both the modern species distribution and the fossil record for Baetiscoidea *s.s.* fossils, in conjunction with palaeo-climatic and tectonic conditions, which will have affected the success and evolution of each species over time. This is used to formulate hypotheses to explain the distribution of the crown-group species. The phylogenies derived in Chapters 5 and 6 are used as the starting point for discussion of various biogeographic scenarios, which consider both vicariance and dispersal models. Comparisons of distributions of other non-related freshwater taxa are used to draw parallels in area relationships. Parameters governing ecological preferences are recorded and compared. The exercise starts with examining the distribution of currently known species.

8.2 Methods and deductions used to assess global distribution patterns

The collecting localities of the extant species were mapped (Figures 8.1-8.4) using data from museums (AMGS, FAMU, MZL and PERC), databases (Global Biodiversity Information Facility 2008), colleagues (Jean-Marc Elouard, Lauret, Languedoc-Roussillon, France, pers. comm., 2005; Santiago Robles, Madrid, Spain, pers. comm., 2001, 2008; Manuel Toro, Department of Aquatic Environment, Center for Hydrographic Studies, Madrid, Spain, pers. comm., 2008; Martin Schletterer, Institute of Ecology, Innsbruck, Austria, pers. comm., 2008, 2010) and the literature (Vayssière, 1893; Lieftinck, 1932; Gillies, 1954; Peters, 1967; Alouf, 1977; Pearson and Penridge, 1979; Soldán and Braasch, 1984; Campbell and Hubbard, 1988; Sartori and Gattolliat, 2003; Tong and Dudgeon, 2000; Zhou and Zheng, 2004; Dalkıran, 2009). Grid references were estimated as closely as possible from maps following site descriptions if the sites were not geo-referenced in these papers. Additional information was taken from publications which indicated range extensions of species already described: Sweden (Trägårdh, 1911; Alm, 1918); Latvia (Katschalova, 1962, 1965); Czech Republic (Ulmer, 1927); Russia (Schletterer and Kuzovlev, 2007); Hungary (Újhelyi, 1966); Greece (Hoffmann, 1994), Austria (Vayssière,

1890b), Germany (Ulmer, 1927); France (e.g. Joly, 1871; Vayssière, 1882, 1890b; Lafon, 1952); Portugal (Terra, 1984); and Italy (Bellmann, 1988). A more detailed account of the European species, including a more comprehensive list of localities, can be seen in Schletterer and Füreder (2009). Additional distribution records of undescribed species which are not included in these maps and discussions include records from the Hindu Kush-Himalayan region (Ofenböck *et al*., 2008) and Bangladesh (Alam, *et al*., 2008; Bari, *et al*., 2008). Schletterer (pers comm., 2010) has recently received material from Bhutan.

Ages in terms of the geological timescale, quoted in the discussion, are from the International Stratigraphic Chart (International Commission on Stratigraphy 2006).

Six approaches are applied to unravel prosopistomatid biogeography. The questions posed in each case are put forward in this section, and ways of addressing them, with discussions, are presented in section 8.3.

8.2.1 The influence of palaeo-climates and palaeo-landmass distributions through time on Prosopistomatidae distribution patterns

The methods used here are based upon a synthesis of published palaeontological information. The aim is to project backwards in time to see what variables may have influenced current distributions, without considering phylogeny. Evidence of fossil species belonging to the Baetiscoidea *s.s*., palaeo-landmass arrangements and changing palaeo-climatic conditions are used to aid the unravelling of modern distribution patterns. What does the fossil record show of distribution of the immediate ancestral members of this lineage? What factors may have caused the fossil lineages in Brazil, southern Australia, North America and the Baltic region of Europe (for references see Table 8.1) to die out? Palaeo-climatic conditions are reviewed in relation to changing continental landmass arrangements, with implications for the cause of the demise of stem-group lineages and the events which produced the current species. Fossil distribution is considered in comparison to palaeo-landmass arrangements. This hypothetico-deductive approach provides a novel way of viewing evolution and biogeography in Prosopistomatidae and their immediate stem-group ancestors.

8.2.2 Vicariance as a result of continental drift, with an African-centred Gondwanan origin

The hypothesis tested here is that the extant species' distribution may be primarily due to continental vicariance, with subsequent dispersal. These relationships are interpreted in

terms of vicariance-driven speciation, based on geological evidence of tectonic rifting. This is a separate approach to the use of the dated nodes from the molecular phylogeny, as the latter still has an element of uncertainty due to the limited scope of genes which amplified successfully and the small number of species tested. Morphological evidence cannot be discounted because of conflict with the preliminary molecular results, and deserves interpretation. Thus the ages of tectonic breakup on Gondwana are synthesized and applied to the morphology-derived phylogeny to produce an interpretation of the extant species' distributions in the context of Gondwanan vicariance.

8.2.3 Cenozoic recolonisation from an Angara craton refuge, and later dispersal from Asia

An alternative hypothesis to the vicariant distribution from a Gondwanan origin is based on the dated nodes on the molecular phylogenies of Chapter 6. The relative ages of some of the extant lineages, indicating that the oldest extant lineage is found in Asia, is fundamental to this interpretation. One way to assess the area distribution of each species and their area of occurrence is to produce a phylogeny reflecting area of occurrence. In this case, a spreadsheet was constructed reflecting the presence / absence data for each *Prosopistoma* species, using the regions Africa, Madagascar, Europe, India, South East Asia, Pacific Islands and Australasia. Due to the high degree of endemism, most of the "character states" are autapomorphic, so are not informative, and thus a meaningless cladogram is generated. It is not possible to use a finer scale resolution due to the limited knowledge of distribution of each species globally. Therefore, instead, the old area cladogram approach was used (e.g. Brundin, 1966; Nelson and Platnick, 1981; Page, 1988). In this method, species names on the molecular phylogeny are replaced with the area of occurrence. This provides a dendrogram showing implied historical relationships between geographic areas, rather than phylogenetic relationships among taxa, i.e. the phylogenetic relationships are assumed to reflect geographic changes. A disjunct relationship shown in the area cladogram is further investigated in section 8.2.4. Nelson and Platinck (1981) considered that cladograms for different taxa which inhabit the same area should provide a general biogeographic summary.

8.2.4 Disjunct distribution between Europe and Southern Africa

The molecular phylogeny (Chapter 6) shows a disjunct distribution between two closely related European and southern African *Prosopistoma* species. Such a disjunct distribution has previously been recorded for a number of species across many taxa (see Bologna *et al*.,

2008, and publications therein). The possible causes of this disjunction are investigated by synthesizing knowledge on palaeo-climate in Africa and Europe during the Pleistocene. In particular, extreme climate conditions such as glaciations and drought are sought as an explanation to account for unexpected relationships.

8.2.5 Track analysis and area relationships

This section is based on the idea of comparable area cladograms reflecting relationships between geographic areas, and on the concept of analysing and comparing the distributions of unrelated organisms to see if a general trend occurs. The comparison of distributions, and tracing hypothetical pathways between them, was first introduced formally by Leon Croizat (1958) in what became known as the "Panbiogeography" approach to biogeography. This approach looks for taxa which share the same biogeographic history (cenocrons), using congruent distributions to support vicariance models for evolution. The premise is that dispersal is less likely to have resulted if multiple taxa achieve the same distribution patterns, although the effect of prevailing winds and oceans currents should not be overlooked. Distributions of each taxon group under investigation are plotted on a map, and areas are linked according to the distributions suggested by the phylogenies in each reference. Overlapping of individual tracks provides generalised tracks, which allow the hypothesis that ancestral biotas were fragmented by tectonic changes, or their range changed due to external factors such as changing climate (e.g. Croizat, 1958, 1964; Morrone and Crisci 1995; Craw *et al*. 1999; Toledo *et al*., 2007). Track analysis aims to identify ancestral biotas by investigating current distributions. Eight other monophyletic aquatic groups with similar distributions to Prosopistomatidae were selected. Some of these groups also have trans-Antarctic and trans-Holarctic tracks which do not reflect recent prosopistomatid distribution, but they do tie in with known stem-group Baetiscoidea fossil distributions.

Strength of association between different biogeographic regions represented by the aquatic organisms was investigated using Sørensen's coefficient (SC) of biotic similarity (Sørensen, 1948).

$$SC = 2a / (2a+b+c)$$

where:

a = the number of taxa common between two regions,
b = the number of taxa unique to the first region,
c = the number of taxa unique to the second region

These associations were further tested using a Bray-Curtis index of similarity, and visualised using Multi-Dimensional Scaling (MDS) (sometime referred to as perceptual mapping), using PRIMER version 6 (Clarke and Gorley, 2001). In this case, regions occupied by each taxon used in the track analysis are being compared. The Bray-Curtis index reflects the proportion of the total in which two objects differ (Podani, 2000), and the table of similarities thus produced is used by the MDS to produce a spatial representation of the data, revealing the relationships between the areas.

8.2.6 Adaptation to specific river habitat conditions

Is there any relationship between the distribution of species and the type of river they inhabit? What abiotic factors play the biggest role in influencing their distribution? Do all Prosopistomatidae prefer similar ecological conditions? Why are they, as a group, so sensitive to environmental change? This was investigated by synthesizing all known ecological parameters pertaining to each species, including river size, flow preference, altitude, substrate type and size, water temperature and pH. These abiotic environmental parameters (Appendix Table 8.2) associated with each species were compiled from museum records and from literature (same sources as distribution data, section 8.2). Ordination was carried out using Principal Component Analysis (PCA) and Canonical correspondence analysis in CANOCO (ter Braak, 1988). As some of the values were ranges, all values obtained were coded in a similar way as for a phylogenetic analysis except that coded values started at 1 (Appendix Table A8.3). The ordinates were plotted to visualise species distribution patterns relative to the abiotic parameters. The correspondence analyses of parameters and species distribution from the PCA were overlaid.

8.3 Results and Discussion

8.3.1 Effects of palaeo-climates and palaeo-landmass arrangements on species distributions

This approach looks at distributions seen today and projects backwards in time to see how these may have been brought about by changes in habitat suitability for the evolving organisms as land mass positions and climates have changed. To recap, Chapter 7 looked back to the origin of mayflies as a group. The earliest lineages of true mayflies were already well established in the Triassic, with their stem-group relatives active in the late Carboniferous through to the Permian. Figure 7.3 compared the position of the continental

plates with respect to the developing lineages, showing that precursor mayflies were already established on the Pangaean landmass. The comparison of wing venation in the baetiscoid group of fossil pre-mayflies and mayflies with extant mayflies (Figure 7.5) suggested that the baetiscoid lineage, with the typical Posteritorna venation, may have separated earlier from a stem-group ancestor than the remaining stem-group mayflies.

At this point it is necessary to investigate whether any Prosopistomatidae or at least Baetiscoidea *s.s.* fossil evidence is available. Figure 8.5 shows the localities of known fossils for the group. There are no prosopistomatid fossils *per se* known at present, since the fossil reported by Sinitshenkova (2000a) from Burmese amber as a prosopistomatid, approx 100 million years old, has been redetermined as belonging to *Palaeocloeon*, a member of the Baetidae (Kluge, 2004). In support of Kluge's decision to remove this fossil from the Prosopistomatidae based on comparative morphology with extant Prosopistomatidae (Chapter 2), the wing venation does not have the characteristic position of the tornus relative to CuA and CuP (i.e. Sinitshenkova's fossil belongs with the Anteritorna). Furthermore, the legs and claws illustrated by Sinitshenkova (2000a) do not fit the structure known for Prosopistomatidae.

Several fossils for the Baetiscoidea have been found (Table 8.1), covering a surprisingly large geographic range, indicating a Pangaean origin of this lineage. The oldest known fossils indicate that the stem group of the Baetiscidae originated at least 200 Ma (Pescador *et al*., 2009). If modern Prosopistomatidae and Baetiscidae are truly sister lineages, this implies that the ancestral Prosopistomatidae lineage existed as far back as the Triassic, before Pangaea split. Stem line fossils of the Baetiscidae have been reported from the Crato Formation in Brazil, dating back to the Upper Aptian stage (125-112 Ma) of the Lower Cretaceous (Staniczek, 2007). The Koonwarra fossil beds in Victoria, Australia, have also yielded a baetiscoid fossil from the Lower Cretaceous (Late Aptian, some 118-115 Ma). Although provisionally assigned to Siphlonuridae (Jell and Duncan, 1986), Staniczek (2007) places it as a member of the Baetiscoidea, belonging to the stem group of Baetiscidae, extending the distribution of these precursor taxa considerably. Sinitshenkova (2000b) described a fossil from New Jersey, North America, from the upper Cretaceous (Turonian stage, 93.5-89.3Ma), which McCafferty (2004) transferred from its original designation in †Cretomitarcyidae, to the Baetiscoidea as a putative Baetiscidae precursor. Other more recent Baetiscidae fossils have been found in Eocene Baltic Amber, some 45 Ma (Staniczek and Bechly, 2002; Godunko and Krzemiński, 2009), indicating a more widespread distribution of this family than it presently occupies, and that the lineage was

well established at that time, and present in Europe. The purported Baetiscidae fossil of Zamboni (2001) is placed as *incertae sedis* by Staniczek (2007).

The shaping of the continents as they moved over time, and the consequential change in palaeo-climate, will have played a major role in shaping the evolution of the current species from these precursors. This leads to further questions. If the family arose from a tropical Gondwanana lineage, they may be expected to still have a preference for warmer waters (e.g. Edmunds (1972) indicated that Siphlonuroidea, considered to be a primitive mayfly lineage, are largely adapted to cooler waters, having a largely holarctic and amphinotic distribution). Appendix Table 8.2 shows the recorded habitat conditions for current *Prosopistoma* species, as far as can be ascertained, and this is discussed further in section 8.3.6.

It is necessary to look at fossil distribution in relation to the landmasses as they were arranged in the past to interpret current distribution patterns with fossil distributions in mind. Figure 8.6a shows the current distribution patterns of the Baetiscoidea, and these are transposed onto a palaeo-landmass arrangement (120 Ma) with the fossil localities superimposed (Figure 8.6b). The northern hemisphere fossil species are younger than the southern hemisphere fossils, and having only four fossils makes it difficult to draw generalised conclusions. Fossil evidence from Africa, Madagascar, Antarctica and India would help to complete this jigsaw, but is unlikely to be found. From the arrangements of the palaeo land masses and the prevailing climate over each epoch, it can be deduced why some of the lineages represented by fossils may have died out. Table 8.2 summarises the global climate from the Devonian to present, and simultaneously depicts changes in landmass arrangements (information from Dickens (1993), Eskov (2002) and Scotese (2003)). The Permian-Triassic (P-T) extinction event took place some 251 Ma, a time of mass extinction forming the boundary between the Permian and Triassic periods (Bowering *et al*., 1998). Davis *et al*., (2010), based on supertree analysis of multiple taxa combining fossil dating, indicate that order-level diversity peaked just before the end-Permian extinction, and that at least 11 more lineages survived this extinction than implied by fossils alone.

A forward-projecting synthesis of the world following the P-T extinction offers a way to interpret faunal and floral distribution patterns resulting from changes in global climates and landmass arrangement. Prior to the P-T extinction event, around 300 Ma, much of Gondwana was covered by glaciers (McCarthy and Rubidge, 2005), but by the late Permian, both Gondwana and Angara were without frost and the world was generally warm

(Dickens, 1993). It is almost certain that the stem-groups of insects arose in more tropical parts of the globe (Meyen, 1987; Rasnitsyn, 1989; Eskov, 1996). The synthesis of fossil and molecular evidence (Chapter 6) shows that the baetisocid lineage would have been well established by the Cretaceous (some 145 Ma). Although the oldest Baetiscoidea fossil is only 125-112 Ma (Table 8.1), the lineage of "true" mayflies goes back at least to the Triassic in the Mesozoic. It is thought that mayflies attained their highest diversity during the Mesozoic (Brittain and Sartori, 2003). What is known of life on earth at that time? Stem group mayflies that had evolved during the late Permian and had survived the P-T extinction event, were faced with arid conditions across much of the landmass of Pangaea during the Triassic (Table 8.2d), and it is possible that only the polar areas, which were warm temperate, offered refuge to aquatic insects such as mayflies. During the Jurassic (Table 8.2c), Gondwana and Laurasia began to be separated by the Tethys Sea, and the nacent Indian and Atlantic Oceans began to separate Gondwana. The equatorial zone was no longer arid due to the associated changes in ocean circulation, and the wetter climate produced an environment more suitable for mayflies. During the Cretaceous (145.5 – 65.5 Ma) (Table 8.2b), the landmasses became fragmented, and the climate around the world was relatively homogeneous, which would have allowed widespread dispersal of the fauna of that time. This is probably a critical period in the development of the mayflies as we know them today. All the Baetiscoidea fossils, with the exception of the Baltic amber fossils (which are more recent), fall into this period, and the prevailing ambient climatic conditions concur with the widespread distribution of the fossils. Figure 8.7 and Table 8.2 sections 'a' and 'b' help to visualise the timing of the more recent plate tectonic events in relation to the time of occurrence of the fossil species.

This broad review of the palaeo-climate offers no reason for the current absence of the Baetiscoidea in South America. They were present in the Brazilian Crato Formation, which is a snapshot in time, and are likely to have continued to survive in this area for some time after the formation of these fossils. One can only speculate as to how widespread their range was. When did this lineage die out? A possible explanation is that they were victims of the Cretaceous-Paleogene (K-P) extinction event. McCafferty (1990) reports that the Mesozoic mayfly fauna was considerably different from the Cenozoic fauna, following the mass K-P extinctions. Brazil (the last outpost of South American Baetiscidae) was relatively close (some 6000 km) to the meteorite impact site which is said to have been the cause of the mass extinctions of this period (Alvarez *et al.*, 1980). Alternatively, these ancestral mayflies may have died out, or survivors migrated from this

region, driven by the prolonged extreme drought conditions (Table 8.2b) which were prevalent in the interior of Gondwana before rifting began (Smith and Botha, 2005). Due to the distance of the interior landmass from the ocean while it was a single mass of land, little to no rain fell, and the climate was therefore extremely dry and inhospitable. Only after the landmasses began to separate would the ocean currents have changed their pattern of circulation, bringing rain and making what is now the western side of Africa and eastern side of South America less desert-like, and therefore more suitable for *Prosopistoma* colonization. It is therefore proposed that the species on the African mainland migrated westwards across Africa after the split with South America, and therefore South America was not recolonised.

The baetiscoid lineage managed to survive in what is now North America (Sinitshenkova, 2000b; McCafferty, 2004), producing the Baetiscidae of today, and also survived for some time in Europe, as shown by the Eocene Baltic amber Baetiscidae. This lineage was probably spread over the Laurasian landmass during the Jurassic, surviving well into the Cenozoic, having survived the K-P extinction. Their demise in Europe was probably the result of more recent Pleistocene glaciations. It has been suggested that due to the east-west arrangement of mountain ranges in Europe, many species went extinct during the glacial periods, while in North America, the north south orientation of mountain ranges allowed a corridor of escape to warmer climates further south (Barber-James *et al*., 2008). What of Prosopistomatidae *per se*? These are discussed in greater detail in the following section.

8.3.2 Vicariance due to continental drift and subsequent dispersal

What is known about the dates for the breakup of the supercontinents, and do all authors agree? The understanding of the breakup of Pangaea and the relative placements over time of the more recent palaeo-landmasses (Gondwana, Laurasia and Angara) is essential in understanding the historical biogeography of any organism and their relative propinquity of descent. Consensus must be reached about the arrangement of the landmasses from the time when the complete supercontinent Gondwana existed, placement of the separating landmasses with respect to the poles and equator, and times of separation.

Earlier and widely accepted models of plate tectonics (since the 1950's) have indicated that by the end of the early Cretaceous, there were six major continental land masses, largely separated from each other by deep ocean passages (Figure 8.8a). Barron (1987) provided a synthesis of the models of plate tectonic reconstructions up to that date,

and came up with some notable modifications to classic tectonic plate theory with respect to times of separation of the landmasses. Most notable was the work of Rabinowitz *et al*., (1983) who proposed a much earlier separation of Madagascar from Africa than previously accepted, saying that Madagascar separated at approximately 160 Ma, and reached its current position with respect to Africa by 120 Ma. Another big change was shown by Cande and Mutter, (1982) who indicated an earlier separation of Australia and Antarctica. Veevers (1986) put the breakup of Australia and Antarctica estimated as mid-Cretaceous (95 ± 5 Ma). Craig (2003a) also provided a useful summary of the different positions of constituent components of Gondwana at various time scales according to different authors.

The following list summarises the approximate times for the beginning of separation of landmasses, based on Barron's (1987) summary, with subsequent refinements as indicated:

Africa – Madagascar 160 Ma (Barron, 1987); 158 - 165 Ma (Briggs, 2003; Yoder and Nowak, 2006); 166 Ma (Ali and Aitchison, 2008)

India – Antarctica (160 Ma Barron, 1987)

Africa – South America (125 Ma Barron, 1987)

Africa – Antarctic (125 Ma Barron, 1987)

India – Madagascar (90 -100 Ma Barron, 1987) (85 - 90 Ma Aitchison *et al*., 2007)

Australia – Antarctica (90 - 110 Ma Barron, 1987) (previously widely accepted as Eocene, approximately 55 Ma (e.g. Scotese)

India/Seychelles – Madagascar 84 - 96 Ma (Briggs 2003)

India – Seychelles 64 Ma Barron (1987)

While these authors were debating the time of continental rifting, Hay *et al*. (1999) proposed alternative plate tectonic reconstructions for the Cretaceous, not only considering times of separation, which are comparable to other models, but also relative placement of the landmasses. They indicated three major continental landmasses instead of six, with large contiguous landmasses surrounded by shallow epicontinental seas (Figure 8.8b). Most models assumed that the major continental blocks (Eurasia, Greenland, North America, South America, Africa, Madagascar-India, Australia, and Antarctica) had separated from one another by the end of the Early Cretaceous (Fig. 8.8a). These models also proposed that deep ocean passages connected the Pacific, Tethyan, Atlantic, and Indian Ocean

basins. The revised view of the Early Cretaceous put forward by Hay *et al*. (1999), refined by Hay (2009), is that by the Early Cretaceous, there were three large continental blocks (North America – Eurasia; South America – Antarctica – India –Madagascar – Australia; and Africa), and that large contiguous land masses were surrounded by shallow epicontinental seas (Fig. 8.8b). As Africa and South America began to split, the Madagascar-India-Antarctica-Australia landmass moved southwards in an arc. The Antarctic Peninsula remained in contact with the southern Andes until the Oligocene. In the early Cretaceous, India rotated away from Antarctica and slid past Madagascar, remaining in contact with Antarctica *via* a land bridge consisting of the Kerguelen Plateau, Sri Lanka and the Ninetyeast Ridge, until final separation during the late Cretaceous. India and Madagascar also separated at this time. It is interesting to note that Hay's concept of three large continental landmasses reflects the ideas of earlier biogeographers (e.g. Jeannel (1960)), who proposed the landmasses of Laurasia, Angara and Gondwana, although these early ideas were based only on speculation and observation of fossil and present day faunal distribution, not on geological evidence. Ali and Aitchison (2009) criticize Hay *et al*. (1999), their research showing that the Kerguelen Plateau could not have provided a terrestrial causeway between East Antarctica and India during the Cretaceous. However, the Kerguelen Plateau, parts of which were exposed at around 99.6 Ma (the boundary between Early and Late Cretaceous), is thought to have played a role in providing a conduit for terrestrial floras and faunas with which to cross the considerable ocean which separated India–Seychelles–Madagascar from Australia–Antarctica (Ali and Aitchison, 2008).

Considering connections between North Africa and southern Europe, Barrow (1978) (quoted in Duval *et al*., 1978), indicated that the Apulia-Anatolia plate rifted from northern Africa at 160 Ma and became part of Europe during the Albian Cenomanian (Albian 112 - 99.6 Ma, Cenomanian 99.6 - 93.5 Ma). More recent connections are discussed in section 8.2.4.

While the precise detail of the breakup of the landmasses, and Gondwana in particular, are still being refined, the broad conclusions are sound and useful for determining the history of any group of taxa. Once the landmasses had separated, the conditions on each separate landmass changed relative to the others, leading to allopatric speciation. For example, India moved relatively quickly (in approximately 70 Ma (Smith and Sandwell, 1997)) from its southern most position after rifting from Antarctica and Australia in the southern hemisphere to its current position.

Thus the breakup of Gondwana began approximately 165 Ma, considerably after the earlier Baetiscoidea precursor taxa were established, which offers the tectonic vicariance of Gondwana as an attractive model for the dispersal of the Prosopistomatidae. Edmunds (1972, 1979), considering the relationship between Afrotropical and Oriental mayflies, was the first to postulate a Gondwanan origin of *Prosopistoma*, proposing that India carried the group to Asia. The results of the nymphal and adult morphological phylogeny (Barber-James, 2009; Chapter 5) support Edmunds' hypothesis. Additionally, two clades were postulated within the family, the "African" clade and the "*P. variegatum*" clade, so named because of characters shared with all of the African species, or with *P. variegatum*, the type species for the family. The "African" clade dominates Africa, and co-exists with the "*P. variegatum*" clade in Madagascar, Asia and the Pacific Islands (Figures 8.2-8.4). This distribution points to them evolving on the Gondwanan landmass before the Lemurian landmass (consisting of Madagascar, Deccan plate and associated fragments - *sensu* van Steenis, 1962) broke off. The absence of the "*P. variegatum*" clade from Africa suggests that lineage may have had a later Lemurian origin. India collided with Asia some 34 Ma (Aitchison *et al*., 2007) possibly introducing both clades to what is now the Oriental region. Evidence of an intervening landmass (a Neotethyan intraoceanic arc) with which India collided some 57 Ma may have allowed earlier dispersal into Asia (Ali and Aitchison, 2008), prior to the collision of the Deccan plate.

Subsequent colonization of the Palearctic by the precursors of the "*P. variegatum"* clade, and migration and dispersal across Southern Asia and the Indo-Pacific islands to Papua New Guinea and Australia by both groups, provides a plausible explanation of the current distribution of this family. This does not, however, account for the absence of members of the "*African*" clade in Europe, and no explanation is apparent at present. It is highly likely that unknown species of *Prosopistoma* will still be found to occur in the largely unexplored rivers of countries between Asia and Europe, and several have recently been discovered (e.g. M. Schletterer (pers. comm. July 2010) now has material from Bhutan). Knowledge from studying such species will fill in some of the current gaps and refine the phylogeny.

The distribution of the known species on a world map (Figure 8.1) shows that the "*P. variegatum*" clade is widespread across Europe (represented by the questionably single species, *P. pennigerum*), and the species in the Middle East (*P. orhanelicum* and *P. oronti*) are also of this lineage. It continues to have a rather disjunct distribution, being represented in Sri Lanka (*P. lieftincki*), China (*P. sinense* and *P. trispinum*), the Philippines (*P. boreus*),

Papua New Guinea and the Solomon Islands (*P. sedlaceki*), and Australia (*P. pearsonorum*). It is worth noting that Sri Lanka is part of the original Lemurian landmass, breaking off some 109 Ma (Smith and Sandwell, 1997; Briggs, 2003). Interspersed with these species are members of the "*African*" clade, with *P. indicum* in India, *P. funanense* in China and Vietnam, *P. wouterae* extending through Thailand, Malaysia, Sumatra and Java, *P. olympus* in Borneo and *P. palawana* in the Philippines (Figure 8.3). Barber-James (2003) suggested that the distribution of *Prosopistoma* species amongst the Indo-Pacific islands follows Huxley's modification of Wallace's line, with Palawan having a different species to the main Philippine Islands. *Prosopistoma boreus* ("*P. variegatum*" clade) is found on Mindanao, while *P. palawana* ("*African*" clade) occurs on Palawan. However, the distribution pattern no longer seems as clear, as members of both the "*P. variegatum*" and "*African*" clade are found on either side of this hypothetical line. To investigate this more thoroughly, recent geological and climatic history of these islands and associated mainland need to be related to the distribution pattern seen today. This would require a much larger database of distribution records than is currently available for the Prosopistomatidae. However, some trends can be extrapolated from what is known. Some parts of the Philippine islands have areas which are over 25 million years old (van der Weerd and Armin, 1992), for example Mindanao (where *P. boreus* is found); others are much younger, from 10 million to 100 000 years only. During the Miocene (24-13 Ma) and again during the Pliocene (5.5-4.5 Ma) (Woodruff, 2003), the sea levels in this area were around 100m higher than present, which would have resulted in much of the land being submerged more than once, thus causing vicariance and speciation in isolated areas that were not submerged, and subsequent secondary dispersal when the sea levels were lower.

The Isthmus of Kra region, a narrow strip of land between Malaysia and Thailand, is a widely recognised biogeographic boundary, representing the transition zone between the Sundaic and Indochinese biotas (*sensu* Woodruff, 2003). The species assemblages of many groups of flora and fauna differ to the north and south of this region (de Boer and Duffels, 1997); e.g. cicadas (Yaakop *et al*., 2005), butterflies (Corbet, 1941), birds (Hughes *et al*., 2003), and frogs and snakes (Inger, 1996; Inger and Voris, 2001). Such studies have resulted in the postulation that the isthmus was breached during the Neogene marine transgressions discussed by Woodruff (2003), allowing speciation of separated populations on either side. Phylogeographic analyses of freshwater decapod species (de Bruyn *et al*., 2005) showed that this biogeographic transition zone between the Sundaic and Indochinese biotas held true for freshwater organisms as well as terrestrial. Since *P. wouterae* straddles

this region (Figure 8.3) (instead of there being one species above the transition zone and a second below it as suggested by the examples above), this would imply that *P. wouterae* may have dispersed across the transition zone after the sea level dropped, indicating that it may be a relatively young species. Has it been present prior to the increased Neogene sea level, the separated populations on either side would be expected to have diverged and speciated as in the many other examples, above. This is another point of conflict between the morphological and molecular phylogenies, where *P. wouterae*, the only Oriental species represented in the molecular phylogeny, is the oldest of the extant species. The Neogene covers a period of 2.58 Ma to 23.03 Ma (Ogg *et al*., 2008), and the lineage represented by *P. wouterae* is estimate to be between 60.43 and 131.49 Ma (Chapter 6). A thorough molecular investigation of *P. wouterae* at the population level with samples from several sites throughout its distribution is needed to resolve these questions.

The origins of islands in the Indonesian region can be useful in interpreting faunal distribution patterns amongst these islands. The region is formed from three major tectonic plates, the southeast Asian plate, the Indo-Australian plate and the Pacific plate, along with several smaller platelets (Katili, 1989 as cited by MacKinnon *et al*., 1996). It was initially thought that the western part (the Malay Peninsula, Sumatra, Java, Borneo and western Sulawesi) was derived from Laurasia (250-200 Ma, during the Triassic), while the eastern islands, including the rest of Sulawesi, were derived from Gondwana much later (Audley-Charles, 1981 as cited by MacKinnon *et al*. (1996). However, more recent palaeontological and geological discoveries suggest that this western part was not part of Laurasia but separated from Gondwana much later, in the mid-Jurassic (190-160 Ma) and Cretaceous (140-65 Ma) (Audley-Charles, 1987; Burrett *et al*., 1991, both cited by MacKinnon *et al*., 1996).

Considering the position of what is now Australia in the late Jurassic, the Australian plate was still in contact with the east side of the Deccan Plate (Table 8.2c). This could imply that ancestors of *P. pearsonorum*, the Australian species, rather than dispersing from the north, could have been carried on the Australian landmass after its breakup from Gondwana. Subsequent dispersal to Papua New Guinea could have occurred (both *P. pearsonorum* and *P. sedlaceki* are of the "*P. variegatum*" clade). This coincides with the age of the Koonwarra fossil. However, it seems more likely that the current Australian species arrived more recently by dispersal from the north. Campbell (1990) considers that some Australian mayflies have a strongly tropical component, with a recent northern origin, and includes *Prosopistoma* with this group. Most Australian mayflies, however,

have a stronger link with temperate South America, indicating a southern connection *via* Antarctica, and very few have a true tropical Gondwanan component.

A species of uncertain lineage that still needs investigating is the one occurring in North Africa (Figure 8.1, 8.4), in Algeria (Gagneur and Thomas, 1988; Thomas, 1998) and the Atlas mountains of Morocco (Touabay *et al*., 2002). It is expected to be more closely related to the European species than to other African species, due to the Palaeartcic nature of the North African fauna. In the Western Mediterranean, the Alboran Arc, a small subsea plate, drifted westwards up against both Spain and North Africa during the Cenozoic (Duggan *et al*., 2004). At the same time, deep magmatic upwelling slightly raised the profile of the entire region, resulting in the sealing off of the western end of the Mediterranean for about 600 000 years. During this time, the Mediterranean Sea virtually dried up, although the resulting valley was very saline. This may have allowed a greater interchange of fauna between northern Africa and southern Europe, especially at the western end of the Mediterranean basin, where conditions were less harsh and the two adjacent landmasses were at close proximity. Thus the North African species is postulated to have colonised from Europe, and would be expected to be more closely related to *P. pennigerum* than to the nearest African species. However, until material becomes available, relationships remain speculative. The molecular phylogeny implies that the African species are the result of dispersal from Europe prior to the formation of the Sahara desert, and only further molecular analyses using more rapidly evolving genes like CO1 (which was not successfully amplified for this thesis), and with many more species represented, will resolve these questions.

It would also be valuable to examine further material from different sites across Europe (Figure 8.4). While initial molecular results indicate that *P. pennigerum* is a single widespread species, different genes may give a different interpretation (especially CO1). Considering the climate difference between a more northerly record such as southern Sweden (Trägårdh, 1911), and a southerly record such as Spain (S. Robles, pers. comm. 2002, 2008), it would not be surprising to find that there is more than one species here.

Koch (1988), based on mandibular structure of the extant *Prosopistoma* nymphs known at that time, proposed a division of the family into a northern and southern group, the result of vicariance caused by the Tethys Sea during the Upper Cretaceous. While this may indeed have had a role in their speciation, his theory does not take into account certain species found in Madagascar, namely members of the "*P. variegatum*" clade (Chapter 5; Barber-James, 2009), which would fit into his northern group. He postulated a subsequent

colonization of Asia via India by his southern group (which corresponds to the "*African*" clade), and supports the idea of vicariance due to continental drift. Based on the two clades evident in the morphological phylogeny, a tropical Gondwanan origin is hypothesized, with a Lemurian origin of a second clade within the family. Fossil evidence and the molecular phylogeny (also dated with fossils), suggest that the stem group species are old enough to allow this assumption. Figure 8.9 depicts the proposed biogeography of the Prosopistomatidae, based on the two clades shown by morphology, and following the concept of vicariance due to continental drift carrying species from Africa on to Asia.

Edmunds (1979) suggested that although there are a number of families with an African origin, including the Prosopistomatidae, Oligoneuriidae and Tricorythidae, the interchange of mayfly fauna between the Afrotropical and Oriental realms has predominantly been from Asia to Africa via the Middle East. Based on relative diversity in each geographic region, he proposed that families such as the Caenidae, and genera like *Ephoron* (Polymitarcyidae) and *Ephemera* (Ephemeridae) spread from the Oriental realm to Africa. He suggested that the Oriental region served as a refuge for subtropical Laurasian flora and fauna during the Paleogene when other areas went through periods of climatic unsuitability (drought or cold). Jeannel (1960) suggested that the great eastern palaeo-landmass known as Angara (now Siberia and northern China), served as a refuge for ancestral stock of many organisms during times of glaciation and drought as this region was always situated in a relatively equitable climate (Table 8.2a-g). Furthermore, the craton representing what was Angara has not been covered by the sea since Cambrian times (Eskov, 2002).

A third scenario to consider with regard to distribution of mayflies between Asia and Africa is the possibility of a two-directional interchange. Thus, a lineage occurring originally in Africa may have diversified in Asia and subsequently dispersed back into Africa, e.g. certain Lepidoptera (Kodandaramaiah and Wahlberg, 2007), frogs (Kosuch *et al*., 2001) and snakes (Kelly *et al*., 2009). This dispersal was facilitated by a connection between Africa and the Arabian Peninsula, and Eurasia, during the Oligocene (Gheerbrant and Rage, 2006).

Although dispersal has without doubt played a role in the distribution of the modern *Prosopistoma* species, the question is whether the current distribution pattern is more the product initially of tectonic vicariance, and if so, to what extent. Edmunds (1972) maintained that the Ephemeroptera as a whole are very poor dispersers, using their absence from oceanic islands with suitable aquatic environments as an example. However, there are

several examples of mayfly dispersal to islands, especially of baetid mayflies, e.g. Brinck and Scherer (1961) found mayflies on both Madeira and the Azores, which are 1000 km from Madeira and 1500 km from the nearest mainland. Müller-Liebenau (1971) documented baetids on the Canary Islands. Gattolliat (2004) has shown the dispersal of mayflies from Africa to Reunion, a distance of over 700 km. Monaghan *et al.* (2005), comparing the molecular makeup of Baetidae on Africa and Madagascar, concluded that at least Baetidae disperse more than originally thought. Gattolliat *et al*., (2008) also noted *Cloeon* and *Baetis* species which had dispersed to Madeira. While the Australian *P. pearsonorum* almost certainly reached Australia by dispersal from Asia, this was most likely by gradual dispersal along the island chain, moving between adjacent islands (Barber-James, 2003), and no large ocean barriers were crossed in achieving this.

Did the recent Prosopistomatidae really have a Gondwanan origin, with subsequent dispersal as proposed here? Could they perhaps, instead, have spread from an Asian refuge (Angara) to Europe and via the Middle East to Africa (section 8.3.3)? The absence of the "*P. variegatum*" clade from sub-Saharan Africa implies that they probably did not evolved on Africa (although they could have become extinct). Their presence on Madagascar and Asia becomes difficult to explain without invoking the moving Deccan plate rafting the lineage northwards. With the oldest of the extant species dating at around 130 Ma (Chapter 6), this is plausible. The Gondwanan vicariant dispersal hypothesis should not be discounted in favour of later overland dispersal from Asia, despite preliminary molecular evidence to the contrary. The vicariance-dispersal model offered here assumes that the concept of the two prosopistomatid clades is sound; the limited molecular evidence so far does not support this, and this needs further research in the future. An alternative model is discussed in the following section.

8.3.3 Cenozoic recolonisation from an Angaran refuge; molecular clock implications and dispersal as the main model for *Prosopistoma* speciation

To resolve the relative ages of the crown-group species of modern Prosopistomatidae, and the age of the stem group of the Baetiscoidea, a molecular phylogeny (Chapter 6) was constructed. This suggests a secondary late Cretaceous or early Cenozoic dispersal from the Oriental region in two directions. Palaeontology and geology have wide margins of imprecision, so to minimise errors in incorrect fossil calibration, several independent calibrations were included in these analyses. Rather than just testing the age within the Baetiscoidea, a range of molecular data representing a number of other mayfly lineages

was taken from GenBank, calibrated with a number of fossil dates, both baetiscoid and others. The results from morphology and from molecular data are conflicting, giving very different topologies in the phylogenies derived from each and the Baetiscidae are not recovered as a sister lineage to Prosopistomatidae in the molecular phylogeny. Comparing these results with Ogden and Whiting's (2005) molecular phylogeny and Ogden *et al*.'s (2009) combined molecular-morphological phylogeny, a similar scenario is observed. The molecular phylogeny alone did not support the monophyly of the Baetiscoidea, while with the addition of morphological characters, the lineage is recovered as monophyletic.

The most useful result from the prosopistomatid molecular phylogeny (Chapter 6) was determining the relative ages of prosopistomatid species that were investigated, which, despite considerable age ranges at each node depending on the calibration, gave the oldest species in Asia and the youngest in Africa and Europe. This implies dispersal from Asia in the direction of prevailing winds, from the Angaran refuge which is now part of Siberia, to Europe and Africa, and simultaneously down the Pacific Island chain to northern Australia. This agrees with the part of the discussion in section 8.3.2, where with numerous other studies have indicated dispersal from Asia, including other mayflies (e.g. certain Polymitarcyidae, Ephemeridae and Caenidae (Edmunds, 1979)). The area cladogram (Figure 8.10) shows the relationships between the areas according to the molecular phylogeny.

Dispersal has undoubtedly played a major role in increasing the range of prosopistomatid species, with subsequent evolution of species from populations that have become allopatric. Dispersal requires a series of progressive steps to allow range expansion, or in some cases, a massive leap. It is most likely that some dispersal occurred between Africa, Madagascar and India while these landmasses were still in closer proximity, during the Cretaceous. As mentioned earlier, the occurrence of the current *Prosopistoma* species in Australia seems to be via more recent dispersal from Asia rather than continental vicariance, despite the baetiscoid fossil representative of Gondwanan age in Australia (Table 8.1). This does not refute the age of the lineage, but would rather point to a more recent secondary diversification and dispersal of crown-group species than suggested by the continental drift model. The research of Dijkstra (2007) on the dispersal of dragonflies from Asia offers an interesting perspective. The dragonflies were shown to have dispersed westwards across the Indian Ocean, with oceanic islands providing points of settlement and a source for further dispersal. Present-day prevailing winds across the Indian Ocean blow westwards from South East Asia and the Pacific Islands towards Madagascar.

Madagascar is thought to have moved into the belt of easterly trade winds during the Paleocene, becoming warmer and wetter, reaching the full effect of the winds around 30 Ma during the early Oligocene. The monsoon system present now only became established in the last 8 Ma (Wells 2003). Warren *et al*. (2010), in a synthesis of molecular phylogenies comparing Asian and Madagascan flora and fauna, found that 78 % of the divergences studied post-date the separation of India and Madagascar by up to 87 Ma. Indian winter monsoon winds and periods of low sea levels during which chains of islands occurred between the Seychelles, Mascarenes and India, are used to account for the higher than excepted Asian influence in Madagascar for lineages which are too young to be explained by continental drift. This offers an alternative hypothesis for the presence of the "*P. variegatum"* lineage in Madagascar, suggesting that it may have originated in Asia rather than on the Lemurian landmass as postulated in section 8.3.2. While, unlike dragonflies (Dijkstra, 2007), *Prosopistoma* species have very low flight capability, it can be postulated that a dead female with fertilized eggs may have been blown across to Madagascar from Asia. The possible age range of *P. variegatum* of 7 to 15 Ma (Chapter 6) allows this hypothesis. Further molecular analysis of Asian, Pacific Island, Madagascan and African species would be needed to investigate the ages of and relationships between species inhabiting these areas, and see if there is evidence of introgression. A further test would be to simulate the conditions of long distance jump dispersal such as this by testing the vialibility of eggs from a dead female which has been subjected to laboratory tests simulating dispersal conditions. The presence of species on islands which may have acted as stepping stones in range spreading and speciation, as seen in the dragonflies, would also need to be confirmed.

If dispersal from Asia, by whatever means, has played such a large role in the biogeography of the Prosopistomatidae, why do orders such as the Plecoptera (Fochetti and Tierno de Figueroa, 2008) or Megaloptera (Cover and Resh, 2008) have such a high diversity in Asia, and such a low diversity in Africa? Why have these not dispersed in a similar way, either via the Middle East or by wind? Gheerbrant and Rage (2006) give the isolation of Africa from the Mid-Cretaceous (Albian-Aptian) to Early Miocene, a period covering some 75 million years, as a cause of absence or poor diversity of some taxa. Specific ecological requirements may play a role in facilitating dispersal of particular groups of taxa, but distribution patterns brought about by dispersal are more random, as prevailing wind directions, for example may change direction over time. Vicariance

produces repeatable patterns, though these may be obscured by subsequent dispersals and extinctions.

The molecular clock approach is currently very popular, and can provide a useful yardstick by which to estimate lineage divergence times. However, the results must still be treated with some caution. While a molecular phylogeny based on a number of different genes gives a useful reflection of relationships, the dating of clocks still shows wide error margins (as seen in the great range of possible dates of lineage splitting shown in Chapter 6). Pulquério and Nichols (2006) found that different molecular clock methods can give dates that differ up to 20 fold. However, the relaxed clock method introduced and developed by Drummond and Rambaut (2003, 2007); Drummond *et al*. (2006) provides a better estimate than some of the earlier methods as it does not rely only on assumptions of nucleotide substitution rate, which differs in different organisms, and takes different evolutionary models into account.

It is important to consider the different usage of the term "dispersal" by ecologists, who just use it to mean movement from one place to another, e.g. of a propagule, while for disperalist biogeographers, it means differentiation and speciation, as well as movement. If dispersal over a barrier can happen once, it is possible that it may continue to occur across a barrier in both directions, thus preventing differences in a gene pool from accumulating, preventing speciation. Even if dispersal occurred over a barrier in only one direction (e.g. as the result of a prevailing wind), this may dilute changes in the gene pool of the second population, preventing speciation. On the other hand, vicariance splits a population, and if the barrier is large enough (e.g. an increasingly large stretch of ocean), the populations eventually become far enough separated over time to stop gene flow that may otherwise occur if the barrier is not too great. Both processes can lead to isolation by distance, but dispersal may not lead to speciation, while vicariance usually does.

8.3.4. The disjunct relationship between European and southern African species

The results of the preliminary molecular phylogeny (Chapter 6) show an unexpected close relationship between the widespread European species, *P. pennigerum* and the relatively widespread southern Africa species, *P. crassi.* Assuming this to be an accurate reflection, there are three possible scenarios accounting for this pattern. The ancestral species to both may have occurred in Africa and moved northwards; it may have had an equatorial African origin, dispersing simultaneously northwards and southwards to the more temperate zones now occupied by both species; or it may have been Laurasian and moved southwards.

The concept of a disjunct European-Southern African distribution is not a new one, for example, Jeannel (1957) noted this for certain Carabidae (Coleoptera). A recent focussed research program to investigate this disjunct distribution pattern, particularly between European Mediterranean and southern African fauna, has shown that this pattern is prevalent among a wide range of different taxa. These combined studies (Bologna *et al*., 2008) concluded two models within this pattern, firstly complete disjunction (where intermediate species are assumed to have become extinct), secondly disjunction with intermediate relicts in the Sahara and East African regions. As no *Prosopistoma* material from East Africa produced molecular results, and the material in North Africa has never been examined beyond family-level recognition in ecological studies by other authors (Gagneur and Thomas, 1988; Thomas, 1998; Touabay *et al*. 2002) and is not available for study, the Prosopistomatidae cannot categorically be placed in either of these two models at present.

A disjunct pattern can be explained by a number of historic events. A widespread continuous distribution during the Palaeogene of an ancestral species may have become fragmented due to drier climatic conditions which caused fragmentation of forest ecosystems in East Africa and development of the Sahara desert during the Pliocene. Additionally, Miocene landbridges between Europe and Africa which formed after the Tethys Sea closed some 18 Ma, allowed much range expansion (McGuire and Kron, 2005). Bologna *et al*. (2008) refer to corridors which periodically connected Europe and Africa over the last 20-25 Ma. Dapporto *et al*. (2009) suggest that Mediterranean islands may have functioned as refugia during cold periods. The *P. crassi-P. pennigerum* lineage is given as being at most 5 Ma (Chapter 6), indicating recent dispersal between the two continents. Relationships between the other African *Prosopistoma* species needs further investigation using molecular techniques with fresh material to determine both lineage ages and relationships to clarify the evolutionary patterns behind this distribution.

8.3.5 Track compatibility analysis applied to Prosopistomatidae distributions patterns

The aim of this exercise is to see whether there are distribution patterns amongst other unrelated freshwater organisms which are congruent with the distributions seen in Prosopistomatidae, both in time (geological age) and space (distribution). Congruency of distribution patterns implies a common history, driven by similar evolutionary forces (for example, splitting of a previously wide distribution range by formation of barriers, causing vicariance, or dispersal due to corridors of suitable habitat or a prevailing wind). This

method is based on the panbiogeographic approach of Croizat (e.g. Croizat 1958, 1964). It involves delineating tracks linking distribution patterns of members of one taxon on a map, and comparing this to tracks for different taxa with similar distribution patterns. This allows the deduction of generalised tracks which show whether many unrelated taxa have arrived at their current distribution via similar pathways or not. The significance of a generalized track depends on the tracks being based on phylogenetic principles, reflecting a similar time period. If the track is repeated frequently, it has deterministic value rather just reflecting stochastic events, which would be little more than descriptive. Croizat *et al*.'s (1974) premise is that biogeographical hypotheses must be congruent for a wide range of organisms. A flaw in this approach is that current distributions may not reflect historical patterns. This has been shown to be the case in several examples looking at plant community change over time, e.g. Livingstone (1967) and Davis (1983). However, the aim is to use current distributions to estimate past distributions and to explain what is likely to have influenced these distributions.

Although the examples selected below are at different taxonomic ranks, they are all monophyletic lineages, the main rationale needed to allow such a comparison, as these represent units of evolution. While Croizat's panbiogeographic approach has been heavily criticized (e.g. Mayr, 1985; Seberg, 1986), it allows reflection on whether other taxa may have reached their current distribution in a similar way. This may point to refugia or offer other solutions which may not be immediately apparent from studying only one confined group. Craw *et al*. (1999) see panbiogeography as a tool to emphasize the spatial dimensions of biodiversity, and this is how it is intended here. It allows assessment of distributions, uncluttered by preconceived ideas of geographical affinities. Although criticized due to its lack of method and consequently largely narrative approach, many researchers have continued to expand and apply methods to panbiogeographical theory (e.g. Grehan, 1988, 2001; Heads, 2005a). Phylogeography (Avise *et al.*, 1987) deals with similar theoretical concepts, focussing on spatial arrangements of genetic lineages among closely related species. Due to the limited success with gene amplification in prosopistomatids, this approach could not be used in this thesis.

Since Ephemeroptera are restricted to freshwater ecosystems, other freshwater organisms were chosen for comparison. Distributions of freshwater fish and non-insect invertebrates make particularly useful comparisons as they are less able to disperse than freshwater insects. It is most constructive to compare the distribution of other taxa which occurred far back in geological time, rather than comparison with taxa which are too recent

for continental drift to have had any effect on the formation of their species. This may seem to presuppose vicariance by continental drift as a driving factor behind the evolution of these groups, but if only younger lineages are chosen, vicariance is eliminated by default. It is important to be aware of the age of each lineage, otherwise there is no common underlying event. Some lineages considered to be older than the breakup of Gondwana were selected, and some which are younger but having a similar distribution were investigated as a comparison to see whether dispersal only would produce a similar pattern. The examples were initially chosen by similarity of distribution pattern rather than by age. Lineage age determination is important as it can be critical in deciding whether a distribution may be attributed to tectonic vicariance (or other geographic barriers), or whether to more recent dispersal. If geographically separated but related taxa can be shown to be older than a barrier (e.g. older than the age of separation or two landmasses, or formation of a chain of mountains which can separate two river systems), then vicariance is implied, unless dispersal over or around that barrier is still possible. If it is of the same age or younger than the barrier, vicariance is falsified. A vicariance hypothesis can be verified if examples of other unrelated organisms follow the same pattern, as animals evolve with the earth as it changes.

8.3.5.1 Example 1. Sisoroidea catfish (Figure 8.11)

The Sisoroidea catfish share a similar distribution pattern to the Prosopistomatidae, although they are absent from Madagascar, Australia and Europe. Their absence from Madagascar and Australia indicates that a Gondwanan origin is unlikely (though they may have become locally extinct in the regions of absence). The group is thought to have originated in the Cretaceous, which post-dates the splitting of Gondwana. Hora and Silas (1952) indicated an Asian origin (Yunnan, China), based on the fact that the highest number of species are known from this area. The molecular phylogeny of Sullivan *et al*. (2008) supports an Asian origin and dispersal. This offers an explanation for the absence from Madagascar and Australia; their presence on these landmasses could have been anticipated if the distribution was the result of tectonic vicariance. Thus a superficially similar modern distribution does not indicate a similar origin.

8.3.5.2 Example 2. Cichlidae fish (Figure 8.12)

The global relationships between cichlid species (excluding more recent African great lakes species) has been based on the assumption of the influence of continental drift vicariance

biogeography, with the age of divergence of major lineages being given as Mesozoic, based on geological evidence for the time when the landmasses began to separate. The African and South American taxa are considered monophyletic, and sister to the Madagascan and Indian taxa, offering a classical tectonic vicariance model (Stiassny, 1991; Farias *et al*., 1999, 2000). Bănărescu (1990) recorded a Miocene fossil representative, indicating a relatively young age for this group, although this does not exclude the possibility of the existence of older fossils. Farias *et al*. (2000) produced a total evidence analysis which concluded vicariance events associated with the fragmentation of Gondwana as the explanation for cichlid biogeography. They found Malagasy/Indian cichlids to be the most basal lineages, with a sister-group relationship to monophyletic African and Neotropical clades. In contrast, a dated molecular phylogeny calibrated using the age of cichlid radiations in the great lakes of Africa produced by Vences *et al*. (2001) implies that a more recent Cenozoic dispersal is more likely. They suggest a Cenozoic recolonisation of Madagascar following the extinction of the original faunas at the time of the K-P mass extinctions. The question this raises is how do mouth-brooders disperse? There are no propagules to be taken on bird's feet, and this suggests that the dispersal conclusion needs to be investigated further.

8.3.5.3 Example 3. Anura (Figure 8.13)

Amphibians have been used as a classical example of vicariance biogeography (e.g. Richards and Moore, 1996; Bossuyt and Milinkovitch 2001; Van Bocxlaer *et al*., 2006) due to the long-held belief that they are not able to disperse over ocean barriers because of their intolerance to salt water. Vences *et al*. (2003) provide evidence from molecular data of dispersal of frogs (Mantellidae) from Madagascar to the Comores Islands (which have a volcanic origin). They also show the dispersal of Hyperoliidae from Africa to Madagascar and from Madagascar to the Seychelles (Figure 8.13). They indicate that three dispersals from Asia to Africa took place in the Tertiary (Rhacophoridae tree-frogs in the Eocene, and of *Rana* and *Hoplobatrachus* in the Miocene), possibly crossing the Arabian Peninsula or using land bridges in the Tethys Sea, or even rafting. These frogs fall within the suborder Neobartrachia, which have a predominantly South and Central American distribution, though a few widespread families are known (e.g. Ranidae) and several regionally endemic families (e.g. Myobatrachidae from Australasia, Hemisotidae from Africa). Such a distribution could be interpreted as being due to tectonic vicariance, but fossil evidence is against this (e.g. the Leptodactylidae fossil from amber, 37 Ma *(Cogger and Zweifel, 1998)*

– though this may not represent the oldest member of the lineage, which is thought to have arisen in the Mesozoic). Gao and Shubin (2003) give an age between 164-167 Ma for a fossil representing the Cryptobranchoidea. These provide further examples of localised dispersal playing a greater role than originally thought, although it cannot be used to dispute that vicariance has played a significant role in global frog distribution, with each monophyletic clade having its own particular pattern. The role of vicariance was further supported by Bossuyt *et al*. (2006), focussing on Ranidae (Figure 8.13), who showed endemism of each major clade with one Gondwanan plate. Another example of speciation due to tectonic vicariance of Gondwana can be seen in the Indian burrowing frog and its only sister group which is isolated on the Seychelles (Biju and Bossuyt, 2003). This ancient extant lineage of neobartrachian frog dates back some 130 Ma.

8.3.5.4 Example 4. The Syncarida (Figure 8.14)

The Syncarida of the Malacostraca (Higher Crustacea) are an archaic group dating back to the Carboniferous, and since the Triassic they have occurred only in freshwater. They have very limited dispersal capacity due to their mesopsammal habit (i.e. ground-water inhabiting). Although the Bathynellacea lack a fossil record, their geographical distribution indicates that they must have originated not later than the Upper Palaeozoic (245 Mya) (Schram,1981; Cho, 2005). A primitive species described from Australia (Cho, 2005) comes from a region which has not been submerged by the sea since the Palaeozoic, attesting to the antiquity of this lineage. Schminke (1974) indicated that this crustacean lineage dispersed from East Asia to Europe, through Africa and on to South America, with another lineage dispersing from East Asia through to Australasia and via Antarctica to South America in the Mesozoic. He took the most primitive taxa in the Far East as being taken as the source, thus the direction of dispersal is seen as towards the locality of more apomorphic forms (broadly dotted lines, Figure 8.14). He saw dispersal within this group is ancient, not recent in the context of the joined Gondwanan landmass which fragmented with these lineages. The historical biogeography of the cosmopolitan genus *Hexabathynella* Schminke, 1972 was investigated by Camacho (2003), who concluded tectonic vicariance since the time of Pangaea to account for all species (solid lines, Figure 8.14) except the New Zealand species, which is the product of dispersal (finely dotted line, Figure 8.14). This ancient group may have survived better than the baetiscoid stem group hiding in subglacial refugia under the ice and in interstitial places during glacial periods.

8.3.5.5 Example 5. Gerromorpha (Hemiptera) (Figure 8.15)

Møller Andersen (1982) synthesized the phylogeny and distribution of all of the members of this aquatic heteropteran group. Based on his phylogenies, he produced generalised tracks to suggest common pathways of dispersal or patterns of vicariance. This is summarised in Figure 8.15, focussing on the area concerning Prosopistomatidae distribution. Møller Andersen concluded that ancestral gerromorphans were widespread during the Mesozoic (200 Ma), with lineages within this diverging after continental break-up occurred. These vicariance events formed the basis of the modern day distribution, with details being obscured by extinction and more recent dispersal. Grimaldi and Engel (2005) put the Gerromorpha as having a Mesozoic (Jurassic) origin, some 170 Ma, which gives credence to Møller Andersen's hypothesis (referring back to section 8.3.2 where dates are given for Gondwana breakup).

8.3.5.6 Example 6. Ecnomidae (Trichoptera) (Figure 8.16)

The Ecnomidae have a wide distribution globally, occurring in every biogeographic region except Antarctica (de Moor and Ivanov, 2008). Johansen and Espeland (2010) suggest, based on molecular phylogeny and distribution patterns, that this family had a Gondwanan origin. Molecular dating of species in New Caledonia (Espeland and Johansen, 2010) indicate that Ecnomidae in New Caledonia are about 65 Ma, while *Agmina* species have radiated on this island within the last 20 million or so years. Looking at the origins of New Caledonia, lineages may be expected to be older due to the link to continental Gondwana. Instead, geology reveals some twists which make these biogeographic interpretations very interesting. New Caledonia was once part of the eastern margin of Gondwana with Australia, rifting in the late Cretaceous between 83 and 54 Ma. It was still at that stage part of a larger landmass, Zealandia, which also comprised what are now New Zealand and the Chatham Islands (Neall *et al.*, 2008). Now largely submerged, Zealandia protrudes above sea level as the islands of New Zealand, the Chatham Islands and New Caledonia. Although originally formed from continental crust, local tectonic activity has contributed further to the structure of these islands, so the geological history only extends back prior to 40 Ma. Terrestrial plant fossils from a nearby seamount indicate that another large island existed there between 38 and 21 Ma (Meffre **et al**. 2006, in Neall *et al*., 2008), showing that these islands are in fact relatively unstable and influenced by volcanic activity, thus having more the nature of a volcanic island than a continental remnant. Islands can, however, be colonised by lineages older than the islands themselves (Heads, 2005a), as earlier islands in

a chain of volcanic islands may have gradually become submerged, with their fauna dispersing to adjacent younger islands prior to submergence of the older islands.

Grimaldi and Engel (2005) place the stem group Ecnomidae at some 155 Ma, this being younger than the beginning of the breakup of Gondwana, although the component landmasses were still closely associated at that time, with only the African and Madagascan-Indian-Antarctica landmass having begun separation. Thus the Australia-New Zealand-Antarctica-South American component was still intact, which may account for the apparently amphinotic Chilean-New Zealand distribution observed today. Espeland and Johansen (2010) have shown a great species radiation in Ecnomidae on New Caledonia in a relatively short time, showing that while a stem group may be ancient, the crown-group may have arisen relatively recently. This ties in well with what has been found for the Prosopistomatidae.

8.3.5.7 Example 7. Stenopsychidae (Trichoptera) (Figure 8.17)

Ulmer (1912) reports Stenopsychidae fossils from Baltic amber, which gives an age of around 45 Ma. Grimaldi and Engel (2005) place the Stenopsychidae stem group as originating some 185 Ma (Jurassic), indicating a much longer existence of the lineage than the amber fossil alone would give. In the Stenopsychidae, 65 species occur in the Oriental region (extending into the eastern Palaearctic), 10 in Australia, with only one Afrotropical and one Neotropical species (de Moor and Ivanov, 2008). This dominance of species in Asia suggests an Asian origin for this family, and implies dispersal rather than tectonic vicariance. However, the age of the lineage and the amphinotic distribution of Australia and South America could allude to this being a remnant of a wider Gondwanan distribution, linked via Antarctica

8.3.5.8 Example 8. Chironomidae (Diptera) (Figure 8.18)

The Chironomidae are an old lineage, dating back to the Triassic (210 Ma) (Krzemiński and Jarzembowski, 1999). Brundin (1966) hypothesized that the distribution of the Chironomidae (Diptera) was influenced by the breakup of Gondwana. This was later supported by the discovery of a fossil from about 130 Ma (Lower Cretaceous) (Schlee and Dietrich, 1970), which is prior to the initiation of the South Atlantic, and to have a fossil of this age, the lineage must have been in existence for some time before 130 Ma. Brundin's research laid a sound foundation for biogeographic reconstructions based on phylogeny. Within the Chironomidae, a number of distribution patterns have been clearly identified

(Sæther, 2000). Sæther did not include Madagascar in his figure, but only one fifth of Madagascan species are endemic (Sæther and Ekrem, 2003) indicating a strong dispersal influence. The African species tend to be represented by warm-eurythermic species, and exclude the cold-stenothermous genera and species more common in Europe. Only one fifth of the species on Madagascar are endemic to that area, and more than half of the species on the Seychelles are limited to the Malagasy region (Sæther and Ekrem, 2003), suggesting that most of the species are the result of dispersal, and the tracks of Sæther (2000) do not include Madagascar. However, a link between chironomid fauna of tropical Africa and the South and East Asian subregions of the Sino-Indian Region is evident (Sæther and Ekrem, 2003). Both Gondwanan vicariance patterns and dispersal can be seen to account for current chironomid distributions.

8.3.5.9 Synthesis of track analyses

The above set of examples provides but a few comparisons of organisms with more or less congruent modern distributions. Many more examples could be selected, which could result in different inferences being drawn. This limited literature survey has highlighted a few trends. One is that the crown group members of several taxa are now considered to be younger than previously thought, shown by dated molecular phylogenies (e.g. Vences *et al*., 2001; Vences *et al*., 2003) and the molecular phylogeny of *Prosopistoma* (Chapter 6) shows this too. Tectonic vicariance of Gondwana (Figure 8.19) is seen to have played a major role in producing the distribution of taxa seen, but there is also a frequent pattern of colonisation by dispersal from Asia rather than from Africa. Thus both vicariance and dispersal are shown to play an important role in producing the current distributions of species from old lineages (Figure 8.19). These vicariances and dispersal routes agree with aspects of both the morphological and molecular phylogeny-based interpretations of *Prosopistoma* distribution.

Is there any way of further testing these distributions and drawing conclusions whether their relationships have any meaning? In an attempt at rationalising the above examples, areas of occurrence were used to determine relationships between the areas. Deriving comparable area cladograms for each taxon was not feasible as the phylogenies of each group were at different geographic resolutions (i.e. some global, some more localised), and did not include all regions for each example. Instead, presence or absence in each designated area for each taxon was recorded without trying to determine direction of range expansion. Appendix Table A8.1 reflects the presence / absence data as derived from

the mapped distributions shown in Figures 8.11-8.18, with the inclusion of Prosopistomatidae distribution.

Sørensen's coefficient (SC) of biotic similarity was used to assess the strength of association between the different regions based on these taxa (Table 8.3). While it may have been better to use recognized bioregions for the matrix used in these assessments, the approach taken here gives more detail, for example comparing Madagascar and Africa rather than just considering the Afrotropical realm, and recognizing the Middle East as a separate region rather than including it with the Palaearctic. This better reflects the original components of Gondwana and the identity of other palaeo-landmasses. The Middle East represents an important dispersal route (Botosaneanu, 1992), so should be considered as an entity. The same matrix (Appendix Table A8.1) was assessed using Bray-Curtis analysis of similarity, ordinated using MDS (Figure 8.20).

The results of the Sørensen's similarity (Table 8.3) and MDS analyses (Figure 8.20) show strongest affinities between Africa and India (complete similarity at the taxon level investigated). This strong link between Africa and India is thought to be due to the Gondwanan connection of these land masses (discussed in section 8.3.2). The tendency for dispersal from an Asian stronghold (discussed in section 8.3.3) and the dispersal of taxa to Australia via the Pacific Islands, resulting in a strong link between Australia and these islands, are generally reflected by the relative values of the Sørensen's index. The relatively close association between Europe and Australia (Table 8.3) may reflect dispersal from Asia simultaneously to Europe and to Australia, resulting in shared taxa, although the lower association between Europe and Asia does not agree with this. The affinity between Africa and Madagascar is not as strong as may have been expected, which may perhaps reflect the high degree of endemicity known on Madagascar for many different organisms. Table 8.3 shows a surprisingly high affinity between the Pacific Islands and South America and Australia and South America, possibly reflecting dispersal between these regions. The strong European – North American affinity reflects their shared Holarctic history. Overall, the above results using Sørensen's coefficient provide a useful way to compare the distribution ranges of the taxa studied. One problem when comparing these diverse taxa between Madagascar and Africa is that there are many close relationships which are unique at a number of levels from generic to family, and at taxonomic lower levels they may not be revealed

A final thought on track analysis - does sharing a similar distribution pattern really mean that unrelated organisms reached this position by the same means and at the same

time, as implicit in Croizat's approach? While this may reflect a similar history, they may alternatively have arrived at their current distribution by very different means or at different times. Also, current distributions may not be a good reflection of past distributions.

8.3.6. Adaptation to specific river habitat conditions

The ordinates derived from the correspondence analyses produced plots of species relationships based on abiotic parameters (Figures 8.21), examining patterns between species based on physical abiotic parameters.

While the underlying geology of a river system may be assumed to affect the nature of the river substrate, the effects of this on species distribution are shown here to be negligible, and species from regions with similar geology do not necessarily group together. For example, India and Africa are both largely formed from Precambrian igneous and metamorphic rock, forming stable continental shield (see references under "geology"), but *P. indicum* does not group with the African species under these criteria. Despite similar basic geology, the formation of the Himalayas, causing river rejuvenation, and the Monsoon climate, will have had a greater effect than shared geological history. Africa (and Australia) has much more weathered landscapes with corresponding effects on the river courses. Thus, species have adapted with the rivers in which they live.

The PCA (Figure 8.21) indicates that temperature, pH and current speed are important criteria in determining the presence of most *Prosopistoma* species. Substrate is more important for some species, these including several of the southern African species (*P. africanum, P. crassi, P. amanzamnyma, P. mccaffertyi*), the Moroccan species, P. *orhanelicum* (Turkey) and *P. olympus* (Borneo). Width of the river does not seem to affect distribution at all.

Section 8.2.1 alluded to a preference for warmer waters if *Prosopistoma* had a tropical Gondwanan origin. While most of the species occur in warmer tropical waters, the northern European and Russian species are an exception. While the Mediterranean populations occupy rivers with a relatively equitable annual temperature range, the Volga River is known to freeze in winter, and rivers in Sweden are also subjected to harsh winter temperatures. Some of the localities in Asia are also subjected to snow in winter. Thus, while some species are adapted to cooler climates, there is a greater tendency for *Prosopistoma* species to occupy warmer, more tropical climates (Appendix Table A8.2) while the Baetiscidae tend to occupy rivers with cold winter minimum temperatures. Possibly cold-tolerance is a derived adaptation, and topicality plesiomorphic in this family.

8.4 Conclusions

What is the vagility (dispersal capacity) of the Ephemeroptera? Mackerras (1970b) suggested that mayflies are particularly good subjects for biogeographic studies because of their short-lived dispersal stage, and in many cases, narrow range of ecological tolerance. Edmunds (1972) also pointed out that the dispersal abilities of Ephemeroptera is particularly poor, making them good candidates for studying biogeography. Although a few more recent studies have shown that mayflies may disperse more than is intuitively obvious (e.g. Monaghan, *et al*., 2005), this applies mostly to Baetidae. Edmunds (1972) also recognised Baetidae as relatively good dispersers due to their occurrence on volcanic islands. Certainly Prosopistomatidae, due to their exceptionally short time in the winged stage (Gillies, 1954; personal observation) and restricted lotic water biotope requirement, do not seem to be good dispersers. Their occurrence on volcanic islands such as the Comores archipelago, for which dispersal seems to be the most likely explanation, does however indicate that they are capable of limited dispersal. Paulian (1961) reported an unnamed *Prosopistoma* species (material not seen) from Mohéli Island (also known as Mwali Island) from the Comores. Starmühlner (1976) reported a second *Prosopistoma* species (Comores sp. 1) from the island of Anjouan (also known as Nzwani Island), the nymph of which has been examined for this thesis. The Comores archipelago forms a chain of volcanic islands in the northern Mozambique Channel, with Anjouan itself being an extinct volcano (Esson *et al*., 1970; Flower, 1972). Schlüter and Trauth (2008) place Anjouan at approximately 3.9 million years old, which could be taken as a minimum age for the Anjouan species. Due to the volcanic origin of these islands, the Comores species can only have arrived by dispersal. Several studies have shown that a species on a volcanic island may be much older than the island itself (Craig, 2003b; Heads, 2005a), as the current fauna may have already existed on a previous, now submerged island within that archipelago chain.

Dispersal between Africa and Madagascar has been shown to play an important role in accounting for the present day distributions of a number of insects groups. For example, Torres *et al*. (2001), based on mitochondrial DNA data, postulated dispersal from Madagascar to Africa for a group of Lepidoptera (Nymphalidae). Monaghan *et al*. (2005) showed that dispersal between Africa and Madagascar plays a larger role than previously thought in baetid mayflies, and the presence of *Prosopistoma* on the Comores archipelago implies that this is true for the Prosopistomatidae. Bearing in mind the short adult life span

(less than an hour) of *Prosopistoma* species, it is surprising that dispersal over this distance has occurred, although possibly a dead female with fertilised eggs could be carried in the wind. The youngest of the Comores islands are closest to Africa, with the age of the islands increasing eastwards (Schlüter and Trauth 2008); possibly colonisation may have occurred on islands which were formed closer to the African mainland than they are now situated. Heads (2005a) pointed out that dispersal should not necessarily be assumed for the occurrence of species on the Comores islands as they are seen now, as during the Eocene-Miocene, other emergent land may have been present along the Davies Fracture Zone of the Mozambique Channel (McCall, 1997, in Heads, 2005a). Another important consideration is that although today the prevailing winds and ocean currents move in an east-to-west direction towards Africa, Ali and Huber (2010) have determined that during the Palaeogene period, strong ocean surface currents flowed from northeast Mozambique and Tanzania eastward towards Madagascar. This ties in well with the position of the Comores relative to Africa, and offers an explanation that the ancestral species may have rafted over to the precursor Comores island archipelago.

An important consideration when investigating distribution and abundance of organisms over time is that a large number of variables, biotic and abiotic, have interacted over a long period of time, resulting in the current distribution patterns. The obligatory freshwater aquatic stage for organisms such as mayflies, introduces a different component to those organisms which are marine or terrestrial. Although the stability of particular freshwater ecosystems may change over time, the very fact that much of the life cycle is in water, thereby experiencing less extreme temperature variations than a terrestrial ecosystem would, may have allowed mayflies to survive climatic changes which terrestrial organisms did not.

The ongoing debate over whether distribution patterns are more the product of dispersal or vicariance is one that must be tempered with wisdom. It is not constructive for science when one particular view point or method dominates to the exclusion of others, and biogeography seems to be particularly prone to this. Many examples have been published where a widely accepted vicariance distribution has been abandoned as currently favoured new approaches give another picture entirely. A widely quoted example is that of members of the Proteaceae. Traditionally quoted as the supreme example of vicariance biogeography since the time of Hooker (publications 1844-1860), sister-group lineages between Africa and Australia have been confirmed using molecular phylogenies (e.g. Waratahs (Emobthriinae) - Weston and Crisp (1994)). More recent studies however, e.g., Barker *et*

al. (2007), Mast *et al*. (2008), Sauquet *et al*. (2009), using dating methods based on fossil pollen, cast doubt that vicariance accounts for the distribution pattern seen in all modern members of the Proteacea. Molecular studies have shown that divergence between sister lineages in Africa and Australia occurred only 40-50 Ma, making this too recent to be accounted for by continental drift, inferring instead that dispersal accounts for most of the distribution patterns seen in the Proteacea today. What is not taken into consideration in such studies is that there may have been long periods of stasis, with minimal genetic change, and then sudden divergence. As Eldredge and Gould (1972) pointed out in their punctuated equilibrium hypothesis, changes in evolution are not necessarily gradual but can occur in random bursts. Modern coalescent models are starting to take this into account.

The molecular approach to phylogeny has provided a wealth of information and interpretation to many biogeographical questions, but molecular clock dating methods still seems to have pitfalls, and are sensitive to data quality and quantity (hence the large range in dates for each node in Chapter 6). A plethora of new software is continually being developed to solve the ever circular arguments which dominate the science of biogeography; positively, this has succeeded in turning aspects of biogeography from a narrative philosophy into a science with statistical backing. How much has dispersal influenced the distribution patterns seen today? While more recent dispersal events may cause range spreading, much of the initial foundation of lineages was the product of the breakup on Gondwana. These are two time scales which are often confused, and may lead to misleading interpretations.

The distribution of resources is also important for shaping distribution, as destination localities must have conditions suitable for the dispersing taxon. If the Prosopistomatidae had reached their current distribution predominantly by dispersal, what were the mechanisms for dispersal, and what routes could they have taken? The fossil literature synthesis undertaken for this thesis shows that the stem-group fauna was present already in Pangaean times, when species may have been widespread due to an equable and homogenous climate during the early Triassic. Tectonic vicariance and rafting played a big role in separation of and subsequent speciation of earlier lineages, with allopatric speciation occurring on each landmass after separation. Further dispersal and subsequent splitting of populations resulted in further allopatric speciation, which continues to this day. In Africa, this occurred through a series of river capture events (Barber-James, 2003). Along the Pacific archipelagos, speciation was brought about as a result of rising and falling sea levels causing periodic isolation (e.g. McCafferty, 1999; Craig *et al*., 2001). Glaciations are

most likely the cause of the reduced distribution and diversity of the family in Europe. Dapporto *et al*. (2009) suggest that Mediterranean islands may function as refugia during cold periods for nymphalid butterflies, with recolonisation to the mainland when climate conditions improved.

Evolution and biogeography are intricately interconnected, with changes of physical features of the earth, including climate change, interplaying with the fitness of a species to occupy a particular biotope and adapt with appropriate features to enable it to live there successfully. The two schools of though, vicariance and dispersalist biogeography, work together to produce the distribution patterns of organisms seen today. While the paradigms of practicing biogeographers may seem to change, the final result, hopefully, is a reasonable interpretation of the past history of a group of organisms, which may have important implications to understanding future patterns, and thereby enabling management and conservation of species under threat of extinction.

It can be concluded from the evidence presented in this chapter that the biogeography of Prosopistomatidae species can be explained by both vicariance and dispersal. Further molecular studies, focusing on Asian species, would help to resolve many still unanswered questions. Figure 8.22 shows two possible biogeographic models to explain the current distribution of Prosopistomatidae species, Figure 8.22a shows vicariance from an Africa-centred Gondwanan origin and subsequent dispersal (strongly supported by morphological phylogeny); Figure 8.22b (based on weakly supported molecular phylogenies) shows a Jurassic dispersal from an Angaran refuge to Australasian region, and to Europe and Africa, with secondary recolonisation of Europe from Africa following Pleistocene glaciations. This does not explain why species with the characters of the "*P. variegatum*" lineage (*sensu* Barber-James 2009) do not occur in Africa, although there seems to be a one way trend of dispersal from Africa to Madagascar (Vences *et al*., 2001, 2003). Thus, the biogeographic affinities of the Prosopistomatidae species are not yet fully resolved. The comparisons of other species sharing the same biotope as each *Prosopistoma* species, and the relative distributions of species within these communities, may be another future route to follow.

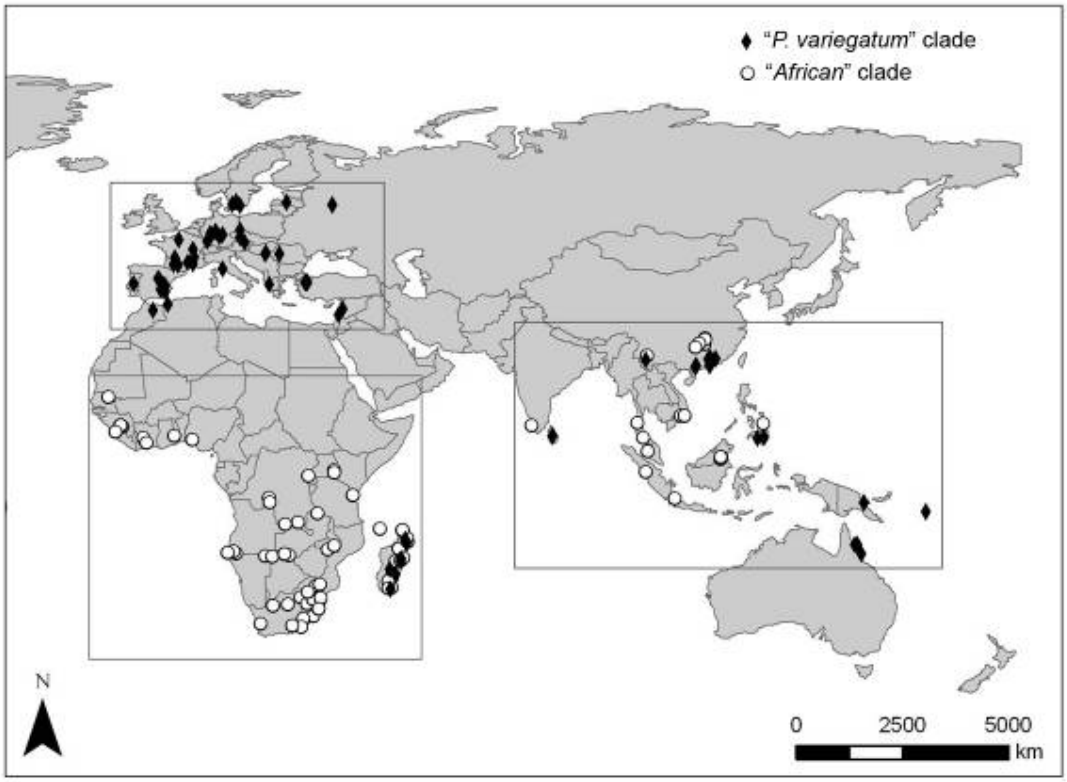


Figure 8.1. Global perspective of distribution of *Prosopistoma* species, showing the occurrence of the two lineages proposed by the morphological phylogeny.

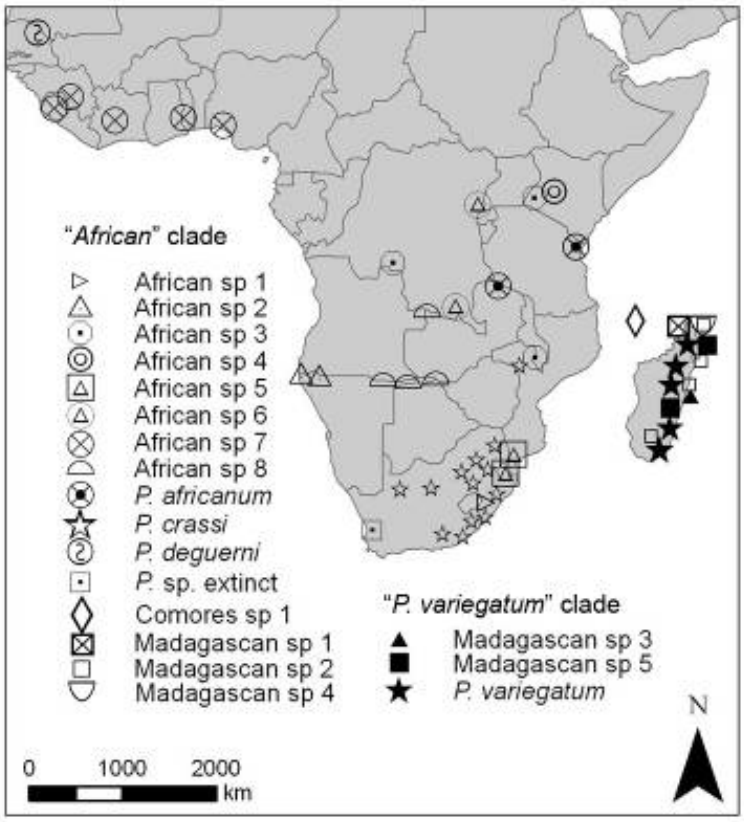


Figure 8.2. Localities of the species known within the Afrotropical region.

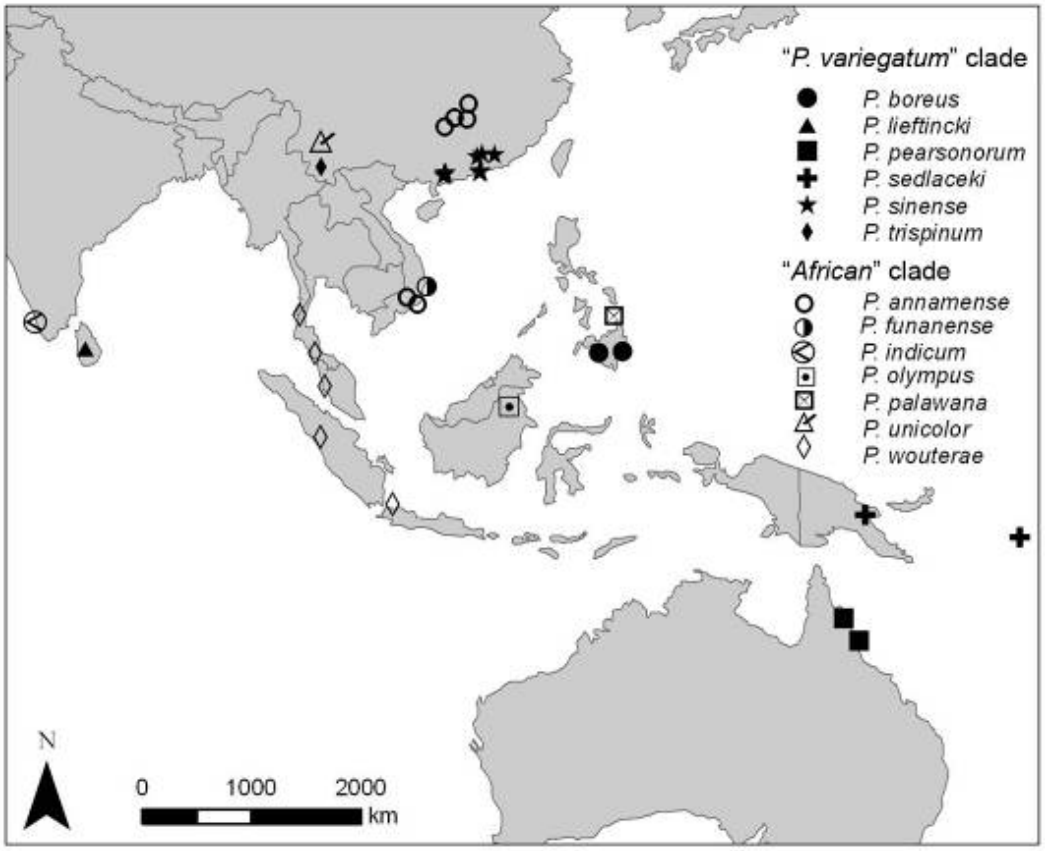


Figure 8.3. Localities of the species known within the Oriental and Australasian regions.

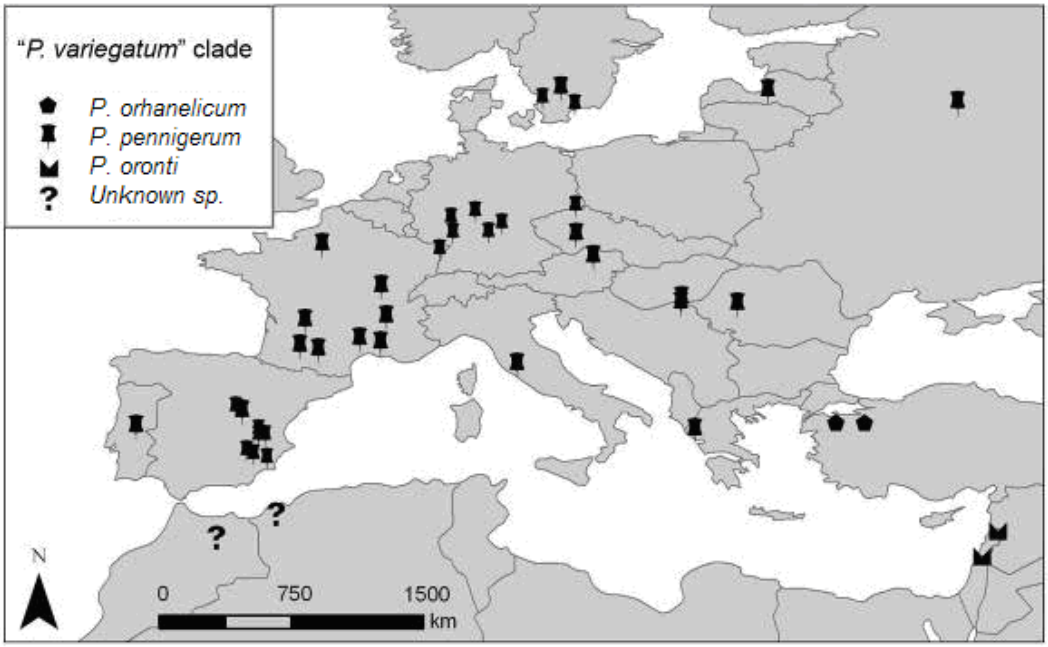


Figure 8.4. Localities of the species known within the Palaearctic region.

Table 8.1. Currently recognized stem-group Baetiscoidea fossils records.

Superfamily	Genus, species	Stage (adult or nymph)	Era	Period	Age (Ma)	Site (modern)	Author
Baetiscoidea	†*Balticobaetisca velteni*	Adult female	Cenozoic	Tertiary (=Paleogene)	54.8-33.7	Baltic	Staniczek and Bechly (2002)
Baetiscoidea	†*Balticobaetisca stuttgardia*	Adult male	Cenozoic	Tertiary (=Paleogene) Eocene	59.8-39.9	Baltic	Godunko and Krzemiński (2009)
Baetiscoidea	†*Protobaetisca bechlyi*	Nymph	Mesozoic	Lower Cretaceous	125-112	Crato fossil formation in Brazil	Staniczek (2007)
Baetiscoidea	*Unnamed* (?Siphloneuridae gen nov by Jell and Duncan; Stanickzek moved to Baetiscoidea.)	Nymph	Mesozoic	Lower Cretaceous	118-115	Koonwarra fossil beds in Victoria, Australia	Jell and Duncan (1986); Staniczek (2007)
Baetiscoidea (†Cretomitarcyidae)	†*Cretomitarcys luzzii*	Adult male	Mesozoic	Upper Cretaceous	93.5-89.3	New Jersey, North America	Sinitshenkova (2000b), McCafferty (2004)

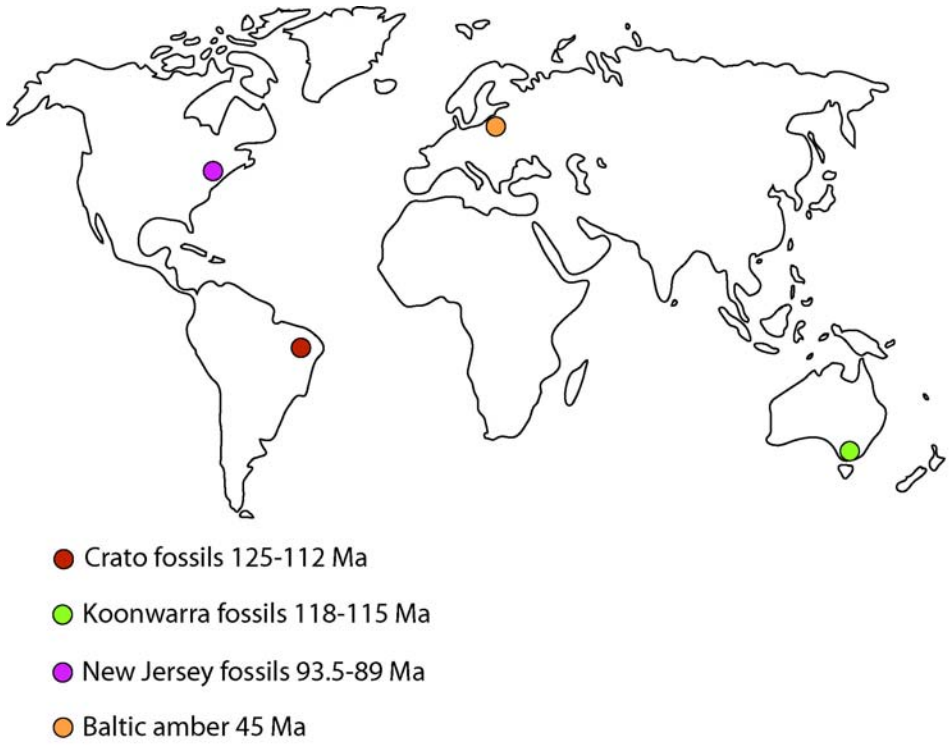


Figure 8.5. Localities of known stem-group fossils (Baetiscoidea), and dates of occurrence.

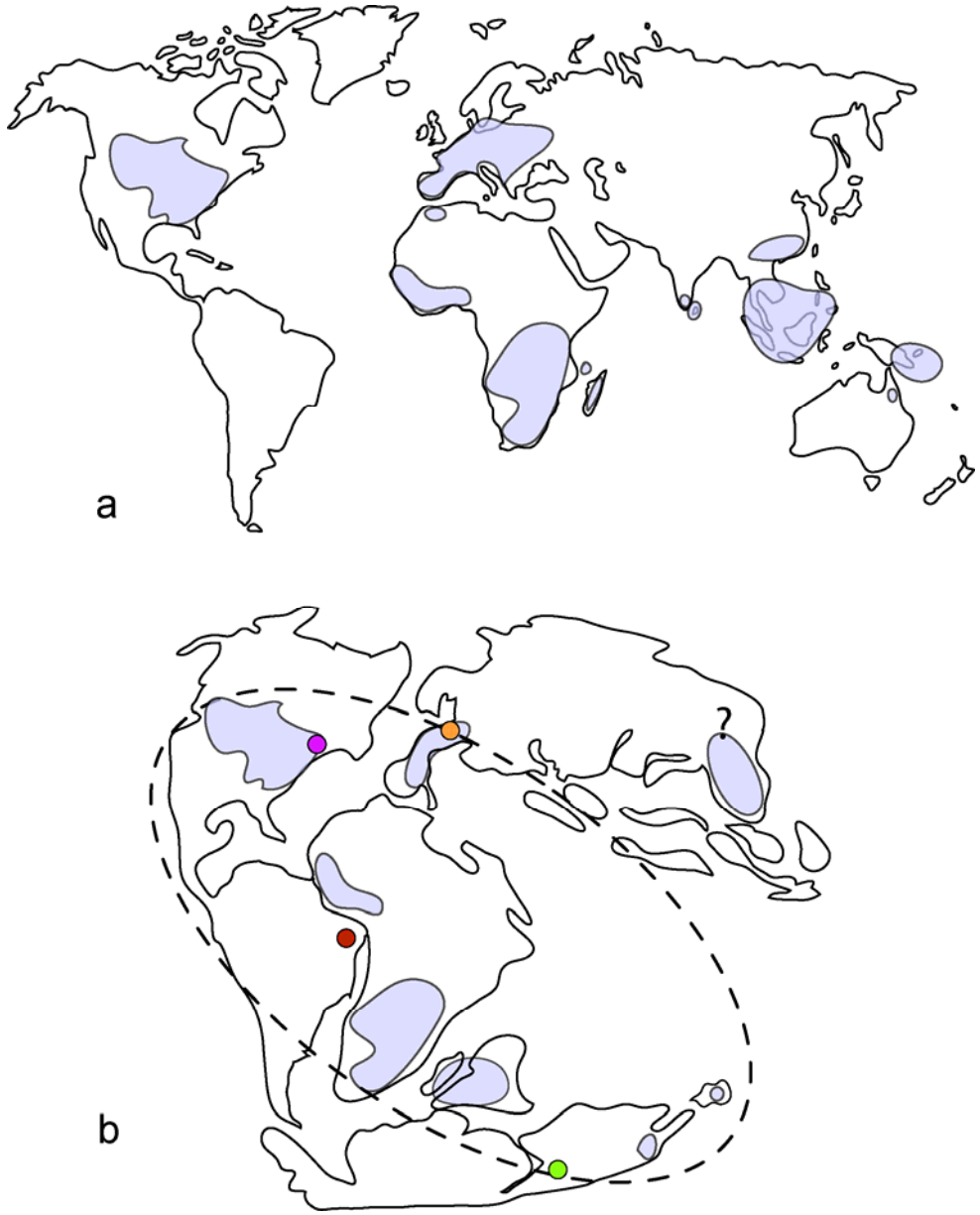
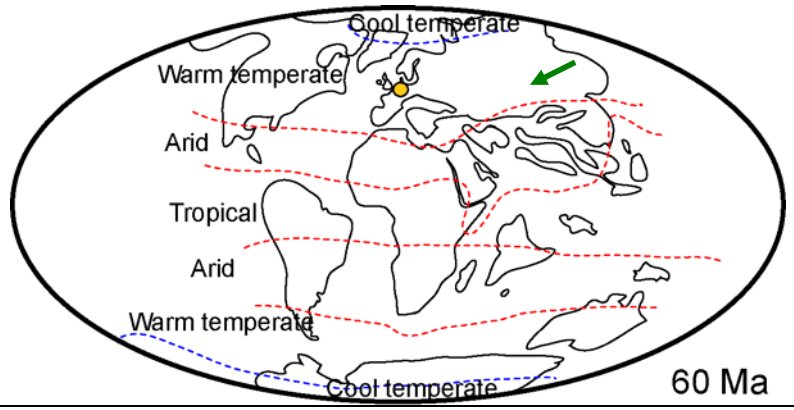
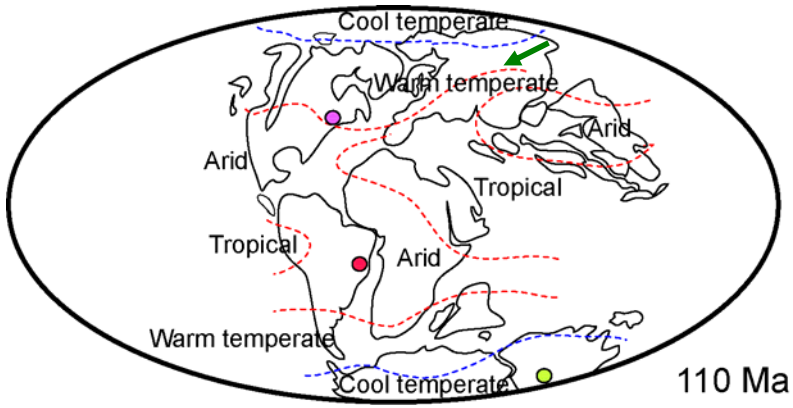
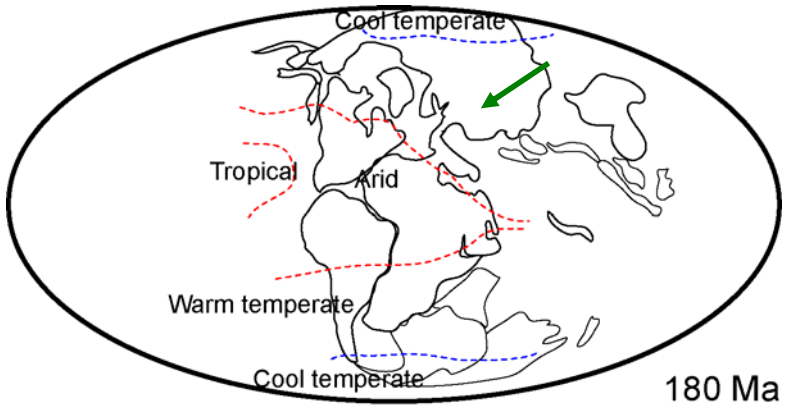
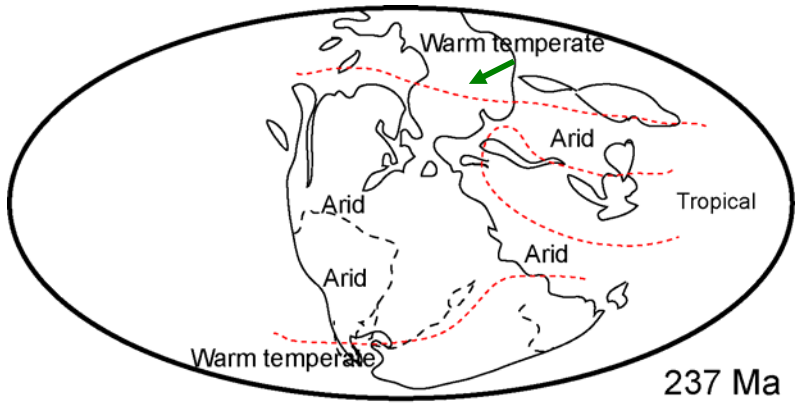
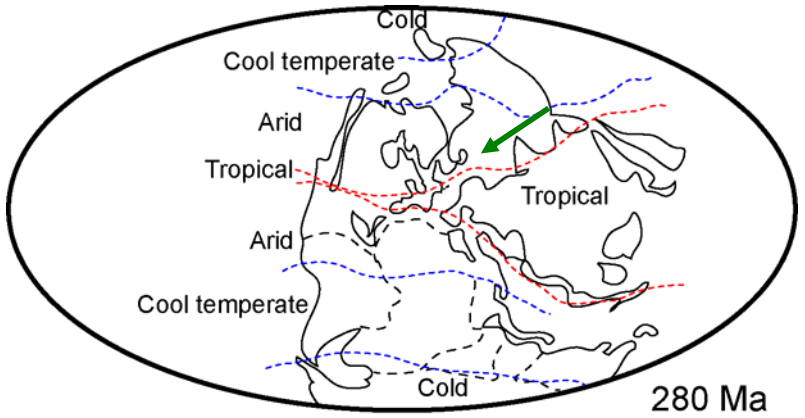
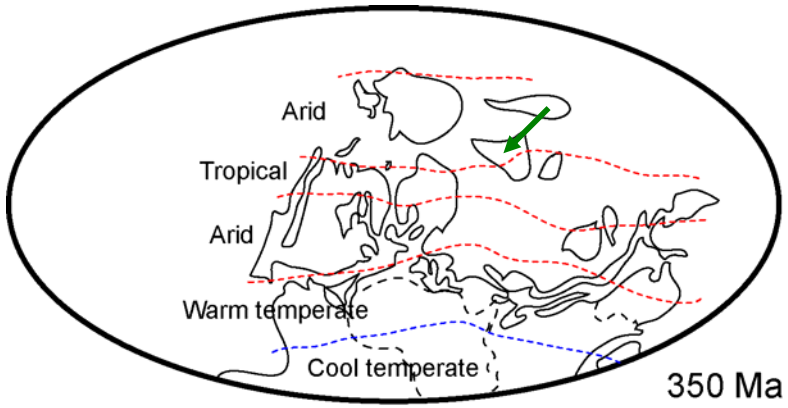


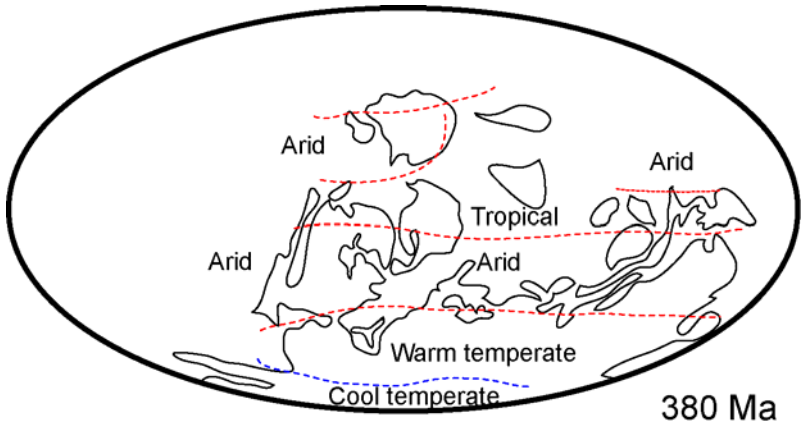
Figure 8.6. (a) Current distribution of the Baetiscoidea (in grey), (b) superimposed upon an approximately 120 Ma landscape (after Scotese). Known baetiscoid fossils as in Figure 8.5. The dotted line encompasses the area of possible distribution suggested by these distributions. There is no fossil evidence that they were present in Asia as such at this time, and no evidence to the contrary, hence the question mark.

Table 8.2. Climatic zones of the world during different paleontological periods, complied from Scotese 2003. Approximate position of Angaran craton indicated with dark green arrow. Dotted lines demarcate approximate climatic boundaries.

Climatic details for each Period	Arrangement of landmasses and climate
a. Cenozoic (Paleogene and Neogene) 65.5 Ma to present The Paleogene was still warm. The Neogene went back to sharp thermal gradients and polar glaciers ● Baltic amber fossil 45 Ma	
b. Mesozoic - Cretaceous 145.5 – 65.5 Ma (map 110 Ma) Landmasses more fragmented, climate more or less homogeneous "thermal era". Climate subtropical to warm temperate. Central regions still relatively arid. This is the period ended with the K-P (Cretaceous-Paleogene) mass extinction event 65.5 Ma. ● Crato fossils 125-112 Ma ● Koonwarra fossils 118-115 Ma ● New Jersey fossils 93.5-89 Ma	

Climatic details for each Period	Arrangement of landmasses and climate
c. Mesozoic - Jurassic 199.6 – 145.5 Ma (map 180 Ma) Much tectonic activity and separation of land masses. Tethys separated Gondwana and Laurasia. Indian and Altantic oceans began to form and divide Gondwana (South America, Antarctica, Australia and New Zealand) and Lemuria (*sensu* Van Steenis) Africa, India, Madagascar. During this period, Angaraland mayflies moved to more equatorial zones (which were less arid due to changed climate due to splitting of Pangaea) (Eskov, 2002).	
d. Mesozoic - Triassic 251 -199.6 Ma (map 240 Ma) Formation of Pangaea. Large arid equatorial belt. Small climatic gradients. Allowed wide dispersal of plants and insects. Subtropical central Asia was centre of evolution and dispersal at this time (Eskov, 2002). In the Early Triassic no equatorial humid zone can be identified and whereas the tropical zone was hotter than at present, the polar zones were also warm. This period appears to have been almost universally dry. The climate remained warm during the Middle and Late Triassic but humid zones can be recognized. The climate also showed some apparent asymmetry (Dickens, 1993).	

Climatic details for each Period	Arrangement of landmasses and climate
e. Palaeozoic - Permian 299 Ma – 251 Ma (map 280 Ma) Early Permian: Continental glaciations on Pangaea; Sharp climatic zonation, continuation of "cryo-era". During the Early Permian the world became warmer probably with some fluctuation in climate and with evidence for seasonal ice but not for glaciers. Late Permian: reduced temperature contrasts, climate of Gondwana and Angara both without frost. During the Late Permian the warming continued, and a new kind of provincialism developed within the broad tropical and subtropical zone. By the end of the Permian, the earth was very warm (Dickens, 1993). The Permian-Triassic (PT) boundary, 251 Ma, marks period of mass extinction.	
f. Carboniferous 359.2 – 299 Ma (map 340 Ma) Sharp climatic zonation "cryo- era" Euamerican and Cathaysian realm tropical Gondwana and Angaraland cool temperate to glacial (Eskov, 2002) During the Late Carboniferous the world became colder and the faunas and floras more provincial. Ice, probably mountain ice, appeared in Boreal and Austral regions. Widespread glaciation occurred in the earliest Permian but it had a very asymmetrical character. Whether continental ice sheets like those of the Pleistocene were present is not proven (Dickens, 1993).	

Climatic details for each Period	Arrangement of landmasses and climate
g. Palaeozoic - Devonian 416 – 359.2 Ma (map 380 Ma) The Late Devonian apparently had a warm climate. The climate of the Early Carboniferous was warm and equable. No cold climate can be identified, and apparently no ice was present at the poles. Even a cold-temperate climate is difficult to identify. On the other hand the tropical zone does not appear to have been particularly hot. Apparently this reflects an ocean current system and a weather system different from the present ones (Dickens, 1993).	

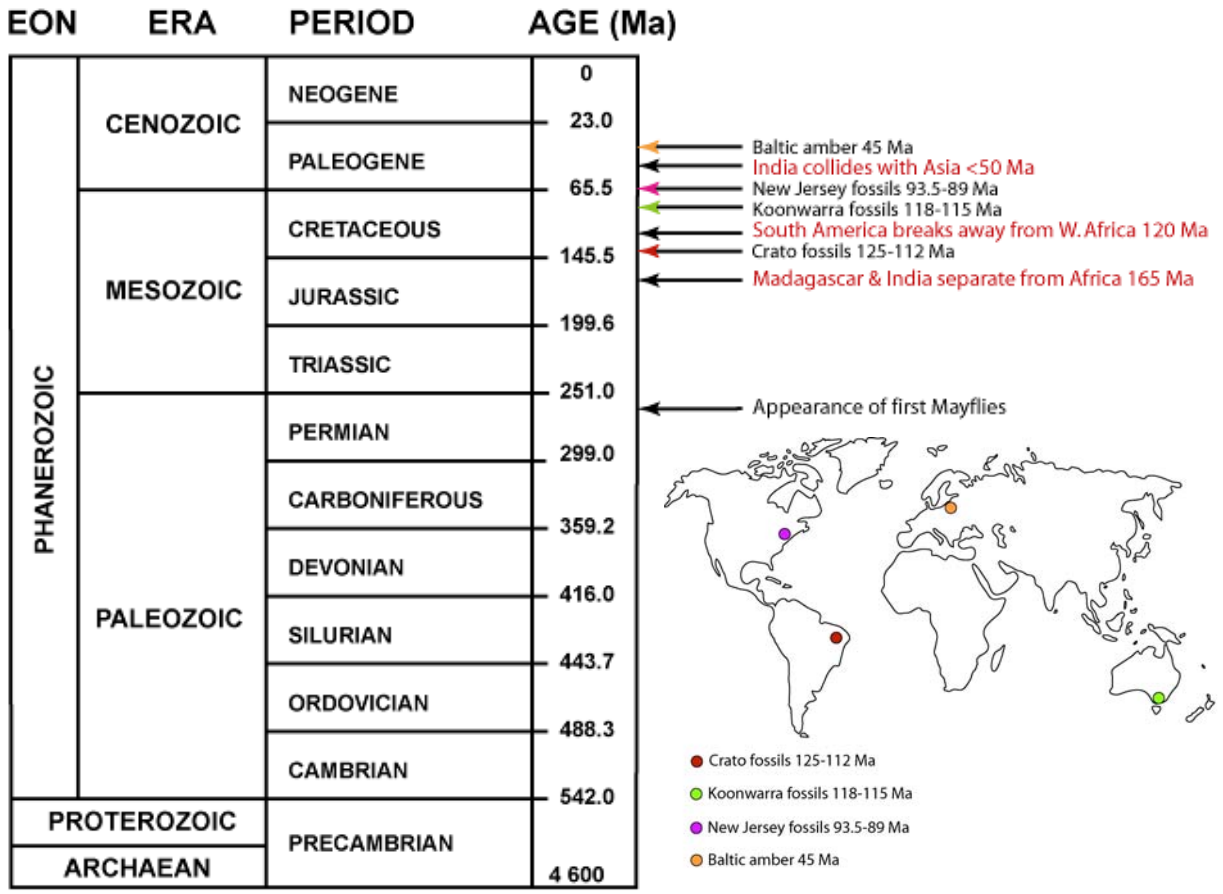


Figure 8.7. Stratigraphic chart (after International Stratigraphic Chart 2009 *www.stratigraphy.org*) showing timing of the more recent plate tectonic events in relation to the time of occurrence of the fossil species.

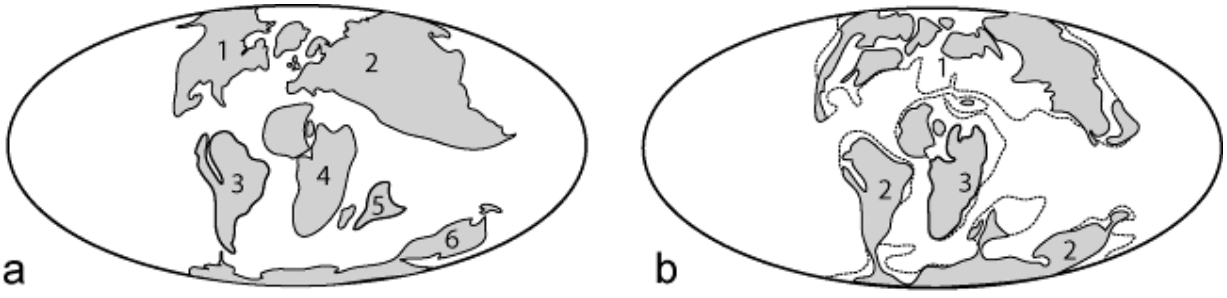


Figure 8.8. Alternative reconstructions for mid-Cretaceous paleogeography (80 Ma). (a) after Scotese, showing six major continental landmasses, (b) after Hay *et al*. 1999, showing three major continental landmasses, with large contiguous landmasses surrounded by shallow epicontinental seas (dotted lines).

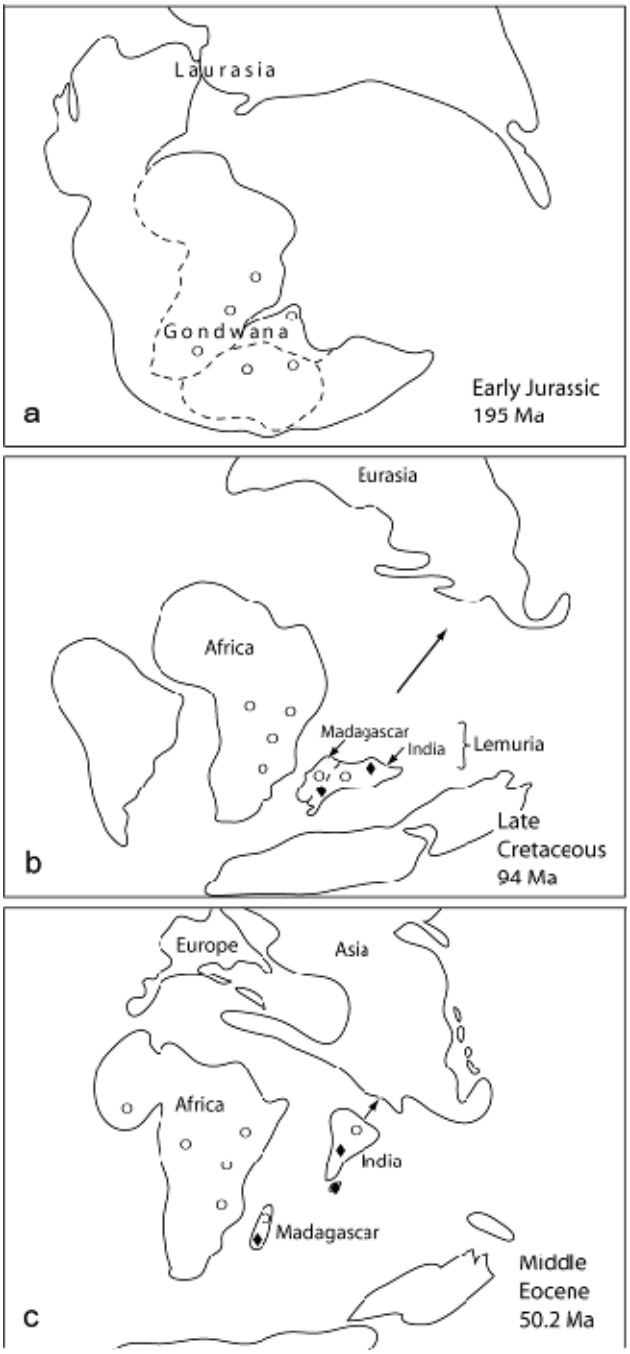


Figure 8.9. Proposed dispersal of precursor *Prosopistoma* lineages, based on currently known distribution and morphological phylogeny (maps adapted from Scotese (2003). (a) Gondwanan landmasses still connected, (b) Madagascar and India are still joined, forming "Lemuria", (c) India rafts northwards to join up with Asia. ○ "African lineage", ♦ "*P. variegatum* lineage" sensu Barber-James (2009).

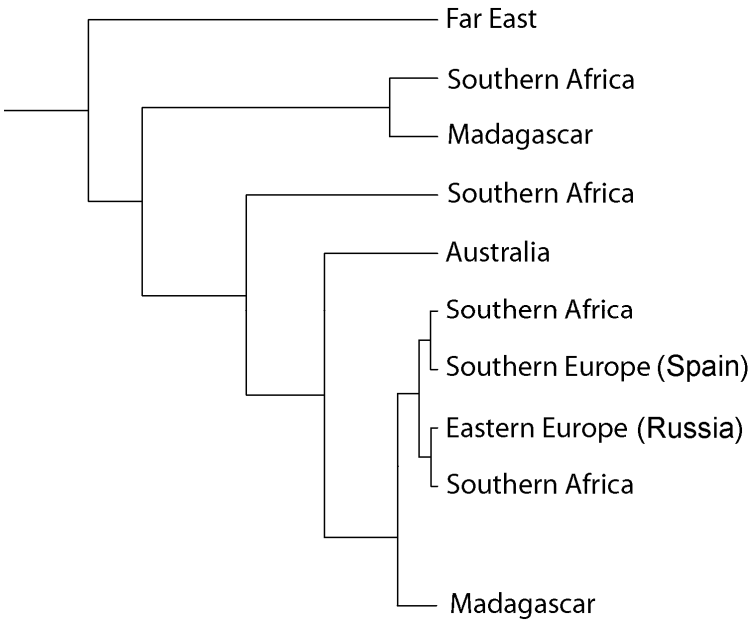


Figure 8.10. Area cladogram with localities based on relationships from the relaxed molecular clock phylogeny.

Figure 8.11. Global distribution of Sisoroidea catfish (group origin Cretaceous). Map adapted from Bănărescu (1990). Approximated dispersal routes shown by dotted lines.

Figure 8.12. Global distribution of Cichlid fish (after Farias *et al.,* 2000). Proposed vicariant dispersal showed by solid lines, and more recent dispersal by dotted line.

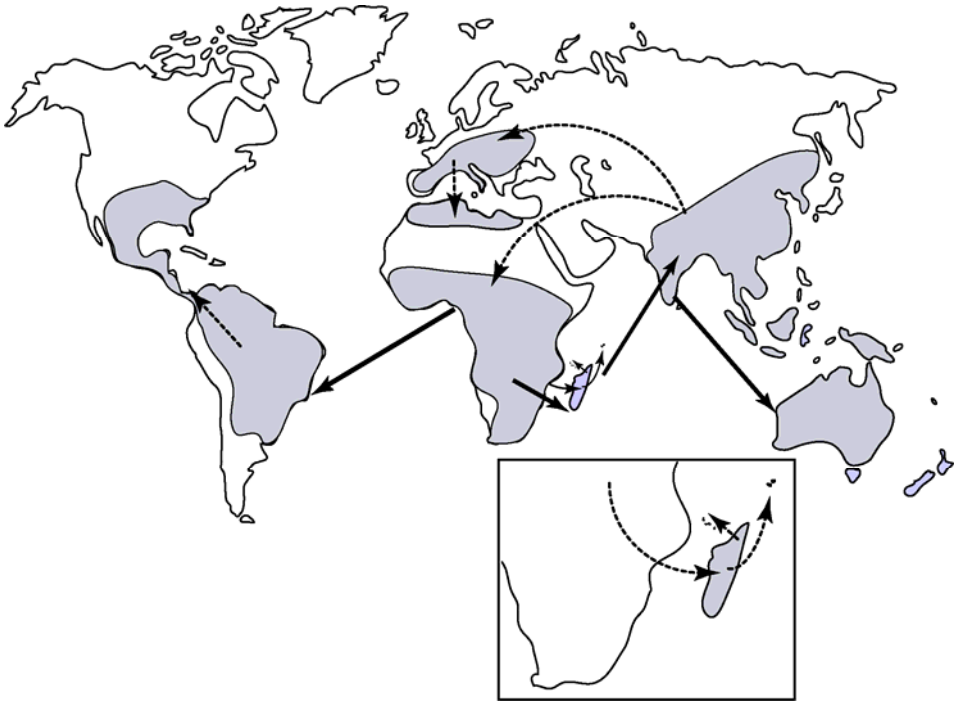


Figure 8.13. Generalized distribution of Neobartrachian frogs. More recent dispersals noted by Vences *et al*. (2003) between Asia and Africa, and from Africa to Madagascar and Comores and Seychelles (inset) are shown with dotted lines, as well as other small-scale dispersals. Bossuyt *et al*. (2006) indicate predominantly Gondwanan vicariant relationships for Ranidae (solid lines).

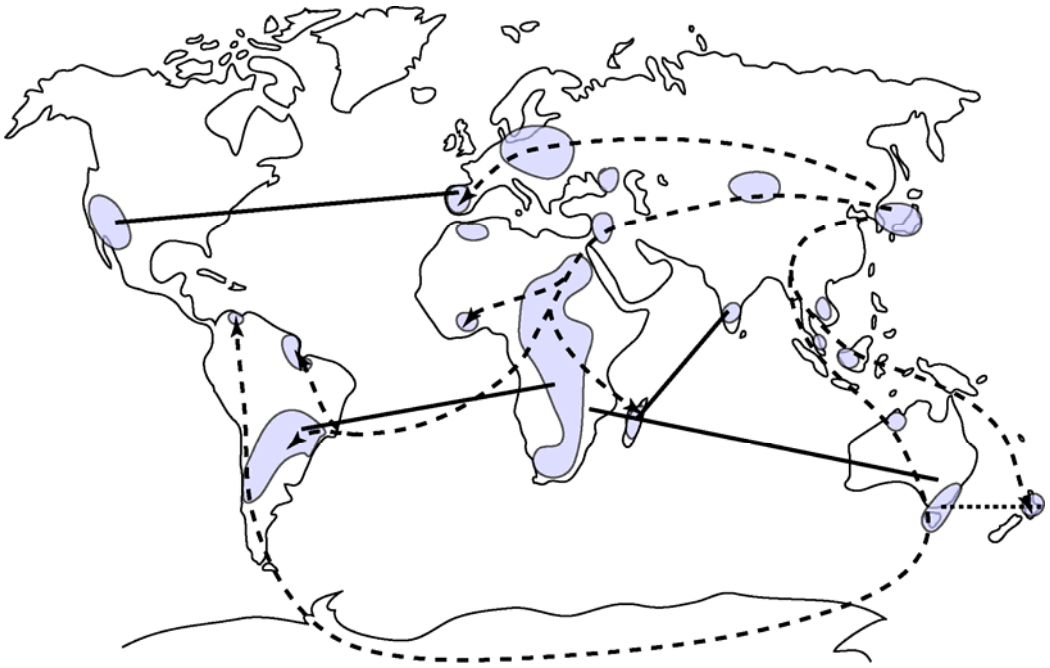


Figure 8.14. Parabathynellidae (Syncarida) distribution (after Bănărescu, 1990), with distributions as proposed by Schminke (1974) (broadly dotted lines), and vicariance relationships after Camacho (2003) (solid lines; dispersal to New Zealand shown by finely dotted line). At the time of Schminke's publication, North American and Indian species were not known. This broad distribution indicates a primitive cosmopolitanism during Pangean times.

Figure 8.15. Gerromorpha, show tracks modified from Møller Andersen (1982). (Gerromorpha are also present in the Middle East – species described since 1982).

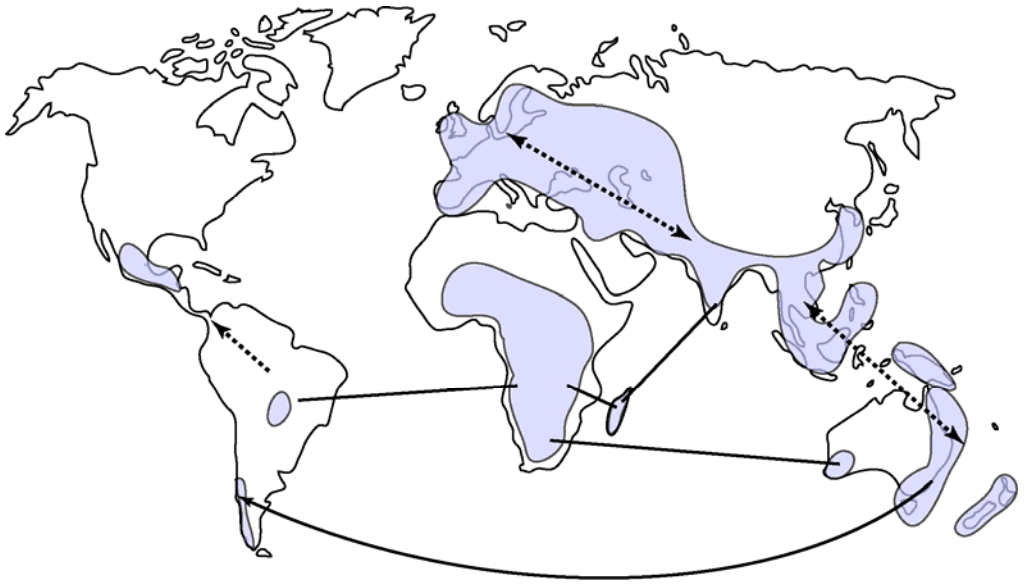


Figure 8.16. Ecnomidae (Trichoptera) (distribution after Bănărescu (1990), with solid lines showing vicariant dispersal from the Gondwanan origin proposed by Johansen and Espeland (2010). Dotted lines show possible dispersal. This family has been show to be capable of rapid diversification.

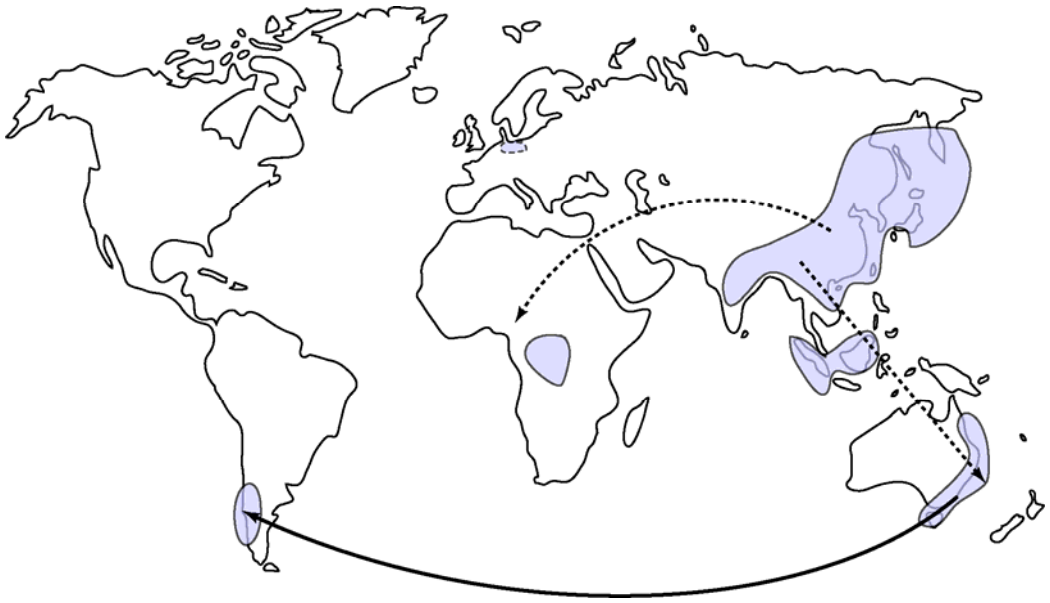


Figure 8.17. Stenopsychidae (including Baltic amber *Stenopsyche* fossil) (distribution after Bănărescu (1990). An Asian dispersal is indicated (dotted line), with remnant Gondwanan amphinotic link shown (solid line).

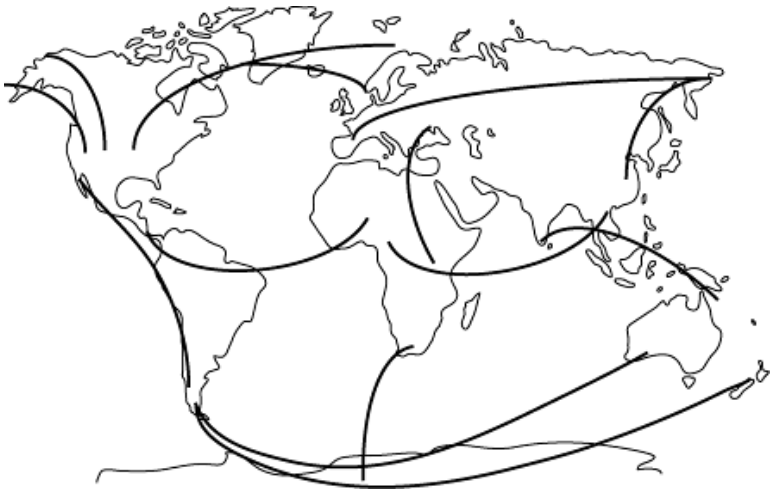


Figure 8.18. Generalised tracks of Chironomidae (after Sæther, 2000).

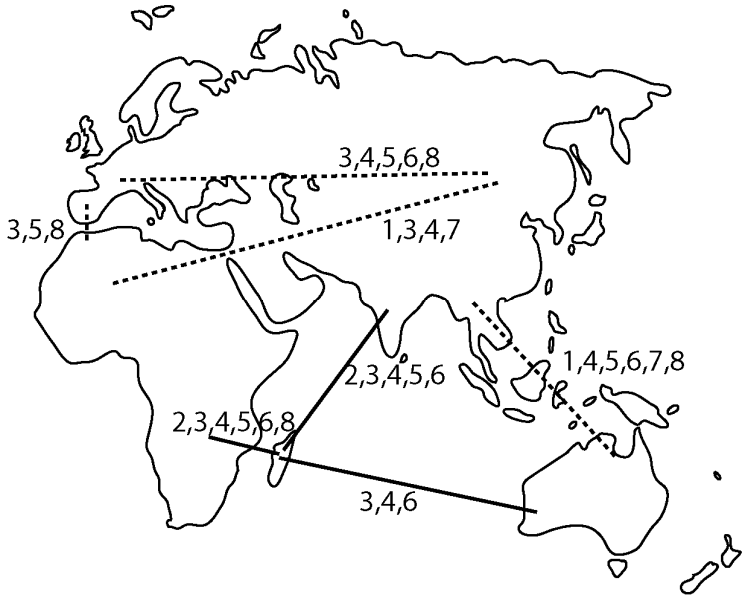


Figure 8.19. Synthesis of tracks investigated, focusing only on region which relates to current Prosopistomatidae distribution. Numbers refer to the taxon example number in the text. Gondwanan vicariance is shown with a solid line, dispersal with a dotted line.

Table 8.3. Sørensen's index showing relative affinities for each region based on taxa used in the track analysis exercise. The higher the value, the more similar are the faunas. Relationships with a value above 0.85 are shown in bold.

	Africa	Mada-gascar	Middle East	Europe	Asia (Far East)	India	Pacific Islands	Austra -asia	South America	North America
Africa	1.00	0.77	0.82	0.66	0.62	**1.00**	**0.87**	0.82	0.82	0.57
Madagascar		1.00	0.62	0.72	0.74	0.75	0.57	0.77	0.77	0.43
Middle East			1.00	0.83	**0.88**	0.82	**0.93**	0.71	0.43	0.54
Europe				1.00	0.71	0.67	0.77	0.83	0.66	**0.86**
Asia (Far East)					1.00	**0.95**	**0.94**	**0.88**	0.75	0.62
India						1.00	**0.89**	0.82	0.82	0.57
Pacific Isl.							1.00	**0.94**	**0.85**	0.50
Australasia								1.00	**0.85**	0.43
S America									1.00	0.72
N America										1.00

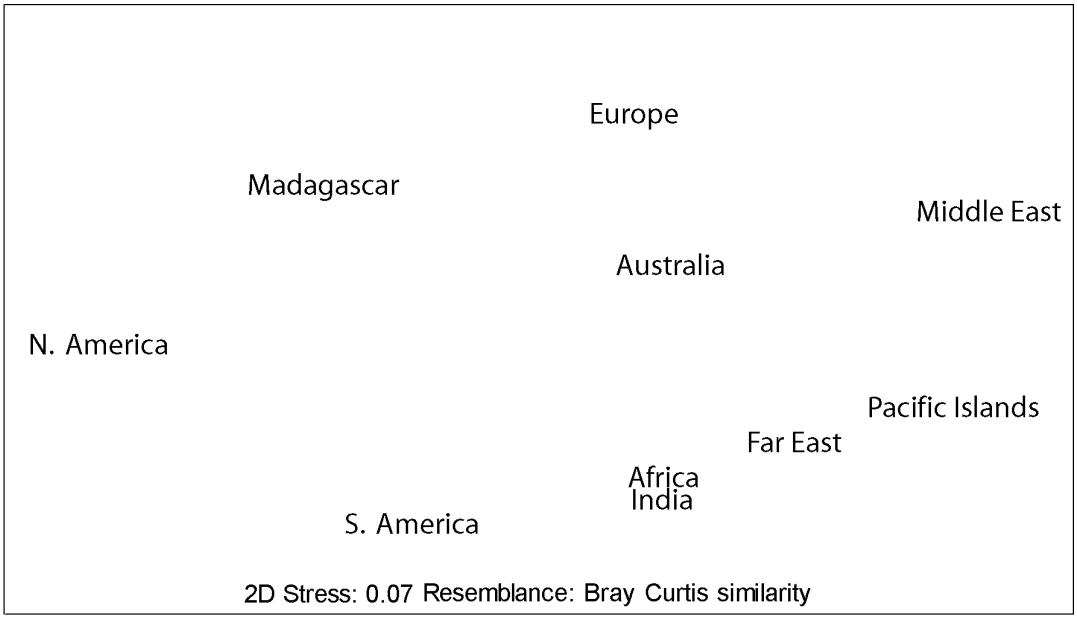


Figure 8.20. Bray Curtis analysis of similarities between regions derived from the examples used in the track analysis (Figures 8.11 – 8.18), shown as two-dimensional MDS plot of area relationships based on similarities (India and Africa overlapped completely, but the labels were separated for legibility).

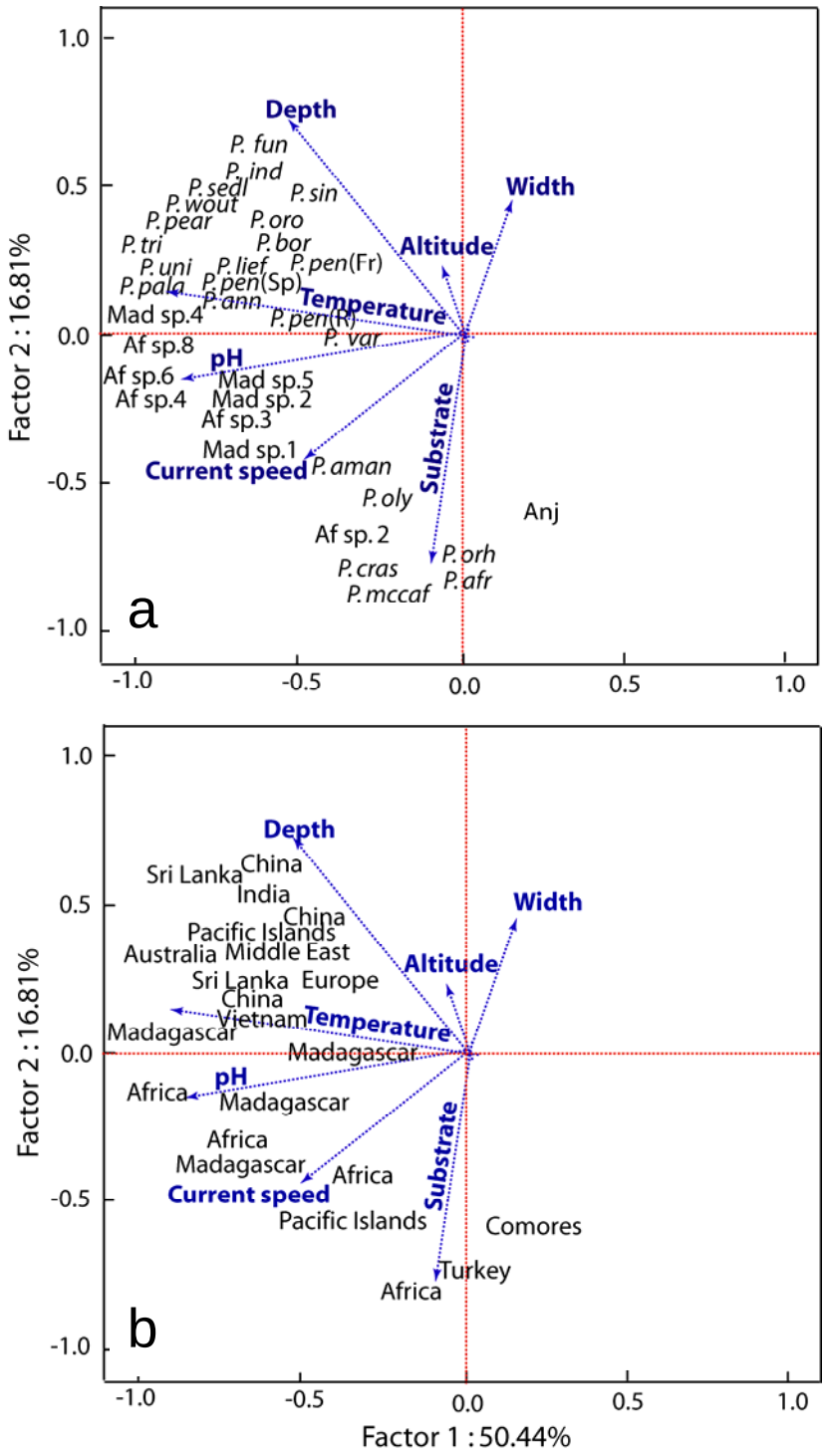


Figure 8.21. PCA showing affinities of *Prosopistoma* species relating to abiotic parameters (Appendix Table A8.2), derived from coded transformation (Appendix Table A8.3). (a) Plot of the first two components of the PCA analysis of abiotic parameters showing species and effects of parameters, (b) generalized areas replace species names. Eigenvalues of correlation matrix used for PCA indicate that Factor 1 accounts for 50.44 % of total variation and Factor 2 for 16.81 %.

Table 8.4. Eigenvectors and Eigenvalues of PCA correlation matrix for abiotic variables, showing first six factors, and cumulative variance (%).

Variable	Factors					
	1	2	3	4	5	6
Depth	-0.516	0.624	0.064	0.294	0.294	0.396
Width	0.131	0.429	0.309	-0.831	0.034	0.093
Current	-0.500	-0.380	-0.520	-0.253	-0.341	0.391
Substrate	-0.116	-0.697	0.272	-0.125	0.625	0.142
Temperature	-0.897	0.123	0.121	0.054	0.032	-0.212
pH	-0.854	-0.141	0.218	-0.1800	-0.127	-0.231
Altitude	-0.071	0.201	-0.819	-0.211	0.430	-0.228
Eigenvalue	20.18	6.73	4.21	3.78	3.21	1.89
Cumulative variance (%)	50.44	67.25	77.79	87.24	95.27	100.00

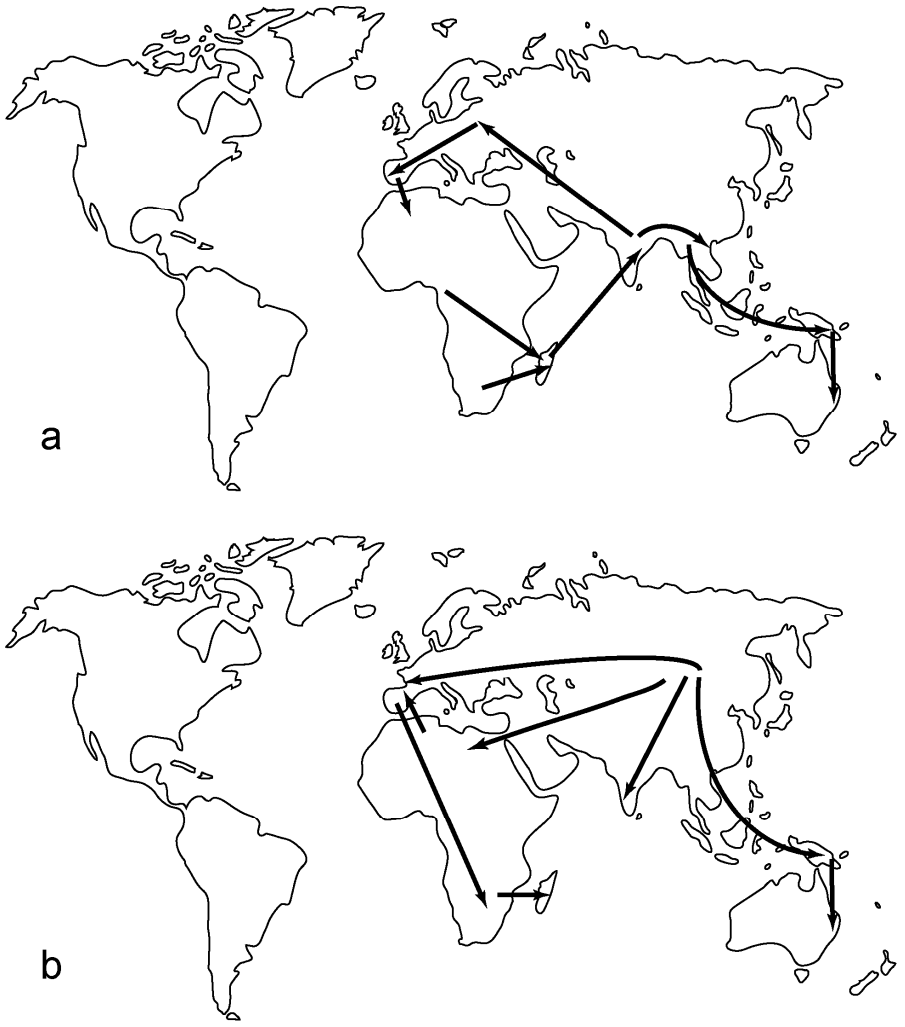


Figure 8.22. Comparison of two alternative biogeographic models to explain the distribution of the Prosopistomatidae species (a) vicariance from African Gondwanan origin and subsequent dispersal (based on morphological phylogeny), (b) Jurassic dispersal from Angaraland refuge to Australasian region, and to Europe and Africa, with secondary recolonisation of Europe from Africa (or Mediterranean island refugia, Dapporto *et al*., 2009) following Pleistocene glaciations.

Chapter 9

General Discussion, Conclusions and Future Research Directions

"Be yourself and think for yourself; and while your conclusions may not be infallible, they will be nearer right than the conclusions forced upon you."

Elbert Hubbard 1856-1915

Synopsis

This final chapter reviews the findings of the thesis, drawing final conclusions to the questions posed at the beginning of the thesis. Most of the questions asked initially have been addressed. Absence of adult material for many species, and lack of fresh material for molecular analysis, were both short-comings. As with any subject of research, it seems that the more that is known, the more there still needs to be discovered. Future research directions are suggested.

9.1 Summary of findings

9.1.1 Prosopistomatid Morphology

In Chapter 2, morphological structures found in the nymphs of Prosopistomatidae were extensively examined, and redefined in terms of their component parts and origins. Thus, nymphal head sutures, antennal length and relative segment sizes, mouthpart structures, gill details and tibial setal structure and arrangement, were some of the characters that were elucidated and described. In particular, in the labium, the glossae and paraglossae are shown to be fused within the prementum rather than lost. The structure of the hypopharynx indicated that the superlinguae of the hypopharynx are fused, and are in a ventral position, rather than laterally situated as in other mayflies. Studies on subimaginal and adult morphology, in particular changes in claw structure between the subimaginal and imaginal stages of the male, were linked with structures on the female thorax and gonopore area. In the male, no lengthening of the forelegs between the subimaginal to imaginal moult was found, which is very unusual in mayflies. These observations provide the basis for a new interpretation of an unusual mode of mating for the Prosopistomatidae, unique in the

Ephemeroptera. These structures are potentially useful characters for phylogenetic analysis, if adults were known for more species.

9.1.2 Prosopistomatid Diversity

Chapter 3 showed that morphological measurements can be used for species delimitation in Prosopistomatidae. Morphometric comparisons dealing with measurements and shape were analysed using Principal Component Analysis (PCA). Components derived from the measurements were plotted, producing groupings of specimens showing successful species delimitation, although there is some overlap between species. Using Discriminant Function Analysis (DFA), head width and carapace shape were shown to have the strongest influence in separating nymphs of different *Prosopistoma* species. Additionally, certain ratios, such as carapace length to width, remained constant throughout the life of the nymph, making this, in combination with other characters, a useful criterion for species delimitation for any instar. The gradual allometric growth of the carapace of *Prosopistoma* nymphs contrasts with the patterns observed in *Baetisca* (Pescador and Peters, 1974), an important difference when looking at the relationships between these families, indicating a difference in early ontogeny.

9.1.3 Prosopistomatid Identification

The Artificial Neural Network approach taken in Chapter 4 was applied to nymphal morphological characters. This produced a successful method for distinguishing species using a computer-driven artificial intelligence search. This has powerful implications for future methods to provide easy-to-use electronic identification aids. The development of such methods requires the expert knowledge of a given taxon to provide the criteria to feed into a neural network programming system such as MATLAB. Programming skills are needed initially but a stand-alone product with a user-friendly front that can be used for taxon identification by non-expert para-taxonomists can be produced

9.1.4 Prosopistomatid Phylogeny

Phylogenetic analysis of morphology (Chapter 5) showed that the family is represented by two distinct clades, and this is supported independently by characters of the adult and nymphal stages. This strongly suggests that the family should be divided into two genera, with *P. variegatum* as the type of one clade, and possibly *P. africanum* as the type of the second clade. However, the preliminary molecular phylogenetic studies cast doubt on this,

and splitting of the family into two genera cannot be decided conclusively until further molecular research is carried out using more suitable genes. This would require more individuals from each species, with a greater representation of species from all areas within their geographic range, especially from Asia, and alternative primers for genes such as CO1 need to be tested. The molecular analysis indicated that the more recent (i.e. crown-group) *Prosopistoma* species have dispersed from Asia to their current distribution, and that the European species may be the result of a secondary colonization from Africa. While the stem-group ancestors date back to the Carboniferous, the oldest crown group lineage represented by *P. wouterae* dated back to the Lower Cretaceous (Chapter 6). The investigation of characters of stem-group fossils (Chapter 7) provided support for the hypothesis that the clade comprising the Prosopistomatidae and Baetiscidae diverged from the main line Ephemeroptera at an early stage, and a simple parsimony analysis of selected characters, mostly wing-based, supported this.

9.1.5 Prosopistomatid Biogeography

The biogeography of the family (Chapter 8) was tackled using several different approaches, and explored whether the modern distribution is predominantly the result of Gondwanan vicariance (as suggested by the morphological phylogenies) or later dispersal (as implied by the molecular phylogenies). This was also discussed taking palaeo-climatic changes and contemporary reconstructions of palaeogeography into account. Distributions of other unrelated freshwater organisms were compared to see if a generalized pattern of distributions supported congruent distribution patterns. This would imply a common history, driven by similar evolutionary forces. It was concluded that there was a tendency (from the literature assessed data) for dispersal from Asia, and that crown-groups were often found to be younger than previously thought. However, tectonic vicariance was also shown to have played a fundamental role in determining distributions. Sørensen's coefficient (SC) of biotic similarity, used to compare the association of geographic regions occupied by the organisms investigated in this analysis, showed a very strong relationship between Africa and India, which supports vicariance of Gondwana as the main factor causing speciation on these landmasses. In contrast, the strong correlation between South-East Asia, the Pacific Islands and Australia indicates a greater likelihood of dispersal between these regions. Finally, abiotic conditions characterising the ecological preferences of each species were tested to see which factors, if any, had the greatest influence on *Prosopistoma* distribution patterns. Temperature, pH and current speed had the strongest

influence on the species distribution, with substrate type apparently more important to the Turkish and some of the African species than to others, while depth of river was more influential in some of the Asian species.

9.2 Discussion

In Chapter 5, attention was drawn to the plethora of species concepts (Wilkins, 2002, defines 26) that have been developed since the founding concepts of Ray (1686), Linnaeus (1735), Wallace (1858), Darwin (1859) and Mendel (1866). Why have so many species concepts arisen? As de Queiroz (2007) points out, the nature of the definition of species depends largely on the needs being addressed. There are two main lines of thought; (a) how do we recognise a species, describe it and name it (based on the idea that phenotypic variation is discrete and not continuous); (b) what defines a species from an evolutionary perspective? One aim is to categorize and identify organisms; often species have been defined purely on structural characters, so that they can be categorised as separate from others. The second is to try and understand the evolutionary processes that give rise to species, and this recognises them as an evolving unit.

In assessing the question of whether Prosopistomatidae should be divided into two genera based on morphology, the first question is whether the species themselves are well delimited, as ranking is subjective. Are the named entities which have been dealt with here really species? Can the identity of an organism be decided upon by looking at morphology only? Do the relationships derived from morphological characters give an accurate estimate of the evolutionary processes behind species formation? Morphology, as a reflection of evolution, may allow assumptions to be made about identity and relationships, but behavioural observations are also necessary to determine functional aspects such as conspecific mate recognition. The Specific Mate Recognition System (SMRS) concept of species, developed by Paterson (e.g. 1978, 1980, 1982, 1985, 1989) advocates the driving force of evolution to be the recognition of conspecifics as a mate. Would each of the "species" studied in this thesis hold true under this concept? The sexually active conspecific males and females must recognise each other as mates, the first step being synchronised emergence. The emergence and mating of *Prosopistoma* is amongst the shortest in all mayflies. Males emerge at the first light of dawn, and shed their subimaginal skin. In *P. africanum*, the mid and hind legs are atrophied in the adult male, the forelegs remaining functional as these are used to grasp the female (Gillies, 1954). The females of

all known *Prosopistoma* species are sexually mature as subimagos, and have non-functional legs. In *P. africanum*, females emerge slightly after the males. Gillies (1954) reports that in *P. africanum*, the entire adult stage is over within the first 45 minutes after the break of dawn. Males of *P. africanum* have been noted to have a peculiar jerky flight motion (Gillies, 1954), and this is possibly a signal to the female to recognise him as a mate. Further investigation of the flight and mating behaviour of a number of species would be useful to show whether flight pattern may be a mate signalling process in these mayflies. Given how difficult it is to find these animals, and especially to time their emergence dates, it may be many years before this is achieved. One problem with the mate recognition concept is that it implies that natural section acts only on the short lived adult stage, and ignores the longer lived nymph, a problem when considering mayflies. However, Paterson (1985, 1989) mentions that species are an incidental consequence of adaptation. Thus adaptive pressures on a mayfly nymph may causes changes in the nymph, but unless a change also influence the SMRS, the species is not affected. Conversely, a change in the nymph may disrupt mate recognition, and that change will therefore either be eliminated, or perpetuated in the form of a new mate recognition signal.

Dobzhansky (e.g. 1940, 1951, 1970, 1973), Dobzhansky *et al*., (1977), recognised the role of reproductive isolation in species formation, based on the realisation that natural selection operates through mutation in genes. These ideas were built on by Mayr (1942, 1963, 1982, 1985, 1988), who developed the so called “Biological Species Concept”. This proposed that species were not just morphologically similar, but reproductively able to breed with only conspecifics, i.e. are reproductively isolated units. If a part of a population was separated, it would start to differ over time (due to genetic drift and natural selection acting on genetic changes caused by mutations), resulting in a new species. He advocated the development of isolation mechanisms in the subpopulations, to protect the integrity of each developing gene pool. Are there are any behavioral processes or physical structures that could be considered as isolating mechanisms in mayflies? What keeps the integrity of a mayfly species? Could there be any unique factors keeping species of prosopistomatids from interbreeding? Time of emergence (noted above) is an important behavioural consideration, especially as the adult stage is so short lived in *Prosopistoma*, and could offer a means of temporal allopatry. However, this is only important if two closely related species are geographically sympatric. Some *Prosopistoma* species are sympatric, e.g. *P. crassi* and *P. mccaffertyi* in South Africa, *P. annamnese* and *P. funanense* in Vietnam, and some of the Madagascan species. Selection pressure has to act upon the breeding stage of a

population and species to effect evolutionary changes. In Ephemeroptera, the nymphal stage occupies a relatively long portion of the life cycle, while the adult stage is short-lived. This is particularly the case in Prosopistomatidae. Therefore adaptive changes (to habitat) are more likely to occur in the nymph, but evolutionary changes are more likely to affect the breeding stage. This may be of relevance when examining morphological characters for phylogenetic studies, suggesting that more emphasis should be placed on adult rather than nymphal morphology. Further investigation using more adult *Prosopistoma* species, focusing on the details in leg morphology and structure of genitalia observed in Chapter 2, would give a more comprehensive adult phylogeny.

Many tropical aquatic insects are only on the wing at dusk, fewer at dawn (personal observation). All species of *Prosopistoma* in which the adult stage is known have emergence at dawn. This behaviour is confined to only a few mayfly groups (e.g. Caenidae and Prosopistomatidae). However, as all *Prosopistoma* species studied as adults so far are dawn emergers, whether from a cold European climate or from tropical Africa, this cannot function as a way to keep sympatric species from mixing. The precise time of flying may still be relevant, but not enough details are known of their emergence behaviour; do they perhaps emerge at slightly different times of the year, for example? If the genitalia of the known species are compared, differences can be observed, but are they enough to act as physical isolating mechanisms to prevent intraspecific breeding in *Prosopistoma*? This is conceptually problematic, as it implies that differences in genitalia developed in order to keep species separate, which is clearly not the case. Male genitalia are notoriously unhelpful in species diagnosis in certain mayfly families, which probably means little specialisation. Indeed, it seems unlikely that physical isolating mechanisms function in *Prosopistoma* to keep species as discrete entities, while temporal and geographic isolation may reinforce these species. The trouble with the concept of isolating mechanisms is that this gives Mayr's concept a teleological implication; evolution and speciation cannot be teleological as there is no particular goal.

Some *Prosopistoma* species (e.g. *P. pearsonorum*) are tentatively thought to be parthenogenetic (Campbell and Hubbard, 1998), as males have never been collected (though may still be found). This may also occur in other *Prosopistoma* species (e.g. Madagascan sp. 2, *P. mccaffertyi*) where the nymphs' eyes are all small and to date only females have been collected. If a species is asexual and parthenogenetic, this causes it to become isolated as it doesn't need to find a mate. This begs the question; do these species sometimes produce males? If so, the SMRS can apply to them, but if not then some other

evolutionary driving force must be at work. What could that be? Asexual development can occur if the chance of finding mates is very small. The short adult longevity and flight duration period in *Prosopistoma* are pertinent. If strong winds blow periodically this would diminish the potential to find a mate, favouring parthenogenesis. A good example of facultative parthenogenesis is seen in Cladocera, where males are produced only during times of hardship, and a resting ephippial egg stage is produced (e.g. Banta *et al*., 1926; Gibson *et al*., 1998). Normal good conditions produce only parthenogenetic females. This has not yet been recorded in Ephemeroptera.

If a mutation occurred in a parthenogenetic female, that trait would, if successful, be passed on fully to the parthenogenetic offspring, introducing a genetic variation in the population. Whether these apparently parthenogenetic *Prosopistoma* species are haploid or diploid is a question beyond the scope of this thesis, but establishing ploidy would be necessary to confirm parthenogenesis. Soldán and Putz (2001) investigated the karyotypes of several species in a number of mayfly families and found that these were mostly constant within a family but varied between families. An investigation of this nature with the Prosopistomatidae and Baetiscidae would not only help to evaluate their placement in higher level ephemeropteran phylogeny, but would give valuable insights into the parthenogenetic status of members of these families.

More recent approaches to defining species involve the use of genetics and gene sequences, dealt with in Chapter 6. With the study of genetics has come the recognition of the effects of hybridisation and introgression. Templeton and colleagues e.g. Templeton (1989, 1998), Crandall and Templeton (1993), Castelloe and Templeton (1994), Templeton *et al*. (2001), proposed the Cohesion Concept, which accounts for organisms that reproduce sexually, asexually and parthenogenetically. Templeton defines species as “the most inclusive group of organisms having the potential for genetic and/or demographic exchangeability” (Templeton, 1989), and approaches his studies from a genetic perspective. A cohesion species is "an evolutionary lineage that serves as the arena of action of basic microevolutionary forces, such as gene flow (when applicable), genetic drift and natural selection" (Templeton, 1994). Thus the cohesion concept resembles the evolutionary species concept of Simpson (1961) by considering species as lineages. It emphasizes population genetics and the causes of phenotypic similarity within species, including the influence of genetic drift.

The effects of introgression must also be considered. Introgression involves the movement of a gene from one species to another by backcrossing a hybrid with one of the

parent species. Ordinarily, a hybrid of two species is not fertile, but periodically sympatric species have hybridized and produced an F1 hybrid that, although possibly not as fit as the parent species, may never-the-less survive to reproduce with one of the parent species, producing first generation back-cross hybrids. Successive back-crossing with the parent species can result in the establishment of a new species with genetic traits different from both parent species. This is more complex than straight hybridization, and does not produce a 50-50 mix of the genes of the two parent species. Usually hybrids are an evolutionary dead end, but occasion successful introgression can result in new gene combinations which may be successful. Seehausen (2004) provides an excellent synthesis of how introgressive hybridization influences evolution. He notes that hybridization is more common when a population invades a new environment. This occurs between closely related species with the same chromosome numbers, allowing hybrid bisexual populations. It has recently been shown that there are many more species of hybrid origin than previously realized (e.g. Dowling and Secor, 1997; Rieseberg, 1997; Smith *et al*., 2003). So far, this has most commonly been recorded in plants and fish. Two ecologically divergent species may be brought together by an event where the habitat has been perturbed (e.g. flooding bringing together two normally well separated but closely related aquatic species). The hybrid may be fitter than either parent in a new niche, resulting in adaptive radiation. Adaptive introgression occurs when a hybridisation has resulted in favourable characters, which are then selected for. Arnold and Martin (2009) discuss four case studies where adaptive introgression has brought about good qualities from both parents, to give a unique organism with its own characteristics derived from both parents (it could equally well have been a bad character combination, in which case they would not survive). The concept of horizontal gene transfer, resulting from the study of metagenomics, has also resulted in a mind shift in terms of understanding evolutionary processes and speciation. In this approach, genomes rather than species are the unit of study (e.g. Marco, 2008). The effect of introgression has not been widely documented in mayflies, and is currently limited to a few examples, e.g. it has been shown in Baetidae (Ståhls and Savoainen, 2008). Barber-James *et al.* (2008) suggest introgression of genes into adjacent bioregions as a factor explaining the extant distribution of genera, e.g. introgression from the Palaearctic to Oriental, or from the Neotropics to the Nearctic.

The ecological approach used to interpret species relationships in Chapter 8 investigated whether ecological parameters such as river size, flow, current speed, substrate type and pH affect species distribution patterns. On the global scale investigated,

the resolution was very coarse, and this approach would be more useful when comparing ecological conditions in more detail over a smaller geographic area. Additionally, rivers often do not reflect the environment around them, e.g. if running through arid area, they still have some riparian vegetation. Geology of an area may affect the size of the components of the substrate on which the mayflies live (boulders, cobbles), and may also influence water chemistry, e.g. pH, TDS and salinity. Much more detailed ecological information is needed for all species before they can be clearly defined ecologically. Schletterer and Füreder (2009) recorded the historic distributions of the European populations of *P. pennigerum* and categorised them according to ecological demands as lithophil, reophil, or potamophil, thus identifying substratum and current as key factors for their distribution. They concluded that the distribution of *P. pennigerum* is linked to basin scale and temperature regime. This approach provides a much more refined assessment of ecology, and is recommended at this resolution for each bioregion.

Species are not just similar entities that can be separated from other discrete entities over time, but bear relation to each other. This implies that a species does not have lasting boundaries, leading to the question – how long can a species remain relatively constant? What are the chances that the species collected from a stream today is significantly altered compared to specimens occurring, say, 5000 years ago? Can they change significantly in such a short time? One would tend to think the answer is no, they will not change, much. However, there are a number of examples of speciation occurring over short periods of time (e.g. Kontula *et al*. (2003) showed that 33 species of a sculpin fish evolved in Lake Baikal during the Pleistocene from their original riverine ancestor; Johnson *et al.* (2000) and Nagl *et al.* (2000) record that some 500 species of cichlids have arisen in the Lake Victoria region within 15 000–200 000 years); Mallet (2008b) recorded rapid speciation in Lepidoptera due to hybridization and introgression). The rate of speciation is variable; each situation is unique and unpredictable, depending on the environmental circumstances in which the species is living. If it is in a stable environment, it is less likely to undergo marked change for thousands of years, or longer. The molecular clock methods applied in Chapter 6 showed relatively recent evolution of some of the species, with the crown-group species ranging in age from a maximum of 131.5 Ma to a minimum of 1.2 Ma.

What are the practical reasons for wanting to define species? Without clearly knowing whether a distinct entity is being dealt with, it is difficult, if not impossible, to understand relationships, which has consequence for conservation issues. Having clearly delimited species with an understanding of relationships between them is the first step to

listing and conserving natural biodiversity. This is also important in understanding a species from an evolutionary aspect – how they have evolved and what is likely to affect their future evolution. Knowing a species' geographic distribution, and having strong evidence of what caused the species to be as it is, allows prediction of what factors may affects the success of *Prosopistoma* species in future .

Different species concepts could possibly give a different interpretation of diversity of species and /or higher taxa. An incorrect understanding could have a detrimental effect on environmental management. It is important that conservationists share the same conceptual framework to implement management practices relating to species.

9.3 Future Directions

The research done for this thesis has answered many questions and raised several more as the work has unfolded. One question is whether Baetiscidae and Prosopistomatidae are really as closely related as is currently believed. A sister-group relationship between these two families was favoured at the start of the thesis (Chapter 1), based on morphological work of many taxonomists (e.g. Vayssière, 1934; Gillies, 1954; Edmunds and Travers, 1954; Fontaine, 1958; Kluge, 2004), comparing apparently homologous characters (particularly carapace, gill structure, and some characters of wing venation). This conclusion is supported by several molecular phylogenies (Ogden and Whiting 2005, Ogden *et al.* 2009). However, evidence gathered during the research for this thesis indicates that perhaps they are not as closely related as is thought. Evidence opposing their close relationship includes the difference between the two families in the way the carapace develops in the nymphs. The dated molecular phylogenies derived from the few genes that amplified (Chapter 6) also provides evidence against their putative sister group relationship as they two families do not group together. Further molecular research using genes additional to those used in Chapter 6, is needed to investigate this further. A comparison of egg structure between the two families reveals a further difference, with *Prosopistoma* eggs (Chapter 2) having no polar cap, while Pescador and Berner (1981) show a distinct polar cap in *Baetisca.* Additional focused research on these two families, including detailed comparative anatomy and histological comparisons, may help to clarify this relationship. Comparisons of egg structure for the purpose of comparing a wider selection of *Prosopistoma* species (providing a useful additional character for morphology based phylogeny within the family), and *Baetisca* species, for comparison between the families would be useful.

Fresh material for many more species, especially across Asia to the Middle East needs to be sought to develop a more secure molecular phylogeny. Additional genes should also be isolated, with different primers tried for some of the genes, e.g. CO1; possibly specific primers for *Prosopistoma* CO1 genes need to be designed. The fact that standard primers for CO1 (HCO and LCO) did not work for Prosopistomatidae, but worked for Baetiscidae (Ball *et al*. 2005) may also reflect that the two families are not as closely related as previously thought. The apparently close relationship between *P. pennigerum* in Europe and *P. crassi* in southern Africa, suggested from the examination of the molecular phylogeny, needs to be investigated further. The support for these results is not strong, and there are gaps in the datasetss, so this relationship may not hold under more rigorous testing, including the investigation of many more of the African species. While it is possible to conjecture about recolonisation of Europe from African populations after Pleistocene glaciations, implied by the molecular phylogeny, this does not really seem feasible with the observed morphological differences between the two species.

More adult and nymphal associations need to be made to facilitate further species descriptions using all life history stages, preferably based on several specimens. Having a bigger range of material for each species would also improve reliability of morphological phylogeny investigations, and from a molecular perspective, would enable further studies of population genetics that would facilitate the understanding of gene flow and have implications for speciation.

With regard to biogeography, the distributions of Prosopistomatidae species can at best be seen to be the product of both Gondwanan vicariance and dispersal. The molecular clock methods are still controversial, and have been considerably criticized. Sanmartín and Ronquist (2004) tried to resolve the discrepancies in molecular clock results by analysing a dataset of 54 animal and 19 plant phylogenies. They found that animal distributions tend to be congruent with the vicariance of Gondwana, while dispersal plays a larger role in plant distributions. Heads (2005b) provides a critique of the calibration of molecular clock methods, pointing out that while fossil dates provide a minimum age for a particular lineage, these dates are often wrongly taken as absolute ages of divergence. He also criticizes the approach which deems older geological events to be irrelevant, and points out that in fact the younger events may be irrelevant, particularly with respect to biota on islands associated with hot spots and subduction zones, as the biota may be much older than the younger islands. To quote Heads "*Problems with calibrating the nodes, as well as with substitution models, mean that phylogeography's claim to be able to test between*

vicariance and dispersal is not justified". Nelson and Ladiges (2009) refer to the molecular clock approach as being a futile assessment, reminiscent of earlier phenetic studies. They refer to several dated molecular phylogenies which relate to organisms previously attributed to having a Gondwanan distribution, now shown to be the product of more recent post-Gondwanan dispersal, for example in the southern beech trees, *Nothofagus* (Knapp *et al.,* 2005), and *Adansonia*, the baobab trees (Baum *et al*., 1998). Perhaps the tempering approach of Gibbs (2006) is more constructive "*...the chronological information that even a crude clock provides can tell us whether organisms came to New Zealand with the land itself, or whether they have arrived since*."

In assessing genetic contributions to mayfly research since this type of work first started, Monaghan and Sartori (2009) point out that newer genetic methods do not necessarily give better results than earlier methods. They emphasize that it is important that any approach should be set in the framework of an objective and repeatable species concept. In the case of the Prosopistomatidae, interesting options have been offered as a result of the dated molecular phylogenies, for example, the Asian species being the oldest of the crown-group species, implying dispersal from this region. Before anything categorical can be concluded from the molecular results, considerably more research is needed. Ages of more of the African species would help, for example, to clarify the disjunct European-African relationship indicated in the molecular phylogeny.

If dispersal is used as an argument to explain the distribution of mayflies, it could be expected that the crown-group Prosopistomatidae should be more widespread than they are. *Prosopistoma* species should, by this reasoning, also be found in the Neotropical and Nearctic regions. A broader distribution could also be expected if they are the remnants of a once more widespread stem-group lineage of Pangaean origin (as suggested by the fossil record). In such an ancient lineage, populations of *Prosopistoma* species have been moulded through time, with some species becoming extinct and others forming, these patterns being obliterated with time, leaving only a few snatches in time represented in the fossil record as evidence of their earlier existence.

At this point, they are seen as a vulnerable group of mayflies, and may be on their way to extinction due to man-induced environmental change. For example, they are considered extinct in the Red List of threatened mayflies of Germany (Malzacher *et al*., 1998). In Sweden, *P. pennigerum* has not been recorded since 1916 (Engblom, 2001). Whether they as a family are threatened to the point of extinction may be debatable, but they have certainly disappeared from many rivers in which they were previously recorded.

Schletterer and Füreder (2009) noted that *P. pennigerum* is extinct in most of the major European rivers where it was once recorded. Populations are still known from the Volga River (Russia) (Schletterer, pers. comm., August 2010) and from three rivers in Spain (the Cabriel, Secura and Mundo Rivers – Robles, pers. comm., April 2009). Hydroelectric schemes (e.g. on the upper Rhine River), which affect flow, habitat alteration and pollution, are the main variables that have negatively affected populations of Prosopistomatidae in Europe (Schletterer and Füreder, 2009). In South Africa, abstraction of water for irrigation, building of impoundments on rivers that changes their flow patterns, and spraying of pesticides and herbicides seem to be the main causes of their decline. For example, one undescribed species from the Olifants River, Western Cape, appears to have become extinct before it could be described taxonomically. This area is intensively cultivated with citrus orchards, and abstraction of water for irrigation and aerial crop spaying negatively affects the riverine fauna (personal observation). In Madagascar, large scale habitat destruction, deforestation, jamming of newly cut hardwood logs in rivers down which the cut trees are rafted, and silting due to road building and irresponsible mining practices (Draper, 2010) also negatively affect all river-dwelling biota. What is the future of the family? Will they survive as relict populations in suitable rivers which are less disturbed? How many species remain undiscovered? Further collecting in Africa, Madagascar and Asia will without doubt find undescribed species, but it is unlikely that they have been overlooked in the Neotropics and Nearctic. In some cases only one individual of a species has been collected (e.g. a unnamed Madagascan nymph), or the species is described from early instar nymphs (e.g. *P. deguernei* from Senegal), and more specimens are needed. It would be desirable to get fresh material for each species within Africa and to establish a more detailed phylogeny of these. Their distribution and phylogeny could be interpreted in greater detail in terms of palaeodrainage patterns and known uplift and river capture events (a preliminary assessment of this was provided by Barber-James, 2003). Greater understanding of their evolution and ecology could help to devise conservation and management strategies to protect this unique group of mayflies and ensure that they are still present for future generations to appreciate. As an ecologically sensitive group, they may prove to be useful as umbrella species to protect the environment for other less sensitive organisms with which they coexist. New (1993) discusses the value of selecting priority taxa to maximise conservation efforts, and seeks to find optimal focal groups which can represent communities. *Prosopistoma* species may

offer such a function, their presence representing a state of good conservation of rivers in which they are recorded.

9.4 Concluding Remarks

It is possible that further refinement of our understanding of tectonic events may yet cause us to have another paradigm shift in our understanding of relationships between organisms and the ever-changing landmasses on which they live. Biogeography has reached a stage where both dispersal and tectonic vicariance are seen to interact together to mould the biota sharing our planet today. However, it seems that many molecular investigations contradict the previously accepted theories of tectonic vicariance, and perhaps a balance has not yet been reached. Mooi and Gill (2010) use examples from fish systematics to suggest that molecular phylogenetics is causing scientists to lose touch with real animals, and that synapomorphies are no longer the currency of investigation when using molecular techniques. They go as far as saying that fish systematics is in a crisis because of this. It is a warning that needs to be heeded, and while molecules provide valuable extra data, the biology of the organism under investigation must not be forgotten.

There is still a great deal of work to be done on Prosopistomatidae, from discovering and describing all life cycle stages of new species, to investigating their mating behaviour and life history in greater detail. Ecological work such as comparing seasonal trends and possibly using this to further support decisions about what really defines each species, both morphologically and genetically, is also needed. For example, if two very similar sympatric species have different breeding seasons or flight time of adults, this would help to resolve the species status and indicate that there really are two species and not one.

The synthesis of a large number of papers by many authors for the chapter on biogeography has lead to the personal realization that all too often researchers are too closely focused on their own group, and forget to look at the bigger picture. Without putting a phylogeny and current species' distribution patterns into an historic context, much of the value of the phylogeny is lost. It is like doing a statistical analysis without interpreting the data. The hows and whys of a phylogeny are what make it meaningful, highlighting the difference between descriptive and explanatory science, between pattern and mechanism. The research presented here has answered some questions, yet raised others that need further study. New approaches to examining the phylogenetic relationships

including looking at past, present and future scenarios, including effects of changing climate, can offer guidelines for conservation of this valuable and unique family of mayflies.

--- The End ---

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List of Appendices

Appendices

Each Appendix is numbered after the Chapter number to which it refers. There are no appendices for Chapter 9.

Appendix for Chapter 1

Appendix Table A1.1 Coden abbreviations used in this thesis, using the convention set by Evenhuis (2008) in the *Insect and Spider Collections of the World*, updated on the Bishop Museum website, *http://hbs.bishopmuseum.org/codens/codens-inst.html.*

Institution	**Coden**
The Albany Museum, Grahamstown, South Africa	[AMGS]
The Natural History Museum, London, England	[BMNH]
The Museum National d'Histoire Naturelle, Paris, France	[MNHN]
The Musée de zoologie, Lausanne, Switzerland	[MZL]
The Purdue University Entomological Research Collection, West Lafayette, Indiana, USA	[PERC]
Florida A and M University, Tallahassee, Florida, USA	[FAMU]
Musee Royal de l'Afrique Centrale, Tervuren, Belgium	[RMCA]

Voucher specimens (including holotypes, neotype and paratypes examined or erected), and other material examined, come from or are deposited in these institutes, as sited in Appendix Table A2.1.

Appendix for Chapter 2

Appendix Table A2.1. List of *Prosopistoma* and *Baetisca* material examined, with locality details. Material used in molecular analysis in blue. "n" refers to the nymphal stage; "sub" indicates subimago; "molecular" indicates that specimens were used for molecular analysis. All material is in ethanol unless otherwise stated.

Museum	Catalogue	Species name	Country	Locality	Date	Latitude	Longitude	Collectors	No of specimens	Status
BMNH	1954-125	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	14-09-1952	-5.0833	38.6667	M.T. Gillies	1 n	holotype
BMNH	1954-125	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	18-01-1953	-5.0833	38.6667	M.T. Gillies	3 ♂	paratypes
BMNH	1954-125	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	25-01-1953	-5.0833	38.6667	M.T. Gillies	3 ♂	paratypes
BMNH	1954-125	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	01-02-1953	-5.0833	38.6667	M.T. Gillies	1 ♂, nymphal shuck	paratypes
BMNH	1954-125	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	00-06-1952	-5.0833	38.6667	M.T. Gillies	6 n	paratypes
BMNH	1954-125	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	25-01-1953	-5.0833	38.6667	M.T. Gillies	4 ♂	paratypes
BMNH	1954-125	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	11-01-1953	-5.0833	38.6667	M.T. Gillies	1 ♂	paratypes
BMNH	1954-125	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	18-01-1953	-5.0833	38.6667	M.T. Gillies	3 ♂	paratypes
BMNH	1954-125	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	00-01-1953	-5.0833	38.6667	M.T. Gillies	1 ♂	paratypes
BMNH	1954-125	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	00-09-1952	-5.0833	38.6667	M.T. Gillies	4 n	paratypes
BMNH	1954-125	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	18-01-1953	-5.0833	38.6667	M.T. Gillies	1 ♂	paratypes
BMNH	1954-125	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	11-01-1953	-5.0833	38.6667	M.T. Gillies	1 ♀	paratypes
BMNH	A941-50	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	01-02-1953	-5.0833	38.6667	M.T. Gillies	8 ♂	-
BMNH	A847-56	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	21-10-1954	-5.0833	38.6667	M.T. Gillies	9 ♂ imago	-

Museum	Catalogue	Species name	Country	Locality	Date	Latitude	Longitude	Collectors	No of specimens	Status
BMNH	A953-6	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	1953-1954	-5.0833	38.6667	M.T. Gillies	3 ♀, 1 ♂	-
BMNH	A951-2	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	00-01-1953	-5.0833	38.6667	M.T. Gillies	2 ♀, 1 nymphal shuck	-
BMNH	A932-40	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	21-10-1954	-5.0833	38.6667	M.T. Gillies	9 ♂ imago	-
BMNH	B430	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	05-03-1985	-5.0833	38.6667	M.T. Gillies	1 ♀ subimago	-
PERC	unnumbered	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	25-01-1953	-5.0833	38.6667	M.T. Gillies	5 n (3 headless)	-
PERC	unnumbered	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	05-02-1953	-5.0833	38.6667	M.T. Gillies	1 n	-
PERC	unnumbered	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	25-01-1953	-5.0833	38.6667	M.T. Gillies	3 ♂	-
PERC	unnumbered	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	08-01-1953	-5.0833	38.6667	M.T. Gillies	1 ♂	-
PERC	unnumbered	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	25-01-1953	-5.0833	38.6667	M.T. Gillies	1n 1♂ (both in part)	-
PERC	unnumbered	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	00-01-1953	-5.0833	38.6667	M.T. Gillies	1♀ 1n (previously dissected by T. Fink)	-
PERC	unnumbered	*P. africanum*	Tanzania	Sigi River, Amani, East Usambara Mountains	08-05-1953	-5.0833	38.6667	M.T. Gillies	1 ♂	-
AMGS	CAW 78V	*P. africanum*	N. Zambia	Lunzua River at Mbala-Mpulunga road	13-04-1994	-8.8642	31.1486	R. Bills	1 n	-
AMGS	GEN 355C	*P. amanzamnyama*	South Africa	Krom River,between Zwartberg-St Bernhard's Peak	17-11-1959	-30.1042	29.1875	NIWR	3 n	
AMGS	GEN 1840B	*P. amanzamnyama*	South Africa	Hlatikulu Vlei	15-09-2005	-29.2375	29.7847	M. Graham	1 n	-
AMGS	GEN 1841B	*P. amanzamnyama*	South Africa	Amanzamnyama River	17-11-2005	-30.6006	29.7436	M. Graham	1 n + 4 n	Holotype + 4 paratypes
AMGS	GEN 1863A	*P. amanzamnyama*	South Africa	Weza River	01-04-2009	-30.6060	29.7557	M. Graham	1 n	-
AMGS	GEN 1866A	*P. amanzamnyama*	South Africa	Ngwangwane River	01-09-2009	-29.9552	29.5220	M. Graham	9 n	-

Museum	Catalogue	Species name	Country	Locality	Date	Latitude	Longitude	Collectors	No of specimens	Status
AMGS	GEN1870B	*P. amanzamnyama*	South Africa	Mzimkhulu River at Horseshoe bend	04-09-2009	-30.6285	30.2443	M. Graham	4 n	2n molecular
AMGS	McC SA 41A	*P. crassi*	South Africa	Olifants River KNP 15km from tar Fig tree	29-10-1990	-24.0458	31.3831	W.P. McCafferty, F.C. de Moor, H.M. Barber	2 n	-
AMGS	McC SA 35A	*P. crassi*	South Africa	Sabie River 7km N of Skukuza	23-10-1990	-24.9708	31.4514	W.P. McCafferty, F.C. de Moor, H.M. Barber	1 n	-
AMGS	VAL 342C	*P. crassi*	South Africa	Vaal River, above Lindeques Drift	27-02-1958	-26.7389	27.6028	F.M. Chutter	1 n	-
AMGS	VAL 364A	*P. crassi*	South Africa	Vaal River, below Loch Vaal Barrage	31-03-1958	-26.7653	27.6833	F.M. Chutter	1 n	-
AMGS	VAL 365B	*P. crassi*	South Africa	Vaal River above Lindeques Drift	31-03-1958	-26.7389	27.6028	F.M. Chutter	1 n	-
AMGS	VAL 390D	*P. crassi*	South Africa	Vaal River above Lindeques Drift	29-05-1958	-26.7389	27.6028	F.M. Chutter	4 n	-
AMGS	VAL 432C	*P. crassi*	South Africa	Waterval River, Balfour-Standerton Rd	16-10-1958	-26.8542	28.8889	F.M. Chutter	1 n	-
AMGS	VAL 435A	*P. crassi*	South Africa	Vaal River above Lindeques Drift	30-09-1958	-26.7389	27.6028	F.M. Chutter	2 n	-
AMGS	VAL 446A	*P. crassi*	South Africa	Vaal River, above Lindeques Drift	28-10-1958	-26.7389	27.6028	F.M. Chutter	1 n	-
AMGS	VAL 463A	*P. crassi*	South Africa	Wilge River, at Frankfort	18-09-1958	-27.2819	28.4889	F.M.Chutter	4 n	-
AMGS	VAL 466C	*P. crassi*	South Africa	Vaal River, Morgenzon-Amersfoort Rd.	15-09-1958	-26.8514	29.6972	F.M.Chutter	17 n	-
AMGS	VAL 477C	*P. crassi*	South Africa	Vaal River, above Lindeques Drift	25-11-1958	-26.7389	27.6028	F.M.Chutter	1 n	-
AMGS	VAL 556B	*P. crassi*	South Africa	Wilge River, Warden-Reitz Rd. bridge	13-10-1958	-27.8111	28.7861	F.M.Chutter	5 n	-
AMGS	VAL 558A	*P. crassi*	South Africa	Meul River, Warden-Harrismith Rd.	13-10-1958	-28.0250	28.9986	F.M.Chutter	5 n	-
AMGS	VAL 561E	*P. crassi*	South Africa	Wilge River, at Frankfort	13-10-1958	-27.2819	28.4889	F.M.Chutter	18 n	-
AMGS	VAL 566B	*P. crassi*	South Africa	Vaal River, Morgenzon-Amersfoort Rd.	18-10-1958	-26.8514	29.6972	F.M.Chutter	1 n	-
AMGS	VAL 569D	*P. crassi*	South Africa	Below Nestle Effluent, Standerton	16-10-1958	-26.9625	29.2417	F.M.Chutter	4 n	-

Museum	Catalogue	Species name	Country	Locality	Date	Latitude	Longitude	Collectors	No of specimens	Status
AMGS	VAL 571M	*P. crassi*	South Africa	Wilge River, at Swinburne	18-10-1958	-28.3472	29.2806	F.M.Chutter	7 n	-
AMGS	VAL 606F	*P. crassi*	South Africa	Klip River, Vrede-Volksrust Rd. bridge	14-01-1959	-27.3556	29.4833	F.M.Chutter	1 n	-
AMGS	VAL 626D	*P. crassi*	South Africa	Wilge River, at Swinburne	10-02-1959	-28.3472	29.2806	F.M.Chutter	1 n	-
AMGS	VAL 645C	*P. crassi*	South Africa	Wilge River, at Frankfort	09-02-1959	-27.2819	28.4889	F.M.Chutter	1 n	-
AMGS	VAL 686A	*P. crassi*	South Africa	Wilge River, at Frankfort	08-12-1958	-27.2819	28.4889	F.M.Chutter	2 n	-
AMGS	VAL 700B	*P. crasfsi*	South Africa	Vaal River, Morgenzon-Amersfoort Rd.	20-11-1958	-26.8514	29.6972	F.M.Chutter	1 n	-
AMGS	VAL 733E	*P. crassi*	South Africa	Klein Vaal River, at Goedehoop Farm	21-07-1959	-26.8194	30.1333	F.M.Chutter	5 n	-
AMGS	VAL 737S	*P. crassi*	South Africa	Klein Vaal River, at Goedehoop Farm	21-07-1959	-26.8194	30.1333	F.M.Chutter	5 n	-
AMGS	VAL 745C	*P. crassi*	South Africa	Vaal River, Standerton-Villiers Rd.	20-07-1959	-27.0153	29.0250	F.M.Chutter	9 n	-
AMGS	VAL 746C	*P. crassi*	South Africa	Above Nestle effluent, Standerton	20-07-1959	-26.9611	29.2431	F.M.Chutter	2 n	-
AMGS	VAL 749A	*P. crassi*	South Africa	Vaal River, Morgenzon-Amersfoort Rd.	21-07-1959	-26.8514	29.6972	F.M.Chutter	4 n	-
AMGS	VAL 752C	*P. crassi*	South Africa	Below Standerton sewage works	21-07-1959	-26.9792	29.2375	F.M.Chutter	1 n	-
AMGS	VAL 765B	*P. crassi*	South Africa	Vaal River, Standerton-Villiers Rd.	18-08-1959	-27.0153	29.0250	F.M.Chutter	5 n	-
AMGS	VAL 772C	*P. crassi*	South Africa	Klein Vaal River, at Goedehoop Farm	19-08-1959	-26.8194	30.1333	F.M.Chutter	2 n	-
AMGS	VAL 796M	*P. crassi*	South Africa	Vaal River, Morgenzon-Amersfoort Rd.	19-08-1959	-26.8514	29.6972	F.M.Chutter	24 n	-
AMGS	VAL 811A	*P. crassi*	South Africa	Vaal River, Standerton-Villiers Rd.	22-09-1959	-27.0153	29.0250	F.M.Chutter	15 n	-
AMGS	VAL 817D	*P. crassi*	South Africa	Vaal River, Morgenzon-Amersfoort Rd.	23-09-1959	-26.8514	29.6972	F.M.Chutter	26 n	-
AMGS	VAL 822A	*P. crassi*	South Africa	Klein Vaal River, at Goedehoop Farm	23-09-1959	-26.8194	30.1333	F.M.Chutter	2 n	-
AMGS	VAL 831B	*P. crassi*	South Africa	Vaal River, Ermelo-Piet Retief Rd.	09-11-1959	-26.6444	30.1514	F.M.Chutter	3 n	-

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AMGS	VAL 838B	*P. crassi*	South Africa	Klein Vaal River, at Goedehoop Farm	10-11-1959	-26.8194	30.1333	F.M.Chutter	4 n	-
AMGS	VAL 841A	*P. crassi*	South Africa	Wakkerstroom-Heyshope Dam Rd bridge	11-10-1959	-27.0208	30.1583	F.M.Chutter	1 n	-
AMGS	VAL 842C	*P. crassi*	South Africa	Vaal River, at Klipbank Farm	11-10-1959	-26.7014	30.0833	F.M.Chutter	4 n	-
AMGS	VAL 843A	*P. crassi*	South Africa	Vaal River, Ermelo-Amsterdam Rd.	23-09-1959	-26.5653	30.2139	F.M.Chutter	3 n	-
AMGS	VAL 890B	*P. crassi*	South Africa	Vaal River, Standerton-Villiers Rd.	20-10-1959	-27.0153	29.0250	F.M.Chutter	8 n	-
AMGS	VAL 899F	*P. crassi*	South Africa	Vaal River, Morgenzon-Amersfoort Rd.	20-01-1960	-26.8514	29.6972	F.M.Chutter	3 n	-
AMGS	VAL 906A	*P. crassi*	South Africa	Vaal River, Ermelo-Amsterdam Rd.	22-01-1960	-26.5653	30.2139	F.M.Chutter	1 n	-
AMGS	VAL 943B	*P. crassi*	South Africa	Vaal River, Morgenzon-Amersfoort Rd.	10-12-1959	-26.8514	29.6972	F.M.Chutter	2 n	-
AMGS	VAL 944A	*P. crassi*	South Africa	Below Standerton sewage works	11-12-1959	-26.9792	29.2375	F.M.Chutter	2 n	-
AMGS	VAL 946B	*P. crassi*	South Africa	Vaal River, Morgenzon-Amersfoort Rd.	10-12-1959	-26.8514	29.6972	F.M.Chutter	3 n	-
AMGS	VAL 959A	*P. crassi*	South Africa	Klein Vaal River, at Goedehoop Farm	10-12-1959	-26.8194	30.1333	F.M.Chutter	2 n	-
AMGS	VAL 961A	*P. crassi*	South Africa	Klein Vaal River, at Goedehoop Farm	10-12-1959	-26.8194	30.1333	F.M.Chutter	1 n	-
AMGS	VAL 1053B	*P. crassi*	South Africa	Vaal River, Standerton-Villiers Rd.	23-03-1960	-27.0153	29.0250	F.M.Chutter	4 n	-
AMGS	VAL 1156C	*P. crassi*	South Africa	Vaal River, Standerton-Villiers Rd.	03-06-1960	-27.0153	29.0250	F.M.Chutter	1 n	-
AMGS	VAL 1221A	*P. crassi*	South Africa	Vaal River, Morgenzon-Amersfoort Rd.	20-07-1960	-26.8514	29.6972	F.M.Chutter	1 n	-
AMGS	VAL 1226A	*P. crassi*	South Africa	Below Standerton sewage works	21-07-1960	-26.9792	29.2375	F.M.Chutter	2 n	-
AMGS	VAL 1230C	*P. crassi*	South Africa	Vaal River, Standerton-Villiers Rd.	21-07-1960	-27.0153	29.0250	F.M.Chutter	1 n	-

Museum	Catalogue	Species name	Country	Locality	Date	Latitude	Longitude	Collectors	No of specimens	Status
AMGS	VAL 1281A	*P. crassi*	South Africa	Below Standerton sewage works	21-07-1960	-26.9792	29.2375	F.M.Chutter	1 n	-
AMGS	VAL 1329B	*P. crassi*	South Africa	Wilge River, Warden-Reitz Rd. bridge	11-08-1961	-27.8111	28.7861	F.M.Chutter	5 n	-
AMGS	VAL 1333H	*P. crassi*	South Africa	Wilge River, at Swinburne		-28.3472	29.2806	F.M.Chutter	1 n	-
AMGS	GEN 109J	*P. crassi*	South Africa	Mataffin, near Nelspruit	26-07-1959	-25.4500	30.9458	NIWR	1 n	-
AMGS	GEN 112H	*P. crassi*	South Africa	Mataffin, near Nelspruit	07-07-1959	-25.4500	30.9458	NIWR		-
AMGS	GEN 131J	*P. crassi*	South Africa	Crocodile river, at Malelane	07-07-1959	-25.4833	31.5000	NIWR	20 n	-
AMGS	GEN 146A	*P. crassi*	South Africa	Suid Kaaprivier, near Barberton	06-07-1959	-25.7333	30.9833	NIWR	1 n	-
AMGS	GEN 149J	*P. crassi*	South Africa	Olifants river, near Mica	09-07-1959	-24.2208	30.8167	NIWR	4 n	-
AMGS	GEN 157E	*P. crassi*	South Africa	Sabie river, at Sabie bungalows	08-07-1959	-25.0917	30.7667	NIWR	1 n	-
AMGS	GEN 223F	*P. crassi*	South Africa	Mataffin, near Nelspruit	20-11-1959	-25.4500	30.9458	NIWR	2 n	-
AMGS	GEN 249E	*P. crassi*	South Africa	Lower Sabie, Kruger National Park	18-11-1959	-25.0250	31.9833	NIWR	5 n	-
AMGS	GEN 257D	*P. crassi*	South Africa	Crocodile River, at Rietvlei Farm	21-11-1959	-25.3833	30.5500	NIWR	2 n	-
AMGS	GEN 307F	*P. crassi*	South Africa	Lower Sabie, Kruger National Park	19-11-1959	-25.0250	31.9833	NIWR	1 n	-
AMGS	GEN 395D	*P. crassi*	South Africa	Komati river, near Badplaas	07-07-1960	-25.9167	30.6250	NIWR	1 n	-
AMGS	GEN 400C	*P. crassi*	South Africa	Crocodile River Dickon Hall, Nelspruit	24-08-1960	-25.4542	30.9750	NIWR	1 n	-
AMGS	GEN 402E	*P. crassi*	South Africa	Crocodile river, at farm Rietvlei	23-08-1960	-25.3833	30.5500	NIWR	1 n	-
AMGS	GEN 411D	*P. crassi*	South Africa	Crocodile R.,Dickon Hall, Nelspruit	25-08-1960	-25.4542	30.9750	NIWR	3 n	-
AMGS	GEN 420E	*P. crassi*	South Africa	Crocodile river, at Schagen	25-08-1960	-25.4333	30.7917	NIWR	2 n	-
AMGS	GEN 471G	*P. crassi*	South Africa	Olifants River Rest Camp, K.N.P.	28-06-1960	-24.0083	31.7417	NIWR	1 n	-
AMGS	GEN 679E	*P. crassi*	Zimbabwe	Mazoe river, near Changara	24-06-1961	-16.5417	33.2792	NIWR	2 n	-
AMGS	GEN 798G	*P. crassi*	South Africa	Usutu River, 1,2km above Usutu Pulp Co	20-08-1981	-26.5431	31.0028	NIWR	1 n	-

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AMGS	MOI 29BG	*P. crassi*	South Africa	Klein Mooi River, at Durleigh Farm	15-03-1995	-29.2283	29.8997	C. Dickens	2 n	-
AMGS	LIM 5G	*P. crassi*	South Africa	Upstream of Crocodile river	05-09-1991	-25.4775	30.7028	H.M Barber, N.P.E James	1 n	-
AMGS	TUG 6J3	*P. crassi*	South Africa	Stn.10, at Colenso	22-09-1953	-28.7333	29.8167	W.D.Oliff	1 n	-
AMGS	TUG 14J3	*P. crassi*	South Africa	Stn.14, at Tugela Ferry	24-09-1953	-28.7333	30.4500	W.D.Oliff	1 n	-
AMGS	ECR 699A	*P. crassi*	South Africa	Inxu River	06-06-2003	-31.1993	28.4335	P. Maseti	11 n	-
AMGS	ECR 700A	*P. crassi*	South Africa	Inxu River	06-06-2003	-31.1993	28.4335	P. Maseti	1 n	-
AMGS	ECR 701A-C	*P. crassi*	South Africa	Inxu River	28-10-2003	-31.1993	28.4335	P. Maseti	11 n	-
AMGS	ECR 702A-B	*P. crassi*	South Africa	Inxu River	28-10-2003	-31.1993	28.4335	P. Maseti	4 n	-
AMGS	ECR 703A-C	*P. crassi*	South Africa	Inxu River	07-06-2003	-31.2480	28.5308	P. Maseti	28 n	-
AMGS	ECR 704 A-B	*P. crassi*	South Africa	Inxu River	29-10-2003	-31.2480	28.5308	P. Maseti	10 n	3 n molecular
AMGS	ECR 705A	*P. crassi*	South Africa	Inxu River	26-10-2003	-31.1744	28.1217	P. Maseti	3 n	-
AMGS	ECR 706A	*P. crassi*	South Africa	Gatberg River	26-10-2003	-31.2497	28.1539	P. Maseti	1 n	-
AMGS	ECR 707A	*P. crassi*	South Africa	Inxu River	08-06-2003	-31.2027	28.2445	P. Maseti	2 n	-
AMGS	ECR 708A	*P. crassi*	South Africa	Inxu River	08-06-2003	-31.2198	28.6308	P. Maseti	9 n	-
AMGS	ECR 709A	*P. crassi*	South Africa	Inxu River	29-10-2003	-31.2198	28.6308	P. Maseti	9 n	-
AMGS	ECR 710A	*P. crassi*	South Africa	Inxu River	29-10-2003	-31.2198	28.6308	P. Maseti	2 n	-
AMGS	ECR 711A	*P. crassi*	South Africa	Gatberg River	05-06-2003	-31.2169	28.1539	P. Maseti	1n	-
AMGS	uncatalogued	*P. crassi*	South Africa	Buffalo R.at weir on farm Amanzi	30-10-2003	-32.9917	27.7758	R.W. Palmer	?	lost
AMGS	ORP 20E	*P. crassi*	South Africa	Orange River Gifkloof	06-04-1992	-28.4333	21.7500	R.W. Palmer	1 n	-
AMGS	ORP 26F	*P. crassi*	South Africa	Orange River Gifkloof	08-04-1992	-28.4333	21.7500	R.W. Palmer	1 n	-
AMGS	ORP 47D	*P. crassi*	South Africa	Orange River Gifkloof	17-08-1992	-28.4333	21.7500	R.W. Palmer	2 n	-
AMGS	ORP 49W	*P. crassi*	South Africa	Orange River Gifkloof	07-09-1992	-28.4333	21.7500	R.W. Palmer		-
AMGS	ORP 62I	*P. crassi*	South Africa	Orange River Gifkloof	07-12-1992	-28.4333	21.7500	R.W. Palmer	1 n	-
AMGS	ORP 119 AD	*P. crassi*	South Africa	Orange River Gifkloof	04-10-1993	-28.4333	21.7500	R.W. Palmer	1 n	-
AMGS	uncatalogued	*P. crassi*	South Africa	Mthatha River, Kambi Forest	26-10-2005	-31.4713	28.6151	B.R. Madikizela	1 n	-
AMGS	WAR 75A	*P. crassi*	South Africa	Vaal R.Prinsloo Bridge Warrenton	18-10-1963	-28.1264	24.8083	F.M.Chutter		-
AMGS	GEN 1836C	*P. crassi*	South Africa	Gatberg River	21-09-2003	-31.2228	28.2628	M. Graham	27 n	6 n molecular
AMGS	GEN 1837A	*P. crassi*	South Africa	Umzimvubu River	24-10-1996	-30.5667	29.2000	B.R. Madikizela	3 n	-
AMGS	uncatalogued	*P. crassi*	South Africa	Tina River	24-10-1996	-30.5833	29.4167	B.R. Madikizela	1 n	-

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AMGS	uncatalogued	*P. crassi*	South Africa	Tina River	24-10-1996	-30.7000	28.7500	B.R. Madikizela	1 n	-
AMGS	GEN 1838A	*P. crassi*	South Africa	Umkomaas River at Shozi Weir	17-11-2004	-30.1356	30.6737	B. Wynne	7 n	-
AMGS	GEN 1848A	*P. crassi*	South Africa	Great Fish River	01-06-2005	-32.7506	25.8103	N. Rivers-Moore	1 n	1 n molecular
AMGS	GEN 1868A	*P. crassi*	South Africa	Mzimkhulu R at main bridge, Mzimkhulu	02-09-2009	-30.2591	30.2443	M. Graham	4 n	-
AMGS	GEN1869A	*P. crassi*	South Africa	Mzimkhulu R site 6	03-09-2009	-30.3565	30.0482	M. Graham	1 n	-
AMGS	GEN 1870A	*P. crassi*	South Africa	Mzimkhulu R at Horseshoe bend	04-09-2009	-30.6285	30.2443	M. Graham	1 n	-
AMGS	TUG 6J3	*P. crassi*	South Africa	Tukhela River, Stn.10, at Colenso	22-09-1953	-28.7333	29.8167	W.D.Oliff	1 n	-
AMGS	TUG 14J3	*P. crassi*	South Africa	Tukhela River, Stn.14, at Tugela Ferry	24-09-1953	-28.7333	30.4500	W.D.Oliff	2 n	-
AMGS	TUG 19J7	*P. crassi*	South Africa	Tukhela River, at Bond's Drift	31-10-1953	-29.1731	31.4167	W.D.Oliff	4 n	-
AMGS	TUG 79J5	*P. crassi*	South Africa	Tukhela River at Mandini	17-08-1954	-29.1667	31.4083	W.D.Oliff	3 n	-
AMGS	TUG 81J31	*P. crassi*	South Africa	Tukhela River, Stn.19, at Mandini	04-09-1954	-29.1667	31.4083	W.D.Oliff	1 n	-
AMGS	TUG 85J18	*P. crassi*	South Africa	Tukhela River, Stn.18, at Middle Drift	04-12-1954	-28.9000	31.0333	W.D.Oliff	1 n	-
AMGS	TUG 93J27	*P. crassi*	South Africa	Tukhela River, Stn.19b, at Mandini	20-09-1955	-29.1667	31.4083	W.D.Oliff	1 n on slide	-
AMGS	TUG 105U	*P. crassi*	South Africa	Thukela River at Middle Drift	04-09-1985	-28.9411	31.02528	B. Fowles	1 n	-
AMGS	TUG 123Z	*P. crassi*	South Africa	Thukela River at Tugela Estates	17-08-1999	-25.7569	30.1583	F.C. de Moor, C. Dickens, F.M. Chutter, M. Coke	3 n	-
AMGS	TUG 146E	*P. crassi*	South Africa	Thukela River 1.5 km above Jamesons Drift	06-10-1999	-28.7744	30.8917	F.C. de Moor, C.R. Lugo-Ortiz	1 n	-
AMGS	MOI 29BG	*P. crassi*	South Africa	Klein Mooi River, at Durleigh Farm	15-03-1995	-29.2283	29.8997	C. Dickens	2 n	-
AMGS	UMK 33AL	*P. crassi*	South Africa	Umkomaas River at Lundy's Hill	10-10-1996	-29.7417	29.8722	F.C. de Moor, C. Dickens, T. Mawela	1 n	-
AMGS	UMK 80AH	*P. crassi*	South Africa	Umkomaas River at top weir, above Saicor	30-04-1996	-30.1697	30.6986	F.C. de Moor, C. Dickens, T. Mawela	1 n	-

Museum	Catalogue	Species name	Country	Locality	Date	Latitude	Longitude	Collectors	No of specimens	Status
AMGS	UMK 91AF	*P. crassi*	South Africa	Umkomaas River at Vergelegen Nat res	06-05-1996	-29.5528	29.4917	F.C. de Moor, C. Dickens, M. Graham	2 n	-
AMGS	UMK 94C	*P. crassi*	South Africa	Umkomaas River near Impendle	10-10-1997	-29.6247	29.7414	F.C. de Moor, H.M. Barber-James, C. Dickens	3 n	-
AMGS	UMK 99S	*P. crassi*	South Africa	Mkomozana River near confl with Umko	09-10-1996	-29.6361	29.7417	F.C. de Moor, H.M. Barber-James	3 n	-
AMGS	UMK 102AA	*P. crassi*	South Africa	Umkomaas River at at Lundy's Hill	11-10-1996	-29.7414	29.8747	F.C. de Moor, H.M. Barber-James	2 n	-
AMGS	UMK 103AQ	*P. crassi*	South Africa	Umkomaas R at Lundy's Hill	10-10-1996	-29.7417	29.8722	F.C. de Moor, H.M. Barber-James	6 n	-
AMGS	UMK 121AG	*P. crassi*	South Africa	Umkomaas River at Staebraes, side ch	11-10-1996	-29.9861	30.1547	F.C. de Moor, H.M. Barber-James	1 n	-
AMGS	UMK 106AN	*P. crassi*	South Africa	Umkomaas R at top weir, above Saicor	12-10-1996	-30.1697	30.6986	F.C. de Moor, H.M. Barber-James	19 n	-
AMGS	UMK 118AE	*P. crassi*	South Africa	Umkomaas River at confl with Lufafa	12-10-1996	-30.0167	30.1778	F.C. de Moor, H.M. Barber-James	9 n	-
AMGS	UMK 119X	*P. crassi*	South Africa	Umkomaas River at Staebraes	14-10-1996	-29.9861	30.1547	F.C. de Moor, H.M. Barber-James	8 n	-
AMGS	UMK 131AB	*P. crassi*	South Africa	Umkomaas River at Hella Hella	14-10-1996	-29.9181	29.9250	F.C de Moor, H.M. Barber-James, C. Dickens	7 n	-
AMGS	UMG 1040Q	*P. crassi*	South Africa	Umgeni River upstream of Wartburg Road	16-10-1996	-29.4646	30.4620	M. Graham, C. Dickens	4 n	-
AMGS	UMG 1041W	*P. crassi*	South Africa	Umgeni River upstream of Wartburg Road	15-10-2003	-29.4646	30.4620	M. Graham, C. Dickens	2 n	-
AMGS	UMG 1045G	*P. crassi*	South Africa	Umgeni River above Mpolweni confluence	15-10-2003	-29.4447	30.4467	M. Graham, C. Dickens	1 n	-
AMGS	UMG 1059A	*P. crassi*	South Africa	Umgeni River above Mpolweni confluence	13-10-2003	-29.4447	30.4467	M. Graham, C. Dickens	3 n	-
AMGS	UMG 1072F	*P. crassi*	South Africa	Umgeni River upstream of Wartburg Road	01-03-2004	-29.4646	30.4620	M. Graham, C. Dickens	1 n	-
AMGS	UMG 1075G	*P. crassi*	South Africa	Umgeni River above Mpolweni confluence	17-06-2004	-29.4447	30.4467	M. Graham, C. Dickens	3 n	-
AMGS	AD 2J5	*P. crassi*	South Africa	Verdruk Dam, near Hattingspruit, Dundee	23-09-1953	-28.0833	30.1500	W.D. Oliff	1 n	-

Museum	Catalogue	Species name	Country	Locality	Date	Latitude	Longitude	Collectors	No of specimens	Status
AMGS	BFO 18J19	*P. crassi*	South Africa	Ingogo River	29-05-1959	-27.5833	29.9250	W.D. Oliff	1 n	-
PERC/ AMGS	McC SA 40	*P. mccaffertyi*	South Africa	Sabie River at Lisbon Estates	27-10-1990	-24.9861	31.4514	W.P. McCafferty, F.C. de Moor, H.M. Barber	1 n on slide, 5 n PERC, 5 n AMGS	Paratypes
PERC	McC SA 40A	*P. mccaffertyi*	South Africa	Sabie River at Lisbon Estates	27-10-1990	-24.9861	31.4514	W.P. McCafferty, F.C. de Moor, H.M. Barber	1 ♀ sub	-
AMGS	GEN 112H	*P. mccaffertyi*	South Africa	Mataffin, near Nelspruit	07-07-1959	-25.4500	30.9458	NIWR	9 n	-
AMGS	GEN 244E	*P. mccaffertyi*	South Africa	Usutu River, near Big Bend	24-11-1959	-26.8583	31.9000	NIWR	1 n	-
AMGS	GEN 249E	*P. mccaffertyi*	South Africa	Lower Sabie River, Kruger National Park	18-11-1959	-25.0250	31.9833	NIWR	5 n	-
AMGS	GEN 251E	*P. mccaffertyi*	South Africa	Lower Sabie River, Kruger National Park	18-11-1959	-25.0250	31.9833	NIWR	3 n	-
AMGS	GEN 269C	*P. mccaffertyi*	South Africa	Sabie river, at Sabie bungalows	24-11-1959	-25.0917	30.7667	NIWR	1 n	-
AMGS	GEN 307F	*P. mccaffertyi*	South Africa	Lower Sabie River, Kruger National Park	19-11-1959	-25.0250	31.9833	NIWR	3 n	-
AMGS	GEN 536D	*P. mccaffertyi*	South Africa	Lower Sabie River, Kruger National Park	01-07-1960	-25.0250	31.9833	NIWR	1 n	-
AMGS	GEN 674C	*P. mccaffertyi*	South Africa	Usutu River, 11km below Pulp mill, St7A	13-07-1961	-26.5833	31.0833	NIWR	1 n	-
AMGS	GEN 674D	*P. mccaffertyi*	South Africa	Usutu River, 11km below Pulp mill, St7A	13-07-1961	-26.5833	31.0833	NIWR	1 n	-
AMGS	GEN 677G	*P. mccaffertyi*	South Africa	Usutu River, at Mlambanyati, St1A	12-07-1961	-26.4417	31.0667	NIWR	1 n	-
AMGS	GEN 798G	*P. mccaffertyi*	South Africa	Usutu River, 1.2 km above Usutu Pulp mill	20-07-1981	-26.5431	31.0028	F.C. de Moor	1 n	-
AMGS and PERC	LIM 17L; PERC_SA40	*P. mccaffertyi*	South Africa	Sabie River at Lisbon Estates	27-10-1990	-24.9861	31.4514	W.P. McCafferty, F.C. de Moor, H.M. Barber	1 n + 11 n + 1 ♀	Holotype n; paratype 11 n, 1 ♀
AMGS	LIM 29K	*P. mccaffertyi*	South Africa	Sabie River above Skukuza at measuring weir	24-10-1990	-24.9764	31.5847	H.M. Barber, F.C. de Moor	1 n	-
AMGS and PERC	LIM 31T PERC-SA35	*P. mccaffertyi*	South Africa	Sabie River 7 Km above Skukuza	23-10-1990	-24.9803	30.5853	W.P. McCafferty, F.C. de Moor, H.M. Barber	2 ♀; 4 ♀	-

Museum	Catalogue	Species name	Country	Locality	Date	Latitude	Longitude	Collectors	No of specimens	Status
AMGS	KUN 17D	African sp 2	Namibia	Cunene River at Stein Guard Post	12-11-1997	-17.4319	13.9847	F.C. de Moor, N. Kohly	15 n	-
AMGS	KUN 32AL	African sp 2	Namibia	Cunene River downstream of Ondurusu Falls	13-11-1997	-17.3828	13.9136	F.C. de Moor, N. Kohly	1♂ sub	-
AMGS	KUN 34A	African sp 2	Namibia	Cunene River, near Ohangonga Guard Post	14-11-1997	-17.2992	13.7803	F.C. de Moor, N. Kohly	5 n	-
AMGS	KUN 36K	African sp 2	Namibia	Cunene River, Omapapurawe Guard Post	15-11-1997	-17.2186	13.6458	F.C. de Moor, N. Kohly	2 n	-
AMGS	KUN 48A	African sp 2	Namibia	Cunene River at Etemba Guard Post	16-11-1997	-17.1853	13.5978	F.C. de Moor, N. Kohly	3 n	-
AMGS	KUN 59A	African sp 2	Namibia	Cunene River, Oonjana 'Palm Grove Camp site'	17-11-1997	-17.0050	13.4311	F.C. de Moor, N. Kohly	3 n	-
AMGS	KUN 79F	African sp 2	Namibia	Cunene River, Gorge below Epupa Falls	19-11-1997	-17.0008	13.2456	F.C. de Moor, N. Kohly	1 n	-
AMGS	KUN 81D	African sp 2	Namibia	Cunene River, Gorge below Epupa Falls	19-11-1997	-17.0008	13.2456	F.C. de Moor, N. Kohly	1 n	-
AMGS	KUN 85H	African sp 2	Namibia	Cunene River, Gorge below Epupa Falls	19-11-1997	-17.0008	13.2456	F.C. de Moor, N. Kohly	1♂ sub	-
AMGS	KUN 117Q	African sp 2	Namibia	Cunene River, Sera Cafema Camp site	25-11-1998	-17.2111	12.2028	F.C. de Moor, R. Tharme	1 n	-
AMGS	KUN 120H	African sp 2	Namibia	Cunene River, downstream of Baynes Mts	25-11-1998	-17.1464	12.7625	F.C. de Moor, R. Tharme	1♂ sub	-
AMGS	KUN 121F	African sp 2	Namibia	Cunene River,at Baynes Mts	25-11-1998	-17.0194	12.9578	F.C. de Moor, R. Tharme	3♂ sub	-
AMGS	KUN 122D	African sp 2	Namibia	Cunene River, above proposed Baynes dam site	25-11-1998	-16.9872	13.0928	F.C. de Moor, R. Tharme	1♂ sub	-
AMGS	KUN 149C	African sp 2	Namibia	Cunene River at Omapapurawe Guard Post	29-11-1998	-17.2186	13.6458	F.C. de Moor, R. Tharme	1 n	-
AMGS	KUN150E	African sp 2	Namibia	Cunene River at Omapapurawe Guard Post	29-11-1998	-17.2186	13.6458	F.C. de Moor, R. Tharme	1 n	-
AMGS	KUN 157G	African sp 2	Namibia	Cunene River	21-01-1998	-17.1736	12.6975	K. Roberts	3♂ sub, 1♂ imago	-

Museum	Catalogue	Species name	Country	Locality	Date	Latitude	Longitude	Collectors	No of specimens	Status
AMGS	KUN 171C	African sp 2	Namibia	Cunene River, Sera Cafema Camp site	14-11-2000	-17.2164	12.2025	K. Roberts	11 n	-
AMGS	GEN 683G	African sp 3	Malawi	Shire River, at Mpatamanga gorge	25-06-1961	-15.7250	34.7292	NIWR	2 n	-
AMGS	CAW 381H	African sp 3	DRC	Kasai River upstream of Mbelenge min	15-08-2007	-5.5886	20.9188	M. Graham	3 n	2 n to molecular
AMGS	CAW 382P	African sp 3	DRC	Kasai River at Lumbembe confluence	16-08-2007	-6.6101	21.0699	M. Graham	1 n	-
AMGS	CAW 181A	African sp 3	Kenya	Awach nr Gendia	26-06-1996	-0.4000	34.6667	P. Martin	1 n	-
AMGS	CAW 388A	African sp 4	Kenya	Isiukhu River	10-11-2007	0.2544	34.7497	M. Graham	3 n, 1 slide	3 n to molecular
RMCA	DC 284,286	African sp 6	DRC	Lukafu River	00-12-1930	-10.8592	27.1047	de Witte	1 n, 1 slide	-
MZL	-	African sp 6	DRC	Park Virunga, route Ishango-Butambo	23-02-1981	-0.9997	29.2500	J-M. Elouard	8 n, 1 slide	-
MZL	W2	African sp 7	Ivory Coast	Dimbokro	1975-12-16	6.6364	4.7056	unknown	10 n	-
MZL	1201	African sp 7	Guinea	Boussoulé, Milo River, trib of Niger	?	9.4042	-10.0289	unknown	3 n	-
MZL	Ek	African sp 7	Ivory Coast	Eutomokzo, Maraoue	1979-11-21	7.0783	-5.7150	unknown	8 n	-
MZL	RCI	African sp 7	Togo	Amou oblo	?	7.3831	0.8667	unknown	2 n	-
MZL	1101	African sp 7	Guinea	Sassambaya, Niandan, trib of Niger River	1984-12-23	9.6039	-10.5297	J-M. Elouard	1 n	-
MZL	1101	African sp 7	Guinea	Sassambaya, Niandan, trib of Niger River	1985-03-01	9.6039	-10.5297	J-M. Elouard	10 n	-
MZL	1101	African sp 7	Guinea	Sassambaya, Niandan, trib of Niger River	1985-06-29	9.6039	-10.5297	J-M. Elouard	2 n	-
MZL	1101	African sp 7	Guinea	Sassambaya, Niandan, trib of Niger River	1988-04-07	9.6039	-10.5297	J-M. Elouard	2 n	-
MZL	1101	African sp 7	Guinea	Sassambaya, Niandan, trib of Niger River	1985-01-25	9.6039	-10.5297	J-M. Elouard	6 n	-
MZL	1101	African sp 7	Guinea	Sassambaya, Niandan, trib of Niger River	1985-03-27	9.6039	-10.5297	J-M. Elouard	10 n	-
MZL	1101	African sp 7	Guinea	Sassambaya, Niandan, trib of Niger River	1988-04-06	9.6039	-10.5297	J-M. Elouard	1 n	-
MZL	3000.1	African sp 7	Ivory Coast	Tiassalé, Bandama River	1978-03-11	5.9292	-4.9936	J-M. Elouard	6 n	-
MZL	97000.1	African sp 7	Sierra Leone	Yrafilaia, Selu River, trib of Rocke	1989-02-07	8.2594	-11.6072	J-M. Elouard	3 n	-

Museum	Catalogue	Species name	Country	Locality	Date	Latitude	Longitude	Collectors	No of specimens	Status
AMGS	CAW 128J	African sp 8	Zambia	Sakeji River, above school	13-04-1994	-11.2167	24.3167	H.M. Barber-James		-
AMGS	Uncatalogued	African sp 8	Botswana	Okavango River, Popa Falls (E.bank)	28-07-1997	-17.9031	20.1000	E. Taylor	1 n on slide, 1 n	-
AMGS	Uncatalogued	African sp 8	Zambia	Wenela rapids, Zambezi	08-08-1997	-17.4692	24.2375	E. Taylor	2 n	-
AMGS	Uncatalogued	African sp 8	Botswana	Chobe/Zambezi	05-02-1998	-17.7694	25.1547	E. Taylor	1 n	-
AMGS	GEN 1839A	African sp 8	Zambia	Wenela rapids, Zambezi	16-10-2001	-17.4786	24.2448	R.W. Palmer	-	2 n to molecular
FAMU	F/An/15c	Comores sp 1	Comores	Gege River	13-03-1974	-12.2167	44.4333	F. Starmühlner	1 slide	-
MZL	(P0057)	Madagascan sp 1	Madagascar	Ikopa River	10-05-1991	-19.0103	47.7050	J-M. Elouard	1 n	-
MZL	(P0510)	Madagascan sp 1	Madagascar	Manambolo River	18-10-1995	-18.9214	47.9328	T. Pilaka	1 n	-
MZL	(P0519)	Madagascan sp 1	Madagascar	Manambolo River	20-10-1995	-18.9092	47.9147	T. Pilaka, Z.P. Andriambelo	1 n, 1n on slide	-
MZL	Mad 163a	Madagascan sp 1	Madagascar	de Manques	20-11-2001	-12.4833	49.2000	R. Gerecke and T. Goldshmidt	1 n	-
MZL	(P0198)	Madagascan sp 2	Madagascar	Makis River	01-04-1994	-12.5278	49.1692	J-M. Elouard, M. Sartori	1 n	-
MZL	(P0200)	Madagascan sp 2	Madagascar	Makis River	02-04-1994	-12.5278	49.1692	J-M. Elouard, M. Sartori	1 n	-
MZL	(P0494)	Madagascan sp 2	Madagascar	Mananara River	26-10-1995	-18.5778	47.8617	J-M. Elouard, R. Oliarinon, T. Pilaka	4 n	-
MZL	(P0526)	Madagascan sp 2	Madagascar	Manampanihy River	21-11-1995	-24.6769	46.8219	J-M. Elouard, T. Pilaka	3 n	-
MZL	(P2130)	Madagascan sp 2	Madagascar	Madiofasina River	27-11-2001	-18.9231	48.4425		5 n	-
MZL	(P2148)	Madagascan sp 2	Madagascar	Beanamalao River	15-06-2001	-18.8314	48.7692	R. Oliarinony	1 n, 1 n on slide	-
MZL	(P2166)	Madagascan sp 2	Madagascar	Sahaparasy River	19-06-2001	-18.8806	48.3883	R. Oliarinony	1 n	-
MZL	(P2169)	Madagascan sp 2	Madagascar	Sahaparasy River	10-09-2001	-18.8806	48.3883	R. Oliarinony	1 n	-
MZL	(P2170)	Madagascan sp 2	Madagascar	Sahaparasy River	08-10-2001	-18.8806	48.3883	R. Oliarinony	1 n	-
MZL	(P2172)	Madagascan sp 2	Madagascar	Sahaparasy River	28-11-2001	-18.8806	48.3883	R. Oliarinony	2 n	-
MZL	Mad 011	Madagascan sp 2	Madagascar	Ampanakamonty	21-07-2001	-18.5525	47.8708	R. Gerecke and T. Goldshmidt	6 n	-
MZL	Mad 011c	Madagascan sp 2	Madagascar	Ampanakamonty	21-07-2001	-18.5525	47.8708	R. Gerecke and T. Goldshmidt	3 n	-
MZL	Mad 048	Madagascan sp 2	Madagascar	Trib of Mangoky River	25-08-2001	-23.2575	46.1019	R. Gerecke and T. Goldshmidt	1 n	-

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MZL	Mad 138	Madagascan sp 2	Madagascar	Fandramanana	05-11-2001	-18.3267	49.3144	R. Gerecke and T. Goldshmidt	1 n	-
MZL	Mad 165b	Madagascan sp 2	Madagascar	Antomboka	21-11-2001	-12.5002	49.1732	R. Gerecke and T. Goldshmidt	1 n	-
MZL	Mad 154	Madagascan sp 4	Madagascar	de Manques	17-11-2001	-12.4833	49.2000	R. Gerecke and T. Goldshmidt	1 n, 1 slide	-
MZL	Mad 163a	Madagascan sp 4	Madagascar	de Manques	20-11-2001	-12.4833	49.2000	R. Gerecke and T. Goldshmidt	1 n	-
MZL	Mad 165	Madagascan sp 4	Madagascar	Antomboka	21-11-2001	-12.5002	49.1732	R. Gerecke and T. Goldshmidt	1 n	-
MZL	(P2130)	Madagascan sp 5	Madagascar	Madiofasina River	27-11-2001	-18.9231	48.4425	R. Oliarinony		-
MZL	(P2140)	Madagascan sp 5	Madagascar	Belakato River	10-10-2001	-18.8272	48.4206	R. Oliarinony	4 n	-
MZL	(P2141)	Madagascan sp 5	Madagascar	Belakato River	14-11-2001	-18.8272	48.4206	R. Oliarinony	1 n	-
MZL	(P0215)	Madagascan sp 5	Madagascar	Aff. de Namorona	18-04-1994	-21.2611	47.4197	J-M. Elouard, M. Sartori, M.R. Andriamihaja	1 n	
MZL	(P2155)	Madagascan sp 5	Madagascar	Sandrasoa River	08-07-2001	-18.9386	48.5169	Oliarinony R.	1 n	-
MZL	P0400	Unknown adult	Madagascar	Tsiribihina basin, Tributary of Manandona River	18-04-1995	-19.9478	19.94778	J-M. Elouard, M. Sartori, and L. Blanc.	♂ imago ♀ sub	-
MZL	P0249	Unknown adult	Madagascar	Rianila basin, Beforona river.	27-10-1994	-18.9658	18.96583	ORSTOM	♂ imago	-
MZL	(P0165)	*P. variegatum*	Madagascar	Iantara River	17-11-1993	-22.2244	47.0306	ORSTOM	2 n, 2 n on slides, 1 ♂ sub on slides	-
MZL	(P0167)	*P. variegatum*	Madagascar	Sahanivoraky River	19-11-1993	-22.2258	47.0114	ORSTOM	3 n, 1 ♂ sub	-
MZL	(P0526)	*P. variegatum*	Madagascar	Manampanihy River	21-11-1995	-24.6769	46.8219		1 n	-
MZL	(P0543)	*P. variegatum*	Madagascar	Andranohela River	26-11-1995	-24.5964	46.7403	J-M.Elouard	1♀ on slide, 1 ♂ sub on slide	-
MZL	(P0600)	*P. variegatum*	Madagascar	Manambaroa River	24-05-1996	-20.6194	46.3986	J.M Elouard, Sambatra.	2 n	
MZL	(P0623)	*P. variegatum*	Madagascar	Manantenina River	17-10-1996	-14.4325	49.7603	J. Legrand, D. Randriamasimanana	1 n	-
MZL	(P0721)	*P. variegatum*	Madagascar	Sahatandra River	29-04-1998	-19.0256	48.3411	J-M. Elouard, M. Sartori, N. Raberiaka	1 n	-

Museum	Catalogue	Species name	Country	Locality	Date	Latitude	Longitude	Collectors	No of specimens	Status
MZL	(P0722)	*P. variegatum*	Madagascar	Unnamed tributary of Lakato River	29-04-1998	-19.0519	48.3606	J-M. Elouard	1 n	-
MZL	(P0731)	*P. variegatum*	Madagascar	Lakato River	31-05-1998	-19.1425	48.4189	J-M.Elouard	4 ♂ subs, 1 n on slide	-
MZL	(P0762)	*P. variegatum*	Madagascar	Sahatandra River	16-10-1998	-19.0444	48.3619	J. Legrand, N. Raberiaka	1 n	-
MZL	(P0763)	*P. variegatum*	Madagascar	Lakato River	17-10-1998	-19.1389	48.4142	J. Legrand, N. Raberiaka	2 n	
MZL	(P0765)	*P. variegatum*	Madagascar	Tributary of unnamed river	23-10-1998	-19.0992	48.2369	J. Legrand, N. Raberiaka	1n, + 3n, 1♂ imago, 2♂ subs,	Holotype + paratypes
MZL	(P2008)	*P. variegatum*	Madagascar	Lakato River	27-04-2003	-19.1425	48.4189	M.T. Monaghan.	5 n	-
MZL	(P2105)	*P. variegatum*	Madagascar	Antanambotsira River	15-11-2001	-18.8814	48.4303	R. Oliarinony	1 n	-
MZL	(P2115)	*P. variegatum*	Madagascar	Rianasoa River	13-09-2001	-18.4428	48.8383	R. Oliarinony	1 n	-
MZL	(P2118)	*P. variegatum*	Madagascar	Rianasoa River	29-11-2001	-18.4428	48.8383	R. Oliarinony	1 n	-
MZL	(P2127)	*P. variegatum*	Madagascar	Madiofasina River	11-09-2001	-18.9231	48.4425	R. Oliarinony	1 n	
MZL	(P2139)	*P. variegatum*	Madagascar	Belakato River	12-09-2001	-18.8272	48.4206	R. Oliarinony	4 n	-
MZL	(P2141)	*P. variegatum*	Madagascar	Belakato River	14-11-2001	-18.8272	48.4206	R. Oliarinony	1 n	-
MZL	(P2147)	*P. variegatum*	Madagascar	Belakato River	04-06-2002	-18.8272	48.4206	R. Oliarinony	1 n	-
MZL	(P2148)	*P. variegatum*	Madagascar	Beanamalao River	15-06-2001	-18.8314	48.7692	R. Oliarinony	4 n	-
MZL	(P2149)	*P. variegatum*	Madagascar	Beanamalao River	13-09-2001	-18.8314	48.7692	R. Oliarinony	2 n	-
MZL	(P2150)	*P. variegatum*	Madagascar	Beanamalao River	29-11-2001	-18.8314	48.7692	R. Oliarinony	4 n	-
MZL	(P2154)	*P. variegatum*	Madagascar	Sandrasoa River	21-05-2001	-18.9386	48.5169	R. Oliarinony	2 n	-
MZL	(P2157)	*P. variegatum*	Madagascar	Sandrasoa River	17-09-2001	-18.9386	48.5169	R. Oliarinony	5 n	
MZL	(P2160)	*P. variegatum*	Madagascar	Sandrasoa River	25-11-2001	-18.9386	48.5169	R. Oliarinony	6 n	-
MZL	(P2161)	*P. variegatum*	Madagascar	Sandrasoa River	16-01-2002	-18.9386	48.5169	R. Oliarinony	3 n	-
MZL	(P2162)	*P. variegatum*	Madagascar	Sandrasoa River	22-02-2002	-18.9386	48.5169	R. Oliarinony	3 n	-
MZL	(P2164)	*P. variegatum*	Madagascar	Sandrasoa River	12-05-2002	-18.9386	48.5169	R. Oliarinony	5 n	-
MZL	(P2179)	*P. variegatum*	Madagascar	Irihitra River	18-09-2001	-18.9503	48.5375	R. Oliarinony	1 n	-
MZL	(P2180)	*P. variegatum*	Madagascar	Irihitra River	26-11-2001	-18.9503	48.5375	R. Oliarinony	1 n	-
MZL	(P2181)	*P. variegatum*	Madagascar	Analambalo River	12-06-2001	-18.9606	48.5011	R. Oliarinony	1 n	-
MZL	(P2182)	*P. variegatum*	Madagascar	Analambalo River	17-06-2001	-18.9606	48.5011	R. Oliarinony	1 n	
MZL	(P2183)	*P. variegatum*	Madagascar	Analambalo River	25-11-2001	-18.9606	48.5011	R. Oliarinony	1 n	-
MZL	Mad 022	*P. variegatum*	Madagascar	S. of Ionilahy River	12-08-2001	-21.7667	47.7333	R. Gerecke and T. Goldshmidt	1n	-
										-

Museum	Catalogue	Species name	Country	Locality	Date	Latitude	Longitude	Collectors	No of specimens	Status
MZL	Mad 058	*P. variegatum*	Madagascar	Antarantca	04-09-2001	-24.9333	46.6333	R. Gerecke and T. Goldshmidt	3n	
MZL	Mad 060	*P. variegatum*	Madagascar	Antarantsa	05-09-2001	-23.7631	46.9750	R. Gerecke and T. Goldshmidt	1 n	-
MZL	Mad 072	*P. variegatum*	Madagascar	Stream crossing RIP 118 at km 34.5	10-09-2001	-24.9331	46.6333	R. Gerecke and T. Goldshmidt	1n	-
MZL	Mad 107	*P. variegatum*	Madagascar	Left trib Mahiavona River	08-10-2001	-19.4167	47.2000	R. Gerecke and T. Goldshmidt	1n	-
PERC	-	*P. variegatum*	Madagascar	Anevoka River, 15 Km E of Perinet (= Andasibe)	11-10-1971	18.9569	48.2806	G.F. and C.H. Edmunds, F. Emmanuel	2n	
PERC	-	*P. variegatum*	Madagascar	Amboasary River, Perinet	12-10-1971			G.F. and C.H. Edmunds	2n	-
PERC	-	*P. variegatum*	Madagascar	Farimbony River	15-10-1971	18.9069	48.0903	G.F. and C.H. Edmunds	2n	-
FAMU	E2505.4	*P. lieftincki*	Sri Lanka	Rangala, Knuckle Mountains, 12 miles ENE Kandy	11-03-1962	7.4078	80.7778	Lund University expedition	1 n	-
FAMU	E2503	*P. boreus*	Philippines	Mindanao, stream E. side Mt Mckinley nr. Davao	24-08-1946	6.9708	124.1842	F. Werner	3 n	-
FAMU	E2594.6	*P. sedlaceki*	Papau New G	Bulolo River	25-10-1964	-6.6633	146.7294	W.L. and J.G. Peters	1 n	-
FAMU	E2502	*P. palawana*	Philippines	Palawana, Bacungan, nr Puerto Princesa	22-30-03-1947	10.1000	125.4833	F. Werner	2 n	-
AMGS	uncatalogued	*P. pearsonorum*	Australia	South Johnstone River	25-06-2008	-16.4167	146.0333	J.M. Webb	5 n	molecular
AMGS	uncatalogued	*P. pennigerum*	Russia	Volga River	2006,2007	56.2590	34.3203	M. Schletterer	3 n	molecular
AMGS	uncatalogued	*P. pennigerum*	Spain	River Mundo (site Mundo4)	27-03-2008	40.7000	-2.5899	S. Robles and M. Toro	4 n	molecular
AMGS	uncatalogued	*P. pennigerum*	Spain	Cabriel River (site CABP3)	01-12-1999	39.3300	-1.0900	S. Robles and M. Toro	5 n	-
AMGS	uncatalogued	*P. pennigerum*	Spain	River Segura (site SEG5)	27-03-2008	40.4725	-2.2850	S. Robles and M. Toro	6 n	molecular
AMGS	uncatalogued	*P. orhanelicum*	Turkey	Bursa province, Orhaneli stream, Deliballılar site	22-11-2001	39.9183	28.9725	Nurhayat Dalkıran	6 n	molecular
MZL	uncatalogued	*P. oronti*	Israel	Kinnot Valley, River Dan	07-05-1990	33.1000	35.6167	M. Sartori	2 n	-

Museum	Catalogue	Species name	Country	Locality	Date	Latitude	Longitude	Collectors	No of specimens	Status
MZL	uncatalogued	*P. oronti*	Israel	Hula Valley, Upper Jordan River	07-05-1990	33.1035	35.6110	M. Sartori	1 n	-
MZL	uncatalogued	*P. oronti*	Israel	Golan, Ein Jalabina	05-05-1990	32.9818	35.6895	M. Sartori	1 n	-
MZL	0821P	*P. olympus*	Borneo	East Kalimantan, Malinau watershed, Temalat stream	21-06-2000	116.5581	2.9914	P. Derleth	1 n	-
MZL	0521P	*P. olympus*	Borneo	East Kalimantan, Malinau watershed, Tamalang stream	19-08-2000	116.5081	2.9833	P. Derleth	1 n	-
MZL	0531C	*P. olympus*	Borneo	East Kalimantan, Malinau watershed, Bengahau stream	08-08-2000	116.5128	2.9894	P. Derleth	1 n	paratype
-	uncatalogued	Unknown	Vietnam	Dak Lak, Yok Don National Park. DakKlau Creek.	15-02-2001	-	-	D.H Hoang and Y.J. Bae	1 n	molecular
Baetisca material examined.										
FAMU	uncatalogued	*Baetisca rogersi*	USA	South Carolina, Ocomee County, Little River	00-05-1972	-	-	P. Carlson	3 n	
FAMU	uncatalogued	*Baetisca rogersi*	USA	Florida, Gadsden County, Crooked Creek	04-02-2004	30.5828	84.8839	A.K. Rasmussen and B.A. Richard	2 n	molecular
FAMU	13Fla728	*Baetisca becki*	USA	Florida, Okaloosa County, Blackwater River	05-05-1979	-	-	W.L and J.G. Peters	1 ♂ imago	

Appendix Table A2.2. Suitability of different mountants tried for slide-mounting *Prosopistoma* specimens.

Mountant	**Solvent**	**Suitability**	**Negative aspects**
Canada Balsam	Creosote	Solvent destroys soft tissues in *Prosopistoma*	Creosote known to be carcinogenic
Canada Balsam	Clove oil	Suitable for mounting *Prosopistoma*	Slides darken with time, hence poor long term storage prospects
Canada Balsam	Xylene	Suitable	Xylene has unpleasant health side effect (neurotoxin)
Euparal	Euparal essence	Suitable	None
Euparal	Cellosolve	Suitable	Prolonged exposure to Cellosolve can cause hematological and neurological abnormalities

Appendix for Chapter 3

Appendix Table A3.1. Morphometric measurements of nymphs of *Prosopistoma* species.

Catalogue	Species name	Total length (mm)	carapace length (mm)	carapace width (mm)	Distance between eyes (mm)	Head width (mm)	head front/back (mm)	Truss measurements (mm)			
								T1	T2	T3	T4
KUN nov 09	Af sp. 2	2.29	1.22	1.48	0.50	0.80	0.28	0.99	0.94	0.90	1.06
KUN nov 09	Af sp. 2	2.54	1.58	1.70	0.64	0.90	0.31	1.17	1.10	1.09	1.18
KUN nov 09	Af sp. 2	1.97	1.10	1.25	0.48	0.69	0.19	0.85	0.73	0.79	0.81
KUN nov 09	Af sp. 2	1.65	1.10	1.24	0.52	0.73	0.29	0.88	0.92	0.68	0.90
KUN 17D	Af sp. 2	2.33	1.49	1.67	0.50	0.79	0.25	1.10	1.07	1.08	1.16
KUN 34A	Af sp. 2	1.98	1.38	1.46	0.52	0.76	0.28	0.90	0.99	0.98	0.96
KUN 34A	Af sp. 2	2.61	1.80	1.99	0.57	0.88	0.29	1.21	1.20	1.09	1.30
KUN 150E	Af sp. 2	2.19	1.16	1.32	0.62	0.89	0.66	0.90	0.87	0.87	0.89
KUN 150E	Af sp. 2	1.56	1.24	1.51	0.39	0.73	0.27	1.00	0.97	0.97	0.99
KUN 150E	Af sp. 2	1.95	1.16	1.35	0.46	0.73	0.23	0.91	0.88	0.87	0.92
KUN 150E	Af sp. 2	1.80	1.35	1.47	0.46	0.73	0.31	1.00	0.91	0.92	1.01
KUN 150E	Af sp. 2	2.03	1.20	1.39	0.42	0.73	0.35	0.91	0.89	0.90	0.90
KUN 150E	Af sp. 2	1.80	1.12	1.31	0.50	0.73	0.27	0.89	0.88	0.87	0.89
KUN 150E	Af sp. 2	2.34	1.08	1.20	0.46	0.73	0.31	0.83	0.82	0.81	0.82
KUN 150E	Af sp. 2	2.11	1.31	1.51	0.46	0.69	0.31	0.84	0.80	0.80	0.85
KUN 150E	Af sp. 2	2.66	1.04	1.16	0.50	0.77	0.35	0.78	0.77	0.77	0.78
KUN 150E	Af sp. 2	1.95	1.12	1.35	0.46	0.73	0.31	0.89	0.88	0.87	0.89
KUN 149C	Af sp. 2	1.64	1.12	1.35	0.46	0.69	0.35	0.89	0.87	0.88	0.88
Sigi R	*P. africanum*	2.31	1.56	1.58	0.66	0.90	0.24	1.06	1.13	1.13	1.05
Sakeji School	Af sp. 8	2.79	1.91	1.96	0.71	1.02	0.28	1.45	1.50	1.50	1.45
Wenela rapids	Af sp. 8	2.93	1.89	2.47	0.77	1.12	0.27	1.56	1.55	1.56	1.56
Wenela rapids	Af sp. 8	2.08	1.24	1.51	0.58	0.77	0.23	1.09	1.09	1.10	1.09
Wenela rapids	Af sp. 8	3.36	2.28	2.63	0.77	0.77	0.35	1.74	1.75	1.75	1.74
Wenela rapids	Af sp. 8	2.24	1.58	1.89	0.62	0.97	0.27	1.23	1.24	1.23	1.24

Catalogue	Species name	Total length (mm)	carapace length (mm)	carapace width (mm)	Distance between eyes (mm)	Head width (mm)	head front/back (mm)	Truss measurements (mm)			
								T1	T2	T3	T4
Wenela rapids	Af sp. 8	3.05	1.97	2.43	0.31	1.08	0.23	1.56	1.57	1.57	1.56
Wenela rapids	Af sp. 8	3.36	2.36	2.70	0.77	1.12	0.39	1.79	1.80	1.78	1.81
CAW 388A	Af sp. 4	4.27	2.72	3.08	0.92	1.27	0.31	2.06	2.00	1.95	2.16
CAW 388A	Af sp. 4	2.08	1.42	1.59	0.49	0.75	0.21	1.07	1.07	1.05	1.03
CAW 388A	Af sp. 4	3.69	2.57	2.92	0.88	1.26	0.24	2.07	1.83	1.80	2.15
CAW 388A	Af sp. 4	3.40	2.12	2.41	0.81	1.15	0.28	1.63	1.54	1.51	1.83
CAW 388A	Af sp. 4	3.25	2.10	2.38	0.75	1.10	0.28	1.56	1.69	1.55	1.65
CAW 388A	Af sp. 4	2.67	1.66	2.02	0.69	0.95	0.29	1.31	1.35	1.23	1.36
CAW 388A	Af sp. 4	3.14	1.98	2.36	0.77	1.13	0.41	1.55	1.57	1.39	1.62
CAW 381H	Af sp. 3	2.30	1.35	1.50	0.55	0.80	0.17	1.04	1.01	1.02	1.01
CAW 381H	Af sp. 3	2.09	1.26	1.47	0.60	0.79	0.13	1.01	0.89	0.96	1.00
CAW 381H	Af sp. 3	1.77	0.95	1.09	0.43	0.63	0.20	0.83	0.75	0.66	0.71
CAW 382P	Af sp. 3	2.36	1.40	1.32	0.60	0.78	0.21	0.99	0.93	0.95	1.00
	Af sp. 6	4.06	2.93	2.90	0.90	1.29	0.37	2.08	2.03	2.10	2.06
	Af sp. 6	2.83	1.97	2.04	0.71	1.01	0.25	1.43	1.35	1.41	1.49
Bandama R	Af sp. 7	2.82	1.74	1.96	0.67	1.02	0.38	1.23	1.27	1.35	1.28
Bandama R	Af sp. 7	2.70	1.78	1.99	0.66	1.00	0.35	1.37	1.32	1.37	1.29
aManzamnyma R	*P. amanzamnyama*	3.21	2.20	2.06	0.80	1.08	0.34	1.55	1.52	1.50	1.46
	P. amanzamnyama	2.85	1.83	1.74	0.77	0.96	0.40	1.23	1.30	1.24	1.25
	P. amanzamnyama	2.81	1.93	1.73	0.76	1.02	0.31	1.37	1.34	1.16	1.07
	P. amanzamnyama	2.42	1.58	1.45	0.66	0.90	0.21	1.10	1.12	1.06	1.09
	P. amanzamnyama	2.77	1.84	1.70	0.73	0.97	0.31	1.21	1.22	1.29	1.24
GEN 355C	*P. amanzamnyama*	2.30	1.65	1.59	0.93	0.71	0.29	1.09	1.18	1.13	1.17
GEN 355C	*P. amanzamnyama*	1.99	1.43	1.35	0.61	0.81	0.23	0.91	1.04	1.03	1.04
GEN 1866A	*P. amanzamnyama*	2.27	1.55	1.53	0.65	0.88	0.28	1.07	1.06	1.06	1.07
GEN 1866A	*P. amanzamnyama*	2.38	1.64	1.57	0.73	0.94	0.28	1.12	1.16	1.14	1.13
GEN 1866A	*P. amanzamnyama*	1.72	1.67	1.59	0.70	0.89	0.26	1.22	1.12	1.13	1.21
GEN 1866A	*P. amanzamnyama*	2.35	1.57	1.48	0.84	0.62	0.20	1.02	1.25	1.25	1.03
GEN 1866A	*P. amanzamnyama*	2.44	1.62	1.58	0.68	0.93	0.35	1.07	1.23	1.16	1.07

Catalogue	Species name	Total length (mm)	carapace length (mm)	carapace width (mm)	Distance between eyes (mm)	Head width (mm)	head front/back (mm)	Truss measurements (mm)			
								T1	T2	T3	T4
GEN 1866A	*P. amanzamnyama*	2.29	1.65	1.52	0.69	0.90	0.25	1.13	1.20	1.04	1.09
GEN 1866A	*P. amanzamnyama*	2.05	1.38	1.26	0.63	0.83	0.24	0.97	0.95	0.83	0.95
GEN 1866A	*P. amanzamnyama*	2.12	1.35	1.26	0.59	0.84	0.19	1.02	0.99	0.99	1.02
McC SA40	*P.mccaffertyi*	2.28	1.66	1.49	0.70	0.83	0.24	1.03	1.15	1.16	1.07
McC SA40	*P.mccaffertyi*	2.32	1.74	1.51	0.67	0.83	0.22	1.12	1.18	1.18	1.12
McC SA40	*P.mccaffertyi*	2.22	1.47	1.28	0.62	0.83	0.29	0.96	0.99	1.00	1.00
McC SA40	*P.mccaffertyi*	2.05	1.44	1.21	0.57	0.74	0.24	0.99	0.97	0.96	0.93
McC SA40	*P.mccaffertyi*	1.60	1.08	0.98	0.53	0.65	0.24	0.72	0.72	0.77	0.70
McC SA40	*P.mccaffertyi*	2.10	1.48	1.28	0.63	0.80	0.27	0.95	0.96	1.01	0.98
McC SA40	*P.mccaffertyi*	1.92	1.34	1.17	0.63	0.75	0.20	0.88	0.94	0.95	0.83
McC SA40	*P.mccaffertyi*	1.98	1.42	1.24	0.59	0.74	0.18	0.98	0.86	0.89	0.96
McC SA40	*P.mccaffertyi*	1.65	1.10	1.01	0.52	0.67	0.23	0.73	0.80	0.74	0.72
McC SA40	*P.mccaffertyi*	1.91	1.39	1.16	0.58	0.75	0.17	0.98	0.92	0.90	0.82
McC SA40	*P.mccaffertyi*	1.45	0.95	0.82	0.47	0.61	0.21	0.58	0.61	0.67	0.66
McC SA40	*P.mccaffertyi*	1.62	1.12	1.01	0.52	0.68	0.17	0.74	0.78	0.81	0.71
VAL 435A	*P. crassi*	2.74	1.77	2.20	0.74	1.12	0.42	1.40	1.44	1.39	1.39
VAL 446A	*P. crassi*	2.97	1.86	2.29	0.75	1.13	0.40	1.38	1.51	1.51	1.48
VAL 463A	*P. crassi*	1.79	1.08	1.24	0.44	0.73	0.29	0.76	0.74	0.94	0.92
VAL 463A	*P. crassi*	2.40	1.56	1.88	0.64	0.93	0.24	1.14	1.12	1.24	1.38
VAL 463A	*P. crassi*	2.76	1.78	2.19	0.73	1.06	0.29	1.40	1.33	1.42	1.51
VAL 466C	*P. crassi*	2.04	1.27	1.40	0.49	0.73	0.29	0.98	0.83	1.08	0.97
VAL 466C	*P. crassi*	2.04	1.25	1.56	0.52	0.83	0.29	1.06	1.07	0.91	0.95
VAL 556B	*P. crassi*	1.85	1.09	1.34	0.50	0.77	0.28	0.85	0.89	0.84	0.88
VAL 556B	*P. crassi*	2.20	1.40	1.71	0.54	0.90	0.29	1.09	1.11	1.09	1.08
VAL 556B	*P. crassi*	2.24	1.47	1.74	0.63	0.91	0.35	1.10	1.11	1.15	1.14
VAL 556B	*P. crassi*	2.55	1.59	1.96	0.64	0.96	0.36	1.32	1.28	1.23	1.20
VAL 556B	*P. crassi*	3.16	1.95	2.32	0.79	1.14	0.49	1.58	1.49	1.51	1.50
VAL 558A	*P. crassi*	1.52	0.97	1.21	0.46	0.74	0.20	0.79	0.73	0.79	0.84
VAL 558A	*P. crassi*	1.63	1.02	1.32	0.48	0.73	0.28	0.80	0.86	0.82	0.83

Catalogue	Species name	Total length (mm)	carapace length (mm)	carapace width (mm)	Distance between eyes (mm)	Head width (mm)	head front/back (mm)	Truss measurements (mm)			
								T1	T2	T3	T4
VAL 558A	*P. crassi*	1.71	1.01	1.32	0.48	0.73	0.30	0.82	0.87	0.83	0.76
VAL 558A	*P. crassi*	2.13	1.33	1.58	0.57	0.84	0.35	1.15	1.04	0.92	0.98
VAL 561E	*P. crassi*	1.38	0.79	1.00	0.42	0.61	0.21	0.67	0.69	0.58	0.61
VAL 561E	*P. crassi*	1.40	0.87	1.08	0.40	0.64	0.25	0.66	0.65	0.74	0.73
VAL 561E	*P. crassi*	1.40	0.87	1.04	0.39	0.57	0.19	0.62	0.65	0.65	0.66
VAL 561E	*P. crassi*	1.42	0.83	1.05	0.42	0.67	0.18	0.69	0.67	0.68	0.73
VAL 561E	*P. crassi*	1.44	0.86	1.12	0.46	0.67	0.22	0.74	0.72	0.70	0.74
VAL 561E	*P. crassi*	1.66	0.95	1.34	0.50	0.76	0.34	0.86	0.91	0.84	0.72
VAL 561E	*P. crassi*	1.69	1.03	1.33	0.48	0.74	0.28	0.84	0.86	0.87	0.81
VAL 561E	*P. crassi*	1.85	1.16	1.53	0.53	0.85	0.31	0.85	0.99	0.99	0.93
VAL 561E	*P. crassi*	1.88	1.17	1.51	0.49	0.82	0.33	0.94	0.99	0.95	0.92
VAL 561E	*P. crassi*	2.10	1.32	1.58	0.58	0.87	0.30	1.04	1.05	1.02	0.98
VAL 561E	*P. crassi*	2.12	1.32	1.66	0.57	0.88	0.29	1.08	1.02	1.11	1.05
VAL 561E	*P. crassi*	2.12	1.34	1.69	0.60	0.90	0.30	1.08	0.99	1.08	1.07
VAL 561E	*P. crassi*	2.14	1.37	1.71	0.59	0.91	0.31	1.06	1.01	1.07	1.11
VAL 561E	*P. crassi*	2.26	1.45	1.72	0.62	0.92	0.29	1.14	1.07	1.18	1.13
VAL 561E	*P. crassi*	2.27	1.41	1.75	0.55	0.90	0.35	1.11	1.09	1.21	1.13
VAL 561E	*P. crassi*	2.32	1.49	1.78	0.64	0.96	0.34	1.21	1.15	1.17	1.15
VAL 561E	*P. crassi*	2.65	1.79	2.14	0.72	1.05	0.29	1.44	1.39	1.40	1.40
VAL 571M	*P. crassi*	1.84	1.12	1.53	0.47	0.80	0.29	0.89	0.92	0.99	0.89
VAL 571M	*P. crassi*	1.85	1.20	1.55	0.53	0.85	0.20	1.02	0.91	0.87	1.00
VAL 571M	*P. crassi*	2.09	1.30	1.67	0.58	0.85	0.30	1.10	1.07	1.01	1.00
VAL 571M	*P. crassi*	1.84	1.12	1.53	0.47	0.80	0.29	0.89	0.92	0.99	0.89
VAL 626D	*P. crassi*	1.21	0.68	0.93	0.40	0.60	0.23	0.58	0.58	0.55	0.55
VAL 686A	*P. crassi*	1.65	0.98	1.10	0.41	0.61	0.29	0.64	0.62	0.81	0.82
VAL 686A	*P. crassi*	1.88	1.22	1.47	0.53	0.81	0.27	0.96	0.88	0.92	1.07
VAL 700B	*P. crassi*	1.95	1.20	1.46	0.55	0.80	0.22	1.01	1.00	0.87	0.97
VAL 733E	*P. crassi*	1.19	0.72	0.94	0.40	0.58	0.20	0.55	0.55	0.60	0.64
VAL 733E	*P. crassi*	1.68	1.01	1.30	0.49	0.74	0.28	0.78	0.79	0.86	0.80

Catalogue	Species name	Total length (mm)	carapace length (mm)	carapace width (mm)	Distance between eyes (mm)	Head width (mm)	head front/back (mm)	Truss measurements (mm)			
								T1	T2	T3	T4
VAL 733E	*P. crassi*	1.69	1.08	1.33	0.52	0.74	0.28	0.81	0.78	0.89	0.85
VAL 733E	*P. crassi*	1.98	1.27	1.58	0.58	0.87	0.26	0.94	0.96	0.99	1.07
VAL 733E	*P. crassi*	2.18	1.41	1.66	0.61	0.85	0.22	1.02	1.01	1.19	1.16
VAL 838B	*P. crassi*	1.97	1.10	1.37	0.46	0.78	0.31	0.93	0.92	0.80	0.83
VAL 838B	*P. crassi*	1.99	1.22	1.57	0.52	0.83	0.33	1.02	0.97	1.01	0.99
VAL 838B	*P. crassi*	2.19	1.34	1.77	0.61	0.95	0.38	1.13	1.19	1.10	1.03
VAL 841A	*P. crassi*	2.90	1.79	2.04	0.73	1.08	0.36	1.28	1.27	1.35	1.52
VAL 842C	*P. crassi*	1.89	1.21	1.50	0.53	0.82	0.29	0.96	0.94	0.99	1.00
VAL 842C	*P. crassi*	2.01	1.29	1.68	0.57	0.84	0.27	0.99	0.97	1.07	1.13
VAL 842C	*P. crassi*	2.66	1.72	2.08	0.68	1.07	0.39	1.43	1.36	1.30	1.33
VAL 842C	*P. crassi*	3.32	2.21	2.65	0.80	1.20	0.44	1.73	1.70	1.76	1.69
VAL 843A	*P. crassi*	1.32	0.75	1.09	0.37	0.65	0.26	0.60	0.70	0.69	0.69
VAL 843A	*P. crassi*	1.67	1.04	1.36	0.49	0.78	0.32	0.83	0.84	0.93	0.84
VAL 843A	*P. crassi*	1.97	1.24	1.52	0.52	0.83	0.29	1.01	0.99	0.93	0.99
VAL 890B	*P. crassi*	1.36	0.84	1.03	0.43	0.62	0.21	0.71	0.71	0.62	0.65
VAL 890B	*P. crassi*	1.82	1.15	1.41	0.56	0.82	0.30	0.92	0.86	0.91	0.92
VAL 890B	*P. crassi*	1.94	1.23	1.49	0.54	0.75	0.23	0.98	0.86	0.99	1.07
VAL 890B	*P. crassi*	2.17	1.40	1.73	0.61	0.88	0.35	1.10	1.05	1.13	1.18
VAL 890B	*P. crassi*	2.19	1.39	1.61	0.55	0.83	0.27	1.14	1.11	0.94	1.01
VAL 890B	*P. crassi*	2.20	1.38	1.76	0.62	0.93	0.33	1.15	1.13	1.12	1.09
VAL 890B	*P. crassi*	2.24	1.44	1.75	0.62	0.91	0.24	1.11	1.01	1.17	1.23
VAL 890B	*P. crassi*	2.71	1.74	2.02	0.70	1.03	0.38	1.31	1.19	1.46	1.44
VAL 899F	*P. crassi*	4.48	2.87	3.30	0.95	1.35	0.46	2.28	2.21	2.19	2.20
VAL 899F	*P. crassi*	4.52	2.73	3.16	0.86	1.31	0.54	2.12	1.99	2.06	2.04
VAL 899F	*P. crassi*	4.65	2.78	3.15	0.92	1.36	0.51	2.12	2.12	2.07	2.02
VAL 906A	*P. crassi*	3.13	1.96	2.52	0.73	1.16	0.50	1.57	1.62	1.56	1.51
VAL 943B	*P. crassi*	2.66	1.67	2.14	0.67	1.07	0.38	1.36	1.31	1.33	1.36
VAL 944A	*P. crassi*	2.25	1.35	1.72	0.56	0.87	0.32	1.13	1.13	1.05	1.07
VAL 946B	*P. crassi*	2.04	1.26	1.54	0.55	0.83	0.34	0.96	0.94	1.07	1.04

Catalogue	Species name	Total length (mm)	carapace length (mm)	carapace width (mm)	Distance between eyes (mm)	Head width (mm)	head front/back (mm)	Truss measurements (mm)			
								T1	T2	T3	T4
VAL 946B	*P. crassi*	2.04	1.29	1.59	0.57	0.87	0.30	1.04	1.04	1.00	1.06
VAL 946B	*P. crassi*	2.76	1.86	2.20	0.72	1.05	0.34	1.34	1.38	1.49	1.57
VAL 959A	*P. crassi*	2.75	1.80	2.15	0.71	1.08	0.31	1.37	1.36	1.41	1.37
VAL 959A	*P. crassi*	3.90	2.46	2.91	0.86	1.33	0.53	1.90	1.94	1.78	1.97
VAL 961A	*P. crassi*	1.99	1.29	1.60	0.56	0.84	0.31	1.01	1.04	1.00	1.05
VAL 1053B	*P. crassi*	0.81	0.50	0.63	0.31	0.42	0.15	0.41	0.41	0.37	0.39
VAL 1053B	*P. crassi*	1.22	0.75	0.98	0.39	0.58	0.21	0.63	0.62	0.61	0.59
VAL 1053B	*P. crassi*	1.52	0.95	1.22	0.46	0.66	0.24	0.77	0.77	0.79	0.79
VAL 1053B	*P. crassi*	1.62	0.98	1.26	0.46	0.73	0.30	0.83	0.81	0.75	0.81
VAL 1156C	*P. crassi*	2.05	1.31	1.62	0.56	0.82	0.31	1.03	1.01	1.02	1.06
VAL 1221A	*P. crassi*	1.81	1.09	1.43	0.52	0.82	0.33	0.86	0.90	0.89	0.93
VAL 1226A	*P. crassi*	0.89	0.53	0.67	0.31	0.46	0.14	0.41	0.39	0.46	0.45
VAL 1226A	*P. crassi*	1.06	0.63	0.81	0.34	0.52	0.14	0.53	0.52	0.49	0.51
VAL 1230C	*P. crassi*	2.00	1.24	1.58	0.52	0.80	0.28	0.97	1.01	0.98	1.04
VAL 1281A	*P. crassi*	1.38	0.80	0.95	0.40	0.59	0.20	0.57	0.56	0.67	0.68
VAL 1329B	*P. crassi*	1.50	0.91	1.20	0.48	0.66	0.27	0.71	0.76	0.76	0.71
VAL 1329B	*P. crassi*	1.74	1.07	1.40	0.53	0.76	0.30	0.85	0.89	0.88	0.87
VAL 1329B	*P. crassi*	1.81	1.14	1.44	0.59	0.85	0.30	0.96	0.95	0.89	0.87
VAL 1329B	*P. crassi*	1.83	1.16	1.45	0.54	0.82	0.30	0.94	0.95	0.92	0.92
VAL 1329B	*P. crassi*	2.18	1.36	1.68	0.57	0.88	0.33	1.15	1.15	0.99	1.07
UMG 1075G	*P. crassi*	3.14	2.17	2.59	0.83	1.20	0.34	1.64	1.73	1.65	1.72
UMG 1075G	*P. crassi*	2.44	1.43	1.81	0.57	0.94	0.30	1.17	1.11	1.16	1.12
UMG 1075G	*P. crassi*	2.46	1.50	1.94	0.60	0.98	0.33	1.24	1.26	1.20	1.18
UMG 1075G	*P. crassi*	2.37	1.47	1.91	0.61	0.94	0.29	1.27	1.24	1.16	1.18
UMG 1075G	*P. crassi*	2.34	1.47	1.76	0.63	0.89	0.22	1.20	1.17	1.10	1.10
UMG 1075G	*P. crassi*	1.91	1.13	1.47	0.52	0.81	0.18	0.96	0.91	0.89	0.96
UMG 1075G	*P. crassi*	2.03	1.17	1.47	0.51	0.80	0.24	0.88	0.88	1.03	1.00
UMG 1075G	*P. crassi*	2.21	1.25	1.65	0.55	0.85	0.30	1.06	1.02	0.99	1.08
UMG 1075G	*P. crassi*	2.22	1.25	1.66	0.55	0.87	0.29	1.08	1.02	0.98	1.04

Catalogue	Species name	Total length (mm)	carapace length (mm)	carapace width (mm)	Distance between eyes (mm)	Head width (mm)	head front/back (mm)	Truss measurements (mm)			
								T1	T2	T3	T4
TUG 81j31	*P. crassi*	2.25	1.44	1.73	0.62	0.89	0.31	1.14	1.08	1.10	1.17
TUG 93j27	*P. crassi*	1.67	0.98	1.18	0.48	0.70	0.27	0.84	0.80	0.69	0.76
TUG 93j27	*P. crassi*	1.71	1.05	1.31	0.53	0.74	0.18	0.90	0.82	0.76	0.92
TUG 93j27	*P. crassi*	1.76	1.13	1.30	0.51	0.72	0.28	0.92	0.88	0.75	0.85
TUG 93j27	*P. crassi*	3.26	2.07	2.57	0.77	1.18	0.35	1.60	1.55	1.64	1.78
TUG 93j27	*P. crassi*	2.15	1.38	1.67	0.60	0.88	0.26	1.11	1.03	1.08	1.15
TUG 93j27	*P. crassi*	2.72	1.80	2.16	0.73	1.03	0.29	1.45	1.32	1.27	1.51
TUG 93j27	*P. crassi*	2.56	1.66	2.10	0.69	1.01	0.31	1.39	1.27	1.33	1.38
TUG 93j27	*P. crassi*	3.08	2.02	2.21	0.76	1.10	0.36	1.53	1.52	1.43	1.48
TUG 93j27	*P. crassi*	3.12	2.10	2.60	0.81	1.17	0.35	1.74	1.64	1.62	1.68
TUG 105U	*P. crassi*	3.27	2.08	2.19	0.71	1.13	0.37	1.46	1.38	1.64	1.58
TUG123Z	*P. crassi*	1.20	0.66	0.77	0.34	0.50	0.21	0.50	0.52	0.51	0.50
TUG123Z	*P. crassi*	2.49	1.57	1.80	0.69	0.98	0.28	1.23	1.04	1.14	1.34
TUG 146E	*P. crassi*	3.29	1.90	2.34	0.75	1.14	0.39	1.45	1.52	1.50	1.53
ECR 699A	*P. crassi*	2.20	1.45	1.88	0.63	0.94	0.20	1.25	1.15	1.12	1.24
ECR 699A	*P. crassi*	2.24	1.47	1.90	0.63	0.93	0.17	1.25	1.13	1.17	1.25
ECR 699A	*P. crassi*	2.80	1.76	2.15	0.70	1.05	0.34	1.37	1.38	1.41	1.37
ECR 699A	*P. crassi*	2.90	1.84	2.26	0.72	1.10	0.34	1.50	1.43	1.47	1.43
ECR 699A	*P. crassi*	2.51	1.62	2.06	0.72	1.04	0.32	1.39	1.30	1.23	1.29
ECR 699A	*P. crassi*	2.02	1.28	1.60	0.57	0.85	0.33	1.06	1.03	1.00	1.01
ECR 699A	*P. crassi*	1.90	1.20	1.52	0.54	0.84	0.38	0.95	0.96	0.95	0.92
ECR 699A	*P. crassi*	1.69	1.06	1.29	0.49	0.73	0.26	0.91	0.90	0.77	0.78
ECR 699A	*P. crassi*	2.01	1.27	1.62	0.58	0.87	0.33	1.04	1.02	1.03	1.02
ECR 699A	*P. crassi*	1.50	0.92	1.19	0.45	0.68	0.30	0.67	0.71	0.81	0.78
ECR 700A	*P. crassi*	2.18	1.36	1.63	0.61	0.87	0.35	1.14	1.10	1.02	0.93
ECR 711A	*P. crassi*	2.25	1.48	1.85	0.63	0.96	0.39	1.14	1.13	1.24	1.16
Inxu R	*P. crassi*	2.69	1.76	2.15	0.71	1.06	0.36	1.40	1.34	1.41	1.39
Inxu R	*P. crassi*	2.36	1.91	2.15	0.64	0.95	0.26	1.24	1.18	1.14	1.27
Inxu R	*P. crassi*	2.96	1.87	2.27	0.74	1.09	0.34	1.46	1.45	1.41	1.51

Catalogue	Species name	Total length (mm)	carapace length (mm)	carapace width (mm)	Distance between eyes (mm)	Head width (mm)	head front/back (mm)	Truss measurements (mm)			
								T1	T2	T3	T4
Inxu R	*P. crassi*	2.53	1.59	2.00	0.68	0.99	0.29	1.38	1.26	1.21	1.29
Inxu R	*P. crassi*	2.05	1.28	1.62	0.57	0.82	0.35	1.00	1.02	1.02	1.05
Inxu R	*P. crassi*	2.00	1.19	1.48	0.53	0.81	0.35	0.95	0.99	0.91	0.98
Inxu R	*P. crassi*	2.15	1.29	1.63	0.58	0.84	0.30	1.04	1.06	1.01	1.05
Inxu R	*P. crassi*	1.62	0.94	1.20	0.46	0.68	0.29	0.78	0.82	0.82	0.78
GEN 1863C	*P. crassi*	2.91	1.84	2.26	0.67	1.10	0.38	1.44	1.39	1.46	1.46
GEN 1863C	*P. crassi*	2.22	1.45	1.81	0.59	0.92	0.35	1.16	1.10	1.16	1.19
GEN 1863C	*P. crassi*	2.23	1.45	1.81	0.59	0.92	0.35	1.16	1.10	1.16	1.19
GEN 1863C	*P. crassi*	2.08	1.29	1.62	0.53	0.88	0.32	1.05	1.06	1.01	1.03
GEN 1863C	*P. crassi*	2.48	1.54	1.89	0.61	0.94	0.33	1.25	1.17	1.19	1.19
GEN 1863C	*P. crassi*	2.00	1.28	1.62	0.51	0.84	0.29	1.03	1.00	1.05	1.01
GEN 1863C	*P. crassi*	2.05	1.30	1.64	0.52	0.86	0.34	1.06	1.04	1.03	1.08
GEN 1863C	*P. crassi*	1.71	1.08	1.37	0.45	0.79	0.30	0.88	0.90	0.85	0.86
GEN 1863C	*P. crassi*	1.85	1.17	1.55	0.50	0.80	0.32	1.01	0.92	0.96	0.98
GEN 1863C	*P. crassi*	2.30	1.44	1.83	0.57	0.96	0.38	1.12	1.12	1.15	1.18
GEN 1863C	*P. crassi*	1.70	1.38	1.69	0.56	0.91	0.30	1.12	1.04	1.08	1.10
GEN 1863C	*P. crassi*	1.80	1.15	1.47	0.51	0.85	0.32	0.94	0.94	0.94	0.90
GEN 1863C	*P. crassi*	2.76	1.72	2.17	0.61	1.18	0.45	1.31	1.34	1.47	1.37
GEN 1863C	*P. crassi*	2.00	1.26	1.60	0.53	0.85	0.32	1.07	1.03	0.98	1.03
GEN 1863C	*P. crassi*	1.87	1.18	1.49	0.52	0.82	0.32	0.95	0.92	0.94	0.97
GEN 1863C	*P. crassi*	1.52	0.96	1.25	0.45	0.74	0.25	0.80	0.79	0.76	0.78
GEN 1863C	*P. crassi*	1.90	1.20	1.57	0.50	0.83	0.29	1.02	1.00	0.97	0.96
GEN 1863C	*P. crassi*	1.86	1.19	1.55	0.51	0.85	0.25	1.01	0.84	0.97	1.05
GEN 1863C	*P. crassi*	1.73	1.10	1.38	0.49	0.79	0.24	0.87	0.80	0.91	0.92
GEN 1863C	*P. crassi*	1.97	1.22	1.49	0.50	0.81	0.30	0.95	0.90	1.03	0.93
GEN 1863C	*P. crassi*	1.69	1.02	1.31	0.43	0.76	0.30	0.85	0.80	0.82	0.81
GEN 1863C	*P. crassi*	1.62	0.92	1.22	0.42	0.68	0.22	0.78	0.70	0.70	0.84
GEN 1863C	*P. crassi*	1.79	1.07	1.39	0.46	0.76	0.29	0.89	0.84	0.84	0.84
GEN 1863C	*P. crassi*	1.75	1.12	1.40	0.49	0.78	0.30	0.89	0.87	0.87	0.91

Catalogue	Species name	Total length (mm)	carapace length (mm)	carapace width (mm)	Distance between eyes (mm)	Head width (mm)	head front/back (mm)	Truss measurements (mm)			
								T1	T2	T3	T4
GEN 1863C	*P. crassi*	1.36	0.86	1.11	0.44	0.64	0.22	0.73	0.65	0.64	0.70
GEN 1863C	*P. crassi*	1.34	0.84	1.07	0.43	0.61	0.17	0.67	0.68	0.64	0.69
GEN 1863C	*P. crassi*	1.70	1.03	1.34	0.41	0.75	0.26	0.84	0.85	0.84	0.82
GEN 1863C	*P. crassi*	1.79	1.11	1.43	0.48	0.80	0.30	0.91	0.90	0.88	0.87
GEN 1863C	*P. crassi*	1.60	0.90	1.18	0.40	0.70	0.26	0.74	0.75	0.75	0.75
P0198	Mad sp 2	3.92	2.53	2.73	0.82	1.28	0.44	1.93	1.80	1.86	1.85
P0526	Mad sp 2	2.80	1.85	2.20	0.71	1.07	0.40	1.47	1.40	1.37	1.47
P0526	Mad sp 2	2.33	1.36	1.66	0.66	1.02	0.41	1.05	1.17	1.10	1.18
P2148	Mad sp 2	2.75	1.55	1.80	0.58	0.96	0.38	1.26	1.13	1.16	1.21
P2166	Mad sp 2	1.94	1.24	1.41	0.53	0.79	0.27	0.96	0.92	0.90	0.93
P2172	Mad sp 2	2.68	1.57	1.84	0.58	0.91	0.34	1.26	1.08	1.15	1.23
P2172	Mad sp 2	1.96	1.18	1.39	0.44	0.82	0.29	0.93	0.89	0.90	0.91
P0494	Mad sp 2	2.33	1.46	1.65	0.62	0.87	0.25	1.17	1.07	1.03	1.14
P0494	Mad sp 2	2.52	1.52	1.63	0.58	0.80	0.23	1.05	0.98	1.21	1.19
P0494	Mad sp 2	2.19	1.28	1.39	0.46	0.74	0.17	1.10	1.05	1.04	1.10
P0494	Mad sp 2	2.64	1.67	1.77	0.67	0.92	0.34	1.21	1.15	1.30	1.24
P0165	*P. variegatum*	5.06	2.83	3.19	1.45	2.28	0.85	2.06	2.08	2.20	2.14
P0165	*P. variegatum*	5.20	2.97	3.31	1.46	2.37	0.83	2.15	2.14	2.45	2.10
P0165	*P. variegatum*	5.48	3.08	3.43	1.54	2.46	0.95	2.29	2.29	2.48	2.18
P0165	*P. variegatum*	6.42	3.37	3.85	1.87	3.01	1.22	2.48	2.47	2.71	2.60
P0165	*P. variegatum*	6.56	3.38	3.80	1.85	3.08	1.14	2.54	2.58	2.69	2.40
P0165	*P. variegatum*	7.47	4.66	5.18	2.32	3.81	1.46	3.51	3.55	3.78	3.21
P0167	*P. variegatum*	4.52	2.30	2.65	1.30	2.11	0.80	1.79	1.95	1.79	1.43
P0167	*P. variegatum*	5.35	3.40	3.90	1.61	2.83	0.98	2.49	2.60	2.57	2.56
P0167	*P. variegatum*	5.43	3.21	3.55	1.65	2.58	1.17	2.29	2.53	2.53	2.31
P0526	*P. variegatum*	6.24	3.93	4.11	1.95	3.07	0.99	2.79	2.86	2.96	2.67
P0543	*P. variegatum*	3.00	1.80	2.10	0.99	1.56	0.50	1.46	1.45	1.26	1.39
P0543	*P. variegatum*	6.88	4.22	4.69	2.03	3.43	1.26	3.32	3.14	2.92	3.08
P0600	*P. variegatum*	5.60	3.57	3.95	1.88	2.99	0.80	2.55	2.60	2.64	2.73

Catalogue	Species name	Total length (mm)	carapace length (mm)	carapace width (mm)	Distance between eyes (mm)	Head width (mm)	head front/back (mm)	Truss measurements (mm)			
								T1	T2	T3	T4
P0600	*P. variegatum*	5.78	3.65	4.04	2.01	3.07	0.88	2.81	2.75	2.65	2.62
P0623	*P. variegatum*	8.90	5.22	5.19	2.75	4.18	1.27	3.84	3.80	3.49	3.37
P0721	*P. variegatum*	6.42	3.49	4.06	2.01	3.21	1.25	2.70	2.69	2.82	2.54
P0762	*P. variegatum*	8.26	4.54	4.89	2.38	3.78	1.64	2.97	3.16	3.67	3.63
P0763	*P. variegatum*	6.10	3.62	4.14	1.95	3.29	1.07	2.73	2.68	2.77	2.86
P0763	*P. variegatum*	6.25	3.41	3.84	1.58	2.68	1.05	2.58	2.46	2.57	2.61
P2105	*P. variegatum*	1.99	1.14	1.41	0.67	1.09	0.35	0.92	0.90	0.83	0.94
P2115	*P. variegatum*	3.46	2.00	2.19	1.04	1.54	0.62	1.51	1.49	1.47	1.53
P2118	*P. variegatum*	1.50	0.91	1.03	0.51	0.81	0.23	0.70	0.73	0.69	0.65
P2127	*P. variegatum*	4.69	2.48	2.94	1.46	2.29	0.85	1.93	1.83	1.91	2.03
P2136	*P. variegatum*	1.24	0.75	0.90	0.45	0.69	0.15	0.62	0.56	0.52	0.59
P2136	*P. variegatum*	1.52	0.93	1.12	0.56	0.83	0.22	0.78	0.71	0.66	0.70
P2136	*P. variegatum*	2.34	1.39	1.58	0.78	1.17	0.35	1.18	1.14	1.19	1.07
P2136	*P. variegatum*	2.43	1.46	1.67	0.86	1.28	0.36	1.12	1.14	1.13	1.12
P2136	*P. variegatum*	2.53	1.56	1.80	0.84	1.29	0.43	1.18	1.20	1.17	1.12
P2139	*P. variegatum*	3.23	2.07	2.25	1.05	1.62	0.48	1.64	1.61	1.50	1.40
P2139	*P. variegatum*	3.40	1.93	2.28	1.08	1.64	0.63	1.52	1.55	1.43	1.51
P2147	*P. variegatum*	2.50	1.64	1.86	0.91	1.30	0.30	1.29	1.22	1.14	1.30
P2147	*P. variegatum*	2.66	1.66	1.94	0.95	1.41	0.45	1.27	1.23	1.27	1.29
P2147	*P. variegatum*	2.81	1.71	1.87	0.93	1.37	0.52	1.19	1.28	1.32	1.29
P2147	*P. variegatum*	2.82	1.68	1.97	0.98	1.44	0.64	1.28	1.27	1.32	1.28
P2147	*P. variegatum*	4.13	2.72	2.84	1.34	2.25	0.79	1.95	1.91	1.97	1.93
P2149	*P. variegatum*	1.37	0.76	0.91	0.45	0.66	0.22	0.63	0.63	0.53	0.55
P2149	*P. variegatum*	2.70	1.80	1.95	0.98	1.48	0.42	1.28	1.28	1.31	1.30
P2149	*P. variegatum*	2.92	1.89	2.09	1.03	1.55	0.30	1.44	1.33	1.38	1.43
P2150	*P. variegatum*	3.17	1.97	2.10	1.07	1.56	0.55	1.37	1.43	1.47	1.47
P2150	*P. variegatum*	3.47	2.02	2.16	1.02	1.62	0.62	1.47	1.52	1.40	1.47
P2150	*P. variegatum*	3.73	2.52	2.62	1.18	1.76	0.51	1.66	1.87	1.80	1.82
P2150	*P. variegatum*	4.52	3.04	3.00	1.31	1.94	0.59	2.07	2.14	2.05	2.26

Catalogue	Species name	Total length (mm)	carapace length (mm)	carapace width (mm)	Distance between eyes (mm)	Head width (mm)	head front/back (mm)	Truss measurements (mm)			
								T1	T2	T3	T4
P2150	*P. variegatum*	4.77	2.93	2.82	1.33	1.93	0.83	1.83	1.85	2.06	2.17
P2154	*P. variegatum*	1.65	0.87	1.08	0.54	0.86	0.35	0.68	0.70	0.72	0.69
P2154	*P. variegatum*	1.79	1.08	1.28	0.65	1.00	0.43	0.80	0.84	0.78	0.94
P2154	*P. variegatum*	2.08	1.14	1.38	0.66	0.99	0.33	0.92	0.91	0.85	0.95
P2154	*P. variegatum*	2.08	1.04	1.24	0.64	0.94	0.45	0.79	0.82	0.83	0.78
P2157	*P. variegatum*	2.59	1.45	1.83	0.86	1.33	0.41	1.20	1.04	1.19	1.21
P2157	*P. variegatum*	3.06	1.57	2.04	0.92	1.52	0.65	1.28	1.33	1.30	1.24
P2157	*P. variegatum*	3.30	1.88	2.51	1.11	1.76	0.58	1.53	1.57	1.56	1.60
P2157	*P. variegatum*	5.20	3.02	3.14	1.45	2.40	0.98	2.18	2.13	2.14	2.18
P2157	*P. variegatum*	5.24	3.16	3.58	1.38	2.63	0.96	2.40	2.27	2.43	2.39
P2160	*P. variegatum*	1.55	0.76	0.97	0.46	0.70	0.32	0.59	0.60	0.63	0.63
P2160	*P. variegatum*	2.90	1.63	1.88	0.92	1.46	0.63	1.20	1.23	1.28	1.30
P2160	*P. variegatum*	3.05	1.81	1.93	1.00	1.48	0.51	1.17	1.23	1.36	1.48
P2160	*P. variegatum*	4.79	2.65	2.67	1.57	2.40	0.89	1.68	1.92	2.02	1.90
P2160	*P. variegatum*	4.82	2.59	3.06	1.54	2.32	0.94	2.05	1.87	1.95	2.08
P2160	*P. variegatum*	7.55	4.46	4.93	2.38	3.74	1.38	3.36	3.45	3.39	3.18
P2161	*P. variegatum*	2.06	1.12	1.47	0.79	1.18	0.42	0.91	0.91	1.03	0.94
P2162	*P. variegatum*	2.48	1.27	1.73	0.73	1.30	0.54	1.04	1.09	1.05	1.18
P2162	*P. variegatum*	5.33	2.90	3.37	1.50	2.54	1.12	2.19	2.25	2.22	2.27
P2162	*P. variegatum*	6.31	3.69	3.92	1.94	3.05	1.04	2.79	2.75	2.75	2.71
P2164	*P. variegatum*	1.74	0.96	1.17	0.55	0.88	0.23	0.83	0.83	0.73	0.75
P2164	*P. variegatum*	2.09	1.09	1.35	0.70	1.02	0.47	0.85	0.88	0.88	0.87
P2164	*P. variegatum*	3.99	2.26	2.73	1.25	1.98	0.85	1.75	1.74	1.84	1.78
P2164	*P. variegatum*	4.74	2.99	3.35	1.60	2.62	0.93	2.33	2.11	2.23	2.26
P2164	*P. variegatum*	5.06	3.00	3.46	1.71	2.60	1.01	2.08	2.28	2.34	2.28
P2165	*P. variegatum*	1.84	1.04	1.24	0.63	0.92	0.21	0.87	0.86	0.86	0.89
P2165	*P. variegatum*	1.87	1.07	1.33	0.65	0.98	0.38	0.83	0.82	0.90	0.89
P2165	*P. variegatum*	4.37	2.31	2.73	1.37	2.12	0.62	1.76	1.76	1.75	1.80
P2165	*P. variegatum*	4.94	2.64	3.01	1.47	2.28	0.81	2.17	2.00	1.75	1.90

Catalogue	Species name	Total length (mm)	carapace length (mm)	carapace width (mm)	Distance between eyes (mm)	Head width (mm)	head front/back (mm)	Truss measurements (mm)			
								T1	T2	T3	T4
P0057	Mad sp 1	4.26	2.79	2.87	0.91	1.37	0.50	2.04	1.84	1.97	2.02
P0510	Mad sp 1	3.00	1.88	2.14	0.76	1.08	0.43	1.40	1.40	1.44	1.39
P0519	Mad sp 1	3.37	2.14	2.20	0.79	1.17	0.43	1.58	1.54	1.42	1.50
Comores	Comores sp	2.99	2.05	2.17	0.67	1.01	0.23	1.54	1.37	1.55	1.56
France	*P. pennigerum*	4.95	3.29	3.51	1.37	2.12	0.71	2.34	2.39	2.28	2.52
Spain	*P. pennigerum*	3.50	2.17	2.53	1.05	1.55	0.72	1.50	1.81	1.75	1.64
Spain mundo	*P. pennigerum*	3.39	2.09	2.44	0.99	1.57	0.64	1.45	1.71	1.77	1.52
Spain Cabriel	*P. pennigerum*	2.97	1.64	1.90	0.77	1.18	0.39	1.22	1.24	1.21	1.24
Spain	*P. pennigerum*	2.28	1.26	1.50	0.63	0.95	0.38	0.93	1.01	0.97	0.96
Spain	*P. pennigerum*	1.98	1.17	1.43	0.61	0.91	0.28	0.86	0.83	0.93	1.00
Spain	*P. pennigerum*	1.99	1.06	1.29	0.59	0.83	0.22	0.80	0.76	0.84	0.92
Spain	*P. pennigerum*	2.22	1.33	1.53	0.68	0.96	0.36	1.07	1.12	0.92	1.05
Spain	*P. pennigerum*	1.52	0.91	1.08	0.48	0.74	0.26	0.70	0.73	0.67	0.70
Spain	*P. pennigerum*	1.43	0.72	0.92	0.40	0.60	0.20	0.60	0.60	0.54	0.61
Spain	*P. pennigerum*	1.09	0.51	0.71	0.34	0.49	0.12	0.46	0.43	0.44	0.46
Spain	*P. pennigerum*	1.14	0.66	0.70	0.31	0.49	0.19	0.53	0.57	0.58	0.57
Volga	*P. pennigerum*	5.62	3.19	3.42	1.34	1.90	0.76	2.20	2.48	2.40	2.35
Volga	*P. pennigerum*	3.74	2.14	2.41	0.99	1.49	0.58	1.74	1.54	1.51	1.61
Australia	*P. pearsonorum*	4.06	2.31	2.84	1.07	1.57	0.35	1.93	1.62	1.80	1.91
Australia	*P. pearsonorum*	3.98	2.45	2.79	0.92	1.56	0.55	1.71	1.86	1.86	1.82
Australia	*P. pearsonorum*	2.70	1.59	1.90	0.81	1.26	0.50	1.28	1.25	1.23	1.27
Australia	*P. pearsonorum*	2.58	1.52	1.79	0.76	1.16	0.49	1.11	1.10	1.23	1.22
Australia	*P. pearsonorum*	3.85	2.24	2.66	0.99	1.49	0.39	1.76	1.66	1.67	1.80
Australia	*P. pearsonorum*	3.86	2.53	2.80	1.05	1.60	0.47	1.89	1.88	1.82	1.92
Australia	*P. pearsonorum*	3.88	2.52	2.89	1.10	1.65	0.60	1.86	1.96	1.85	1.91
Australia	*P. pearsonorum*	3.22	1.81	2.17	0.84	1.33	0.47	1.43	1.47	1.43	1.48
Australia	*P. pearsonorum*	3.85	2.24	2.66	0.99	1.49	0.39	1.76	1.66	1.67	1.80
Australia	*P. pearsonorum*	3.86	2.53	2.80	1.05	1.60	0.47	1.89	1.88	1.82	1.92
Australia	*P. pearsonorum*	3.00	1.89	2.18	0.76	1.27	0.36	1.40	1.43	1.33	1.47

Catalogue	Species name	Total length (mm)	carapace length (mm)	carapace width (mm)	Distance between eyes (mm)	Head width (mm)	head front/back (mm)	Truss measurements (mm)			
								T1	T2	T3	T4
Australia	*P. pearsonorum*	3.01	1.94	2.09	0.86	1.25	0.46	1.44	1.51	1.52	1.45
Australia	*P. pearsonorum*	3.33	1.91	2.08	0.85	1.23	0.45	1.50	1.41	1.28	1.50
Philippines	*P. boreus*	3.83	2.28	2.32	1.01	1.55	0.65	1.21	1.89	1.89	1.39
Philippines	*P. boreus*	2.95	1.81	1.99	0.92	1.34	0.54	1.10	1.53	1.48	1.19
Philippines	*P. boreus*	2.40	1.40	1.63	0.73	1.13	0.45	0.93	1.23	1.21	0.95
israel	*P. oronti*	4.28	2.75	3.02	1.32	2.03	0.82	1.92	2.09	2.07	1.96
israel	*P. oronti*	4.45	2.72	2.89	1.26	1.92	0.70	1.92	2.06	1.97	1.90
Sri Lanka	*P. lieftincki*	3.08	1.94	2.08	0.92	1.48	0.45	1.22	1.53	1.45	1.39
Philippines	*P. palawana*	2.18	1.36	1.53	0.51	0.82	0.29	0.96	1.12	1.12	0.92
Philippines	*P. palawana*	2.36	1.55	1.70	0.54	0.99	0.33	0.99	1.31	1.25	0.95
New Guinea	*P. sedlaceki*	3.54	1.91	2.14	0.86	1.37	0.41	1.63	1.61	1.64	1.53
Turkey	*P. orhanelicum*	2.94	1.89	2.09	0.88	1.26	0.46	1.44	1.24	1.31	1.53
Turkey	*P. orhanelicum*	2.30	1.39	1.61	0.71	1.01	0.46	1.01	1.21	1.06	0.98
Turkey	*P. orhanelicum*	2.20	1.40	1.60	0.68	1.02	0.24	1.08	0.99	1.03	1.17
Turkey	*P. orhanelicum*	1.89	1.16	1.31	0.59	0.87	0.36	0.86	0.84	0.86	0.79
Turkey	*P. orhanelicum*	2.15	1.38	1.41	0.67	0.97	0.24	1.11	1.02	1.03	1.14
Vietnam	*P. annamense*	2.71	1.78	2.10	0.66	1.03	0.38	1.28	1.45	1.41	1.36
Vietnam	*P. annamense*	2.12	1.37	1.60	0.55	0.84	0.34	1.04	1.21	1.19	1.04
Vietnam	*P. annamense*	1.58	1.05	1.24	0.48	0.72	0.23	0.84	0.78	0.79	0.85
China	*P. trispinum*	3.60	2.17	2.53	1.11	1.57	0.58	1.71	1.74	1.78	1.72
China	*P. unicolor*	3.74	2.43	2.3	0.79	1.18	0.39	1.59	1.68	1.74	1.64

Appendix for Chapter 4

Neural Networks for Classification of Prosopistomatidae nymphs.

Appendix Table A4.1. Input features and their values for the neural network

FN	Feature and codes	Range
1	Carapace with narrow (0) / wide (1) flange	0, 1
2	Distal end of carapace protruding (0) / notched (1)	0, 1
3	Ratio of length of carapace (along median suture):width (0) < 1; (1) ≥ 1	0, 1
4	Ratio of head width : carapace width (0) ≤ 0.5; (1) > 0.5	0, 1
5	Ratio of distance between eyes : maximum head width (0) ≤ 0.6; (1) ≥ 0.61	0, 1
6	Number of antennal segments including scape and pedicel (0) ≥ 7; (1) 6; (2) 5	0, 1, 2
7	Antenna (0) longer than or (1) equal to or (2) shorter than or (3) much shorter than distance from antennal base to anterior margin of head	0, 1, 2, 3
8	Antenna with segment III longer than (0) or subequal to (1) or shorter than (2) or much shorter (3) than remaining segments	0, 1, 2
9	Number of pectinate spines on fore tibia (0) >10; (1) 8-10; (2) 5-7; (3) < 5	0, 1, 2, 3
10	Length of inner canine of mandible compared to outer canine (0) shorter; (1) subequal	0, 1
11	Outer canine of mandible (0) of similar width to inner canine or (1) distinctly broader than inner canine	0, 1
12	Number of setae beneath canines of mandible (0) ≤ 3; (1) 4-9; (2) ≥ 10=2	0, 1, 2
13	Mandible (0) with short, thicker serrated seta preceding rest of setae below canine or (1) without thicker seta	0, 1
14	Setae beneath canine of mandible (0) smooth or (1) serrated	0, 1
15	Number of setae or setal sockets at rear end of mandible (0) < 5; (1) 6-15 (2) 16-40 (3) > 40	0, 1, 2, 3
16	Number of small subapical serrations along inner margin of outer canine (0) ≤ 2; (1) 3-5; (2) 6-10	0, 1, 2
17	Number of small subapical serrations along inner margin of inner canine (0) ≤2; (1) 3-5; (2) 6-10	0, 1, 2
18	Setae of maxilla (0) smooth or (1) serrated	0, 1
19	Length of seta on lacinia compared to length of dentisetae (0) < 0.1 x length of dentisetae; (1) 0.1-0.3 x length of dentisetae; (2) > 0.3 x length of dentisetae	0, 1, 2
20	Maxillary palp (0) extending beyond the notch which marks the separation between the stipes and galea-lacinia or (1) not reaching the notch	0, 1
21	Position of widest part of postmentum (0) widest near central axis; (1) widest near base (proximally)	0, 1
22	Abdominal segments 7-9 posterolateral projection (0) narrow-pointed; (1) broad-pointed; (2) broad-truncated	0, 1, 2
23	Ratio of carapace depth to carapace length (convexity) (0) ≤ 0.3; (1) 0.31-0.4; (2) > 0.4=2	0, 1, 2

FN	Feature and codes	Range
24	Maximum body length (mm) of mature nymph, from proximal point of head to distal point of abdomen (0) > 7; (1) 5-7: (2) < 5	0, 1, 2
25	Shape of anterior margin of carapace (0) strongly projecting forward to lie adjacent to lateral margin of head (1) not projecting forward	0, 1
26	Labrum prominent or not prominent when nymph viewed dorsally (0) not prominent (1) prominent	0, 1
27	Ratio of measurement of distance from distal margin of prosternum to vertex of prosternal plate, to the distance from distal margin of prosternum to margin of first visible abdominal segment (segment VI) (see Figures 3.1b,d; 3.2). (0) ≤ 0.2; (1) 0.21-0.39; (2) ≥ 0.4	0, 1, 2
28	Number of major filaments branching off gill 1 of mature nymph (0) < 10; (1) 10-20; (2) >20	0, 1, 2

Appendix A4.2. The mathematics behind the Artificial Neural Networks classification of Prosopistomatidae nymphs.

An *artificial neural network* (ANN), referred to loosely as a *neural network* or simply a *net*, is a function of the form

$¡^{r} \to ¡^{s}$

where f is the composition of several functions, f_i, of the form:

$f_i(x) = g_i(W^i x + b^i)$

and

The functions g_i are called *transfer functions*, the matrices W^i are called *weight matrices* and the vectors b^i are called *biases*. The action of g_i can be compared to a layer of neurons which process an input pattern and so this is called *layer* i of the network.

If there are just three layers then, for an input vector, p, we have

$a_1 = g_1\left(W^1 p + b^1\right)$

$a_2 = g_2\left(W^2 a_1 + b^2\right)$

$a_3 = g_3\left(W^3 a_2 + b^3\right)$

If we let $a = a_3$ then the *activation*, a, associated with an *input pattern*, p, is given by

$a = f\left(p\right)$

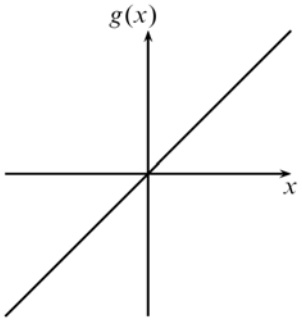
where

$f = f_3 \circ f_2 \circ f_1$.

The transfer functions, g_i, are usually one of the following:

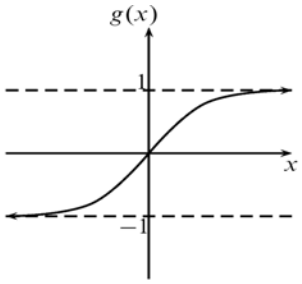
- the *purelin* function

$g(x) = x$



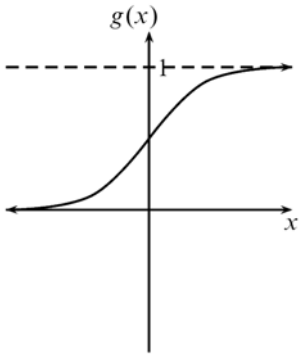
- the *tansig* function

$$g(x) = \frac{e^x - e^{-x}}{e^x + e^{-x}}$$



- the *logsig* function

$$g(x) = \frac{1}{1 + e^{-x}}$$



and a few others, all with the property that they are non-decreasing. Suppose that there is some real-world process which associates target vectors, t_i, with input pattern vectors, p_i and suppose that m such examples are known. We then have data of the form

$p_1 \to t_1$
$p_2 \to t_2$
$p_i \to t_i$
$p_m \to t_m$

If we let

$e_i = | a_i - t_i |$

then e_i is the error between the target i, t_i, and activation i, a_i. Let

$$e = \sqrt{\sum_{i=1}^{m} e_i^{\,2}}$$

Then e is the total error between the activation vector, a and the target vector, t for the particular set of weights: W^1, W^2, W^3 and biases b^1, b^2, b^3. There is a process which attempts to discover those weights and biases for which the error is a minimum or for which the error is within a certain prescribed tolerance. When this is achieved, the function, f, above, which depends on the weights and biases, can be used to simulate the process. This process of discovering the weights and biases is called *training* the network.

Training a Neural Network

The training process is as follows:

Initiate the weights, W^i and biases, b^i randomly.

For each of the m input patterns, p_i produce an activation a_i. Find the total error

$$e = \sqrt{\sum_{i=1}^{m} (a_i - t_i)^2}$$

The total error, e, is a function of the weights and biases. If the components of these weight matrices and bias vectors are listed in some way and represented as $x = [x_1, x_2, \mathrm{K}, x_n]$, then there is some functional relationship between the error, e, and x.

Say

$e = h(x)$.

If x^1 is the first sequence of weights and biases then the next sequence, x^2, is obtained from the first by the updating rule

$x^2 \leftarrow x^1 - r\nabla h(x^1)$.

Here, ∇h is the gradient of the function h and it is evaluated at x^1. The parameter, r, is a *learning rate* and it can be adjusted as the iteration proceeds. At iteration k we have

$x^{k+1} \leftarrow x^k - r\nabla h(x^k)$.

So the updating rule produces x^{k+1} from x^k by forming x^k minus a certain multiple of the gradient at x^k and this ensures that the error diminishes. This is a standard algorithm of numerical analysis.

So the gradient descent method can be used to produce a sequence:

x^1, x^2, K

such that the error decreases with each iteration. This does not guarantee that the errors decrease to zero but a tolerance can be set and the iteration process can be set to continue until this tolerance is met. The way in which the adjustments to the weights and biases are made is very interesting and is called *backpropagation*. It is rather technical and the details are omitted here. The crucial discovery was that it is possible to assign a *sensitivity vector*, σ^i, to layer i and this allows the adjustments of the weights and biases to be performed in a pro--rata fashion, with those weights and biases responsible for the greatest contribution to the error receiving the greatest adjustment.

The process starts with the last layer and progressing to the first, hence the name. There are many ways of doing this with the MATLAB Neural Network Toolbox.

The MATLAB Training Functions

trainb Batch training with weight and bias learning rules.
trainbfg BFGS quasi-Newton backpropagation.
trainbr Bayesian regularization.traincCyclical order incremental update.
traincgb Powell-Beale conjugate gradient backpropagation.
traincgf Fletcher-Powell conjugate gradient backpropagation.
traincgp Polak-Ribiere conjugate gradient backpropagation.
traingd Gradient descent backpropagation.
traingda Gradient descent with adaptive lr backpropagation.
traingdm Gradient descent with momentum backpropagation.
traingdx Gradient descent with momentum and adaptive lr backprop.
trainlm Levenberg-Marquardt backpropagation.
trainoss One step secant backpropagation.
trainr Random order incremental update.
trainrp Resilient backpropagation (Rprop).
trains Sequential order incremental update.
trainscg Scaled conjugate gradient backpropagation.

The scaled conjugate gradient training method is a good workhorse and this is the training method used here.

The Input Patterns: Features

The input patterns for the network are lists of features of the species. Each feature is given a numerical value, as indicated in Appendix Table A4.1. 28 features were selected, and each species is identified by a feature-list (vector) with each component in the range indicated in the table, in which FN denotes the Feature Number.

This information is contained in an Excel spreadsheet in the file: mayflydata.xls. On the third sheet the 28 feature values for each of 33 specimens are given. In the spreadsheet, the species are listed in the first column and the feature values appear as a row of values. The neural networks were constructed in the MATLAB computational environment with the Neural Network Toolbox. The function which is used to construct a neural network is newff. The syntax for this function requires that the input patterns

are presented as *columns* of a matrix, p In this case the matrix p has size 28×33. It has 33 columns (species), each of dimension 28 (features). So p has the form:

$$p = \begin{bmatrix} 0 & 0 & L & 1 & 1 \\ 1 & 0 & L & 1 & 1 \\ M & M & M & M & M \\ 1 & 1 & L & 0 & 2 \\ 1 & 1 & L & 0.38 & 1.22 \end{bmatrix}$$

The features of a specimen are used to classify it as a particular species and some features are more significant than others. Therefore it was decided to rank the features by assigning a ranking from 1 to 10 for each feature, with 1 indicating lowest importance and 10 indicating highest importance. At first, an intuitive ranking was assigned. The feature number and its ranking are listed below:

FN	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Rank	6	6	1	7	9	8	8	9	6	7	10	10	4	10	9	6	7

18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
4	3	3	7	6	5	6	7	8	5	6	7	3	8	9	6	5	8

Thus a ranking vector, r, with components as above, can be used to modify the input vectors for the purpose of training the network.

The Targets: Species

The targets for the network are the 33 species and they are listed in the following table, in which SN denotes the Species Number.

SN	Species
1	*P. africanum* Gillies, 1954 (Tanzania)
2	*P. amanzamnyama* Barber-James, 2010 (KwaZulu-Natal, South Africa)
3	Comores sp 1 (Anjouan, Comores)
4	*P. crassi* Gillies, 1954 South Africa
5	African sp. 2 (Namibia)

SN	Species
6	Madagascan sp. 1 (Madagascar)
7	Madagascan sp. 2 (Madagascar)
8	Madagascan sp. 3 (Madagascar)
9	African sp. 3 (DRC)
10	African sp. 4 (Kenya)
11	*P. mccaffertyi* Barber-James, 2010 (Mpumalanga, South Africa)
12	Madagascan sp. 4 (Madagascar)
13	African sp. 6 (Democratic Republic of Congo)
14	Madagascan sp. 5 (Madagascar)
15	*P. variegatum* Latrielle, 1883
16	African sp. 7 (West Africa)
17	African sp. 8 (Botswana, Zimbabwe)
18	*P. pennigerum* (Müller) 1785 (Europe)
19	*P. orhanelicum* Dalkiran 2009 (Turkey)
20	*P. oronti* Alouf 1977 (Israel)
21	*P. indicum* Peters 1967 (India)
22	*P. lieftincki* Peters 1967 (Sri Lanka)
23	*P. sinense* Tong and Dudgeon 2000 (China)
24	*P. funanense* Soldan and Braasch 1984 (Vietnam, China)
25	*P. annamense* Soldan and Braasch 1984 (Vietnam, China)
26	*P. boreus* Peters 1967 (Philippines)
27	*P. palawana* Peters 1967 (Philippines)
28	*P. wouterae* Lieftinck 1932 (Thailand, Malaysia, Java, Sumatra)
29	*P. olympus* Sartori and Gattolliat 2003 (Borneo)
30	*P. trispinum* Zhou and Zheng 2004 (China)
31	*P. unicolor* Zhou and Zheng 2004 (China)
32	*P. pearsonorum* Campbell and Hubbard 1998 (Australia)
33	*P. sedlaceki* Peters 1967 (New Guinea, Solomon Islands)

To present these species' names as targets for a neural network, they need to be encoded in some way.

Encoding the Targets

Method 1: Natural Numbers

One method is to simply use the numbers: $1, 2, L\ 33$ as targets. However, the network would experience training difficulties with this method as it appears as though there is

an ordering in the targets. Species 2 appears closer to Species 1 than Species 33, for example.

Method 2: Using Binary Numbers

Each number from $1,2,L\ 33$ can be given a binary representation as a 7-dimensional $0,1$ -vector:

$$1=\begin{bmatrix}0\\0\\0\\0\\0\\0\\1\end{bmatrix},2=\begin{bmatrix}0\\0\\0\\0\\0\\1\\0\end{bmatrix}3=\begin{bmatrix}0\\0\\0\\0\\0\\1\\1\end{bmatrix},L\ ,32=\begin{bmatrix}1\\0\\0\\0\\0\\0\\0\end{bmatrix},33=\begin{bmatrix}1\\0\\0\\0\\0\\0\\1\end{bmatrix}$$

This was successful but a slightly simpler method was used for the final network.

Method 3: Using the Phylogenetic Tree

Another method which was tried was to encode the position in the horizontal rooted phylogenetic tree as a vector. Starting from the root of the tree, we move to the species by means of movements: -1 for down and 1 for up. So the species are encoded:

s1=[-1 -1 -1 -1 -1 -1 -1 -1 -1 -1 1]'
s2=[-1 -1 -1 -1 -1 -1 1 -1 -1 -1 -1 1]'
s3=[-1 -1 -1 -1 -1 1]'
s4=[-1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 1]'
s5=[-1 -1 -1 -1 -1 -1 1 -1 1]'
s6=[-1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 1 1]'
s7=[-1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 1]'
s8=[-1 -1 -1 1 -1 -1 -1 -1 1]'
s9=[-1 -1 -1 -1 -1 -1 -1 -1 -1 1]'
s10=[-1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 1]'
s11=[-1 -1 -1 -1 -1 -1 1 -1 -1 -1 -1 -1]'
s12=[-1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 1]'
s13=[-1 -1 -1 -1 -1 -1 -1 1]'
s14=[-1 -1 -1 -1 1 -1 -1 -1 -1 -1 1]'
s15=[-1 -1 -1 -1 1 -1 -1 -1 -1 -1 -1]'

s16=[-1 -1 -1 -1 -1 -1 1]'

s17=[-1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 1]'

s18=[-1 -1 -1 -1 1 -1 1]'

s19=[-1 -1 -1 -1 1 1]'

s20=[-1 -1 -1 -1 1 -1 -1 1]'

s21=[-1 -1 -1 -1 -1 -1 1 -1 -1 1]'

s22=[-1 -1 -1 -1 1 -1 -1 -1 1]'

s23=[-1 -1 -1 1]'

s24=[-1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 1 -1]'

s25=[-1 -1 -1 -1 -1 -1 -1 -1 1 1]'

s26=[-1 -1 1]'

s27=[-1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1]'

s28=[-1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 1]'

s29=[-1 -1 -1 -1 -1 -1 -1 -1 1 -1]'

s30=[-1 1]'

s31=[-1 -1 -1 -1 -1 -1 1 -1 -1 -1 1]'

s32=[1 1]'

s33=[1 -1]'

In this, p' represents the transpose of the row vector p. For example,

$$s_{33} = \begin{bmatrix} 1 \\ -1 \end{bmatrix}$$

The vectors were padded with zeros at the end to make them all the same length. This method worked in the sense that a neural network was trained on these targets and was able to successfully classify all the species. However, as phylogenetic tree may change as new species are discovered, a more simple method of encoding the targets was preferred.

Method 4: The Identity Matrix

The numbers $1, 2, \mathrm{L}\ 33$ can be encoded as 33-dimensional $0,1$-vectors with number j having 1 in position j and 0 s elsewhere. So

$$1 = \begin{bmatrix} 1 \\ 0 \\ \mathrm{M} \\ 0 \\ 0 \end{bmatrix}, 2 = \begin{bmatrix} 0 \\ 1 \\ \mathrm{M} \\ 0 \\ 0 \end{bmatrix}, \mathrm{L}\ 32 = \begin{bmatrix} 0 \\ 0 \\ \mathrm{M} \\ 1 \\ 0 \end{bmatrix}, 33 = \begin{bmatrix} 0 \\ 0 \\ \mathrm{M} \\ 0 \\ 1 \end{bmatrix}$$

This means that the target matrix, t, is simply the identity matrix of size 28 x 33. In other words,

$$t = \begin{bmatrix} 1 & 0 & L & 0 & 0 \\ 0 & 1 & L & 0 & 0 \\ M & M & L & M & M \\ 0 & 0 & L & 1 & 0 \\ 0 & 0 & L & 0 & 1 \end{bmatrix}$$

This is the method which was chosen.

The Neural Networks

Once the input pattern matrix, p_{28x33}, and the target matrix, $t_{33\times33} = I_{33\times33}$ were decided upon, the neural network needed to be constructed and trained, tested and used for simulation. These objectives were achieved by means of the MATLAB scripts:

- maylyutrain
- mayflyutest
- mayflyusim
- mayflytrain
- mayflytest
- mayflysim

The Training Script: mayflytrain

The usual procedure for training a neural network is to partition the data set (the columns of the input matrix p) into two disjoint sets:

- A training set, ptrain
- A test set, ptest.

The target set (the corresponding columns of the target matrix t) is similarly partitioned to form

- ttrain
- ttest

The network is trained with input patterns ptrain with ttrain as targets and then it is tested on the data that it has not seen: ptest, ttest.

The performance of the net, usually the mean--square--error (MSE) or the sum of squares of the error (SSE), is monitored during training on ptrain and training is terminated when a preset goal is attained or the number of training epochs reaches some preset maximum.

The trained net is then simulated on ptest to produce an activation: atest which is compared with the corresponding targets: ttest. If these vectors are 1-dimensional then they can both be plotted on the same graph against their index number and a visual comparison is achieved. Otherwise the sum of squares of the difference between the vectors can be used as a numerical indicator.

Since the performance always decreases with training, it is possible to overtrain a neural network in the sense that its ability to interpolate is compromised in an effort to minimise the performance. This can be avoided by selecting a subset of p, called a *validation set*: pval and the performance of the net on pval is monitored *during training*. The performance on pval will decrease but will start to increase when overfitting starts to occur. At this stage training is prematurely stopped.

In this case, however, the net is being used for pattern recognition. Here, *all* of the input data is used for training, which is tested by noting the outputs after perturbing inputs. Ideally, the trained net is required to classify all of the input patterns correctly. This is what occurred in this case. The worthiness of the trained net is judged by its ability to classify new input patterns. Each of these new input patterns would correspond to a feature vector of a new specimen.

This script uses unranked input patterns and corresponding targets. Once it has been tested, it can be deployed to discover the weighting of the features. This can then be compared with the intuitive weightings assigned earlier.

Pseudocode for the script is as follows:

1. Read the input matrix, p, from the Excel file and take its transpose so that the columns of p are the feature vectors. This gives the input pattern matrix, $p_{28x33.}$

2. Normalise the input patterns so that their components are all of the same order of magnitude. This is achieved with a linear scaling function. There is a MATLAB function: mapminmax which can be used for this purpose. If normalisation is employed, it is necessary to rescale the activations. In this case, the components were all of the same order of magnitude and so normalisation was not necessary.

3. Form the identity matrix, $t = I_{33\times 33}$ as a target matrix.

4. Construct the neural network using the function newff. The function needs to receive as inputs:

- A vector whose components are the minimum and maximum of the matrix p. This is achieved with the function: minmax. So minmax(p) produces a 28 x 2 vector of minimum and maximum values of the rows of the matrix p.
- A vector: $s = [s_1, s_2, s_3]$ whose i^{th} component, s_i is the number of neurons in layer i. It is known that three layers are sufficient for any classification problem. It is necessary to experiment with the layer sizes and sometimes a supervising script is written which systematically varies the values s_i, keeps a record of the performance in each case and selects the structure with the best performance. This was not necessary here since once a net has been found which correctly classifies all input patterns, there is no need for further experimentation. Here the structure vector, s, that did the job was $s = [66, 33, 33]$.
- The number of neurons in the last layer is forced to be the dimension of the target vectors and so we must have $s_3 = 33$
- A MATLAB structure, $\{f_1, f_2, f_3\}$ which contains the names of the transfer functions in each layer.
  Here: f_1=tansig, f_2=tansig, f_3=purelin was chosen. This is a very common configuration.

- The name, tm , of a training method. Here tm=trainscg was used.
- Various training parameters need to be set. The most important of these is the maximum number of epochs allowed for training. Without this, if a goal of zero is set for the performance, the training process will not terminate.

5. Initiate weights and biases in the network. This is achieved with: net=ini(net).

6. Train the network. This is achieved with net=train(net,p,t).

This produces a neural network object, called net, as a variable in the MATLAB workspace.

7. Rename the neural network object, so that it has a unique name and save it. This is achieved with:

- mayflyunet=net and
- save mayflyu.mat

At this stage, a trained neural network, called mayflyunet, is available as a variable in the file mayflyu.mat.

The actual script that was used is as follows.

```
%mayflyutrain
%unranked targets
%uses simple targets
%of form [0, 0, ..., 1, 0 0 0]'
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%
%features are unranked
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%
clear;
clc;

%features
%input matrix on page 3 of the XLfile
```

```
p=xlsread('path\mayflydata.xls',3,'range');
%list features as columns
p=p';

%nf=number of features
%ns=number of specimens
[nf, ns]=size(p);

%make identity matrix for targets
t=eye(ns);

% %Net architecture and training
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%
net=newff(minmax(p),[2*ns,ns],{'tansig','tansig','purelin'},
'trainscg');

%
%Net training
net.TrainParam.epochs=3000;

%number of iterations on validation set with increasing performance
net.trainparam.max_fail=300;
%define validation set
m=size(p,2);
%test and val indices:
tri=[1:m];
%specify data division function and parameters
net.divideFcn='divideind';
net.divideParam.trainInd=tri;
net.divideParam.testInd=[];
net.divideParam.valInd=[1:5:m];

%initiate the weights and biases
net=init(net);

%train the net
net=train(net,p,t);

%rename
```

```
mayfly3unet=net;

%save all info
save mayfly3u.mat
```

The Test Script: mayflyutest

The network now needs to be tested to see whether it can correctly classify all 33 species. The simple script which does this has the Pseudocode:

1. Load the neural network and all the variables used with load mayflyu.mat

2. Use the network, mayflyunet, to simulate on the input patterns, p, to produce an activation, a. This is achieved with a=sim(mayflyunet,p).

3. The activation, a, has columns with 33 components and we round the values which are less than 0.5 to 0 and those which exceed 1 to 1. This produces a 0,1 matrix which can be compared with a target matrix t. If the network correctly classifies the input patterns then we should have a=t. We can have a graphical representation by plotting an open circle, o, for each of the 33 species and simultaneously plotting a star, * for each corresponding activation (Figure 4.2 in text). The plot reveals that the network has correctly classified all 33 species. The script is as follows.

```
%mayfly3u_test
load mayfly3u.mat

%nf=number of features
%ns=number of specimens
[nf, ns]=size(p);

%simulate on ranked inputs
a=sim(mayfly3unet,p);

format short
%find the values in activation which are less than .5
a(a<.5)=0;
```

```
%round those above 1 to 1
a(a>1)=1;

%extract diagonal elements
for j=1:ns
sp(j)=a(j,j);
end

%the species
sp=sp(:);
I=[1:ns]';

%plotting
close all
figure
hold on
plot(I,1,'o')
plot(I,sp,'*')
title('Mayfly NN3: o =target, *=nn classification')
xlabel('species number')
ylabel('activation')
legend('0=target','*=activation')
axis([0 ns 0 1])
hold off
```

Once the network has been trained and tested it can be used to classify other input patterns. If a specimen is obtained which needs classification, its feature list can be presented to the network and its corresponding activation will be its classification in the form its species number.

A modification of the above uses inputs whose components are ranked as mentioned before.

Training with Ranked Inputs: mayflytrain.

This script is essentially the same as mayflyutrain except that a ranking vector, r, is read from the Excel sheet and the input patterns are ranked using this vector. The relevant lines in the script are:

```
fav=xlsread('path\mayflydata.xls',3,'range');

%list features as columns
p=p';

%nf=number of features
%ns=number of specimens
[nf, ns]=size(p);
rr=repmat(r,1,ns);

%ranked inputs
pr=p.*rr/10;
```

The Script: mayflytest

The network, mayflynet, is the corresponding network which is trained on ranked inputs and this was tested as before.

The Script: mayflysim.

At this stage we have neural networks, mayflyunet and mayflynet which can be deployed to classify further input patterns. These input patterns could be feature lists (vectors) of specimens found in the field. For example, if we extract the feature list of Species 1 by calling the first column of the matrix p :

```
p1=p(:,1)
p1 =p(:,1)
    0
    1
    0
    1
    .
```

.
.
2
0
0

If we modify p1 by changing the first feature from 0 to 1 and calling the new feature vector pn:

```
pn = p1;
pn(1)= 1
pn =
    1
    1
    0
    1
    .
    .
    .
    2
    0
    0
```

We can now see how the network classifies this new specimen, pn. The space R^{35} is partitioned into 33 compartments by the network. Classifying this new specimen, pn, involves placing it into one of the compartments and identifying it. This is achieved with the script mayflysim.

```
%mayfly3r_sim
load mayfly3r.mat

pn=input('new input pattern = ')

%ranked inputs
pn=pn.*r/10;

%simulutae on ranked inputs
```

```
an=sim(mayfly3rnet,pn);

format short
%find the values in activation which are less than .5
an(an<.5)=0;

%round those above 1 to 1
an(an>1)=1;

%show an
an

% find species numbers
s=find(an~=0)

%round an
an=an(an>0)

%find corresponding weights
ans=sum(an);

w=an/ans;
out=[s';w'];

%print output
fprintf('species %d weight: %5.4f\n',out)
```

The script prompts the user for an input vector and then attempts to classify it. For example,

```
new input pattern = pn
an =
   0.5312
        0
        0
        0
```

.

.

.

0

0

0

s = 1

an = 0.5312

species 1 weight: 1.0000

The script has identified the species with the modified feature vector, pn, as species 1.

Ranking Features

The trained neural network can be deployed to rank features and then compared to the intuitive rankings. One way of achieving this is as follows:

For each species *j*

for each feature *i*

replace the feature list *p* by a new list *pn* which has feature *i* replaced by the average feature value;

simulate to produce an activation *an*

compute the error between *an* and the corresponding target *t*

store the error

When this has been done we form the average *ea* over all species of the errors caused by replacing a feature value by the average of the feature values. The assumption is that the importance of a feature for classification purposes is proportional to the error caused by replacing it by the average feature value.

The script that achieves this is as follows.

The Script: mayflyuesim

```
%mayflyuesim
clc
```

```
clear

load mayfly3u.mat
 %read intuitive rankings
r=xlsread('path\mayflydata.xls',3,'range');
%normalised
r=r(:)/10;
 %number of species
n=ns;

for j=1:n;
   %new p
   pn(:,:,j)=repmat(p(:,j),1,nf);

   for i=1:nf
      %for species j replace feature i by average value for ns species
      pn(i,i,j)=fav(i);
      an(:,i,j)=sim(mayfly3unet,pn(:,i,j));
      e(i,j)=norm(t(:,j)-an(:,i,j))/norm(an(:,i,j));
   end
end

e=e'; ea=sum(e)/n; ea=ea(:);

ea=round(10*ea); r=10*r;
f=[1:nf]';

out=[f ea r]';

fprintf('fn nn in\n')
fprintf('%2.0f %2.0f %2.0f\n',out)
```

Output from the script lists the feature number, fn, the ranking, nn, by the network and the intuitive ranking, in :

```
fn nn in
```

1 6 6
2 7 6
3 2 1
4 6 7
5 7 9
6 5 8
7 4 8
8 6 9
9 5 6
10 5 7
11 6 10
12 6 10
13 5 4
14 5 10
15 6 9
16 7 6
17 4 7
18 4 4
19 3 3
20 5 3
21 4 7
22 4 6
23 6 5
24 3 6
25 3 7
26 4 8
27 5 5
28 4 6

Deployment of the Network

The trained neural network is a MATLAB object: mayflyunet.mat or mayflyrnet.mat which resides in the workspace after training or after the command: load mayfly.mat, which loads the variables created during training into the workspace. The network consists of the weight matrices and three bias vectors together with three transfer functions. The weight matrices and bias vectors can be recovered with the commands:

```
w1=mayflyunet.iw{:}
w2=mayflyunet.lw(2,1)
w3=mayflyunet.lw(3,2)
b1=mayflyunet.b(1,1)
b2=mayflyunet.b(2,1)
b3=mayflyunet.b(3,1)
```

These can then be written into an Excel spreadsheet using the MATLAB xlswrite function. Once the weight matrices and bias vectors are in a spreadsheet, a function can be written which accepts a column as input and uses the weight matrices and bias vectors along with the corresponding transfer functions to produce an activation as output.

Appendix for Chapter 5

Appendix Table A5.1. Selected characters used for determining the phylogeny of the nymphs of Prosopistomatidae. Outgroup is *Baetisca rogersi*. Species name abbreviations indicated in Table 1.1. Unknown characters marked by "?".

Nymphal Character	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Outgroup	1	0	0	0	0	0	1	0	0	0	1	2	0	0	0	0	2
P. orh	0	1	1	1	1	1	1	0	1	0	1	1	2	0	1	1	2
P. pen	0	1	1	1	1	1	0	1	1	0	1	1	2	0	1	1	2
P. oro	0	1	1	1	1	1	1	2	3	0	1	1	2	1	0	1	?
P. ann	0	1	1	1	1	0	1	1	1	1	0	0	1	?	1	0	?
P. bor	0	1	1	1	1	1	0	1	3	0	1	1	1	0	1	1	?
P. fun	0	1	1	1	1	1	1	1	0	1	0	0	1	?	1	0	?
P. ind	0	1	1	1	1	0	1	2	2	1	0	0	1	0	1	0	?
P. lief	0	1	1	1	1	1	1	1	1	0	1	2	2	1	0	1	?
P. oly	0	1	1	1	1	0	1	1	0	1	0	0	1	0	1	0	?
P. pala	0	1	1	1	1	1	0	1	3	1	0	0	1	0	1	0	?
P. sine	0	1	1	1	1	1	1	2	1	0	1	1	1	?	1	1	2
P. tri	0	1	1	1	1	1	1	1	0	0	1	1	2	?	0	1	2
P. uni	0	1	1	1	1	0	1	2	2	1	0	0	1	?	1	0	?
P. wou	0	1	1	1	1	0	1	1	0	1	0	0	1	0	1	0	1
P. sedl	0	1	1	1	1	1	0	1	1	0	1	1	2	0	0	0	?
P. pear	0	1	1	1	1	1	0	1	1	0	1	1	2	0	1	0	2
P. afr	0	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	0
P. cras	0	1	1	1	1	0	1	1	2	1	0	0	1	0	1	0	0
P. var	0	1	1	1	1	1	1	0	3	0	1	2	2	1	0	1	2
Af sp. 1	0	1	1	1	1	0	1	2	2	1	0	0	1	0	1	0	0
Af sp. 2	0	1	1	1	1	0	0	2	1	1	0	0	1	0	1	0	0
Af sp. 3	0	1	1	1	1	0	1	2	0	1	0	0	1	0	1	0	0

Nymphal Character	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Af sp. 4	0	1	1	1	1	0	1	1	0	1	0	0	1	0	1	0	0
Af sp .5	0	1	1	1	1	1	1	2	2	1	0	0	1	0	1	0	0
Af sp. 6	0	1	1	1	1	0	1	1	0	1	0	0	1	0	1	0	1
Af sp. 7	0	1	1	1	1	0	1	2	1	1	0	0	1	0	1	0	2
Af sp. 8	0	1	1	1	1	0	1	1	2	1	0	0	1	0	2	0	1
Mad sp. 1	0	1	1	1	1	0	1	1	0	1	0	1	1	0	1	0	0
Mad sp. 2	0	1	1	1	1	0	1	1	2	1	0	0	1	0	1	0	1
Mad sp. 3	0	1	1	1	1	1	0	0	3	0	1	2	2	1	0	1	2
Mad sp. 4	0	1	1	1	1	1	0	1	2	0	0	0	2	1	1	0	0
Mad sp. 5	0	1	1	1	1	1	1	0	3	0	1	2	2	1	0	1	2
Comores sp.	0	1	1	1	1	0	1	1	1	0	0	0	1	0	1	0	0

List of nymphal characters and character states:

1, Shape of carapace (0) rounded with entire margin (1) with spiny extensions
2, Carapace development (0) gradual as nymphs mature (1) present even in young instar nymphs
3, Mandible structure (0) non-predatory mandibles (1) predatory mandibles
4, State of glossae and paraglossae (0) partly fused (1) completely fused
5, Third antennal segment (0) not enlarged (1) enlarged
6, Ratio head width: carapace width (0) ≤0.5 (1) >0.5
7, Ratio of distance between eyes : max head width (0) ≤0.6 (1) ≥0.61
8, Number of antennal segments including scape and pedicel (0) ≥7 (1) 6 (2) 5
9, Antenna longer than/ equal to/ shorter than/ much shorter than distance from antennal base to anterior margin of head longer = 0, subequal = 1, shorter = 2, much shorter = 3
10, Length of inner canine of mandible compared to outer canine (0) shorter (1) subequal
11, Outer canine of mandible distinctly broader than inner canine / of similar width to inner canine (0) similar (1) broader
12, Number of setae beneath canines of mandibles (0) ≤3 (1) 4-9 (2) ≥10
13, Regular prostheca (0) thicker, serrated seta preceding rest of setae below canine present (1) absent (2)
14, Position of widest part of postmentum (0) widest near central axis (1) widest near base (proximally)
15, Shape of anterior margin of carapace (0) strongly projecting forward to lie adjacent to lateral margin of head (1) not projecting forward
16, Labrum prominent when nymph viewed dorsally/ not prominent when viewed dorsally (0) not prominent (1) prominent
17, Number of major filaments branching off gill 1 of mature nymph (0) <10 (1) 10-20 (2) >20

Appendix Table A5.2. Selected characters used for determining the phylogeny of the male imago of Prosopistomatidae where known. Outgroup is *Baetisca rogersi*. Species name abbreviations indicated in Table 1. Unknown characters marked by "?".

Adult ♂ Characters	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Outgroup	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0
P. pen	1	1	1	0	0	2	1	3	3	3	3	3	3	1	1	1	0	2
P. afr	0	2	2	0	1	1	1	3	3	3	3	3	3	1	?	1	0	1
P. var	1	1	0	0	1	2	1	2	2	2	3	3	3	2	1	2	0	2
Af sp. 2	0	2	2	1	1	1	1	3	0	3	3	3	3	1	0	0	1	2
Mad sp. 1	0	2	2	1	1	2	1	3	3	0	3	3	3	1	0	0	1	2
Mad sp. 2	0	2	2	0	1	1	1	0	2	0	3	3	3	1	0	1	0	1
(P0400)	0	2	2	1	0	1	1	3	3	4	3	3	3	1	0	0	1	2
(P0249)	0	1	0	2	1	?	1	3	3	3	3	3	3	1	1	1	1	2

List of characters and character states, with second numbers (in parentheses) referring to character states mapped on combined matrix tree:
1,(18), ♂ forewing shape (0) rounded (1) tapering
2,(19), forewing length ♂ (mm) (0) >8.0 mm (1) 5.0-8.0 mm (2) < 5.0mm
3,(20), hindwing length ♂ (mm) (0) > 2.0 mm (1) 1.5-2.0 mm (2) <1.5 mm
4,(21), $\log_{10}$ of ratio of length forewing : hindwing ♂ (0) >0.4 (1) 0.35-0.4 (2) <0.35
5,(22), ♂ intercalary above SC (0) no (1) yes
6,(23), proportional increase in length male sub foreleg: male imago foreleg (%) (0) >30% (1)10-29% (2) < 10%
7,(24), male forelegs elongate/ not elongate (0) elongate (10 not elongate
8,(25), Shape of male subimaginal foreleg claw (0) 1 hooked, 1 blunt (1) paired blunt (2) paired hooked (3) single blunt (4) single blunt plus secondary hook
9,(26), Shape of male subimaginal midleg claw (0) 1 hooked, 1 blunt (1) paired blunt (2) paired hooked (3) single blunt (4) single blunt plus secondary hook
10,(27), Shape of male subimaginal hindleg claw (0) 1 hooked, 1 blunt (1) paired blunt (2) paired hooked (3) single blunt (4) single blunt plus secondary hook

11,(28), Shape of male imago foreleg claw (0) 1 hooked, 1 blunt (1) paired blunt (2) paired hooked (3) single blunt (4) single blunt plus secondary hook
12,(29), Shape of male imago midleg claw (0) 1 hooked, 1 blunt (1) paired blunt (2) paired hooked (3) single blunt (4) single blunt plus secondary hook
13,(30), Shape of male imago hindleg claw (0) 1 hooked, 1 blunt (1) paired blunt (2) paired hooked (3) single blunt (4) single blunt plus secondary hook
14,(31), ♂ no of tarsal segments in foreleg (0) 5 (1) 2 (2) 1
15,(32), ♂ sub flange behind head (0) small (1) large unnotched
16,(33), ♂ imago flange behind head (0) small (1) large unnotched (2) large notched
17,(34), genitalia ♂ imago (0) >½ length of clasper (1) <½ length of clasper
18,(35), state of penes cleft (0) narrow slit (1) v-shaped notch (2) u-shaped notch

Appendix Table A5.3. Selected characters used for determining the phylogeny of the female subimago of Prosopistomatidae where known. Outgroup is *Baetisca rogersi*. Species name abbreviations indicated in Table 1. Unknown characters marked by "?". Character numbering continued from Appendix 5.2.

Adult ♀ Characters	19	20	21	22	23	24	25
Outgroup	1	0	0	0	0	0	0
P. pen	1	1	0	1	1	2	1
P. pear	1	1	0	1	1	1	1
P. afr	1	2	1	1	1	1	1
P. var	1	0	0	1	1	0	1
Af sp. 5	0	2	1	2	1	1	1
Mad sp. 1	1	2	1	1	1	1	1
Mad sp. 2	1	2	1	2	1	1	1
(P0400)	1	2	1	0	1	1	1

19,(36) ♀ forewing shape
20,(37) ♀ forewing length
21,(38) ♀ hindwing length
22,(39) ♀ ratio fw:hw
23,(40) ♀ imago flange behind head
24,(41) ♀ RP development
25,(42) ♀ legs

Appendix for Chapter 6

Appendix Table A6.1. Specimen details for Prosopistomatidae material successfully sequenced. * Heath Ogden's sequences taken from GenBank. (Sequences to be lodged with GenBank once manuscript is accepted for publication). '-' indicates no sequence available.

Species	Date collected	Collector	County	Locality	Museum Catalogue Number	Genbank Accession number		
						16S	18S	Histone 3
P. amanzamnyama	05-12-2005	M. Graham	South Africa	Drakensberg, Glengarry		#####	#####	#####
P. amanzamnyama	01-04-2009	M. Graham	South Africa	Weza River	GEN 1863A	#####	#####	#####
P. amanzamnyama	01/IX/2009	M. Graham	South Africa	Ngwangwane Riv	GEN 1866A	#####	#####	#####
P. crassi	21-09-2003	M. Graham	South Africa	Gatberg River	GEN 1836C	#####	#####	#####
P. crassi	29-10-2003	P. Maseti	South Africa	Inxu River	ECR 704B	#####	#####	#####
P. crassi	18-06-2004	M. Graham, C. Dickens	South Africa	Umgeni River	UMG 1075G	#####	#####	#####
P. crassi	01-06-2005	N. Rivers-Moore	South Africa	Great Fish River	GEN 1848A	#####	#####	#####
P. crassi	03/IX/2009	M. Graham	South Africa	Mzimkhulu River	GEN 1869A	-	-	-
P. crassi	04/IX/2009	M. Graham	South Africa	Mzimkhulu River	GEN 1870A	-	-	-
P. crassi-like	04/IX/2009	M. Graham	South Africa	Mzimkhulu River	GEN 1870B	-	-	-
P. mccaffertyi	27-10-1990	W.P.McCafferty and H.M. Barber	South Africa	Sabie River	SA 40	#####	-	-
African sp. 2	14-11-2000	F.C. de Moor	Namibia	Cunene River	KUN 171G	#####	#####	#####
African sp. 2	13/XI/2009	K. Roberts	Namibia	Cunene River	KUN 192A	#####	#####	#####
African sp. 3	15-08-2007	M. Graham	DRC	Kasai River	CAW 381H	#####	#####	#####
African sp. 4	10-11-2007	M. Graham	Kenya	Isiukhu River	CAW 388A	#####	#####	#####
African sp. 6	11-08-2008	M. Graham	DRC	Ulindi River	CAW 395A	#####	#####	#####
African sp. 7	no date	J.-M. Elouard	Guinea	Boussoule River	-	#####	#####	#####
African sp. 8	09-05-2008	M. Graham	DRC	Magembe River	CAW 400A	#####	#####	#####
African sp. 8	08-10-2008	R.W. Palmer	Zambia	Kafue River	CAW 391A	#####	#####	#####

Species	Date collected	Collector	County	Locality	Museum Catalogue Number	Genbank Accession number		
						16S	18S	Histone 3
P. variegatum	04/IX/2001	J-M. Elouard	Madagascar	Antarantsa River	MAD 058	-	-	-
P. variegatum	27-04-2003		Madagascar	Lakato River	P2008	-	-	-
Madagascan sp. 1	18-10-1995	J-M. Elouard	Madagascar	Manambolo River	P0510	-	-	-
Madagascan sp. 2	30-10-2001		Madagascar	Ankavia River	MAD 129	-	-	-
Madagascan sp. 2	21/VII/2001	R. Gerecke and T. Goldshmidt	Madagascar	Ravoandrina Riv	MAD 011	-	-	-
Madagascan sp. 5	04-06-2002		Madagascar	Belakato River	P2147	-	-	-
Madagascan sp. 6	27-04-2003	M.T. Monaghan	Madagascar	Lakato River	P2008	-	-	-
unknown adult	30-03-1999	J-M. Elouard	Madagascar	Antongombato Basin	P0835	-	-	-
P. pennigerum	27-03-2008	S. Robles	Spain	River Segura	SEG5	-	-	-
P. pennigerum	27-03-2008	S. Robles	Spain	River Mundo	mundo4	#####	#####	#####
P. pennigerum		M. Schletterer	Russia	Volga River	_	#####	#####	#####
P. pearsonorum	25-06-2008	J. Webb	Australia	South Johnstone River	_	#####	#####	#####
P. orhanelicum	22-11-2001	N. Dalkiran	Turkey	Orhaneli Stream	_	-	-	-
Prosopistoma sp	15-02-2001	Y.J. Bae	Vietnam	Dak Lak	_	-	-	-
*Madagascan sp. 2			Madagascar			AY749827	-	-
P. wouterae			Thailand, Malaysia			AY749810.1	AY749882.1	-

Table A6.2. Additional sequences incorporated from GenBank for BEAST molecular clock analysis

Genbank number	Species name	Family	Gene
AY749882	*Prosopistoma wouterae*	Prosopistomatidae	18S ribosomal RNA gene
AY749810	*Prosopistoma wouterae*	Prosopistomatidae	16S ribosomal RNA gene
AY749734	*Prosopistoma wouterae*	Prosopistomatidae	Histone H3 gene
AY749904	*Prosopistoma EP166* (Mad sp 2)	Prosopistomatidae	18S ribosomal RNA gene
AY749827	*Prosopistoma EP166* (Mad sp 2)	Prosopistomatidae	16S ribosomal RNA gene
AY749865	*Baetisca lacustris*	Baetiscidae	18S ribosomal RNA gene
AY338627	*Baetisca sp.* Eph16	Baetiscidae	Histone H3 gene
AY749801	*Lachlania saskatchewanensis*	Oligoneuriidae	16S ribosomal RNA gene
AY338701	*Lachlania saskatchewanensis*	Oligoneuriidae	18S ribosomal RNA gene
AY338623	*Lachlania saskatchewanensis*	Oligoneuriidae	Histone H3 gene
AY749897	*Oligoneuriella rhenana*	Oligoneuriidae	18S ribosomal RNA gene
AY749823	*Oligoneuriella rhenana*	Oligoneuriidae	16S ribosomal RNA gene
AY749745	*Oligoneuriella rhenana*	Oligoneuriidae	Histone H3 gene
AY749869	*Afronurus peringueyi*	Heptageniidae	18S ribosomal RNA gene
AY749790	*Afronurus peringueyi*	Heptageniidae	16S ribosomal RNA gene
AY749843	*Rhithrogena sp.* EP021	Heptageniidae	18S ribosomal RNA gene
AY749765	*Rhithrogena sp.* EP021	Heptageniidae	16S ribosomal RNA gene
AY749704	*Rhithrogena sp.* EP021	Heptageniidae	Histone H3 gene
AY749800	*Ametropus neavei*	Ametropodidae	16S ribosomal RNA gene
DQ648734	*Ametropus neavei*	Ametropodidae	18S ribosomal RNA gene
AY338622	*Ametropus neavei*	Ametropodidae	Histone H3 gene
AY749848	*Baetis tricaudatus*	Baetidae	18S ribosomal RNA gene
AY749771	*Baetis tricaudatus*	Baetidae	16S ribosomal RNA gene
AY749708	*Baetis tricaudatus*	Baetidae	Histone H3 gene
DQ648717	*Caenis youngi*	Caenidae	18S ribosomal RNA gene
AY749763	*Caenis sp.* EP019	Caenidae	16S ribosomal RNA gene
AY338630	*Caenis sp.* EP019	Caenidae	Histone H3 gene
AY555558	*Choroterpes sp.* GG-2004	Leptophlebiidae	16S ribosomal RNA gene

Genbank number	Species name	Family	Gene
AY555525	*Choroterpes sp.* GG-2004	Leptophlebiidae	18S ribosomal RNA gene
AY555569	*Choroterpes sp.* GG-2004	Leptophlebiidae	Histone H3 gene
AM042661	*Cloeon simile*	Baetidae	18S rRNA gene
AJ971774	*Cloeon simile*	Baetidae	mitochondrial 16S rRNA
GQ118267	*Ephemera simulans*	Ephemeridae	18S ribosomal RNA gene
GQ118285	*Ephemera simulans*	Ephemeridae	16S ribosomal RNA gene
GQ118323	*Ephemera simulans*	Ephemeridae	Histone H3 gene
AY749779	*Euthyplocia hecuba*	Euthyplociidae	16S ribosomal RNA gene
AY749855	*Euthyplocia hecuba*	Euthyplociidae	18S ribosomal RNA gene
AY749712	*Euthyplocia hecuba*	Euthyplociidae	Histone H3 gene
AY749761	*Isonychia sp.* EP017	Isonychiidae	16S ribosomal RNA gene,
AY749889	*Isonychia sp.* EP142	Isonychiidae	18S ribosomal RNA gene
AY749740	*Isonychia sp.* EP142	Isonychiidae	Histone H3 gene
AY749793	*Nesameletus ornatus*	Nesameletidae	16S ribosomal RNA gene
AY749872	*Nesameletus ornatus*	Nesameletidae	18S ribosomal RNA gene
AY749724	*Nesameletus ornatus*	Nesameletidae	Histone H3 gene
AY749871	*Siphlaenigma janae*	Siphlaenigmatidae	18S ribosomal RNA gene
AY749792	*Siphlaenigma janae*	Siphlaenigmatidae	16S ribosomal RNA gene
AY749723	*Siphlaenigma janae*	Siphlaenigmatidae	Histone H3 gene
AY749863	*Siphlonurus sp.* EP084	Siphlonuridae	18S ribosomal RNA gene
AY749786	*Siphlonurus sp.* EP084	Siphlonuridae	16S ribosomal RNA gene
AY749718	*Siphlonurus sp.* EP084	Siphlonuridae	Histone H3 gene
GQ118294	*Siphluriscus sp.* BYU IGCEP241	Siphluriscidae	16S ribosomal RNA gene
GQ118276	*Siphluriscus sp.* BYU IGCEP241	Siphluriscidae	18S ribosomal RNA gene
GQ118335	*Siphluriscus sp.* BYU IGCEP241	Siphluriscidae	Histone H3 gene
GQ118281	*Tricorythus sp.* BYU IGCEP206	Tricorythidae	18S ribosomal RNA gene
GQ118297	*Tricorythus sp.* BYU IGCEP206	Tricorythidae	16S ribosomal RNA gene
GQ118338	*Tricorythus sp.* BYU IGCEP206	Tricorythidae	Histone H3 gene
GQ118282	*Oplonaeschna sp.*	Aeshnidae	16S ribosomal RNA gene
GQ118262	*Oplonaeschna sp.*	Aeshnidae	18S ribosomal RNA gene
GQ118317	*Oplonaeschna sp.*	Aeshnidae	Histone H3 gene

Appendix for Chapter 7

Appendix Table A7.1 Overview of putative relationship between Ephemeroptera and other extant insects, according to a number of studies (this is not a complete revision).

Authors	**Morphology molecular**	**(Ephem+Odo) Neop** (Palaeoptera hypothesis)	**Odo (Ephem+Neop)** (Chiastomyaria hypothesis)	**Ephem (Odo+Neop)** (Metapterygota hypothesis)	**Comments**
Börner, 1904	Morphology			*	Introduced term "Metapterygota"
Martynov, 1923, 1924a,b, 1925	Morphology	*			Introduced term "Palaeoptera"
Crampton, 1924	Morphology	*			
Lemche, 1940	Morphology		*		
Riek, 1970	Morphology fossils	*			
Boudreaux, 1979			*		
Hennig, 1981	Morphology fossils	*			
Riek and Kukalová-Peck, 1984	Morphology fossils	*			
Kristensen, 1975	Morphology			*	
Hennig, 1981	Derived Morphology (not symplesimorphic wing resting position)	*			
Kristensen, 1991	Morphology				None conclusive
Kukalová-Peck,	Morphology,	*			

Authors	**Morphology molecular**	**(Ephem+Odo) Neop** (Palaeoptera hypothesis)	**Odo (Ephem+Neop)** (Chiastomyaria hypothesis)	**Ephem (Odo+Neop)** (Metapterygota hypothesis)	**Comments**
1983,1985,1987,1991,1997,2008	fossils				
Brodsky, 1994		*			
Soldán, 1997	Morphology			*	Concluded that 3 paleopterous groups should be considered independently
Whiting *et al.*, 1997					
Carpenter and Wheeler, 1999	Molecular			*	
Willmann, 1999		*			
Fürst von Lieven, 2000	Morphology			*	
Kluge, 2000, 2004	Morphology			*	
Staniczek, 2000	Mandibular muscles a articulation			*	
Wheeler *et al.*, 2001	18S 28S			*	
Grimaldi, 2001	Morphological and developmenta evidence			*	
Hovmöller *et al.*, 2002	18S, 28S	*			
Ogden and Whiting, 2003	18S, 28S, H3			*	
Terry, 2003	18S, 28S, H3	*			
Kjer, 2004	18S		*		
Grimaldi and Engel, 2005	morphology			*	
Yoshizawa and Johnson, 2005	18S		*		
Mallat and Giribet, 2006	18S, 28S		*		
Kjer *et al.*, 2006	18S, 28S, H3, EF-1α, COI, COII, 12S, 16S				"The elucidation of relationships between non-holometabolous neopteran orders is far from resolved"

Authors	**Morphology molecular**	**(Ephem+Odo) Neop** (Palaeoptera hypothesis)	**Odo (Ephem+Neop)** (Chiastomyaria hypothesis)	**Ephem (Odo+Neop)** (Metapterygota hypothesis)	**Comments**
Misof *et al.*, 2007	18S		*		
Hörnschemeyer and Willkommen 2007	Wing sclerites and muscles	*			
Zhang *et al.*, 2008	mt genome			*	
von Reumont *et al.*, 2009	18S, 28S		*		
Simon *et al.*, 2009	Multi gene approach		*		
Kukalová-Peck *et al.*, 2009	Morphology fossils	*			
Meusemann *et al.*, 2010			*		

(i) The Palaeoptera scenario which supports a basal sister group position of Odonata and Ephemeroptera (Odonata+Ephemeroptera, Neoptera), (ii) the Metapterygota scenario (Ephemeroptera basal, Odonata+Neoptera) and (iii) the Chiastomyaria scenario (Odonata basal, Ephemeroptera+Neoptera) (Whitfield and Kjer, 2008).

Appendix Table A7.2. Characters used for parsimony analysis to investigate relationships between stem-group and extant mayfly lineages. A hypothetical outgroup with all primitive characters (0) was used as outgroup in one analysis, and †Triplosobidae was used in a second analysis. Unknown characters marked by "?". Era not included in analysis, but shown for perspective of time.

	(Era)	1	2	3	4	5	6	7	8
†Triplosobidae	Carboniferous	?	1	0	0	0	0	0	0
†Lithoneuridae	Carboniferous	0	1	0	0	0	0	0	0
†Palaeodictyoptera	Carboniferous	1	1	0	0	0	1	1	0
†Misthodotidae	Permian	?	0	0	0	?	0	1	0
†Protereismatidae	Permian	1	0	0	1	0	0	1	0
†Litophlebiidae	Triassic	?	?	1	0	0	0	0	1
Posteritorna	Extant	0	0	1	0	0	0	1	0
Anteritorna	Extant	0	0	1	2	0	0	0	1
Odonata	Extant	2	1	0	3	0	1	1	0

List of characters and character states:
1, Number of cerci (0) 3 (1) 2
2, Costal brace (0) present (1) absent
3, Wings (0) homonymous (1) heternomous
4, Veins CuA and CuP (0) parallel (1) separate but no tornus (2) separated by tornus (3) fused
5, Wing folding (0) cannot fold back (1) can fold back
6, Triadic branching of RP, MA (0) present (1) absent
7, Stems of RA and RP (0) fused near wing base (1) not fused near wing base
8, Archedictyon (0) present (1) absent

Appendix for Chapter 8

Appendix Table A8.1. Coding derived from track analysis patterns, showing presence (1) and absence (0), used for determining Sørensen's coefficient of similarity of areas and for Multi-Dimensioanl Scaling.

Area	Sisoroidea	Cichlidae	Anura	Syncarida	Gerromorpha	Ecnomidae	Stenopsychidae	Chironomidae	Prosopistomatidae
Africa	1	1	1	1	1	1	1	1	1
Madagascar	0	1	1	1	1	0	0	1	1
Middle East	1	0	0	1	1	1	0	1	1
Europe	0	0	0	1	1	1	0	1	1
Asia	1	0	1	1	1	1	1	1	1
India	1	1	1	1	1	1	1	1	1
Pacific Islands	1	0	0	1	1	1	1	1	1
Australia	0	0	1	1	1	1	1	1	1
S. America	0	1	1	1	1	1	1	1	0
N. America	0	0	1	1	1	0	0	1	0

Appendix Table A8.2. Physical parameters of rivers characterising ecological requirements of *Prosopistoma* nymphs. Water temperatures should be treated with circumspection as seasonal variation is not reflected in all cases; some records represent a once-off measurement.

Species	Zoogeographic region	Depth occurring in river (m)	Stream width (m)	Current speed (m/s)	Substrate	Water temp °C	pH	Altitude (masl)	Natural Riparian vegetation	Associated aquatic macrophytes on rocks	Source
P. africanum (Tanzania)	Af	0.4-0.9	≥ 0.3 - 20	Moderate to swift flow	Rocks and stones, no sand and silt	26 - 29	6.2- 7.7	213-853	rainforest	Not recorded	Gillies, 1954
P. amanzamnyama (South Africa)	Af	0.5	3-12	Moderate to swift flow	Stones (doleritic)	14.5 - 25.9	6.7 - 7.2	950-1650	upland grassland with a well-developed woody, yet open, riparian zone.	Not recorded	Mark Graham, pers comm.
P. crassi (South Africa)	Af	0.5-1.0	5-100	0.3-1.1 (pref >0.8)	Extensive rapids, boulder beds, rock bottoms	15 - 28	7.4 - 8.6	420-1200	Thornveld/ savanna	*Hydrostachys* in some Natal streams	Gillies, 1954 Chutter, 1967
African sp. 2 (Namibia)	Af	0.1-0.5	35 - 150	rapids	Bedrock, boulders, cobbles. gravel	29	8.4	180-730	Mopane savanna and riverine woodland	*Hydrostachys* (but seems *Prosopistoma* not associated with this)	de Moor *et al*., 2000
P. deguernei (Senegal)	Af	Not recorded	190 - 310	Not recorded	Not recorded	Not recorded	Not recorded	51	Tropical rainforest	Not recorded	Vayssiere, 1893
African sp. 3 (DRC)	Af	Not recorded	380 - 840	Not recorded	Not recorded	Not recorded	Not recorded	400-500	Open woodland	Not recorded	M. Graham, pers comm.

Species	Zoogeographic region	Depth occurring in river (m)	Stream width (m)	Current speed (m/s)	Substrate	Water temp °C	pH	Altitude (masl)	Natural Riparian vegetation	Associated aquatic macrophytes on rocks	Source
African sp. 4 (Kenya)	Af	Not recorded	26	Not recorded	Not recorded	Not recorded	Not recorded	1468	Not recorded	Not recorded	R. Palmer, pers comm.
P. mccaffertyi (South Africa)	Af	0.3 – 0.8	15 - 95	Moderate to swift flow	Granite bedrock, scattered boulders	11 - 37	7.6-8.3	250-900	grassland and savanna	No macrophytes	Barber-James, 2010
African sp. 6 (DRC)	Af	Not recorded	6-20	Not recorded	"Chutes"	Not recorded	Not recorded	1371-2303	Dense forest	Not recorded	Paulian, 1947; J-M. Elouard
African sp. 7 (West Africa)	Af	Not recorded	30 - 50	Not recorded	Not recorded	Not recorded	Not recorded	80-500	Not recorded	Not recorded	J-M. Elouard
African sp. 8 (Botswana, Zimbabwe)	Af	Not recorded	300 - 850	Not recorded	Not recorded	Not recorded	Not recorded	950-1400	*Brachystegia* savanna	Not recorded	Eliot Taylor
Madagascan sp. 1 (Madagascar)	Af	0.3-0.8	3.5 - 25	1-2	Not recorded	17 - 22	Not recorded	580-1300	Not recorded	Not recorded	Elouard, Sartori, pers comm.
Madagascan sp. 2 (Madagascar)	Af	0.1-1.0	2.5 - 35	0.3-0.8	Not recorded	12.8-28.0	Not recorded	40-1280	Not recorded	Not recorded	Elouard, Sartori, pers comm.
Madagascan sp. 4 (Madagascar)	Af	Not recorded	5 - 10	Not recorded	Riffle, downstream cascade	20 - 21.9	Not recorded	550-850	forest	Not recorded	Elouard, Sartori, pers comm.
Madagascan sp. 5 (Madagascar)	Af	Not recorded	5 - 60	Not recorded	Riffle	19.5-20	Not recorded	70-920	Forest to arid	Not recorded	Elouard, Sartori, *pers comm.*
P. variegatum (Madagascar)	Af	0.1-0.8	2.4-15 12 – 90	0.3-1.0	Riffle	15-28		25-1750	forest	Not recorded	Elouard, Sartori, pers comm.
Comores sp. 1 (Anjouan, Comores)	Af	Not recorded	2-5	0.3-1.0 (Steep gradient)	basaltic boulders and gravel	20-26	8.6	10-150	dense tropical primeval forest	Not recorded	Starmuhlner, 1976

Species	Zoogeographic region	Depth occurring in river (m)	Stream width (m)	Current speed (m/s)	Substrate	Water temp °C	pH	Altitude (masl)	Natural Riparian vegetation	Associated aquatic macrophytes on rocks	Source
Unknown sp. (Algeria)	Pal	Not recorded	6m (in dry season)	slow	Not recorded	Not recorded	Not recorded	300	Arid – scattered woodland only along banks	Not recorded	Thomas, 1998
Unknown sp. (Morocco)	Pal	0.8	5.2	Not stated	Mixed substrate - bedrock, boulders to small pebbles.	9-26	7-8	1680	Dense forest	*Glyceria fluitans* (native to Europe)	Touabay *et al*. 2002
P. pennigerum (France)	Pal	0.5-1.6	5-30	fast	Large rocks	19 (summer)	Not recorded	16-800		Not recorded	Dorier, 1925
P. pennigerum (Spain)	Pal	Not recorded	16	0.21-0.49	Not recorded	Not recorded	Not recorded	614-735	Pine forest	Not recorded	Robles, pers comm.
P. pennigerum (Russia)	Pal	0.5	180	0.6	Gravel and small stones		7.2	158		Not recorded	Schletterer and Füreder, 2009
Unknown sp. (Georgia, Kura River)	Pal	Not recorded	35	Not recorded	Not recorded	Not recorded	Not recorded	800	forested	Not recorded	Kasymov and Agaev, 1986
Koch 1985 Turkey	Pal	Not recorded	160	Not recorded	Not recorded	Not recorded	Not recorded	720	arid	Not recorded	Koch, 1985
P. orhanelicum (Turkey)	Pal	Not recorded	10-40	1.4-2.2	Not recorded	7.7-24.3	7.8–8.6	50-354	Not recorded	Not recorded	Dalkiran, 2009
P. oronti	Pal	0.1-0.46	4-17	2.5	Boulders and rocks	10-20 (summer)		40-660	Natural riparian forest	Not recorded	Alouf, 1977; Thomas, 1988; Koch, 1988; Por *et al*, 1986.

Species	Zoogeographic region	Depth occurring in river (m)	Stream width (m)	Current speed (m/s)	Substrate	Water temp °C	pH	Altitude (masl)	Natural Riparian vegetation	Associated aquatic macrophytes on rocks	Source
P. indicum	Or	≥ 0.5	large river	fast	Small to large rocks	Not recorded	Not recorded	107	Not recorded	Dense algae	Peters, 1967
P. lieftincki	Or	≤ 0.5	small stream	Not recorded	Not recorded	Not recorded	Not recorded	1097-1524	Cloud Forest	Not recorded	Peters, 1967
P. sinense	Or	0.05-0.3	Small rivers	Moderate to fast	Small stones	Not recorded	Not recorded	4-1260	Dense forest	Not recorded	Tong and Dudgeon, 2000
P. funanense	Or	2-4	30	0.1-0..3	Stony bottom	Not recorded	Not recorded	20	forested riparian vegetation	Not recorded	Soldan and Braasch, 1984; Liu *et al.,* 1984
P. annamense	Or	2-4	70	0.1-1.2 (optimal 0.3-0.8)	Gravel bottom riffles, small stones up to 10cm	26.4-29.8	Not recorded	600	Not recorded	Not recorded	Soldan and Braasch, 1984; Liu *et al*.,1984
P. boreus	Or	≤ 0.5	small rocky stream	Not recorded	Gravel bottom rifflles	Not recorded	Not recorded	450-900	forest	Not recorded	Peters, 1967
P. palawana	Or	Not recorded	Small rocky stream	Not recorded	Rocky	Not recorded	Not recorded	near sea level	Not recorded	Not recorded	Peters, 1967

Species	Zoogeographic region	Depth occurring in river (m)	Stream width (m)	Current speed (m/s)	Substrate	Water temp °C	pH	Altitude (masl)	Natural Riparian vegetation	Associated aquatic macrophytes on rocks	Source
P. wouterae	Or	Not recorded	Swiftly flowing mountain streams	Fast flowing current	Gravel and small stones (Lieftinck); large boulders, running deep water; Boulders on sand and small stone substrate	Not recorded	Not recorded	Great range recorded 1050; 10-700	Virgin rainforest	Not recorded	Lieftinck, 1932; Parnrong *et al.,* 2002
P. olympus	Or	0.35	3-8	0.8-0.9	Boulders and cobbles with little gravel and no sand	23.9 - 26.3	5.5-6.0 (dubious –Derleth *pers comm.*)	96	Primary rainforest	Not recorded	Sartori and Gattolliat, 2003; Derleth, 2003
P. trispinum	Or	0.4	55	Not recorded	Boulders, gravel	Not recorded	Not recorded	921	Not recorded	Dense aquatic plants	Zhou and Zheng, 2004
P. unicolor	Or	0.3-0.5	30-60 (braided)	Not recorded	Gravel	Not recorded	Not recorded	1167	Not recorded	Not recorded	Zhou and Zheng, 2004
P. pearsonorum	Au	Not recorded	>40m	Fast flowing current	Large boulders, and cobbles over sand	Not recorded	Not recorded	26-323	Farmland, originally forest. Mitchell R; arid.	Not recorded	Campbell and Hubbard, 1998; Webb, *pers comm*
P. sedlaceki	Au	0.9	2-10	fast	Small to large boulders	Not recorded	Not recorded	638-1609 (11 m on Solomon Island)	Dense forest, including Hoop and Klinki pine	Not recorded	Peters, 1967

Appendix Table A8.3. Abiotic river parameters coded for ordination analysis.

Species	Depth	Width	Curren speed	Substrate composition	Water temperature	pH	Altitude
P.afric	1	1	1	0	2	2	2
P.amanz	1	1	2	1	2	2	3
P.crassi	0	2	1	1	2	3	2
Af sp 2	1	3	2	1	2	3	2
Af sp 2	0	3	0	0	0	0	1
Af sp 3	0	3	0	0	0	0	2
Kenyan sp.	0	1	0	0	0	0	3
Af sp 4	0	2	0	0	0	0	3
P.mccaf	0	2	0	1	2	3	2
Af sp 6	0	2	0	1	0	0	3
Af sp 7	0	2	0	0	0	0	1
Af sp 8	0	3	0	0	0	0	2
Mad sp 1	0	2	2	0	1	0	3
Mad sp 2	0	2	1	0	2	0	2
Mad sp 4	0	1	0	1	1	0	2
Mad sp 5	0	2	0	1	1	0	2
P varieg	2	2	1	0	2	0	2
Comores	0	1	1	2	2	3	1
Algeria	0	1	1	0	0	0	2
Morocco	0	1	0	1	2	3	3
*P.pen*Fr	0	2	2	1	0	0	2
*P.pen*Sn	0	2	1	0	0	0	2
*P.pen*Ru	0	3	1	2	0	2	1
Georgia	0	2	0	0	0	0	3
Turkey	0	3	0	0	0	0	2
P.orhan	0	2	2	0	2	3	1
P.oro	0	2	2	1	1	0	1
P.indic	0	3	2	2	0	0	1
P.lief	0	1	0	0	0	0	3
P.sinense	0	0	1	2	0	0	2
P.fun	0	2	1	2	0	0	1
P.anna	0	2	1	2	2	0	3
P.boreus	0	1	0	2	0	0	2
P.pala	0	1	0	1	0	0	1
P.wout	0	1	2	1	0	0	2
P.olymp	0	1	1	1	2	1	1
P.trisp	0	2	0	1	0	0	3
P.unic	0	2	0	2	0	0	3
P.pears	0	2	2	1	0	0	2
P.sedl	0	1	2	1	0	0	2

List of parameters and parameter states:

1, Depth of river (1) shallow ≤ 0.5 m, (2) deep > 0.5 m

2, Width of river (1) ≤ 10 m, (2) 10-100 m, (3) > 100 m

3, Current speed (1) ≤ 1.0 m/s, (2) > 1.0 m/s

4, Substrate (1) Boulder-bedrock dominance, (2) cobble-gravel dominance

5, Water temperature (1) maximum ≤ 25 °C , (2) maximum > 25 °C

6, pH (1) ≤ 6, (2) 6.1-7.5, (3) > 7.6

7, Altitude (masl) (1) low < 200m, (1) medium 200 - 800 m, (3) High > 800 m.