Evolutionary biology of Gondwanan non-biting midges (Diptera: Chironomidae)

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Keywords

Australia, Chironomidae, continental drift, dispersal, *Echinocladius martini*, *Ferringtonia patagonica*, freshwater invertebrate, gene flow, genetic structure, glaciation events, Gondwana, habitat fragmentation, Lars Brundin, *Naonella forsythi*, New Zealand, Orthocladiinae, Patagonia, phylogenetics, phylogeography, population genetics, population isolation, rainforest contraction, refugia, tectonic uplift, vicariance.

Abstract

The potential restriction to effective dispersal and gene flow caused by habitat fragmentation can apply to multiple levels of evolutionary scale; from the fragmentation of ancient supercontinents driving diversification and speciation on disjunct landmasses, to the isolation of proximate populations as a result of their inability to cross intervening unsuitable habitat. Investigating the role of habitat fragmentation in driving diversity within and among taxa can thus include inferences of phylogenetic relationships among taxa, assessments of intraspecific phylogeographic structure and analyses of gene flow among neighbouring populations.

The proposed Gondwanan clade within the chironomid (non-biting midge) subfamily Orthocladiinae (Diptera: Chironomidae) represents a model system for investigating the role that population fragmentation and isolation has played at different evolutionary scales. A pilot study by Krosch *et al* (2009) indentified several highly divergent lineages restricted to ancient rainforest refugia and limited gene flow among proximate sites within a refuge for one member of this clade, *Echinocladius martini* Cranston. This study provided a framework for investigating the evolutionary history of this taxon and its relatives more thoroughly.

Populations of *E. martini* were sampled in the Paluma bioregion of northeast Queensland to investigate patterns of fine-scale within- and among-stream dispersal and gene flow within a refuge more rigorously. Data was incorporated from Krosch *et al* (2009) and additional sites were sampled up- and downstream of the original sites. Analyses of genetic structure revealed strong natal site fidelity and high genetic structure among geographically proximate streams. Little evidence was found for regular headwater exchange among upstream sites, but there was distinct evidence for rare adult flight among sites on separate stream reaches. Overall, however, the distribution of shared haplotypes implied that both larval and adult dispersal was largely limited to the natal stream channel.

Patterns of regional phylogeographic structure were examined in two related austral orthoclad taxa – *Naonella forsythi* Boothroyd from New Zealand and *Ferringtonia patagonica* Sæther and Andersen from southern South America – to provide a comparison with patterns revealed in their close relative *E. martini*. Both taxa inhabit tectonically active areas of the southern hemisphere that have also experienced several glaciation events throughout the Plio-Pleistocene that are thought to have affected population structure dramatically in many taxa. Four highly divergent lineages estimated to have diverged since the late Miocene were revealed in each taxon, mirroring patterns in *E. martini*; however, there was no evidence for local geographical endemism, implying substantial range expansion post-diversification. The differences in pattern evident among the three related taxa were suggested to have been influenced by variation in the responses of closed forest habitat to climatic fluctuations during interglacial periods across the three landmasses.

Phylogeographic structure in *E. martini* was resolved at a continental scale by expanding upon the sampling design of Krosch *et al* (2009) to encompass populations in southeast Queensland, New South Wales and Victoria. Patterns of phylogeographic structure were consistent with expectations and several previously unrecognised lineages were revealed from central- and southern Australia that were geographically endemic to closed forest refugia. Estimated divergence times were congruent with the timing of Plio-Pleistocene rainforest contractions across the east coast of Australia. This suggested that dispersal and gene flow of *E. martini* among isolated refugia was highly restricted and that this taxon was susceptible to the impacts of habitat change.

Broader phylogenetic relationships among taxa considered to be members of this Gondwanan orthoclad group were resolved in order to test expected patterns of evolutionary affinities across the austral continents. The inferred phylogeny and estimated divergence times did not accord with expected patterns based on the geological sequence of break-up of the Gondwanan supercontinent and implied instead several transoceanic dispersal events

post-vicariance. Difficulties in appropriate taxonomic sampling and accurate calibration of molecular phylogenies notwithstanding, the sampling regime implemented in the current study has been the most intensive yet performed for austral members of the Orthocladiinae and unsurprisingly has revealed both novel taxa and phylogenetic relationships within and among described genera. Several novel associations between life stages are made here for both described and previously unknown taxa.

Investigating evolutionary relationships within and among members of this clade of proposed Gondwanan orthoclad taxa has demonstrated that a complex interaction between historical population fragmentation and dispersal at several levels of evolutionary scale has been important in driving diversification in this group. While interruptions to migration, colonisation and gene flow driven by population fragmentation have clearly contributed to the development and maintenance of much of the diversity present in this group, long-distance dispersal has also played a role in influencing diversification of continental biotas and facilitating gene flow among disjunct populations.

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Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signed:

Matthew Neil Krosch

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Chapter One

General Introduction

The concept that habitat fragmentation and subsequent barriers to migration and gene flow among isolated populations play a crucial role in population dynamics, diversification and extinction hearkens back to the earliest foundations of island biogeography, equilibrium theory and species turnover. Depending on the founding population size and other demographic attributes such as generation time, fecundity and mating strategy, the division of large continuous populations into several smaller patches can produce a variety of responses in refugial populations, including divergence among patches, speciation, inbreeding depression or even local extinction. Moreover, the interruption to colonisation and gene flow that can result from habitat fragmentation can potentially apply to all levels of temporal and geographical scale, ranging from the fragmentation of ancient supercontinents to the isolation of proximate populations of stream-dwelling taxa due to stream hierarchy. The processes that drive fragmentation of continuous populations are considered to thereby represent an integral component in the development and maintenance of global biodiversity (Moritz *et al.*, 2000).

1.1 Habitat fragmentation and phylogeography

Investigating the role of habitat fragmentation in driving diversity within and among taxa thus can incorporate multiple evolutionary scales; including inferences of evolutionary relationships among taxa, assessments of regional intraspecific genetic structure and analyses of genetic structure aimed at detecting gene flow among neighbouring populations. The discipline of phylogeography – essentially a sub-discipline of biogeography –can encompass multiple levels of evolutionary scale and can incorporate data from disciplines including population genetics, geography, geology, demography, life history and/or phylogenetics to test hypotheses regarding the evolutionary history of a given taxon or group of taxa (Avise *et al.*, 1987; Avise, 1998). Phylogeographic theory therefore is built upon the founding principles that govern the reconstruction of evolutionary patterns and

relationships both at the interspecific scale (i.e., phylogenetics) and the intraspecific scale (i.e., population genetics).

Broadly speaking, phylogenetics is concerned with reconstructing the natural hierarchy of relationships among species and investigating the causal elements that drive them (Hennig, 1965). Species relationships have been defined historically by estimates of overall morphological similarity; however this approach often has produced misleading outcomes (e.g., Tate, 1951; Darlington Jr., 1965; Poe, 1996) as morphological similarity does not always represent evolutionary relatedness. Advances in phylogenetic theory have led to the recognition that a comprehensive knowledge of the relative polarity (apomorphy or plesiomorphy) of relevant characters is necessary before the evolutionary history of a particular taxon can be reconstructed accurately (Hennig, 1965; Brundin, 1966). Furthermore, whilst traditional phylogenetic investigations have relied primarily on evaluations of morphological data (e.g., Brundin, 1966; Cracraft, 1981; Benton, 1990; Roberts, 1993; Ponder & Lindberg, 1997), recent developments in molecular genetic technologies, and associated complex statistical analyses developed in parallel, have been quickly integrated into the field (e.g., Waters *et al.*, 2000; Guryev *et al.*, 2001; Bell *et al.*, 2004; Teeling *et al.*, 2005; Allegrucci *et al.*, 2006). Whilst there has been much debate as to which method or methods are 'best' (see Hillis, 1987), one advantage of molecular-based phylogenetics is the ability to allow access to vast datasets that incorporate all heritable information in the genome, spanning from the very recent to the distant past, and can provide phylogenetic resolution for taxa that are morphologically cryptic or phenotypically plastic (Wilcox *et al.*, 1997; Gomez *et al.*, 2002; Baker *et al.*, 2004; Herbert *et al.*, 2004; Bickford *et al.*, 2007). Thus, utilising phylogenetic methods to reconstruct relationships among taxa can provide important insights into the evolutionary history of a particular taxon and its relatives.

By comparison, analyses of population genetic structure test the spatial relationships among unique genetic types (haplotypes) of individuals from multiple populations or geographical regions of a particular species in order to construct gene trees that describe the evolutionary relationships among

those individuals (Avise *et al.*, 1987; Arbogast & Kenagy, 2001). Gene trees then can be overlaid on geographical, geological, botanical, demographic and/or life history data to test particular evolutionary hypotheses. The main objectives of population genetic analyses are thus to determine the patterns and processes that govern both the present geographical distribution of species and the relative connectivity or isolation of populations of the same species (Avise, 1998; Arbogast & Kenagy, 2001). Moreover, comparing phylogeographic patterns among co-distributed taxa can provide crucial information regarding the biogeographical history of a given region, whilst comparing patterns among closely related taxa from different geographical regions can inform inferences of the relative similarity in responses of allied taxa (Arbogast & Kenagy, 2001). At a finer geographical scale, testing population genetic structure by partitioning genetic variation in a hierarchical manner – i.e., within and among neighbouring populations – can reveal patterns of the relative gene flow among populations (Lewontin, 1967). This approach is particularly effective when testing naturally hierarchical systems like those found in riverine ecosystems, whereby the dendritic nature of a stream catchment provides the hierarchy upon which genetic diversity can be mapped. This concept was encompassed in the 'Stream Hierarchy Model' (Meffe & Vrijenhoek, 1988), which proposes that genetic diversity among streams should be greater than within streams, and that among catchments should be greater than within catchments. Testing population genetic structure under the 'Stream Hierarchy Model' has been immensely powerful in resolving patterns of genetic structure within and among populations of many freshwater taxa as movement among populations is bounded by the stream channel in most species (Hughes *et al.*, 2008; Hughes *et al.*, 2009).

1.2 The Orthocladiinae (Diptera: Chironomidae) as a model system

Members of the non-biting midge family Chironomidae (Insecta: Diptera) occur widely across most of the globe and occupy a diverse array of habitats

including peat bogs, cow dung, bromeliad cups and intertidal rock pools, though the vast majority inhabit freshwater streams and lakes (Oliver, 1971; Armitage *et al.*, 1995). The family comprises eleven subfamilies (Orthocladiinae, Chironominae, Tanypodinae, Aphroteniinae, Diamesinae, Prodiamesinae, Buchonomyiinae, Usambaromyiinae, Chilenomyiinae, Podonominae and Telmatogetoninae) within which there is an estimated 10,000 species, of which less than half have been described formally (Armitage *et al.*, 1995). Furthermore, the roots of the Chironomidae run deep, with all subfamilies estimated to have shared a most recent common ancestor in the middle Triassic approximately 248-210 million years ago (mya) (Cranston *et al.*, 2010). Despite a global distribution at the family level, some subfamilies are more restricted in their distributions and habitat preferences. For example, the Diamesinae inhabit only the coldest alpine streams and circumpolar regions, whilst the Telmatogetoninae inhabit areas adjacent to oceans that are subject to tidal forces and receive some freshwater runoff (Brundin, 1966). The Aphroteniinae and the majority of Podonominae (~87%) are found solely in swift mountain streams in the southern hemisphere (Brundin, 1966). The Tanypodinae, Chironominae and Orthocladiinae, however, are more cosmopolitan and species-rich than the aforementioned subfamilies. The Tanypodinae and Chironominae occur on all continents except Antarctica and appear adapted to standing waters of the tropics (although there are exceptions in both subfamilies) (Brundin, 1966). In comparison, the Orthocladiinae are by far the most ecologically diverse subfamily and, whilst these taxa occur globally in all manner of aquatic habitats, members of the Orthocladiinae primarily inhabit cool lotic freshwater environments.

Lotic freshwater chironomids emerge as larvae from an egg cluster laid by a gravid female at the waters' edge (Oliver, 1971; Pinder, 1986). The larval stage is characterised by rapid growth and development through four instars (Oliver, 1971; Pinder, 1986; Lindegaard & Mortensen, 1988; Huryn & Wallace, 2000). While larvae of some chironomid species are free-living predators (e.g., members of the subfamily Tanypodinae), the vast majority attach to a particular instream substrate (rocks, organic matter, etc) – some

even construct protective cases – and feed mainly on fine particulate detritus or algae in the water (Oliver, 1971; Pinder, 1986; Huryn & Wallace, 2000; McKie & Cranston, 2001). The duration of this stage varies greatly across the family and across habitats, ranging from less than two weeks to over two months, and is dependent on many external environmental factors, especially temperature (Oliver, 1971).

Once maturity has been reached, environmental stimuli induce metamorphosis from a larva to a pupa. The pupal stage is extremely brief compared with the larval stage, ranging from only a few hours to a few days (Oliver, 1971; Huryn & Wallace, 2000), and is characterised by the production of an outer casing which protects individuals during rapid growth of wings, legs and other adult structures (Oliver, 1971; Lindegaard & Mortensen, 1988). Once the rapid development of this stage is complete, the pupa floats to the waters' surface at which point the adult emerges from the pupal casing and takes to the wing (Oliver, 1971). Adult emergence in temperate zones is thought to generally follow diel and seasonal patterns (Oliver, 1971; Armitage *et al.*, 1995), while emergence in the tropics is considered to be more asynchronous – a trend observed in several other semi-aquatic insect groups (Boothroyd, 1988; Bunn & Hughes, 1997; Huryn & Wallace, 2000).

The adult stage generally lasts only a few days, although some species can live for a number of weeks (Oliver, 1971; Huryn & Wallace, 2000). The main activity of adults is reproduction, which occurs on the wing, in the midst of a swarm, and after copulation the females depart to rest briefly while the eggs mature, before ovipositing at the waters' edge (Oliver, 1971; McLachlan & Allen, 1987). The adult stage is considered to have the highest potential for dispersal and adult flight patterns can be simplified into three categories: flight to a resting spot immediately after emergence from the pupa; swarming flight; and ovipositional flight (Oliver, 1971; McLachlan & Neems, 1996).

Currently, little definitive data exists that describes the ability of chironomids to disperse among streams. Ovipositional flight – when gravid females

search for an appropriate location to lay an egg mass – is considered to represent the phase at which dispersal away from the natal stream is most likely to occur. Current data regarding wing physiology and flight to body muscle ratios in chironomids from ephemeral systems suggest that they may be capable of extensive dispersal (McLachlan, 1983, 1986; McLachlan & Neems, 1996), though lateral movement away from streams may be restricted by the density of riparian vegetation (Delettre & Morvan, 2000). Anecdotal data also implies that some chironomids may be suited for long-distance transport on wind currents based on the ability of some species to colonise newly created habitats rapidly after their formation (Oliver, 1971) and recovery of chironomids both up to 600m above sea level (White, 1970) and several hundred kilometres from the nearest landmass (Holzapfel & Harrell, 1968). Furthermore, chironomid pupae and larvae often comprise a large proportion of the macroinvertebrate taxa drifting downstream (Williams, 1989; Kerby *et al.*, 1995), implying that passive downstream dispersal may play an important role in the movement of freshwater chironomids. Such ideas have gained additional support from studies that invoke extensive dispersal as the mechanism by which some Australian species have arrived at their present distributions (McKie *et al.*, 2004; McKie *et al.*, 2005). In contrast, existing genetic evidence suggests the opposite may be the case (Martin *et al.*, 2002; Kaiser *et al.*, 2010). In particular, a recent study of the Orthocladiinae species *Echinocladius martini* from the Australian Wet Tropics region suggested dispersal was limited even between neighbouring sites (<1km apart) (Krosch *et al.*, 2009 - Appendix 1).

Krosch *et al* (2009) investigated population genetic structure among populations of *E. martini* from the Wet Tropics bioregion of north-eastern Australia. This area is thought to have consisted of continuous rainforest habitat until widespread conversion to dry sclerophyll forest occurred during the Pleistocene, reaching its maximum extent approximately 38-26 000 years ago (kya) (Kershaw, 1985; Nix & Switzer, 1991; Kershaw, 1994). Contraction of rainforest into small, isolated refugia during periods of sclerophyll expansion likely resulted in the fragmentation of other rainforest endemic taxa, potentially driving genetic divergence in allopatry. In particular, the

hypothesised Black Mountain Corridor (BMC) of north-eastern Queensland is thought to have been a major barrier to dispersal for numerous rainforest endemic species throughout the Pleistocene (Moritz *et al.*, 2000). The BMC is proposed to have reached its greatest extent during the last glacial maximum (~18kya) and extends from around 25km south of Cairns northwards to a maximum width of approximately 75km. The BMC was a large stretch of dry sclerophyll forest and woodland, devoid of rainforest until approximately 8kya. The corridor bisected two major extant rainforest refugia that were distributed around the Atherton Tableland to the south and the Daintree rainforest to the north (Nix & Switzer, 1991; Hugall *et al.*, 2002).

Echinocladius martini populations were sampled across the extant Atherton, Mt. Lewis and Daintree rainforest blocks, along with two sites from another putative historical refuge in the Paluma bioregion northwest of Townsville (Krosch *et al.*, 2009). Using mitochondrial DNA techniques, the study identified several highly divergent lineages that were apparently geographically restricted. Deep divergence was also evident across the BMC which separated lineages from the Atherton Tableland and Paluma Uplands from those sampled from Mt. Lewis and Daintree. Two representative samples from Victoria formed a sister group, however, with the Daintree lineage and one lineage from Mt. Lewis and were nested within the lineages from north of the BMC. Taken together, the study suggested that movement of *E. martini* among sites was restricted and the placement of some northern Wet Tropics lineages with Victorian haplotypes most probably represented the retention of ancestral polymorphisms (Ballard, 2000). Moreover, within one lineage – isolated to the Paluma bioregion – there was evidence for significant population structure among stream sites located only 500 metres apart and which coalesced less than one kilometre downstream. Krosch *et al* (2009) suggested that this may be evidence that *E. martini* dispersal occurs mostly within a stream, with very little movement among streams. These data, however, represent only preliminary results and will require more intensive sampling at a local scale to elucidate more fully the geographical range and extent of *E. martini* dispersal in the Paluma bioregion and to test the relevance of the ‘Stream Hierarchy Model’ to freshwater chironomids. At

a continental scale there is a need to resolve relationships among lineages more accurately and reveal additional diversity in central and southern regions of eastern Australia.

This notwithstanding, *E. martini* has been the focus of almost a decade of intensive ecological study since the original description of the monospecific genus (Cranston, 2000b). *Echinocladius martini* is generally found in leaf packs and riffle sections of cool, shaded, upland freshwater streams that flow through native closed forest and over medium sized cobbles, and has been recorded from such streams along much of the east coast of mainland Australia and Tasmania. This taxon has also been recorded in otherwise atypical locations; including Lake Boomanjin on Fraser Island and Franki's Gulch in the Cooloola section of the Great Sandy National Park in southeast Queensland. Lake Boomanjin is the only known lentic occurrence of *E. martini*, whilst Franki's Gulch is a sandy-bottomed stream that runs through dry *Melaleuca* and *Casuarina* forest; both sites have grossly different habitat characteristics to all other known *E. martini* locations. The recovery of *E. martini* in such locations implies that classical views about the affinities of this taxon to a specific environment may have been incorrect. Indeed, tests of the environmental tolerances of *E. martini* and two other chironomid taxa in controlled laboratory trials by McKie *et al* (2004, 2005), revealed that all three taxa possess quite broad environmental tolerances, potentially explaining the occurrence of *E. martini* in the atypical localities mentioned above. This aside, the general affinity of *E. martini* to cool and shaded environments, along with morphological similarities to Orthocladiinae taxa from New Zealand and South America that possess similar habitat preferences, have led researchers to consider *E. martini* to be a member of a group of Orthocladiinae taxa that may once have been distributed across the ancient supercontinent of Gondwana (Cranston & Edward, 1999; Cranston, 2000b, 2009). Thus, the data of Krosch *et al* (2009) present an ideal framework to compare and contrast phylogeographic patterns and to test specific hypotheses of population structure in Orthocladiinae chironomids thought to be closely related to *E. martini* and that inhabit other austral landmasses.

1.3 Comparative phylogeography across austral landmasses

Comparing and contrasting patterns of population genetic structure among related species forms an integral part of comparative phylogeography (Bermingham & Moritz, 1998; Taberlet, 1998; Arbogast & Kenagy, 2001). Such investigations are generally conducted within a given geographical region to evaluate the relative effect of the biogeographical history of the region on co-distributed taxa and have been immensely powerful in demonstrating the similar effects of particular historical events on population structure in such taxa; for example, Pleistocene glaciations in Europe (e.g., Taberlet *et al.*, 1998; Tribsch & Schönswetter, 2003) and North America (e.g., Brunsfeld *et al.*, 2001), Plio-Pleistocene rainforest contractions across eastern Australia (e.g., Moritz *et al.*, 2000; Hugall *et al.*, 2002; Moritz *et al.*, 2009), and the closure of the Isthmus of Panama (Knowlton *et al.*, 1993; Knowlton & Weigt, 1998). Of equal import, however, is the application of comparative phylogeography to investigating patterns of population structure in closely related taxa that are not co-distributed, to compare and contrast the response of such taxa to particular events throughout their evolutionary history (e.g., Albach *et al.*, 2006).

Two regions in which close relatives of *Echinocladius* are widely distributed, and thus may represent ideal areas in which to compare patterns of phylogeographic structure, are the South Island of New Zealand and southern South America. Both regions are enigmatic, tectonically active fragments of ancient Gondwana that share distinct histories of population fragmentation caused by Neogene mountain range orogeny and Plio-Pleistocene glaciation events. The major topographical feature of the South Island of New Zealand is the Southern Alps mountain range; formed as a result of convergence between the Australian and Pacific continental plates along the Alpine Fault from approximately 6mya (Walcott, 1998). Throughout this period, the South Island is thought to have experienced several glaciation events (Suggate, 1990; Newnham *et al.*, 1999; Suggate & Almond,

2005), including one during the late Pleistocene which covered the majority of the Southern Alps (Mercer, 1983; Suggate & Almond, 2005) and rendered the central South Island a vast tundra (see Trewick & Wallis, 2001). Southern South America shares a superficially similar geological and glacial history to that of the South Island of New Zealand; the subduction of the Nazca plate under the South American plate has driven at least two phases of tectonic uplift over the last 25 million years, initiated most recently in the late Pliocene (~3.5mya), giving rise to the Andes mountain range (Lagabrielle *et al.*, 2004). Additionally, since the initiation of tectonic uplift several glaciation events have covered much of the southern Andes (Mercer, 1983; Rabassa & Clapperton, 1990; Ehlers & Gibbard, 2007).

Biologically, the orogeny of both the Southern Alps and the Andes mountain ranges, in concert with Plio-Pleistocene glaciation events, are believed to have fragmented many populations and thereby dramatically affected the population structure of a diverse array of taxa. In New Zealand, distinct disjunctions have been observed in the distribution of several terrestrial and aquatic taxa, whereby populations of a given taxon are isolated roughly to the northwest (Northern South Island Province (NSIP): Leathwick *et al.*, 2007) and southeast (Southern South Island Province: SSIP) of the South Island, bisected by the Alps (Heads, 1998; Trewick & Wallis, 2001). Similarly, in southern South America, populations of some widespread taxa were bisected by the uplift of the Andes resulting in isolation and marked genetic structure across the mountain range (e.g., Marchelli & Gallo, 2006; Ruzzante *et al.*, 2006; Ramirez *et al.*, 2008; Gonzalez-Ittig *et al.*, 2010). In contrast, in other taxa the genetic signal of population fragmentation across the Andes appears to have been masked by subsequent, or perhaps coincident, Plio-Pleistocene glaciation events (e.g., Allnutt *et al.*, 1999; Palma *et al.*, 2002; Muellner *et al.*, 2005).

As such, both the South Island of New Zealand and southern South America represent ideal regions for testing hypotheses of the relative impact of historical population fragmentation on genetic structure in other Orthocladiinae taxa, as extrapolated from patterns revealed in *E. martini* in

the Australian Wet Tropics. Two such taxa, *Naonella forsythi* Boothroyd and *Ferringtonia patagonica* Edwards, inhabit cool shaded upland streams in New Zealand and southern South America, respectively (Boothroyd, 1994; Sæther & Andersen, 2010). Given their similar habitat preferences, along with shared morphological characters, these taxa are considered to be close relatives of *Echinocladius*. Furthermore, the disjunct distribution of these three genera across their respective austral continents has led researchers to consider them members of a proposed Gondwanan element within the Orthocladiinae subfamily.

1.4 Chironomid connections in Gondwanan biogeography

Purportedly ancient clades that exhibit disjunct austral distributions have been recognised within some subfamilies of the Chironomidae since the early 1960's (Brundin, 1963). Largely using larval and pupal morphology in applying the then newly developed phylogenetic theory (Hennig, 1965), Brundin (1965, 1966) reconstructed phylogenies for putative Gondwanan clades in the Aphroteniinae, Podonominae and Diamesinae subfamilies which showed consistent patterns of relationships among taxa found in Australia, South America and New Zealand. The inferred phylogenies suggested that New Zealand taxa always possessed a sister group in either South America or South America-Australia, but no direct phylogenetic connections between New Zealand and Australian species (Brundin, 1965, 1966). In addition, any Australian group was always the ‘derived’ sister group of an ancestral group in South America. Brundin argued that the phylogenetic patterns recovered represent conclusive evidence for two independent historical connections between the austral landmasses: New Zealand was connected to South America via west Antarctica, with Australia connected to South America via east Antarctica. Brundin (1966) suggested that vicariant evolution after the break-up of the Gondwanan supercontinent, rather than long-distance dispersal, acted as the major force that determined the modern distributions of disjunct austral chironomid groups on the southern continents.

Furthermore, the close correlation of relationships among taxa reported by Brundin (1966) with the geological consensus sequence for the fragmentation of Gondwana, suggests together that the inferred phylogenies for the austral taxa reflect simple vicariant evolution following the break-up of Gondwana.

The supercontinent Gondwana, which rifted from its northern sister Laurasia during the breakup of Pangaea ~215mya, comprised all current austral continents and minor landmasses: South America, Africa, Australia, Antarctica, Zealandia (formerly Tasmantis – New Zealand-New Caledonia), proto-New Guinea, India and Madagascar (Dietz & Holden, 1970; Scotese *et al.*, 1988; McLoughlin, 2001). Gondwana – proposed by some to have been of Precambrian origin (Scotese *et al.*, 1999) – apparently persisted for approximately another 50 million years after rifting off from Pangaea (Cracraft, 1974; Sanmartin & Ronquist, 2004) before fragmentation was initiated around 165mya (Scotese *et al.*, 1988). The final stage of Gondwanan fragmentation involved the rifting of South America from Antarctica approximately 30mya (Barker & Burrell, 1977; Lawver & Gahagan, 2003) and opened the Drake Passage. The expected phylogenetic pattern according to a simple vicariance history, and that which Brundin (1966) described for the three chironomid subfamilies he investigated, is thus that Africa forms a sister group to New Zealand, southern South America and Australia, with New Zealand then sister to southern South America and Australia.

Since Brundin's (1966) analysis, taxonomic descriptions and generic reappraisals of members of the more cosmopolitan Orthocladiinae have also suggested the presence of other disjunct clades of potential Gondwanan origin. Several described genera have been proposed to belong to one of these groups, including the Australian endemic *Echinocladius* (Cranston, 2000b); *Naonella* (Boothroyd, 1994) and *Tonnoirocladius* (Cranston, 2007) from New Zealand; *Ferringtonia* from southern South America (Sæther & Andersen, 2010); *Anzacladius* (Cranston, 2009) – shared by New Zealand and Australia; *Botryocladius* (Cranston & Edward, 1999) and *Limnophyes*

(Sæther, 1990a, b) – known from both Australia and South America; and *Pirara* – shared by Australia, New Zealand and South America (Boothroyd & Cranston, 1995; Cranston, 2000b). These disjunct austral taxa generally inhabit similar environments to the taxa investigated by Brundin (1966) – i.e., cool, pristine, often montane streams – and thus can be considered to represent a novel Gondwanan clade within the Orthocladiinae. Beyond these preliminary suggestions, however, there has been little attention given to resolving the phylogenetic relationships among the proposed Gondwanan orthoclad genera and the few studies that have been conducted have often produced contrasting outcomes (Cranston, 2000b, 2007; Sæther & Andersen, 2010). Thus, currently there is much confusion surrounding the phylogenetic relationships within this group of chironomid genera. Resolving these relationships is important as a comparison not only to morphology-based ideas of the evolutionary affinities of the Gondwanan Orthocladiinae taxa, but to the work of Brundin (1966) and the vast literature base concerning the historical effect of Gondwanan fragmentation on austral taxa. Furthermore, better understanding evolutionary relationships within and among genera will provide essential information for comparative phylogeographic investigations of relatives of *Echinocladius* from other austral continents. The data of Krosch *et al* (2009) can be used as baseline data for testing population structure in allied taxa to address this issue.

1.5 Specific hypotheses of the current study

Chapter Two incorporated data from Krosch *et al* (2009) to assess the relative roles of within and among stream gene flow and, hence, dispersal patterns of *E. martini* within the Paluma bioregion of north-eastern Australia. Expanding the intensity of sampling within the Paluma region to include sites both up- and downstream of the original sampling localities, along with sites in adjacent streams within the same catchment, allowed specific hypotheses regarding the pattern of genetic structure within and among streams to be tested. This fine-scale approach to evaluating levels of relative gene flow

within and among streams thus provided a more comprehensive assessment of *E. martini* population connectivity among streams in the Paluma range of north-eastern Queensland. Importantly, this study also provided information regarding the relative susceptibility of *E. martini*, and its Gondwanan relatives as a whole, to habitat fragmentation, in that if *E. martini* do not readily disperse among streams, their potential for colonising adjacent suitable habitat in response to habitat fragmentation is likely to be restricted.

Chapter Three compared population structure among closely related chironomid taxa that inhabit vastly different and geographically distant landmasses. This study aimed to evaluate the effect of population fragmentation and isolation on *Naonella forsythi* in the South Island of New Zealand and *Ferringtonia patagonica* in southern South America, as a test of predictions based on patterns of phylogeographic structure reported previously for Australian *E. martini*. Furthermore, specific hypotheses concerning the relative impacts of Neogene orogenic tectonic uplift and Plio-Pleistocene glaciation events in both regions were explored to provide novel insights into the evolutionary history of these taxa.

Chapter Four incorporated data from Krosch *et al* (2009) and expanded the geographical range of sampling to provide a broader representation of *E. martini* diversity across the described distribution in eastern Australia. Specifically, this included incorporating additional samples from southeast Queensland, New South Wales and Victoria to resolve patterns of divergence and endemism along the east coast of Australia more fully. The addition of new sites in these regions may help to resolve the anomalous placement of Victorian representatives with lineages from north of the BMC.

At the highest hierarchical level, Chapter Five reconstructed a molecular phylogeny for *E. martini* and its relatives considered to be members of a Gondwanan clade within the Orthocladiinae subfamily. Relevant taxa were collected from Australia, New Zealand and South America, in addition to representative samples obtained from Africa, in order to test Brundin's (1966) predictions of transantarctic sister-group relationships among members of the

austral chironomid fauna. Resolving the evolutionary relationships among the disjunct orthoclad genera allowed inferences to be made about the relative roles of fragmentation of the Gondwanan supercontinent and subsequent transoceanic dispersal in driving the historical radiation of this group across the southern continents. This provided an important comparison with both Brundin's early work on other chironomid subfamilies and the large body of evidence for circumantarctic sister-group relationships in other taxa. This investigation also complemented an existing broad-scale phylogenetic reconstruction of the entire Chironomidae family (Cranston, P. S., pers. comm.), by providing finer-scale resolution to inter- and intrageneric relationships among this subset of the Orthocladiinae subfamily. Resolution of inter-generic relationships among the Gondwanan orthoclads may also provide valuable insights for taxonomists about current taxonomic designations that may require reappraisal.

Taken together, the assessment of evolutionary relationships within and among taxa at the different spatio-temporal scales targeted by the current research aimed to encompass and evaluate the evolutionary history of *E. martini*, and the proposed Gondwanan Orthocladiinae clade as a whole, since initiation of the fragmentation of Gondwana. The overall aim of the study therefore was to reconstruct the evolutionary history of *E. martini* and its relatives since their diversification from a common ancestor and to investigate the processes that have subsequently influenced their phylogeographic structure. This study thus provided a more holistic understanding of the evolutionary history of these taxa, from contemporary dispersal patterns within and among streams, to the impacts of regional and continental scale population fragmentation and isolation on phylogeographic structure.

Chapter Two

Spatial population genetic structure reveals strong natal stream site fidelity in the non-biting midge *Echinocladius martini* Cranston (Diptera: Chironomidae) in northeast Queensland, Australia

2.1 Introduction

Evaluating the role of within and among stream movement of individuals in a riverine landscape is crucial to developing an understanding of stream ecology (Bohonak & Jenkins, 2003). Historically, freshwater habitats were considered to be ecological microcosms that exist in total isolation from the surrounding terrestrial environment and that all processes important in the continuity of such habitats occur below the waterline (Forbes, 1887). In hindsight, this view is clearly simplistic and we now know that freshwater and terrestrial habitats are intrinsically linked, from the nutrients and minerals passed from soils into streams to the contribution of terrestrial primary producers to the benthic food chain. This concept is encompassed by the aquatic-terrestrial flux framework, which describes the two-way interchange of nutrients and energy among the two systems (Gullan & Cranston, 2010). Aside from the movement of these crucial components, one of the most important factors governing the dynamics of populations of aquatic organisms is dispersal (Bohonak & Jenkins, 2003). Whilst the dispersal of most fish and other solely aquatic taxa is generally restricted to within the water column (with some notable exceptions), for those aquatic invertebrates that possess an adult flight stage, movement over land can be just as important in promoting and maintaining population panmixia as underwater processes such as larval drift or positive rheotaxis (Bohonak, 1999; Bilton *et al.*, 2001).

Invertebrate Dispersal

A considerable literature base exists concerning the range and extent of flight in semi-aquatic invertebrates that covers a wide variety of taxa from diverse habitat types (Harrison, 1980; Bilton *et al.*, 2001; Bohonak & Jenkins, 2003). Until recently, much of these data have come from trapping surveys that measured adult flight directly along streams and/or laterally away from streams (e.g., Kovats *et al.*, 1996; Collier & Smith, 1998; Winterbourn &

Crowe, 2001; Petersen *et al.*, 2004; MacNeale *et al.*, 2005). Estimating insect flight in this way, however, can have drawbacks, as trapping is labour-intensive, time-consuming and does not provide information regarding the genetic contribution, and thus gene flow, of effective dispersers to their new population.

Gene flow is generally held to be analogous to average dispersal over time (Bohonak, 1999), in that if individuals are regularly dispersing among sites, they have the potential to interbreed, resulting in gene flow among populations that can potentially homogenise genetic structure spatially (Slatkin, 1985; Ibrahim *et al.*, 1995; Bohonak, 1999). Alternatively, if populations are independent and do not interbreed, a lack of gene flow may result in significant genetic structuring among populations (Slatkin, 1985; Ibrahim *et al.*, 1995; Bohonak, 1999). Indeed, analysing patterns of genetic structure among populations has allowed inferences of the rates of dispersal in a wide variety of aquatic invertebrates, including, for example, caddisflies (Bunn & Hughes, 1997; Baker *et al.*, 2004; Schultheis & Hughes, 2005; Pauls *et al.*, 2006; Smith *et al.*, 2006b), mayflies (Bunn & Hughes, 1997; Hughes *et al.*, 2000; Baker *et al.*, 2004; Smith *et al.*, 2006a), phantom midges (Berendonk & Spitze, 2006), blackflies (Finn & Adler, 2006), stoneflies (Hughes *et al.*, 1999; Schultheis *et al.*, 2002), net-winged midges (Wishart & Hughes, 2003), gastropods (Miller *et al.*, 2006), shrimp (Hurwood *et al.*, 2003), water pennies (Miller *et al.*, 2002) and water bugs (Miller *et al.*, 2002) amongst many others.

Models of population genetic structure in riverine systems

In taxa that exhibit significant spatial genetic structure, discrete patterns can be observed in the partitioning of genetic diversity within and among streams. The classic 'Stream Hierarchy Model' describes one such pattern of genetic structuring where structure is lowest at the smallest spatial scale (within streams) and greatest at the largest spatial scale (among river catchments) (Meffe & Vrijenhoek, 1988). This is an important concept for studies of many

freshwater stream taxa, as stream hierarchy often plays a major role in the distribution of genetic diversity in species that possess low vagility, including some shrimp (Hughes *et al.*, 1995), fish (Hughes & Hillyer, 2006), mayflies (Smith & Collier, 2001; Smith *et al.*, 2006a) and caddisflies (Miller *et al.*, 2002; Smith *et al.*, 2006b).

Even for taxa with comparatively high dispersal capacity, alternative models that can describe their genetic structure within and among streams can be generated. Thus the 'headwater model' was based on patterns of genetic structure among mountain-top populations of the giant water bug *Abedus herberti* Hidalgo (Hemiptera: Belostomatidae) in Arizona, USA (Finn *et al.*, 2007). Populations were isolated in headwater streams by unsuitable lowland habitat and streams from different catchments were located geographically proximate in the same forest block. Often headwater streams from a single catchment were located in several mountain forest blocks. Thus, stream distance among headwater streams was inordinately large, while flight distance was often much reduced. The study demonstrated that genetic structure was partitioned according to forest block, irrespective of stream catchment, implying that movement was restricted only to patches of suitable habitat and thus was dominated by overland dispersal rather than movement in or along the stream reach (Finn *et al.*, 2007).

A different general pattern of population structure has been described from some tropical and sub-tropical systems where adult emergence of aquatic insects is often asynchronous and adult longevity generally limited, potentially resulting in cohorts that are isolated temporally. Thus, any given life stage may represent the progeny of only a few mating events, resulting in a distinct pattern of genetic structure whereby higher divergence is observed at smaller spatial scales due to the effects of genetic drift and lower divergence at larger spatial scales where genetic drift effects are balanced by larger sampling outcomes. This pattern has been formalised as the 'patchy recruitment' model (Bunn & Hughes, 1997), and has been reported for populations of several aquatic insect taxa (e.g., Schmidt *et al.*, 1995; Hughes

et al., 1998; Hughes *et al.*, 2000; Rebora *et al.*, 2005; Schultheis & Hughes, 2005).

Gene flow and dispersal among populations of chironomid midges

To date, few studies have investigated the relative rates of gene flow, and thus effective dispersal, among populations of chironomids. Studies on *Axarus varvestris* (Werle, 2005) and *Chironomus stigmaterus* Say (Hilburn, 1980) in large North American river systems using chromosomal techniques have hinted at the occurrence of some small-scale, generally downstream, gene flow and isolation by distance effects among populations. Similarly, intertidal rock pool populations of European *Clunio marinus* exhibit strong genetic differentiation among fragmented coastal habitats (Kaiser *et al.*, 2010). Populations of taxa in the genus *Austrochlus* Cranston (then *Archaeochlus*), that are restricted to freshwater seeps in Western Australia also exhibit little to no gene flow among populations (Martin *et al.*, 2002). It is evident, therefore, that the geographical range and extent of chironomid gene flow and dispersal requires further study, which would permit broader inferences regarding both the evolutionary and life history of this group.

The sampling design employed in the current study was informed by the results of Krosch *et al* (2009) and greatly expanded the intensity of sampling of *E. martini* populations within the Paluma region to include sites both up- and downstream of the original sampling localities, in addition to sites on separate streams in the same catchment. By employing such a finely detailed sampling design, population genetic structure within and among stream channels was assessed and particular hypotheses concerning the pattern of genetic structure, such as the ‘Stream Hierarchy Model’, were tested. In light of previous evidence, it was expected that *E. martini* populations would largely conform to the ‘Stream Hierarchy Model’ with some gene flow occurring within a stream via downstream larval drift, but with little gene flow evident among streams. Sampling *E. martini* populations in this region at greater geographical intensity and with higher sample sizes was also

expected to resolve genetic diversity within and among sites more fully. Feasibly, this could have included less divergence among streams, implying higher levels of gene flow than was inferred in the earlier study of Krosch *et al* (2009).

Importantly, rainforest assemblages in this region are considered to have remained relatively stable during the dramatic fluctuations in climate that have driven biotic evolution across the Australian continent since the mid-Miocene (Truswell, 1993; Martin, 2006) and particularly in the Wet Tropics throughout the Plio-Pleistocene (Nix & Switzer, 1991; Kershaw *et al.*, 1993). This suggests that any genetic structuring observed among *E. martini* populations has arisen as a consequence of intrinsic physiological or behavioural factors, rather than extrinsic environmental factors. Taken together, the Paluma region represents an ideal model system for investigating the geographical range and extent of chironomid dispersal.

2.2 Methods

Study sites

The two sites of Krosch *et al* (2009) located on Little Birthday and Birthday Creeks (Sites 1 and 6, here) were used as the focal point for the sampling design employed here and additional sites along both stream reaches were also sampled (Table 2.1, Figure 2.1). DNA sequence data from Sites 1 and 6 are accessible under the GenBank Accession Numbers EU670019-EU670033 and were supplemented with additional sampling from these sites. Sites along Birthday Creek were sampled both up- and downstream of the Birthday Creek Falls to assess the effect of this potential dispersal barrier on population genetic structure. In total, four sites were sampled along Little Birthday Creek and four along Birthday Creek, one downstream of the Birthday Creek Falls and three upstream. One site was sampled from the point at which Little Birthday and Birthday Creeks coalesce (henceforth

Table 2.1. Geographical location of sample sites. Site codes match those in Figure 2.1. '*' denotes sites sampled by Krosch *et al* (2009) and sample sizes in parentheses indicates the number of individuals included from their study. Elevation is given as metres above sea level (masl).

Site Name	Site Code	Latitude	Longitude	Elevation (masl)	Sample Size
Little Birthday Creek	1*	18˚58'22"S	146˚09'54"E	850	24(21)
	2	18˚58'24"S	146˚09'45"E	840	23
	3	18˚58'24"S	146˚09'38"E	820	23
	4	18˚58'24"S	146˚09'30"E	790	24
Birthday Creek	5	18˚58'56"S	146˚10'00"E	805	18
	6*	18˚58'49"S	146˚09'50"E	800	31(22)
	7	18˚58'38"S	146˚09'45"E	795	20
	8	18˚58'35"S	146˚09'26"E	760	7
Confluence	9	18˚58'22"S	146˚09'23"E	750	24
Echo Creek	10	18˚59'30"S	146˚09'23"E	750	19

referred to as the 'Confluence'). An additional stream – Echo Creek – which joins with Birthday Creek approximately 3km downstream of the Confluence was also sampled as a reference point and to improve the geographical resolution of the sampling design. Newest site pairs range from 0.19-2.04km apart by Euclidean distance.

Sample collection

Sampling took place in September 2008 and 2009 and involved kick sampling using a 0.9mm x 0.3mm funnel-tapered polyester sweep net and removal by hand of entire leaf packs from riffle sections of each stream site. Instream microhabitats were targeted based on ecological data for *E. martini* which suggests a preference for riffle sections containing trapped organic matter (Cranston, 2000b). Bulk samples were strained through first a coarse grade (~1mm) then a fine grade (~0.2mm) sieve to remove coarse particulate organic matter while retaining chironomid larvae. Samples were transferred immediately to 90% ethanol for long-term storage before transport to Queensland University of Technology, Brisbane, Australia. Chironomid larvae

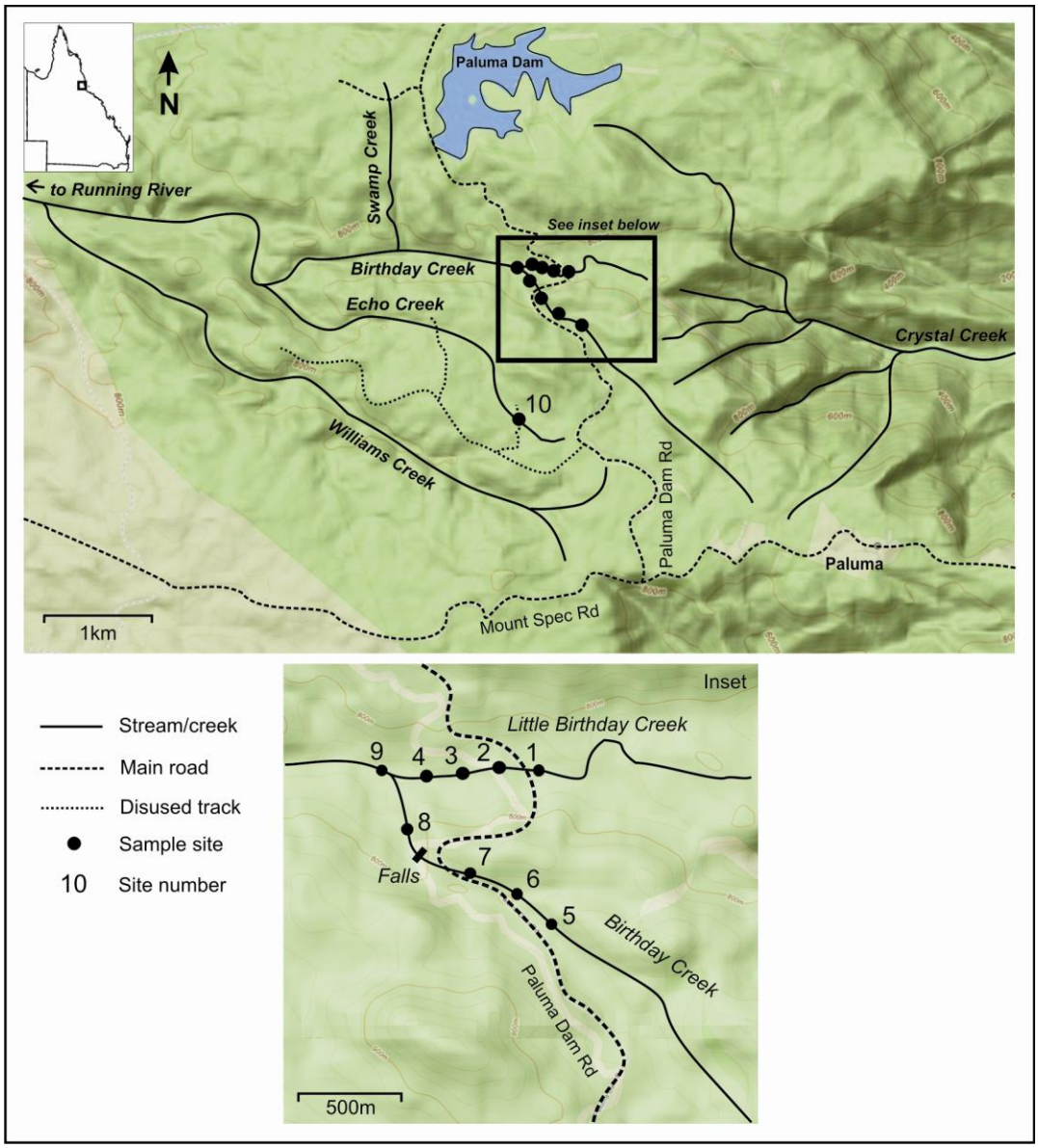


Figure 2.1. Geographical location of sample sites. Site numbers follow Table 2.1.

were removed from bulk samples by eye under low power magnification and head capsules of individual larvae were slide mounted using Hoyer's mountant (van der Meer, 1977) to clear soft tissue. Species identification was subsequently confirmed via examination of head capsule morphology under 400X magnification and cross-examination against a current Lucid Key for the Chironomidae developed by Prof. Peter Cranston. Body sections were stored at 4˚C in 100% ethanol prior to genomic DNA extraction.

Genetic procedures

Total genomic DNA was extracted from larval tissue using the Qiagen DNeasy® extraction kit (Qiagen, Hilden, Germany), following the manufacturer's guidelines. A 639bp fragment of the cytochrome *c* oxidase subunit I (COI) gene was amplified using universal invertebrate COI primers LCO1490 (5'GGT CAA CAA ATC ATA AAG ATA TTG G 3') and HCO2198 (5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3') (Folmer *et al.*, 1994). The COI gene was used for this study as it is fast-evolving and universal primers have been developed, thus rendering it an optimal marker for intraspecific population analysis (Avise, 1986; Moriyama & Powell, 1997). Each reaction tube contained 2 µL of template DNA from extractions, 0.5 µL of each primer (10pmol/µL – manufactured by Geneworks, Adelaide, Australia), 2.5 µL of 10X polymerase buffer (Roche, Mannheim, Germany), 2.0 µL of 25 mM $MgCl_2$ (Fisher, Perth, Australia), 1.0 µL of 10 mM dNTP's (Roche), 0.5 µL of 99% dimethyl sulphoxide (DMSO) (ICN Biomedicals, Inc., Irvine, USA), 0.2 µL of 5U/µL *Taq* polymerase (Roche) and were adjusted to a final volume of 25 µL with dH_2O. The PCR cycle protocol involved initial denaturing at 94˚C for 4 minutes followed by 10 cycles of 94˚C for 30 seconds, 40˚C for 30 seconds and 72˚C for 40 seconds. This was then followed by 25 cycles of 94˚C for 30 seconds, 55˚C for 30 seconds and 72˚C for 40 seconds, before final extension at 72˚C for 10 minutes. Total PCR product was then purified using an UltraClean™ PCR Clean-up Kit (MoBio, Carlsbad, USA) following manufacturer's guidelines. Purified PCR products were amplified using a standard ABI Big Dye® Terminator v.3.1 sequencing protocol and product was cleaned using a standard isopropanol precipitation protocol prior to sequencing in the forward direction at the Griffith University DNA Sequencing Facility (Nathan, Australia). Representative samples were sequenced in the reverse direction to ensure the veracity of the sequence data.

Data analyses

COI sequences were aligned and edited by eye using BioEdit Version 7.0.5 (Hall, 1999). Tests for sequence saturation (an indicator of potential homoplasy) were conducted by calculating the mean ratio of transitions to transversions in MEGA Version 4.0 (Tamura *et al.*, 2007) and by plotting the relationship of transitions and transversions to genetic distance in DAMBE (Xia & Xie, 2001). Tajima's D tests of neutrality were estimated both for each site and for the total dataset using coalescent simulations in DnaSP Version 5.0 (Librado & Rozas, 2009) to determine if sequences were evolving neutrally. Gene diversity (equivalent to expected heterozygosity) and the population parameter, θ_{π}, were calculated using Arlequin Version 3.11 (Excoffier *et al.*, 2005) to estimate genetic diversity within sites. A haplotype network was constructed using a method of statistical parsimony in TCS Version 1.21 (Clement *et al.*, 2000) with a connection limit of 95%. Population expansion was tested using the *g* exponent of population growth as calculated in Lamarc 2.0 (Kuhner, 2006) and by plotting the mismatch distribution (Rogers & Harpending, 1992) of expected and observed frequencies of pairwise differences at each sample site using DNAsp. This included estimating values of θ before ($\theta_{initial}$) and after (θ_{final}) population expansion (Rogers & Harpending, 1992), the rate of expansion (τ) (Rogers, 1995) and the raggedness statistics r (Harpending, 1994) and R_2 (Ramos-Onsins & Rozas, 2002) which relate to the smoothness of the observed distribution of pairwise differences.

Several different methods for partitioning genetic variation among sites were implemented to explore the range and extent of within and among stream dispersal. Exact tests of genetic differentiation ($P<0.05$) based on haplotype frequencies and conventional among-site Φ_{ST} indices ($P<0.05$) incorporating the Tamura-Nei model of evolution (Tamura & Nei, 1993) were estimated in Arlequin. The Tamura-Nei model of evolution is considered to be the most appropriate model for this analysis as it is more sensitive to different substitution patterns than other models implemented in Arlequin (Tamura &

Nei, 1993). Hierarchical analysis of molecular variance (AMOVA: Excoffier *et al.*, 1992) were computed in Arlequin based on Φ_{ST} estimates. AMOVA allows the pooling of data to test particular *a priori* site groupings, for example, the 'Stream Hierarchy Model', while statistical significance was obtained through 1000 random permutations. Nei's genetic chord distance (D_A) (Nei *et al.*, 1983) was estimated among sites using Jukes-Cantor correction (Jukes & Cantor, 1969) in DNAsp and the resulting distance matrix was used to construct an unrooted neighbour-joining population tree in Neighbor (in PHYLIP Version 3.69 - Felsenstein, 1989). The genetic chord distance measure of Nei *et al* (1983), D_A, is considered to be more accurate at estimating population phylogenies than other standard distance measures (Takezaki & Nei, 1996). Incorporating a conservative mutation model (i.e., Jukes-Cantor correction) can reduce bias towards high divergence among certain population pairs sometimes encountered with D_A distance estimates. Corrections for multiple tests were not undertaken here, in accordance with recent concerns regarding their appropriateness for ecological data in which the statistical signal in the data is often subtle and thus potentially obscured by over-conservative alpha corrections (Cabin & Mitchell, 2000; Moran, 2003; Garcia, 2004).

In continuous populations it is possible that, even in the absence of barriers to gene flow, the level of connectivity among populations will decrease as geographical distance increases, referred to as 'Isolation by Distance' (IBD) (Wright, 1943). IBD effects were examined by testing the null hypothesis of no correlation between geographical and genetic distances among sites. Adult flight and larval drift hypotheses for dispersal were tested using straight line and stream distances, respectively. Geographical distances were estimated using the 1:100 000 topographical map for Ingham produced by the Royal Australian Survey Corps in 1986. Mantel tests (Mantel, 1967; Smouse *et al.*, 1986) were implemented in Arlequin to test for correlation between $\log_{10}$-standardised geographical distance and Φ_{ST}, while statistical significance was obtained through 1000 random permutations.

The software package Streamtree (Kalinowski *et al.*, 2008) was used to infer the relative genetic distance represented by each stream section based on a matrix of pairwise Φ_{ST} indices. This algorithm considers the pairwise Φ_{ST} estimates among all sites connected by a given stream section and assigns a genetic distance to each stream section accordingly. Statistical support was provided by calculation of the R^2 coefficient of determination – an estimate of the goodness of fit of the data to the Streamtree model. This algorithm can be used to infer groups of populations and test hypotheses for potential effects of possible instream barriers to gene flow, such as waterfalls.

2.3 Results

In total, 169 new individuals were sequenced from 10 sites, in addition to the 44 individuals available from the previous study of Krosch *et al* (2009) (Table 2.1). This resulted in a total dataset of 213 individuals, representing 58 unique COI haplotypes of 639bp in length. A further site located in the headwaters of the Crystal Creek drainage was sampled to test for differences among catchments, but the only *E. martini* individual collected was a member of a different mitochondrial lineage (see Chapter 4) and thus inappropriate for inclusion here. The ratio of transitions to transversions was 3.697 and the relationship of each substitution type to genetic distance did not show evidence of a plateau (Figure 2.2). This suggests that sequence saturation has not yet been reached and thus the observed genetic diversity is an accurate representation of 'true' diversity (Arbogast *et al.*, 2002).

Population genetic summary statistics

Measures of within-site genetic diversity suggested that populations were highly diverse and the Tajima's D test of neutrality was non-significant for the total dataset (D = -1.68918, p = 0.1000). When tested separately however, two sites (1 and 10) produced statistically significant results (Table 2.2). This

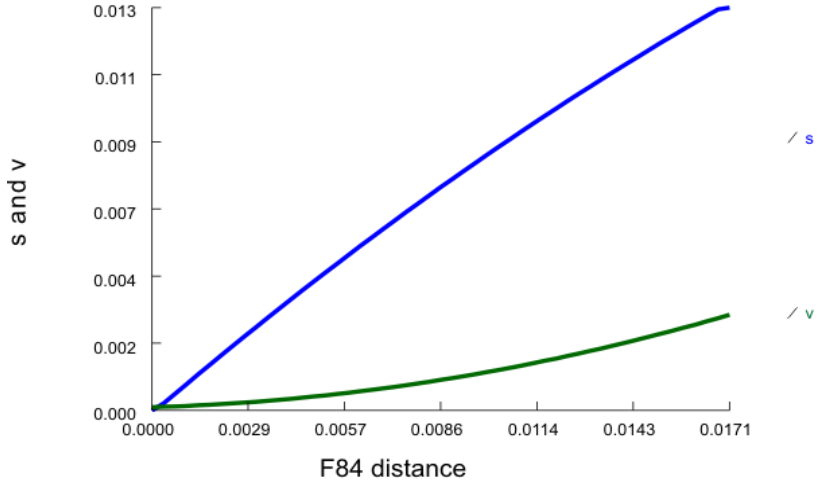


Figure 2.2. The relationship of transitions (s) and transversions (v) to genetic distance.

Table 2.2. Population genetic summary statistics. Values in bold are statistically significant.

Site	Sample size	Tajima's D	D p-value	θ_π	Gene Diversity	# of Haplotypes
1	24	-1.55696	**0.04200**	3.09728	0.7101 +/- 0.1006	10
2	23	-0.58991	0.32400	4.12662	0.8696 +/- 0.0505	11
3	23	-0.84465	0.20700	3.99622	0.8814 +/- 0.0498	12
4	24	-0.62995	0.30900	3.32869	0.8370 +/- 0.0546	10
5	18	-0.51349	0.30900	4.06581	0.8627 +/- 0.0609	9
6	31	0.52479	0.75800	4.68223	0.7720 +/- 0.0618	12
7	20	0.86896	0.84900	4.59623	0.7421 +/- 0.0705	7
8	7	-0.10732	0.47600	4.04046	0.9048 +/- 0.1033	5
9	24	-1.25623	0.10200	3.35691	0.9239 +/- 0.0382	14
10	19	-2.34963	**0.00000**	2.63054	0.8772 +/- 0.0737	13

may be due to large negative Tajima's D values driven by an excess of low frequency haplotypes, possibly indicating recent population expansion or the effects of purifying selection (Excoffier *et al.*, 1992). The *g* exponents estimated for all sites as specific tests of population expansion were all large and positive, suggesting all sites may have undergone dramatic growth. Only Sites 9 and 10, however, produced reliable results as shown by confidence intervals not encompassing zero [Site 9: *g* = 822 (107.8-991.9); Site 10: *g* = 869 (130.1-1002.6)]. Mismatch distribution plots of the frequency of pairwise

differences at sample sites indicated by other analyses to have undergone population growth show only Site 10 to possess the signature of such demographic events (r = 0.0216; R_2 = 0.0666 – Figure 2.3). The multimodal distribution of pairwise difference frequencies at all other sites suggests stable population sizes over time (r = 0.0180; R_2 = 0.0344) (Slatkin & Hudson, 1991; Rogers & Harpending, 1992).

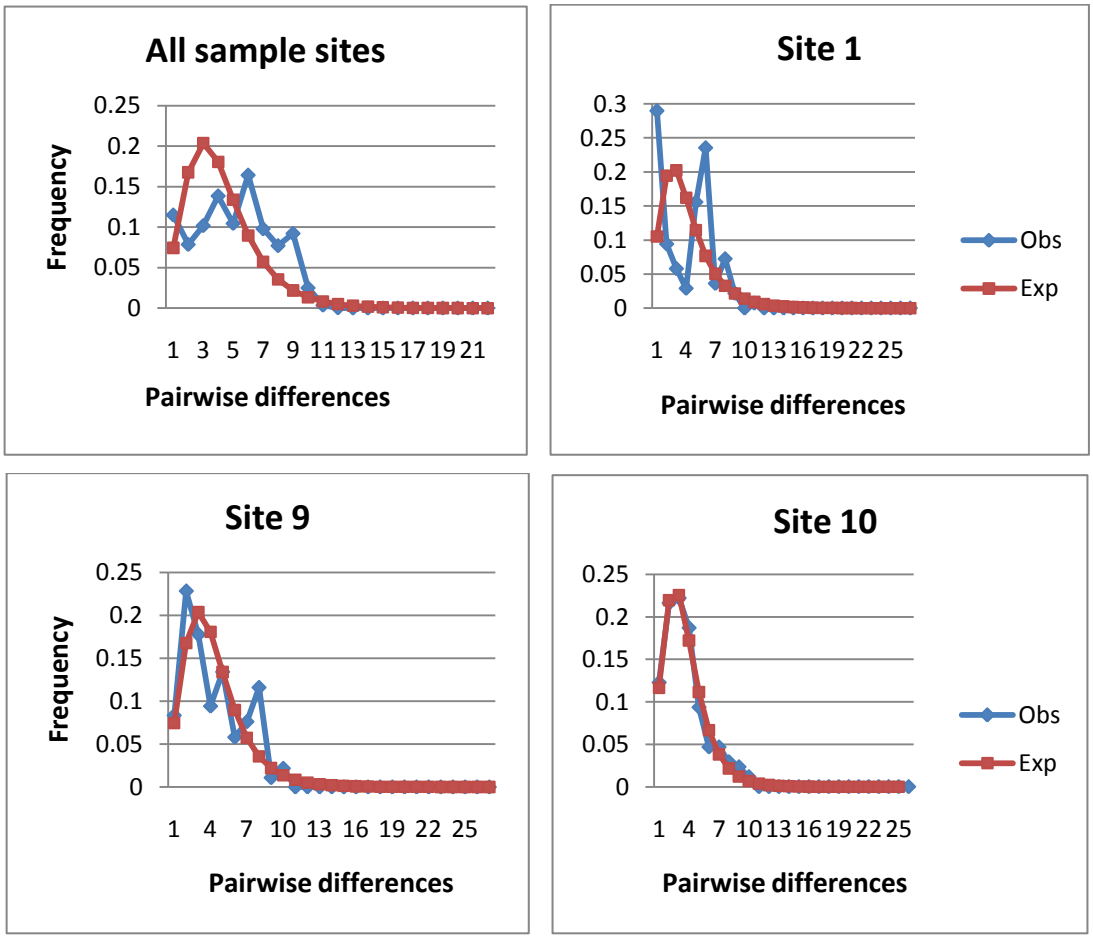


Figure 2.3. Mismatch distribution plots of frequencies of pairwise differences across all sample sites and at Sites 1, 9 and 10 for which other analyses suggested historical population expansion ($\theta_{initial}$ = 1.587, θ_{final} = 1000, τ = 1.655).

Among-site partitioning of genetic diversity

The different methods for partitioning genetic divergence within and among sites produced generally consistent results. Exact tests of differentiation and estimates of Φ_{ST} suggested broadly that the three streams sampled here were significantly different from each other, in that sites along a particular

stream reach did not differ significantly from others along the same stream, but in most instances were significantly different to sites on the other streams (Table 2.3). The most downstream site along Birthday Creek (Site 8) did not differ significantly from any other site except Site 1, whereas its equivalent site on Little Birthday Creek (Site 4) was significantly different from Sites 5, 6, and 7 on Birthday Creek and Site 10 on Echo Creek. This may be due to the low sample size obtained at Site 8 (n=7). The Confluence site (Site 9) appeared to be slightly less divergent from Birthday Creek than Little Birthday Creek, while Site 10 on Echo Creek was significantly different from all sites except Sites 8 and 9, implying some degree of female movement between these sites. Hierarchical AMOVA tests with sites from each of the three streams partitioned in separate groups and the Confluence site in a fourth group, indicated that the majority of variation in *E. martini* populations was present within sites (86.58%), whilst variation among groups was 13.21% and among populations within groups was 0.22%. The unrooted neighbour-joining topology based on Nei's D_A genetic chord distances also supported Little Birthday and Birthday Creek sites forming two separate groups sister to each other, with the Confluence and Echo Creek sites separate (Figure 2.4).

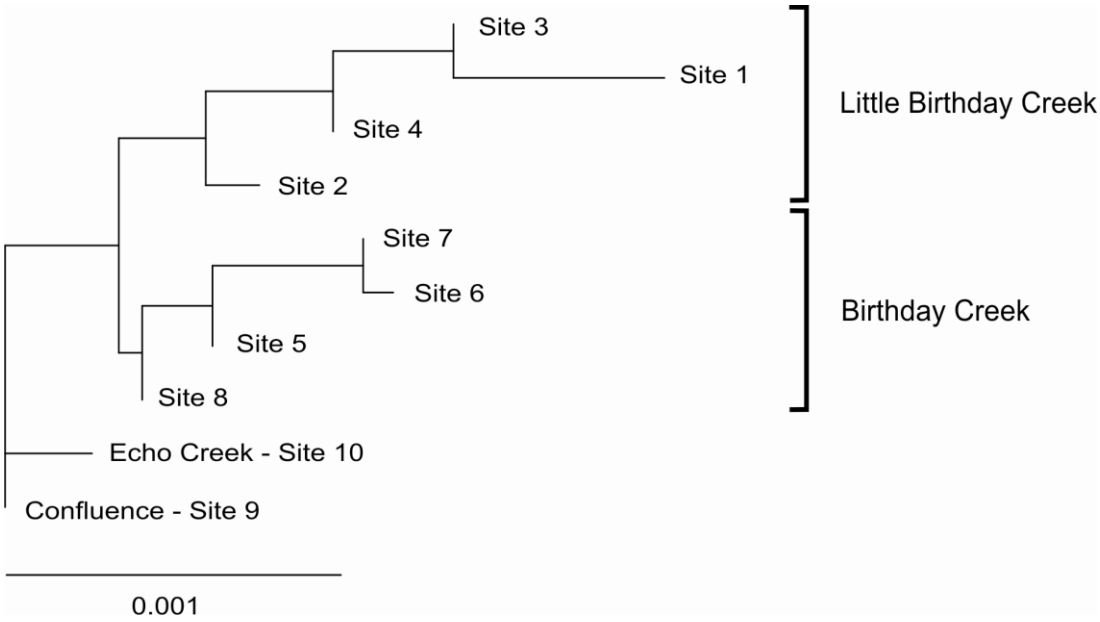


Figure 2.4. Unrooted neighbour-joining topology based on Nei's D_A genetic chord distances among sites.

Table 2.3. Below diagonal shows conventional Φ_{ST} estimates among sites - values in bold represent significant pairwise comparisons (P<0.05). Values boxed in solid lines represent among site Φ_{ST} estimates within Little Birthday Creek (Sites 1-4) and Birthday Creek (Sites 5-8); values boxed in dashed lines represent Φ_{ST} estimates among the two creeks. Pairwise comparisons which produced significant exact test estimates (P<0.05) are denoted by an '*'. Above diagonal shows Nei's D_A indices among sites.

	1	2	3	4	5	6	7	8	9	10
1	-	0.00250	0.00008	0.00020	0.00172	0.00232	0.00185	0.00116	0.00144	0.00201
2	0.04200	-	-0.00018	-0.00006	0.00066	0.00095	0.00055	-0.00002	0.00061	0.00123
3	0.01476	-0.02776	-	-0.00016	0.00063	0.00114	0.00071	0.00009	0.00051	0.00102
4	0.03732*****	-0.01073	-0.02709*	-	0.00044	0.00114	0.00073	-0.00002	0.00030	0.00068
5	**0.23541***	**0.09200***	**0.08941***	**0.07107***	-	0.00024	0.0000	-0.00038	0.00003	0.00036
6	**0.26139***	**0.11438***	**0.13709***	**0.14577***	0.03142	-	-0.00023	0.00011	0.00111	0.00190
7	**0.23566***	0.07379*****	**0.09500***	**0.10545***	-0.00054	-0.03109	-	-0.00022	0.00065	0.00137
8	**0.19073***	-0.00533	0.01503	0.00653	-0.06280	0.00596	-0.03774	-	-0.00024	0.00009
9	**0.21810***	**0.09214***	**0.07984**	0.05250*****	0.00628	**0.14088***	**0.09497***	-0.03678	-	0.00003
10	**0.30042***	**0.17923***	**0.15728***	**0.12142***	**0.06358**	**0.22683***	**0.18956***	0.03938	0.00458	-

Mantel tests of isolation by log_{10}-adjusted distance were significantly different from the null hypothesis of no correlation between geographical and genetic distance among sites for both stream distance (R = 0.543, p = 0.001) and Euclidean distance (R = 0.475, p = 0.003) (Figure 2.5). This result was consistent when Echo Creek (Site 10), the most geographically distant site, was removed for both geographical distance measures (stream: R = 0.073; p = 0.000; Euclidean: R = 0.464, p = 0.011). The two geographical distance measures were used because some sites within a stream were further apart by Euclidean distance, but did not show significantly different Φ_{ST} estimates, compared with some site pairs across streams that were significantly different. These data in concert, suggest that gene flow both within and among stream channels may be limited by geographical distance among sites.

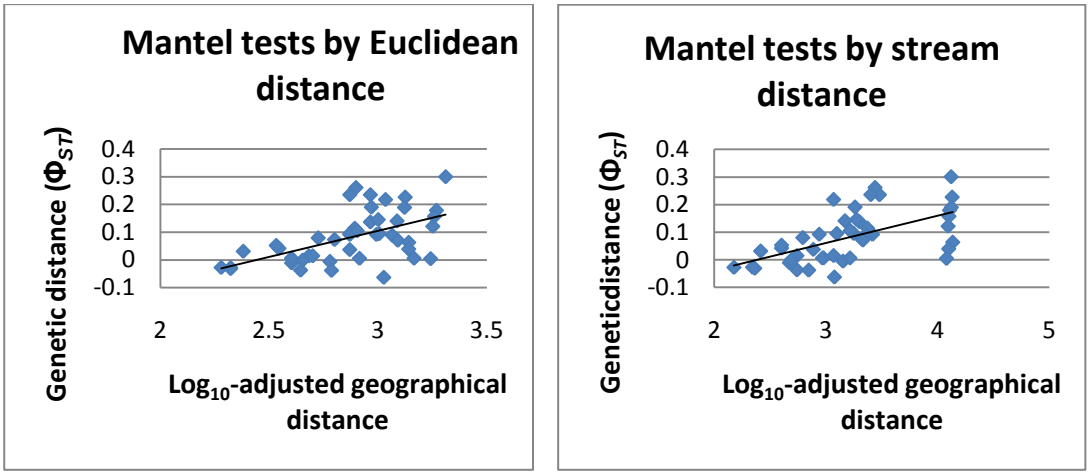


Figure 2.5. Scatterplots showing the relationship between genetic distance (Φ_{ST}) and both Euclidean and stream distance for all pairwise site comparisons.

Partitioning of genetic distances to each stream section followed expectations based on pairwise Φ_{ST} estimates (Figure 2.6), however the statistical support for the fit of the data to the Streamtree model was quite low (R^2 = 0.708). This may be due to the inability of the Streamtree algorithm to account properly for non-stepping stone patterns of differentiation or for highly differentiated populations, both of which can result in the underestimation of genetic distance between sites (Kalinowski *et al.*, 2008). The stream sections that were assigned the greatest genetic distances were between Sites 1 and 2, 4 and 9, 10 and 9 and 8 and 7. The stream section

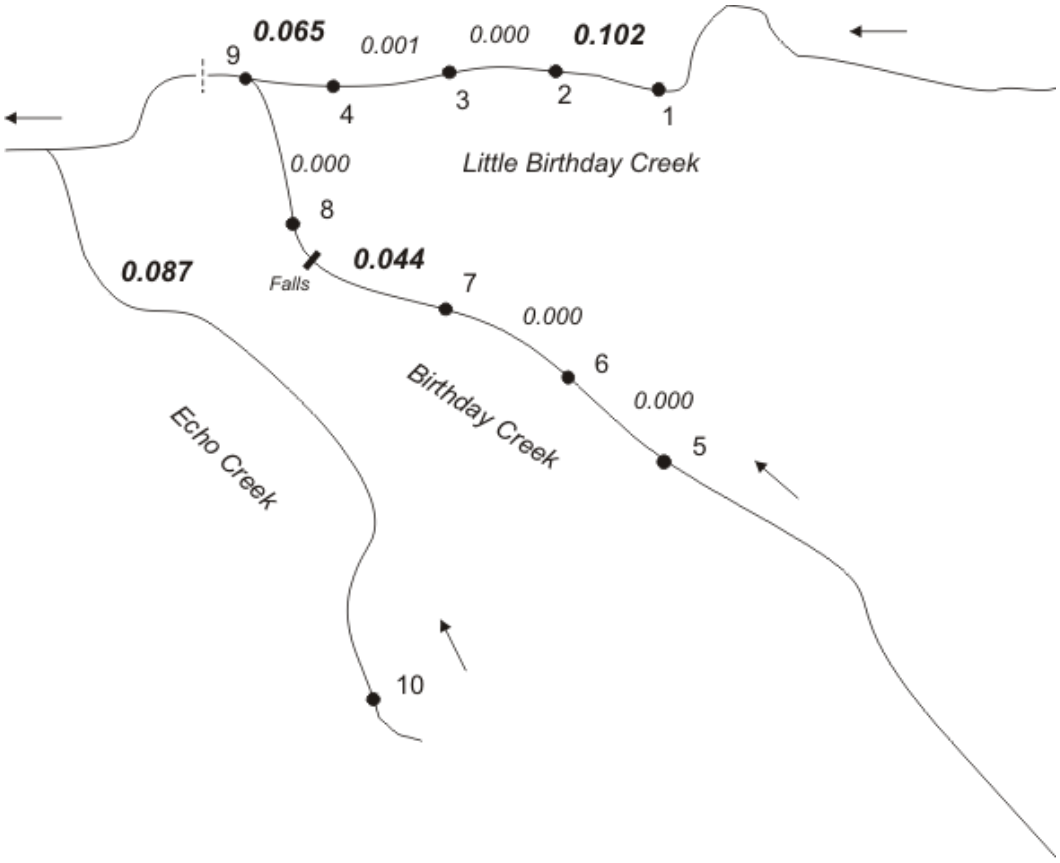


Figure 2.6. Schematic representation of the genetic distance assigned to each stream section (values in italics); i.e., genetic distance of 0.102 assigned to the stream section between Sites 1 and 2. Values in bold indicate stream sections assigned large genetic distances. Direction of stream flow is indicated by arrows.

between Sites 8 and 7 corresponds to the location of the Birthday Creek Falls, an obvious potential barrier to upstream gene flow. Similarly, the stream section that separates Site 10 from all other sites was geographically quite large (~12km) which could have contributed to generally high genetic differentiation among sites.

The genetic distance assigned to the stream section between Sites 4 and 9 likely reflects the relative genetic closeness of the Confluence site to Birthday Creek compared to Little Birthday Creek, implying some impediment to movement between sites on Little Birthday Creek and all other sites. The high genetic distance assigned to the stream section connecting Site 1 and all other sites possibly results from the non-stepping stone pattern of Φ_{ST} estimates among Little Birthday Creek sites, but may also indicate a potential instream barrier to gene flow. Genetic distances assigned to each stream section did not relate to stream gradient (refer to elevation – Table 2.1); for

example, the two sections separating Sites 2, 3 and 4 (the steepest across the study area) and the three sections between Sites 5, 6 and 7 and 8 and 9 (the shallowest across the study area) were all assigned minimal or no genetic distance.

Evolutionary relationships among haplotypes

The haplotype network presented here (Figure 2.7) has revealed additional shared and unique haplotypes compared with the earlier study of Krosch *et al* (2009). The more geographically intensive sampling of the current study has revealed four very common (n>30 individuals) haplotypes shared among sites and an additional eleven shared haplotypes present at lower frequencies (n≤6). One of the four common haplotypes (Haplotype 3) was shared across all three creeks at similar frequencies; indeed, all sites except Site 1 possessed this haplotype. The network also showed a starburst radiation pattern of haplotypes from Haplotype 3, and this, combined with its widespread distribution across sites implies that this may be the ancestral type (Castelloe & Templeton, 1994; Clement *et al.*, 2000). Coalescent theory suggests that shared internal haplotypes represent the ancestral state and that haplotypes radiating from these are more derived. Similarly, shared haplotypes at the edges of the network (tip haplotypes) are considered to represent contemporary gene flow.

Interestingly, all but two haplotypes sampled from Echo Creek (Site 10) other than Haplotype 3 itself were linked directly to this common type, implying colonisation predominantly by individuals of Haplotype 3 followed by subsequent diversification. A similar pattern is observed at Site 6 in the radiation of unique haplotypes from the common shared Haplotypes 1 and 4. The distribution of Haplotype 1 matched that of Haplotype 3, although it was present in much lower frequencies in Little Birthday and Echo Creek sites than in Birthday Creek. It also did not exhibit the same profusion of radiating haplotypes as that for Haplotype 3. In contrast, Haplotype 2 appears highly restricted in distribution, occurring only in Little Birthday Creek and the

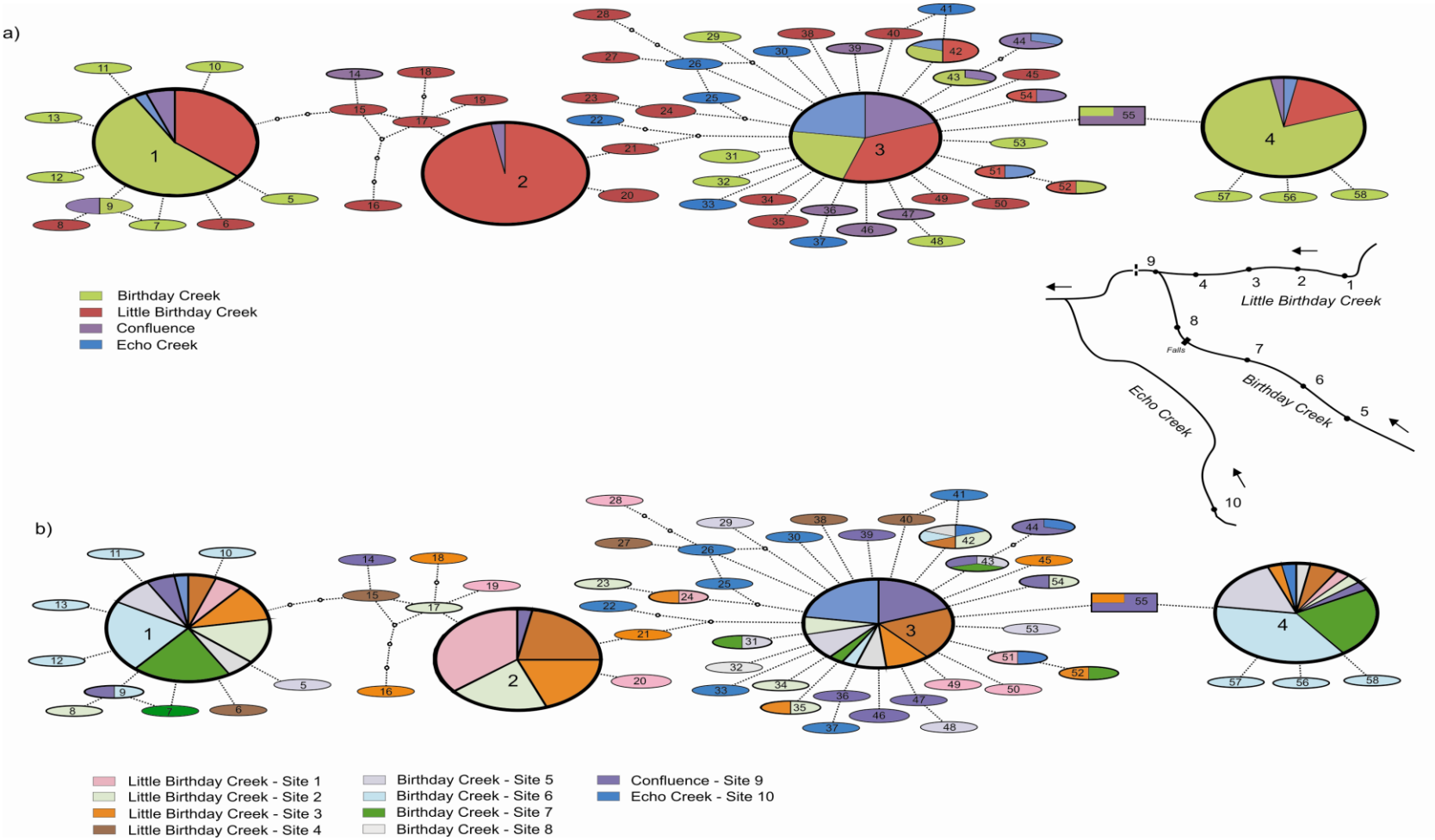


Figure 2.7. COI haplotype network coloured by a) creek of origin (with the Confluence separate), and b) by site of origin. Sizes of nodes and pie segments are proportional to haplotype frequency. Unshaded circles represent unsampled hypothetical haplotypes. Haplotypes are numbered arbitrarily. Schematic map of study regions shows the geographical location of study sites; arrows indicate the direction of stream flow.

Confluence site. Similarly, Haplotype 4, whilst recovered from all sites, occurs at much higher frequency in Birthday Creek sites than in all other sites, implying some degree of historical restriction but with limited movement away from the stream.

Visualising the geographical distribution of shared haplotypes provided a different perspective for determining patterns of gene flow among sites (Figure 2.8). In particular, and as discussed above, the distinct differences in the distributions and frequencies of the four common shared haplotypes among sites imply that individuals in Little Birthday and Birthday Creeks may not readily intermix, but that some degree of movement among streams has occurred. The majority of low frequency shared haplotypes were present at

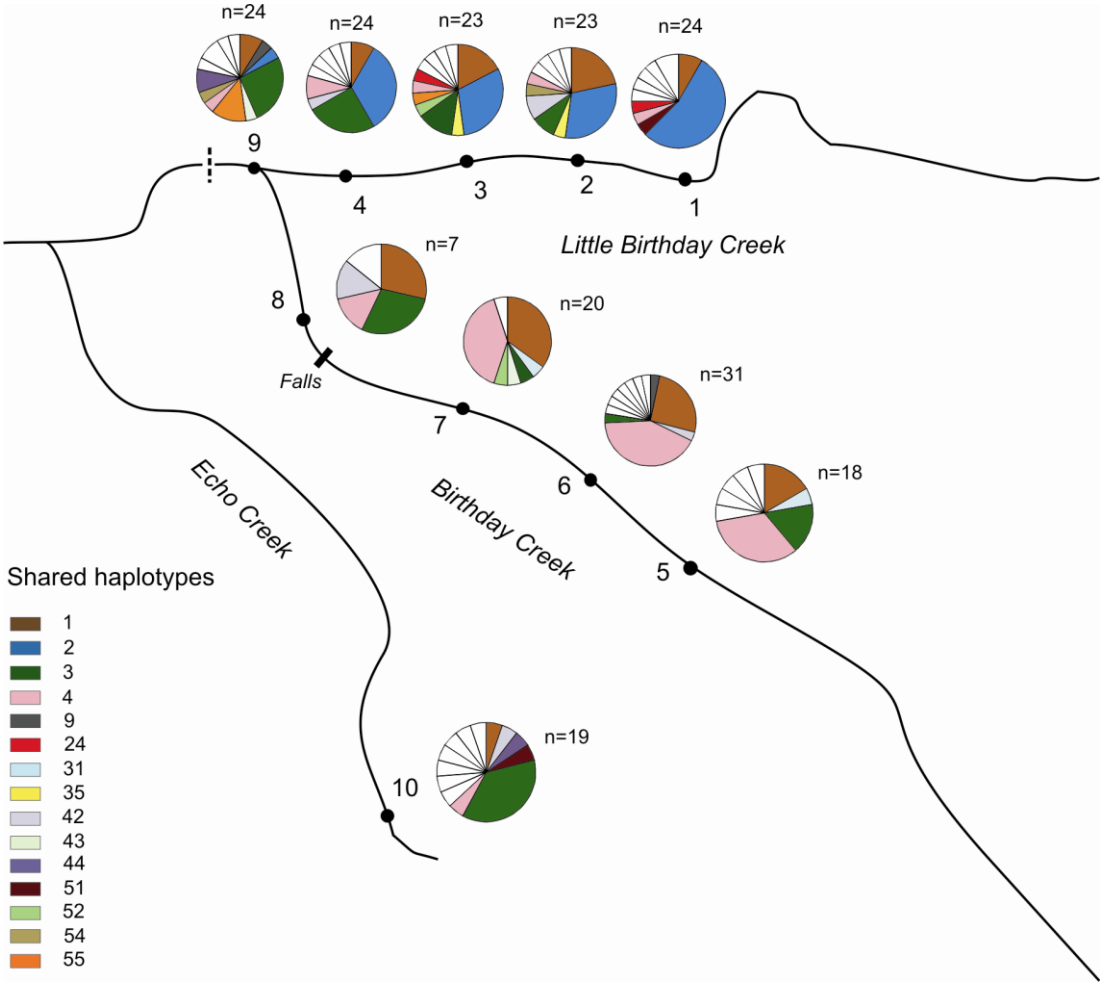


Figure 2.8. Schematic representation of the frequencies of COI haplotypes which are shared among sites in accordance with Figure 2.7. Haplotype numbers follow those in Figure 2.7 and the size of pie segments represents the proportion of sampled individuals that possess a particular haplotype. Unshaded pie segments represent singleton haplotypes.

sites along the same stream (Haplotypes 24, 31, 35) or were shared between individual sites on either Little Birthday or Birthday Creek and the Confluence site (Haplotypes 9, 43, 54, 55), indicating movement along the stream channel. A single shared internal haplotype was sampled from both Site 1 and 10 (Haplotype 51), the two most geographically distant sites, but not in any intervening site. This haplotype appears to have derived from the putative ancestral Haplotype 3 (Figure 2.7), that was absent from Site 1, implying that Haplotype 51 may have originated in Echo Creek and has since dispersed into the upper reaches of Little Birthday Creek. In similar fashion, the low frequency Haplotype 52, which appears to have derived from Haplotype 51, was shared across only Sites 3 and 7. Another derivative of Haplotype 3, Haplotype 42, was distributed across all three creeks, in Sites 2, 4, 6, 8 and 10, but was not found in the Confluence site. Haplotype 44, a derived tip haplotype was shared between Sites 9 and 10, implying relatively recent dispersal among these geographically distant sites.

There was no apparent relationship between the location of a given site along a stream and its complement of haplotypes present; i.e., upstream sites generally resembled downstream sites genetically and are not significantly different at Φ_{ST}. The exception, as mentioned above, was Site 1 which lacked the otherwise widespread Haplotype 3, this haplotype appearing to decrease in frequency with increasing geographical distance from the Confluence. As expected, the frequencies of haplotypes at the Confluence site indicated that this site possessed a mixture of haplotypes from both Birthday and Little Birthday Creeks. Whilst there was no major difference in haplotype composition above and below the Birthday Creek Falls, the sample size at Site 8 was small and thus may not provide an accurate reflection of true diversity at this site.

2.4 Discussion

The results presented here largely conformed with expectations that contemporary gene flow is restricted among sites sampled on Echo, Birthday and Little Birthday Creeks. The improved sampling depth and intensity of the current study has revealed clearer geographical patterns of common endemic haplotypes which dominated Birthday and Little Birthday Creeks, overlain by widespread ancestral haplotypes shared across the three streams. Additionally, the Confluence site comprised a combination of haplotypes from both adjoining streams, implying that dispersal may be restricted to along a stream channel. Nevertheless, the general pattern of significant differentiation among streams notwithstanding, the pattern of shared haplotypes represents clear evidence for limited gene flow mediated by both within and among stream movement. Haplotypes shared between upstream sites and the Confluence were interpreted to be the result of larval drift, whilst haplotypes shared among Echo Creek and some other sites were best explained by movement among streams, most likely via adult flight, since upstream movement of larvae would likely be restricted by stream flow. Whether adults fly overland or along a stream channel, however, cannot be determined here. Whilst there was evidence of isolation by both stream and Euclidean distance, which has been suggested to imply lateral dispersal among streams (Chaput-Bardy *et al.*, 2008), there was no clear evidence for regular headwater exchange through adult flight among upstream sites. Thus, the data presented here suggest that contemporary dispersal of *E. martini* females in streams in the Paluma region is largely, but not completely, restricted to within natal stream channels.

Comparison to general invertebrate dispersal patterns

The natural hierarchical structure of riverine systems provides an obvious framework against which to test patterns of genetic structure (Meffe & Vrijenhoek, 1988) although several alternative models have also been

proposed (e.g., 'patchy recruitment model' - Bunn & Hughes, 1997; 'headwater model' - Finn *et al.*, 2007). Whilst the sampling regime implemented here largely precluded tests of the applicability of alternative models of genetic structure to *E. martini*, the data conform generally to the Stream Hierarchy Model of Meffe and Vrijenhoek (1988), given that the degree of among-stream genetic differentiation was generally greater than that present within a stream. Furthermore, despite the presence of haplotypes shared among streams, there was limited evidence for regular headwater exchange among streams – since sites within a stream did not differ significantly from each other regardless of whether up- or downstream – strongly suggesting that *E. martini* dispersal is largely confined to the stream channel. The frequency and composition of haplotypes observed at the Confluence site also were consistent with expectations for a system dominated by movement generally restricted to within the natal stream. Similar patterns of extremely restricted gene flow have been observed in populations of several other aquatic invertebrate taxa, both in Australia (e.g., Hughes *et al.*, 1995; Hughes *et al.*, 1996; Cook *et al.*, 2007) and globally (e.g., Hughes *et al.*, 1999; Monaghan *et al.*, 2002; Schultheis *et al.*, 2002; Wishart & Hughes, 2003).

Such high levels of differentiation among geographically close streams are unusual for aquatic insects with an adult flight stage. Generally, such taxa exhibit some limited exchange among neighbouring streams and genetic differentiation is consequently greatest at the subcatchment or catchment level (Bohonak & Jenkins, 2003; Hughes *et al.*, 2008). Whilst some studies that have attempted to quantify adult flight suggest that winged aquatic insects are capable of lateral movement over distances equivalent to that between Little Birthday and Birthday Creeks (<2km - e.g., Jackson & Resh, 1989; Kovats *et al.*, 1996; MacNeale *et al.*, 2005), most trapping surveys have demonstrated that lateral dispersal is restricted to distances substantially less than those among sites in the current study (Collier & Smith, 1998; Griffith *et al.*, 1998; Petersen *et al.*, 2004; Winterbourne *et al.*, 2007). The density of riparian vegetation has been proposed to be a limiting factor on flight away from the stream in many taxa (Collier & Smith, 1998;

Delettre & Morvan, 2000). In addition to physiological and behavioural differences, this may explain some of the variation in dispersal abilities recorded among taxa; i.e., higher dispersal rates generally observed in cropland and open woodland than closed forest (but see Petersen *et al.*, 2004).

Quantitative data for adult flight distance in chironomids is available for two species – *Chironomus imicola* and *Polypedilum vanderplanki* – that inhabit ephemeral pools in Africa, and suggest that individuals readily disperse several hundred metres from the home pool to escape diminishing resources and avoid inbreeding (McLachlan, 1983). Investigations of the flight physiology of these two species suggested a propensity for extensive dispersal, combined with distinct sexual dimorphism in which females appear to be better dispersers than males (McLachlan, 1983, 1986). In contrast, the data presented here suggest that while dispersal away from the natal area may well be evolutionarily important for taxa inhabiting ephemeral systems, the same selective pressures may not apply for lotic chironomids. Moreover, given the pattern of highly restricted female dispersal identified here, it is plausible that male chironomids are even more limited in their dispersal ability.

Sample site hydrology and ecology

The three streams sampled in the current study contrast greatly in their hydrology compared with the ephemeral pool system investigated by MacLachlan (1983). The streams flow permanently, although some riffle sections can be quite shallow during the dry season (<2cm in depth - Connolly & Pearson, 2007, Krosch, M. N., pers. obs.), and the general hydrology of the catchment is heavily influenced by seasonal monsoonal precipitation (Soulsby *et al.*, 1997). The streams consist of interconnected riffles and pools flowing over granite substrata and the surrounding environment is dense, highly productive notophyll vine forest (Webb & Tracey, 1981), which ensures an abundance of organic material enters the

stream, while soil runoff is quite low. The dense riparian vegetation typical of these streams may also act to restrict flight of adult chironomids to along the stream channel and limit overland dispersal among streams. The relative habitat stability typical of the Paluma streams contrasts with the highly stochastic nature of the environment inhabited by the two chironomid species that MacLachlan (1983) studied and it might be expected that patterns of movement of individuals differ. Lotic taxa are exposed to different selective pressures than those in lentic systems, with the ephemeral nature of many such habitats presumably driving higher vagility in lentic taxa (reviewed in Marten *et al.*, 2006). Thus, restricted dispersal among populations of *E. martini* in the Paluma bioregion may be driven by a combination of high riparian vegetation density and/or habitat stability and large population size which together may mitigate the requirement to move extensively among streams.

Within-stream movement – 'Stream Drift Paradox'

The relative roles of up- and downstream movement of individuals within a stream channel have been debated widely under the banner of the 'Stream Drift Paradox' (Hershey *et al.*, 1993; Anholt, 1995). The observation that upstream populations of aquatic insects do not go rapidly extinct despite the significant loss of larvae through downstream drift and upstream directional flight bias in some taxa has prompted researchers to suggest that upstream flight could be a compensatory mechanism for ensuring upstream population persistence – dubbed the 'colonisation cycle hypothesis' (Mottram, 1932; Müller, 1954, 1982; Hershey *et al.*, 1993; Williams & Williams, 1993; Winterbourn & Crowe, 2001). In contrast, many have viewed the 'colonisation cycle hypothesis' as simplistic and, thus, alternative hypotheses have been proposed to resolve this drift paradox. Varying degrees of density dependence have been invoked as driving larval drift, e.g., drifting individuals are the result of upstream populations existing above carrying capacity (Waters, 1972), or that stream reaches depauperate of individuals due to downstream drift are rapidly recolonised through upstream flight and thus

new colonisers benefit from reduced intraspecific competition (Anholt, 1995). Furthermore, mathematical modelling of competition between genotypes with differing dispersal types has suggested that upstream flight may be an advantageous strategy ensuring population persistence, but that upstream movement would only be necessary if growth rates were low (Kopp *et al.*, 2001).

Several empirical investigations have attempted to resolve these arguments and have demonstrated that there are numerous biotic and abiotic factors that influence the rate and extent of downstream drift and compensatory upstream movement. Distinct patterns of diel periodicity in drift have been observed in many taxa, generally thought to relate to photoperiod, temperature and predator exposure (e.g., Benson & Pearson, 1987; Flecker, 1992; Jackson *et al.*, 1999). Seasonal patterns in drift and compensatory upstream movement has been recorded in several taxa that is likely to relate to life cycle seasonality and flow regimes (Benson & Pearson, 1987; Williams & Williams, 1993). Analysis of the gut contents of drifting individuals has revealed that individuals are more likely to drift when hungry (Kerby *et al.*, 1995), whilst artificial stream channel experiments have shown that a larval drift response is induced by increasing levels of sedimentation and hypoxia (Connolly *et al.*, 2004; Connolly & Pearson, 2007). Overall, however, much variation in the extent, frequency and duration of downstream larval drift and upstream adult flight have been recorded among taxa, and it is thus unlikely that there will be a single response in all taxa in all environments (Brittain & Eikeland, 1988; Malmqvist, 2002).

Chironomid larvae represent an important component of drifting invertebrates in temperate systems in the northern hemisphere and often make up a significant proportion of the total drift (Benke *et al.*, 1986; Schreiber, 1995) – up to 85% in some streams (Bishop & Hynes, 1969). Furthermore, members of the subfamily Orthocladiinae can constitute a large proportion (up to 91%) of the drifting chironomid taxa (Williams, 1989). Mean diurnal drift density can reach close to 400 individuals per cubic metre, though nocturnal densities up to four times greater have been recorded, and mean upstream compensation

can be up to 56% (Elliot, 1971; Williams, 1989; Williams & Williams, 1993; but see Benson & Pearson, 1987, Schreiber, 1995). In similar fashion, chironomid larvae make up a large part of the invertebrate drift in Australian rainforest streams, sometimes representing up to 25% of the total drifting individuals (Kerby *et al.*, 1995). Furthermore, artificial stream channel experiments have shown that loss of individuals due to drift in *E. martini* populations was ~10% per day (Connolly, N., pers. comm.). It is clear therefore, that chironomid larvae readily enter the moving water column and that downstream drift may play a major role in their dispersal.

The current data provide strong evidence that movement among Paluma *E. martini* populations within a stream channel is likely dominated by downstream drift. The Confluence site possessed the highest number of shared haplotypes (9), four of which (Haplotypes 9, 43, 53 and 54) were shared with only one other site along either Birthday or Little Birthday Creeks. The apparent accumulation of genetic diversity in the Confluence site most likely reflects historical diversification of haplotypes within individual streams driven by restricted gene flow, followed by subsequent downstream movement of individuals, possibly during periods of high rainfall. Furthermore, the distributions of Haplotypes 24, 31 and 35 shared among sites in the same stream imply that movement has been restricted to the natal stream channel, though whether this occurs by downstream drift or upstream flight cannot be tested here. Despite a relatively large genetic distance having been attributed to the stream section representing the location of the Birthday Creek Falls, there was no obvious difference in haplotype composition above or below the falls. Furthermore, Φ_{ST} estimates indicate no significant difference between sites up- and downstream of the falls. Thus, the observed genetic distance partitioned to this stream section may be due not to differences between Site 8 and those upstream of the falls, but to the comparative similarity of Site 8 to the Confluence due to low sample size. Unfortunately therefore, without higher sample size at Site 8, the role of the Birthday Creek Falls in limiting movement of *E. martini* along the stream channel cannot be tested further.

The pattern of haplotypes shared among sites did show some evidence of upstream movement of individuals. The occurrence of Haplotype 4 in much lower frequency in Little Birthday and Echo Creeks than in Birthday Creek suggests that individuals that possess this haplotype may have drifted downstream as larvae, before emerging as adults and flying upstream along a non-natal stream channel. The observed distributions of Haplotypes 42, 44, 51 and 52 among the study sites are likely to have arisen through a similar process and thus represent evidence for upstream movement. An alternative explanation is that adults of this haplotype have dispersed overland among sites; however, this appears unlikely given the apparent overall lack of headwater exchange among upstream sites. It is currently unknown whether *E. martini* is capable of positive rheotaxis as a larva, but given the propensity for downstream drift in this taxon and the family as a whole, it appears more likely that any upstream movement occurs via adult flight. This being said, there was little evidence for regular compensatory upstream flight, as the upstream sites along both creeks do not possess the same overall complement of haplotypes as those further downstream. Indeed, Site 1 lacked entirely the otherwise common ancestral Haplotype 3 and this is likely the reason behind the large genetic distance attributed to the stream section between Sites 1 and 2. Furthermore, upstream sites on both Birthday and Little Birthday Creeks possessed singletons unique to each site and did not share low frequency haplotypes with the neighbouring downstream site. Whilst some rare haplotypes may have been overlooked through random sampling effects, this nevertheless implies that while the loss of individuals from upstream populations to downstream drift is certainly an important factor in the population dynamics of Paluma *E. martini*, population sizes remain large enough to allow persistence in upstream sites in the absence of regular compensatory flight.

Implications for recolonisation and susceptibility to disturbance

The insights into the geographical range and extent of *E. martini* dispersal revealed by this study have important implications for the current

understanding of the ability of chironomids both to colonise new habitat and recolonise existing habitat post-disturbance. Previous experiments involving artificial stream channels revealed that *E. martini* reached peak abundance in newly colonised habitat after ~25 days, in the mid-range for all chironomid taxa studied (Connolly, N., pers. comm.), and similar to the overall mean for chironomids revealed by Benson & Pearson's (1995) trials using artificial substrata. In both studies, the colonisation rates of chironomids were generally more rapid than for most other stream taxa, though some copepod taxa colonise new habitat more rapidly even than chironomids (Connolly, N., pers. comm.). Whilst the artificial stream channels and substrata of the above studies clearly were colonised by individuals already inhabiting the study stream via movement within the water column, little is known of the ability of both *E. martini* specifically and chironomids in general to recolonise new streams after disturbance.

The pattern of generally restricted dispersal among proximate streams observed here suggests that the potential of *E. martini* to recolonise streams even from those that form part of the same catchment may be poor, meaning that catastrophic scouring events, such as severe flooding, may result in local extinction from which *E. martini* populations may not recover. Extrapolating from this, the potential for *E. martini* to recover from local extinction across an entire catchment or rainforest patch would be dramatically lower and may take many generations of chance migrants to recolonise such habitat post-disturbance. This is particularly important given the already highly fragmented state of Australian rainforests. Taken together, these data imply that *E. martini* may be highly susceptible to habitat disturbance (e.g., loss of riparian cover, reduction in water quality) and that once populations are driven to local extinction they are unlikely to recover readily. Moreover, the results of this study reinforce hypotheses of the distinct geographical endemism observed among Wet Tropics *E. martini* populations as having been driven in part by highly restricted dispersal among closed forest refugia. It is likely, therefore, that similar patterns would be observed elsewhere throughout this species' distribution.

Overall, this study represents the most intensive survey of freshwater chironomid population genetic structure conducted to date. The data presented here revealed a distinct pattern of largely restricted gene flow among neighbouring, geographically proximate streams of the same catchment. This is likely to have resulted from a combination of high riparian density physically restricting dispersal and habitat stability and large population size mitigating the requirement for movement among streams. Furthermore, movement among sites within the same stream channel appears to be dominated by downstream larval drift, though there is also clear evidence for some upstream dispersal, likely through adult flight. Large population sizes are also likely to contribute here, by enabling persistence of upstream populations in the absence of regular compensatory upstream flight. Taken together, these data suggest that dispersal of *E. martini* is highly restricted, at a scale of only a few kilometres, and occurs predominantly within the natal stream.

Chapter Three

Phylogeography of two related Gondwanan Orthocladiine (Diptera: Chironomidae) taxa from New Zealand and Patagonia

3.1 Introduction

Investigating the role of population fragmentation and isolation in driving diversification is crucial for the development of a more holistic understanding of organismal evolutionary history and is a founding principle of modern ecology and phylogeography (MacArthur & Wilson, 1967; Avise *et al.*, 1987). The isolation of previously continuous populations in small refugia intersected by unsuitable habitat can interrupt natural dispersal and gene flow in many taxa, potentially leading populations to evolve along separate and sometimes very different trajectories. This process is considered to be an important factor influencing the evolution and maintenance of biodiversity and endemism throughout the world (Moritz *et al.*, 2000). Evaluating the degree to which populations have been affected by such processes has been the focus of a wealth of research that encompasses a variety of historical fragmentation events, including geological orogeny (e.g., Brunsfeld *et al.*, 2001; Ripplinger & Wagner, 2004; Steele *et al.*, 2005), island formation (see Emerson, 2002) and rainforest contraction (see Moritz *et al.*, 2000; Krosch *et al.*, 2009), among others.

Genetic legacy of historical population fragmentation

Significant genetic divergence among populations isolated by such events has been recorded in a diverse array of taxa, from plants to invertebrates to vertebrates. Arguably one of the better-studied groups, however, are freshwater invertebrates, for which several scenarios of population fragmentation have been tested, including Pleistocene glacial cycles (e.g., Ribera & Vogler, 2004; Smith *et al.*, 2006b), mountain orogeny (e.g., Hughes *et al.*, 1999; Arrivillaga *et al.*, 2002) and island formation (e.g., Carson, 1983; Kelly *et al.*, 2001). Global evidence suggests that freshwater invertebrates, particularly those inhabiting lotic systems, are often susceptible to population fragmentation and isolation (Bohonak, 1999; Hughes *et al.*, 2008); however, some taxa that possess high vagility appear to be capable of mitigating any

effects of habitat fragmentation through long-distance dispersal (e.g., Hughes *et al.*, 1998; Hughes *et al.*, 2000; Berendonk & Spitze, 2006). Freshwater invertebrates possess generally large population sizes and short generation times and their distribution in the landscape is restricted largely by available aquatic habitat. Thus, freshwater invertebrate taxa are often optimal model taxa for testing hypotheses of the effects of population isolation.

Two such taxa, the chironomid species *Naonella forsythi* Boothroyd and *Ferringtonia patagonica* Edwards, inhabit pristine freshwater streams in New Zealand and southern South America, respectively (Boothroyd, 1994; Sæther & Andersen, 2010). *Naonella forsythi* is known from upland glacial streams and rivers on both major New Zealand islands (Boothroyd, 1994). This species is highly abundant at all times of the year, with populations peaking during spring and summer (Boothroyd, 1994) and they often dominate the chironomid fauna in some streams (Krosch, M. N., pers. obs.). The recently revised taxon *F. patagonica* has been recorded from upland glacial streams in southern Chile and Argentina (Sæther & Andersen, 2010). Both taxa inhabit tectonically active regions of ancient Gondwana that share distinct histories of population fragmentation due to Neogene mountain range orogeny and Plio-Pleistocene glaciations. Moreover, both these taxa are considered members of a relict Gondwanan clade within the Orthocladiinae subfamily, along with the Australian species *E. martini* for which phylogeographic structure has been inferred previously (Krosch *et al.*, 2009), among others. As such, these taxa represent ideal models for testing hypotheses of the relative impact of historical population fragmentation on genetic structure, as extrapolated from patterns revealed in *E. martini*. More specifically, levels of divergence among populations may be comparable in taxa closely related to *E. martini* that have experienced similar episodes of historical population fragmentation and isolation.

Geological and glacial history of New Zealand

The New Zealand Southern Alps are the major topographical feature of the

South Island, running roughly southwest-northeast along the length of the island. This mountain range is the result of the convergence of the Australian and Pacific continental plates along the Alpine Fault, which until approximately six million years ago (mya), had not undergone significant uplift (Walcott, 1998). The predominant tectonic activity prior to the late Miocene was around 200-500km of strike-slip movement along the fault line caused by different rates of movement of the two plates away from the Antarctic plate (Heads, 1998; Walcott, 1998). From ~6mya the motion of the Australian and Pacific plates altered slightly relative to each other such that significant compression occurred between the plates, subsequently driving the uplift of the Southern Alps (Walcott, 1998). This mountain range is considered to have reached its current average height of around 2000m within the last million years (Mercer, 1983). Throughout the period of tectonic uplift, the South Island also experienced several glaciation events (Suggate, 1990; Newnham *et al.*, 1999; Suggate & Almond, 2005), including one particular event during the late Pleistocene reported to have covered the majority of the Southern Alps (Mercer, 1983; Suggate & Almond, 2005), and rendered much of the remainder of the South Island as tundra (see Trewick & Wallis, 2001).

Genetic structure in New Zealand taxa

Biologically, the orogeny of the New Zealand Southern Alps, in concert with Plio-Pleistocene glaciation events, has been shown to have had a dramatic effect on the population structure of many taxa, mirroring data from northern hemisphere mountain ranges, including the Sierra Nevada, Cascade Range and Rocky Mountains complex in north-western North America (Brunsfeld *et al.*, 2001; Finn *et al.*, 2006), the Himalaya region of Central Asia (Zhang & Jiang, 2006), the Atlas mountains of Morocco (Brown *et al.*, 2002) and, to a lesser extent, the European ‘Alpine Orosystem’ (comprising the Pyrenees, Alps, Carpathians and northern Balkan mountain ranges - Ozenda, 2009). In the South Island of New Zealand, distinct disjunctions have been observed in the distribution of several terrestrial and aquatic plants and animals, whereby

populations of a given taxon are isolated roughly to the northwest (Northern South Island Province (NSIP): Leathwick *et al.*, 2007) and southeast (Southern South Island Province: SSIP) of the South Island, bisected by the Alps (Heads, 1998; Trewick & Wallis, 2001). There are two competing hypotheses as to what has caused these disjunctions; one invokes the strike-slip movement of the two continental plates along the fault and the orogeny of the Southern Alps (Heads, 1998), while the other implies that Pleistocene glaciations rendered the centre of the island unsuitable for many taxa, thereby isolating populations (Trewick & Wallis, 2001). Furthermore, some studies have suggested that populations from the NSIP region may be more closely related to those from the southern North Island, to the exclusion of populations elsewhere on the South Island (e.g., fish - Smith *et al.*, 2005; skinks - Greaves *et al.*, 2007; cicadas - Hill *et al.*, 2009). Some taxa which share this pattern of sister grouping also appear to show patterns of southward population expansion across the Southern Alps post-isolation, implying that the importance of this mountain range as a dispersal barrier may have been reduced in some taxa (e.g., cicadas - Buckley *et al.*, 2001; bats - Lloyd, 2003; snails - Neiman & Lively, 2004). The majority of genetic studies that have attempted to date the divergence of clades in the South Island have estimated population fragmentation to have occurred during the late Pliocene to early Pleistocene, correlating closely with proposed glaciation events.

Currently, however, there is only limited data available regarding population structure within the NSIP, despite recognition that this region possesses both high biological diversity and endemicity, and comparative geological stability, having remained relatively unaffected by historical glaciation events (Wardle, 1963; Leathwick *et al.*, 2007). In terrestrial taxa, the skink species *Oligosoma infrapunctatum* Boulenger has been shown to possess four highly divergent mitochondrial lineages, all of which occur sympatrically in the NSIP (Greaves *et al.*, 2008). Evidence from freshwater systems corroborate this pattern, with marked genetic divergence apparent among major river catchments in the NSIP observed in the galaxiid fish species *Gobiomorphus breviceps* Stokell (Smith et al., 2005). Furthermore, the related galaxiid, *Galaxias vulgaris*,

showed that some Buller River headwater populations in the Nelson region may have originated in catchments on the eastern side of the Southern Alps and that distributions of genetic types could be explained by river capture (Waters & Wallis, 2000). Taken together, these data imply that the NSIP is an ideal region in which to test patterns of relationships among populations that are thought to have mostly avoided the large-scale fragmentation seen elsewhere on the South Island. Thus it is expected that genetic structure will follow predictions based on stream hierarchy; that is, greatest genetic difference observed among catchments ('Stream Hierarchy Model' - Meffe & Vrijenhoek, 1988). Evaluating population genetic structure in *N. forsythi* represents the first detailed genetic survey of any New Zealand chironomid species and the first of any freshwater invertebrate at this scale within the NSIP.

Geological and glacial history of Patagonia

Southern South America shares a superficially similar geological and glacial history to that of the South Island of New Zealand discussed above. Both have experienced significant tectonic uplift and extensive glaciation events since the late Oligocene that have dramatically altered the geomorphology, climate and ecology of the two regions. The active boundary of the South American and Nazca plates is located just off the west coast of the mainland and runs roughly north-south some 5300km, from just north of the equator to around 42˚ South (Gregory-Wodzicki, 2000). The subduction of the Nazca plate under the South American plate has apparently driven at least two phases of tectonic uplift over the last 25 million years, most recently initiated in the late Pliocene, giving rise to the Andes mountain range (Lagabrielle *et al.*, 2004). The Andes extend over 7000km along the west of the continent, reaching an average elevation of more than 4000m to form the most significant topographical feature in South America. Initial orogeny began approximately 20mya with a period of uplift lasting 5-6 million years, followed by a second and still current orogenic phase beginning from around 3.5mya (Lagabrielle *et al.*, 2004). The initial Andean uplift phase has been suggested

to have driven significant changes in climate in southern South America, resulting in a distinct rain shadow effect on the eastern side of the mountain range from approximately 14mya (Montgomery *et al.*, 2001; Blisniuk *et al.*, 2005). In addition, since the initiation of tectonic uplift, several glaciation events covered much of the southern Andes in thick ice sheets, extending from the Pacific coast eastward to the Argentine plains (Mercer, 1983; Rabassa & Clapperton, 1990; Ehlers & Gibbard, 2007). The three major geological formations indicative of glaciations known from this region have been dated to the late Miocene (~7mya), the middle Pliocene around 3.6mya, and the late Pliocene to early Pleistocene (~2.2-1mya) (Mercer, 1983; Rabassa, 2008). There is further evidence from radiocarbon data that extensive glaciation in the southern Andes occurred up until the start of the Holocene (~10kya - McCulloch *et al.*, 2000; Rabassa, 2008).

Genetic structure in Patagonian taxa

The dramatic geological and glacial history of southern South America appears to have affected populations of numerous endemic taxa in a similar fashion to that observed in the South Island of New Zealand, although the current literature base for this region is more limited. Populations of some widespread taxa were bisected by the uplift of the Andes resulting in isolation and marked genetic structure across the mountain range, although estimates of when divergence occurred vary from mid-Pliocene to late-Pleistocene (e.g., southern beech - Marchelli & Gallo, 2006; freshwater perch - Ruzzante *et al.*, 2006; fruit flies - Ramirez *et al.*, 2008; pygmy rice rats - Gonzalez-Ittig *et al.*, 2010). In many other taxa, however, the genetic signal of population bisection across the Andes appears to have been masked by subsequent, or perhaps coincident, historical glaciation events – a pattern also observed among populations fragmented by the European Alpine Orosystem (Taberlet *et al.*, 1998). Several plant and animal taxa show evidence for isolation in refugia from which they have since recolonised newly available habitat, which do not correspond to Andean bisection (e.g., conifers - Allnutt *et al.*, 1999; opossums - Palma *et al.*, 2002; asters - Muellner *et al.*, 2005). Furthermore,

many taxa appear to exhibit patterns of population genetic structure that are correlated with latitude, interpreted as representing multiple historical refugia located at the glacial margin (e.g., *Nothofagus nervosa* - Marchelli & Gallo, 2006; small marsupials - Himes *et al.*, 2008; guanaco - Marin *et al.*, 2008; *Nothofagus antarctica* - Pastorino *et al.*, 2009; *Calceolaria polyrhiza* - Cosacov *et al.*, 2010). In addition, data from a species of podocarp demonstrated that population genetic structure may be related to substrate type (Quiroga & Premoli, 2010), whilst three related lizard taxa exhibit patterns that imply post-recolonisation differentiation associated with particular environmental gradients that contributed to further population divergence (Victoriano *et al.*, 2008). As with studies that have invoked Andean orogeny as driving population divergence, estimates of divergence times in the above studies have ranged from mid-Pliocene to late-Pleistocene.

Data for freshwater organisms from streams in the southern Andes in general is extremely limited, although recent investigations of population genetic structure in various fish taxa have reported evidence for historical population isolation and several river reversal events, from east- to west-draining and vice versa (e.g., perch - Ruzzante *et al.*, 2006; galaxiids - Zemlak *et al.*, 2008). Thus, it will be important to obtain further data regarding population connectivity, particularly for freshwater organisms, to achieve a more holistic understanding of the biogeographical and evolutionary history of the southern Andes. As such, studies of aquatic insect taxa may provide an important comparison with existing data from populations in the region, as they possess generally high population sizes, potential dispersal abilities and rapid generation times, life history traits that are often considered to buffer populations somewhat against any effects of population fragmentation. Moreover, investigating population genetic structure in *F. patagonica* will represent the first detailed genetic study of any Neotropical chironomid species and any freshwater invertebrate in the southern Andes. Comparing and contrasting patterns of population genetic structure among related species is an integral part of comparative phylogeography (Bermingham & Moritz, 1998; Taberlet, 1998; Arbogast & Kenagy, 2001).

Such investigations are generally conducted within a given geographical region in order to evaluate the relative effect of the biogeographical history of the region on co-distributed taxa. In the current study, however, the focus was instead on a comparison of patterns of genetic structure among populations, and thus the responses to historical population fragmentation, of closely related species in different geographical regions; specifically, on separate continents that were connected historically. The chironomid taxa included in the current study – *N. forsythi* and *F. patagonica* – are known relatives of *E. martini* and the three genera are considered to be members of a putative Gondwanan clade within the Orthocladiinae subfamily (Boothroyd, 1994; Cranston, 2000b; Sæther & Andersen, 2010).

Broadly, this study tested expectations regarding the impact of fragmentation and isolation on populations of *N. forsythi* and *F. patagonica*, as extrapolated from patterns reported previously in *E. martini* by Krosch *et al* (2009). Specifically, this study aimed to determine whether *N. forsythi* and *F. patagonica* share with *E. martini* the pattern of several highly divergent, geographically endemic mitochondrial lineages within a single recorded species across similar geographical ranges. Furthermore, specific hypotheses concerning the biogeographical processes (e.g., orogenic uplift, glacial cycle-driven habitat fragmentation) that have been active historically on *N. forsythi* and *F. patagonica* were investigated in order to provide important and novel insights into the evolutionary history of these taxa. In particular, it was expected, given data for *E. martini*, that gene flow among streams within a catchment would be largely restricted in *N. forsythi* and that populations from the North and South Islands of New Zealand would be distinctly differentiated. Likewise, *F. patagonica* populations currently bisected by the Andes mountain range were also expected to exhibit significant genetic structuring. This study represents the first detailed analysis of population genetic structure in New Zealand and Neotropical chironomids and is a novel application of comparative phylogeographic theory for investigating population structure among closely related species inhabiting vastly different and geographically distant landmasses.

3.2 Methods

Study sites

Naonella forsythi

Larvae and pupae of *N. forsythi* were collected from six sites on the South Island of New Zealand (Figure 3.1). Sample sites were located mostly in the Nelson Lakes region, with one site located on the Lewis Pass (Site 6), with all sites contained within the Buller River catchment. Generally, site choice was based on previous surveys of chironomid assemblages in New Zealand in combination with existing evidence that suggests *N. forsythi* exhibits similar habitat preferences to its putative Gondwanan relatives; i.e., prefers riffle sections of cool, shaded, pristine streams (Boothroyd, 1994). Nevertheless, the distributions of these taxa appear to be patchy and while the sampling design of the current study attempted to accommodate for this by targeting particular habitat types, it was not always successful. Furthermore, the geographical range of current sampling was informed by Krosch *et al* (2009) – which suggested dispersal in the related taxon *E. martini* occurs over only limited distances – but does not encompass the entire distribution of *N. forsythi*. The density of riparian vegetation varied among streams, from dense *Nothofagus* forest enclosing narrow streams to more open rivers lined with introduced willow (*Salix* sp.). Sample collection took place predominantly during 2007, though supplementary sampling of the Owen River (Site 4) was undertaken in 2009 in order to acquire a larger sample size from this site. Representatives were also sourced from one location on the North Island (Site 7), along with additional pupae from South Island sites; these collections were carried out during 2008 and 2009 by Prof. Peter Cranston. One individual from each of two related orthoclad taxa (*Anzacladius kiwi* and *Botryocladius freemani*) and a putative second species of stream-dwelling *Naonella* (*N.* sp. n. 1 – Cranston, P. S., pers. comm.) were sequenced as outgroups.

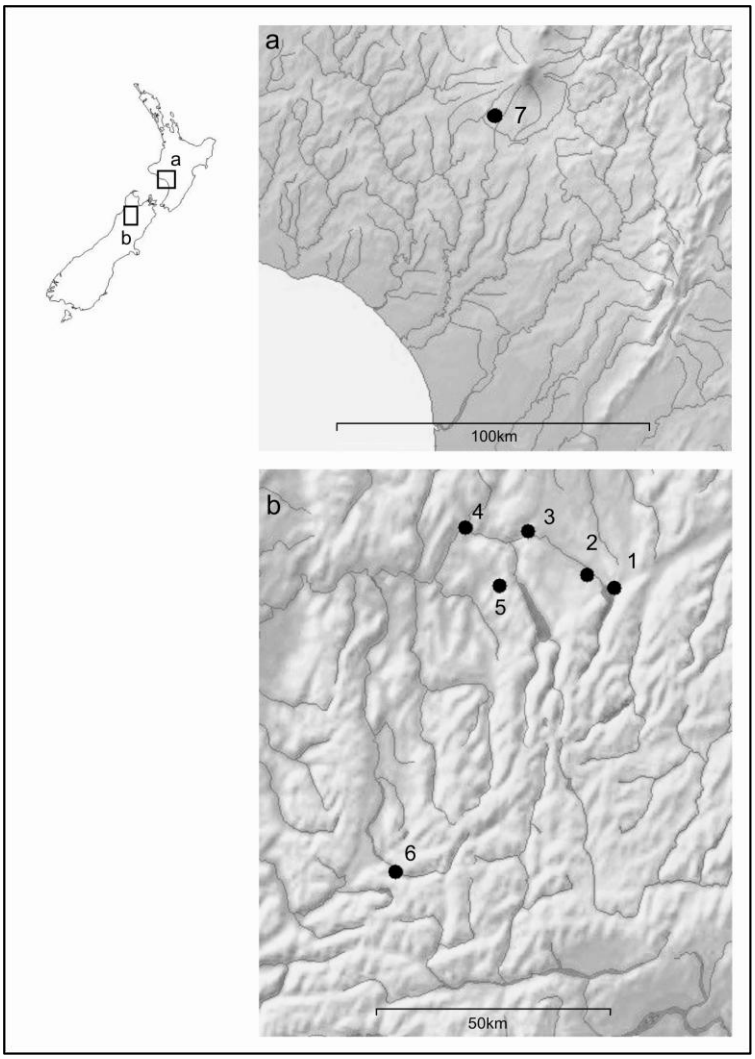


Figure 3.1. Geographical location of *N. forsythi* sample sites in New Zealand. Site numbers are as follows: 1: Borlase Creek, 2: Speargrass Creek, 3: Hope River, 4: Owen River, 5: unnamed Creek near Lake Rotoroa (henceforth referred to as Rotoroa Creek), 6: Jackson Creek, 7: unnamed creek near Ohakune (henceforth referred to as Ohakune Creek).

Ferringtonia patagonica

Larvae and pupae of *F. patagonica* were collected from eight sites in southern South America (Figure 3.2). Six of these sites were located in the Lakes District of Patagonian Chile and two in the Neuquen Province of Argentina. The majority of individuals were collected in October 2007, though nine pupae from various locations were sourced from previous collections made by Prof. Peter Cranston. As with *N. forsythi*, sample sites were targeted according to both previous collections and the general ecology of other related Gondwanan taxa and possessed similarly varied riparian

vegetation densities. Similarly, the geographical scale of sampling was informed by the data of Krosch *et al* (2009), but does not cover the entire range of the species. Individuals of two related genera (*Botryocladius edwardsi* and *Echinocladius martini*) were sequenced as outgroups.

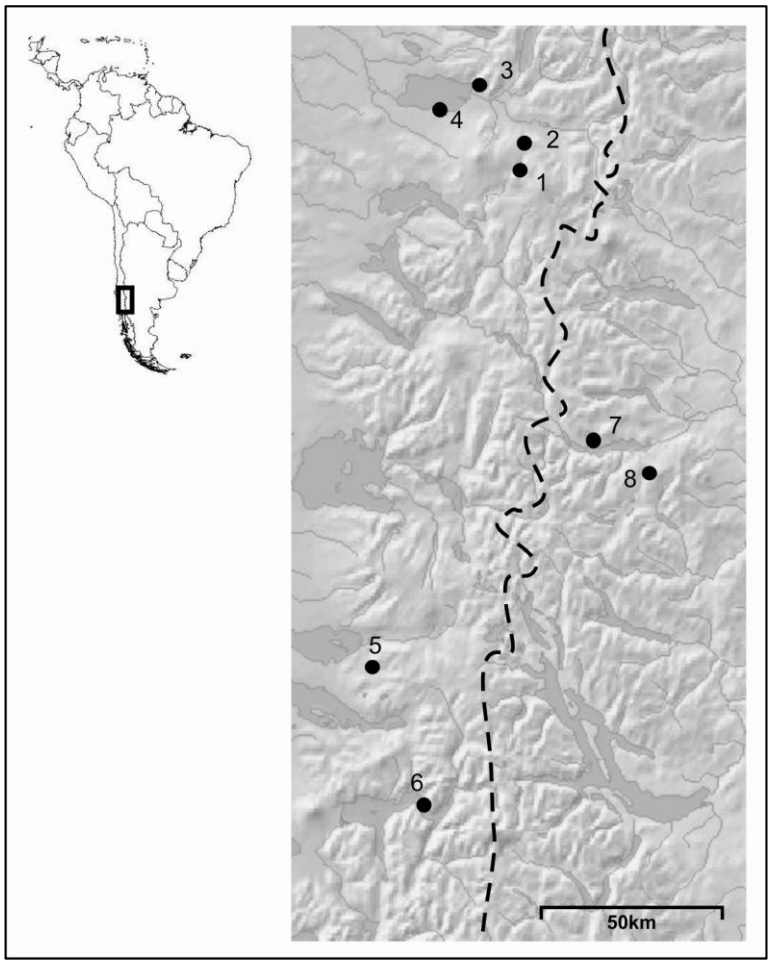


Figure 3.2. Geographical location of *F. patagonica* sample sites in southern South America. Dashed line represents the national border separating Chile and Argentina and roughly follows the Andean ridgeline. Site numbers are as follows: 1: Rio Palguin, 2: Rio Don Baucha, 3: Estero La Casilla, 4: Estero Correntoso, 5: Rio Chaleufu, 6: unnamed creek near Peulla, 7: Arroyo Quilanlahue, 8: Arroyo Partido. Geographical distribution of mitochondrial lineages is indicated by circled sample sites.

Sample collection

Sample collection, preservation, identification and storage was similar for both taxa and followed the process outlined previously (Chapter Two), but also included use of drift or standard sweep nets left half-submerged overnight to recover drifting pupae.

Genetic procedures

Total genomic DNA was extracted as outlined in Chapter Two. A 734bp fragment of the cytochrome *c* oxidase subunit I (COI) gene was amplified using universal invertebrate COI primers COI-s2183 (5' CAA CAT TTA TTT TGA TTT TTT GG 3') and COI-a3014 (5' TCC AAT GCA CTA ATC TGC CAT ATT A 3') (Simon *et al.*, 1994). Each reaction tube contained 4 µL of template DNA from extractions, 0.6 µL of each primer (10pmol/µL), 2.5 µL of 10X polymerase buffer, 3.0 µL of 25 mM $MgCl_2$, 2.0 µL of 10 mM dNTP's, 0.2 µL of 5U/µL *Taq* polymerase and were adjusted to a final volume of 25 µL with dH_2O. The PCR cycle protocol involved initial denaturing at 95˚C for 1 minute followed by 39 cycles of 95˚C for 30 seconds, 49˚C for 1 minute and 72˚C for 90 seconds, before final extension at 72˚C for 3 minutes. Total PCR products were then purified and sequenced as outlined in Chapter Two.

Data analyses

COI sequences were aligned and edited by eye using BioEdit Version 7.0.5 (Hall, 1999). Tests for sequence saturation (an indicator of homoplasy) and Tajima's D tests of neutrality were conducted as outlined in Chapter Two. Clock-like evolution was assessed in Tree-Puzzle Version 5.2 (Schmidt *et al.*, 2002) under a Hasegawa-Kishino-Yano (HKY) model of sequence evolution, which was determined to be the most appropriate model available for the dataset in this software package. Mean among-lineage corrected pairwise divergences were calculated in MEGA under the Tamura-Nei model of evolution, again the most appropriate model available in MEGA.

Between-lineage analyses

The model of nucleotide substitution that best fitted the COI dataset was determined under the Akaike Information Criteria (AIC) and using the online resource FindModel (http://www.hiv.lanl.gov/ content/sequence/ findmodel/findmodel.html); this model was used for all subsequent

phylogenetic reconstruction wherever software options allowed. Given that the various methods of phylogenetic inference treat data differently, multiple methods were used here for both taxa, including neighbour-joining (NJ), Bayesian (B), maximum likelihood (ML) and maximum parsimony (MP – *F. patagonica* only; computation time for this method was prohibitive for *N. forsythi*). Neighbour-joining (10,000 bootstraps) and maximum parsimony (1,000 bootstraps) phylogenetic inference were implemented in PAUP* Version 4.0 (Swofford, 2001). Bayesian reconstruction (5 million generations, 25% burnin) was performed in MrBayes Version 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) under the General-Time Reversible (GTR) model of sequence evolution, incorporating a gamma distribution of nucleotide frequencies. Convergence of the Bayesian analysis was maximised by ensuring the standard deviation of split frequencies fell below 0.01 and by performing multiple runs. Maximum likelihood (1,000 bootstraps) reconstruction was performed using RAXML Version 7.0.3 (Stamatakis, 2006) under the GTRMIX model of sequence evolution and a 50% majority rule consensus tree calculated in Mesquite Version 2.71 (Maddison & Maddison, 2007). Lineages were defined as having strong statistical support across several methods of phylogenetic reconstruction with greater than 2% corrected pairwise distance among lineages.

Times to most recent common ancestor (tmrca) for relevant nodes were estimated for both taxa using the BEAST Version 1.4.8 software package (Drummond & Rambaut, 2007) under the GTR model of evolution. Bayesian consensus topologies for each taxon were used as starting trees to reduce computational time. The chironomid mitochondrial divergence rate of 1.5% per million years calculated from divergence time estimates of Nearctic and Palearctic *Chironomus* species (Martin *et al.*, 2002) was fixed as the initial mean rate of molecular evolution under a relaxed lognormal molecular clock and the tree prior was set to ‘Speciation: Yule Process’. For *N. forsythi*, four runs of 50 million generations each were performed and the log files combined to produce a total run of 180 million generations after burn-in. For *F. patagonica*, three runs of 40 million generations each were performed and the log files combined to produce a total run of 108 million generations after

burn-in. Different run lengths between the two datasets reflect the difference in the number of generations required for estimates of tmrca to obtain appropriate support (e.g., effective sample sizes >200).

Within-lineage analyses

Patterns of genetic structure among populations within relevant lineages in both *N. forsythi* and *F. patagonica* were investigated using the following analyses which are described in detail in Chapter Two. Tajima's D tests of neutrality, gene diversity and the population parameter, θ_π, were calculated for individual sites as estimates of within-site genetic diversity. A haplotype network was constructed with a connection limit of 90% and differentiation among sites was estimated by calculating conventional among-site Φ_{ST} indices ($P<0.05$). Mantel tests of isolation by distance were implemented using Euclidean distances among sites for *F. patagonica* and both Euclidean and stream distances among sites for *N. forsythi*; all geographical distances were estimated using Google Earth. Stream distances could not be calculated for *F. patagonica* as sites were located in different catchments. For *N. forsythi*, the relative genetic distance represented by each stream section was estimated using Streamtree based on a matrix of pairwise Φ_{ST} indices and statistical support was given by the calculation of the R^2 coefficient of determination. A neighbour-joining population tree was constructed in MEGA using Nei's genetic chord distance (D_A) among sites for *N. forsythi*.

3.3 Results

New Zealand: *Naonella forsythi*

In total, 184 individuals of *N. forsythi* from seven sites were sequenced for COI, producing a read length of 734bp, representing 128 unique haplotypes (Table 3.1). The ratio of transitions to transversions was quite high at 6.309

and the relationship between genetic distance and each substitution type showed a plateau in transitions as genetic distance increased (Figure 3.3).

Table 3.1. Geographical location of New Zealand sample sites and numbers of *N. forsythi* sourced from each site. Elevation is given as metres above sea level (masl).

Site Name	Site number	Latitude	Longitude	Elevation (masl)	Sample Size
Borlase Ck	1	41°48.24'S	172°50.45'E	678	32
Speargrass Ck	2	41°46.50'S	172°46.30'E	562	27
Hope River	3	41°41.38'S	172°37.05'E	360	41
Owen River	4	41°41.02'S	172°27.14'E	245	25
Unnamed creek near Lake Rotoroa	5	41°48.11'S	172°32.31'E	675	30
Jackson Ck	6	42°22.17'S	172°16.01'E	500	27
Unnamed creek near Ohakune	7	39°24.20'S	175°27.15'E	578	2

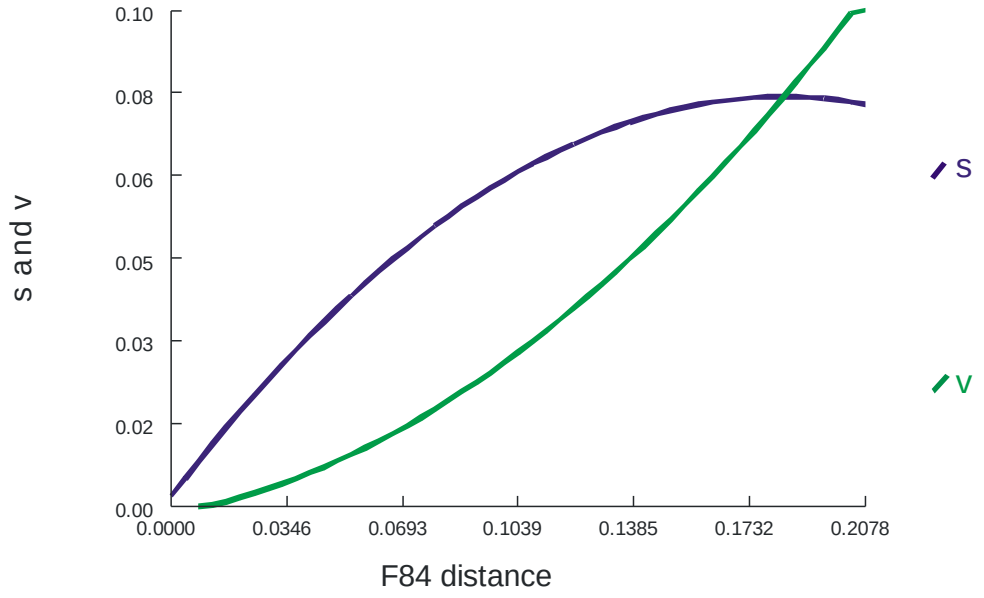


Figure 3.3. The relationship of transitions (s) and transversions (v) to genetic distance in *N. forsythi*.

Both provide potential evidence of sequence saturation, implying that multiple substitutions may have occurred at a particular site and thereby potentially underestimating true divergence (Arbogast *et al.*, 2002). Tajima's D tests of neutrality were non-significant (D = 1.3154, p = 0.925), suggesting that the

sequences had not evolved under selection. Assumptions of clocklike evolution were rejected (p = 0.000) and thus a relaxed clock model was used to infer divergence times.

Phylogenetic reconstruction

Topologies produced by the different methods of phylogeny reconstruction were generally concordant, although levels of resolution varied (Figure 3.4). Four highly divergent, morphologically cryptic evolutionary lineages were well-supported by two of the three methods; ML interestingly did not resolve Lineage 4 as monophyletic. Levels of support for nodes connecting lineages were generally high across methods, particularly the Bayesian reconstruction where only one major node showed a posterior probability below 1.00. All methods consistently placed the putative novel species of *Naonella* (*N*. sp. n. 1) as sister to all *N. forsythi*; a placement which, in concert with morphological differences (Cranston, P. S., pers. comm.), lent support to the notion that this specimen represented a novel species-level taxon. The pattern of relationships among lineages was consistent among methods; Lineage 2, 3 and 4 shared a common ancestor, sister to that of Lineage 1, which diverged to form the ancestor of Lineage 2 and the common ancestor of Lineages 3 and 4. Corrected mean pairwise divergence among lineages ranged from 5.30% between Lineages 3 and 4 to 10.1% between Lineages 1 and 2 and 1 and 3 (Table 3.2). Divergence estimates between the four lineages and the putative second species were slightly higher, ranging from 12.2% to 13.4%.

Distributions of the four divergent lineages identified here generally were not congruent with geographical location as each lineage was comprised of individuals from multiple streams that were often separated by large geographical distances (Figure 3.1 and 3.5). In particular, the widely distributed Lineage 1 was recovered from all seven sample sites, including Ohakune Creek, whilst Lineage 3 was sampled from four sites on the South

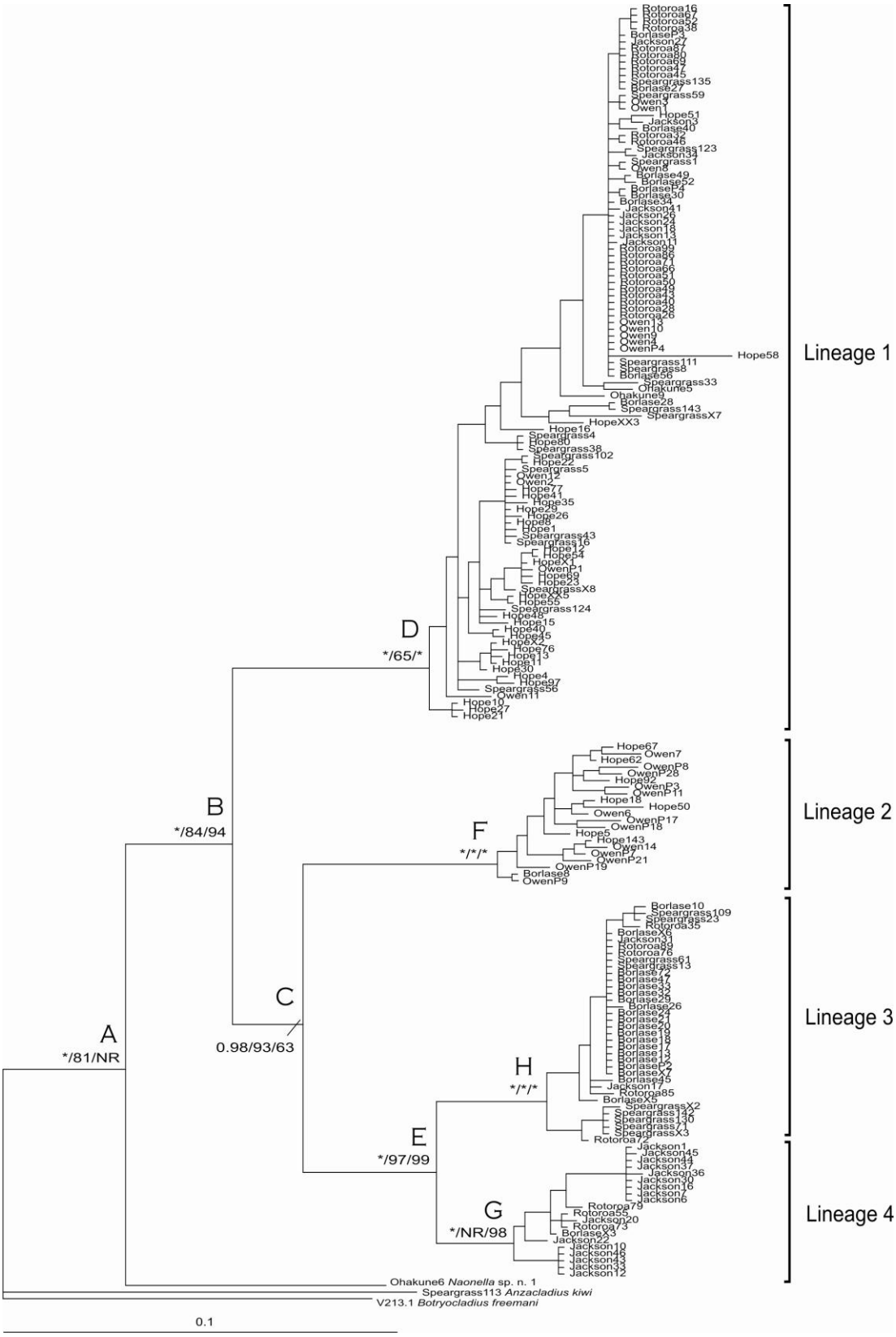


Figure 3.4. Consensus Bayesian topology for *N. forsythi* COI sequences with branch lengths representing expected substitutions per site. Node-associated support values for major nodes are as follows: Bayesian posterior probability/ML bootstrap/NJ bootstrap probabilities; '*' represents posterior probabilities of 1.00 or bootstrap probabilities of 100, 'NR' indicates a node that was not resolved. Haplotypes are named by both site and molecular voucher code. Lettered nodes are those for which tmrca was estimated.

Island. Lineages 2 and 4 were sampled from three sites each, however for both lineages one site (Borlase Creek) was represented by only a single individual. In Lineage 2, the two remaining sites, Hope and Owen Rivers, were geographically proximate (<15km), whereas the remaining two sites in Lineage 4, Jackson Creek and Rotoroa Creek, are separated by

Table 3.2. Corrected mean among-lineage pairwise divergence for *N. forsythi*.

	Lineage 1	**Lineage 2**	**Lineage 3**	**Lineage 4**	***Naonella* sp. n. 1**
Lineage 1	-				
Lineage 2	10.1	-			
Lineage 3	10.1	9.90	-		
Lineage 4	9.80	9.30	5.30	-	
***Naonella* sp. n. 1**	12.2	13.0	13.4	12.4	-

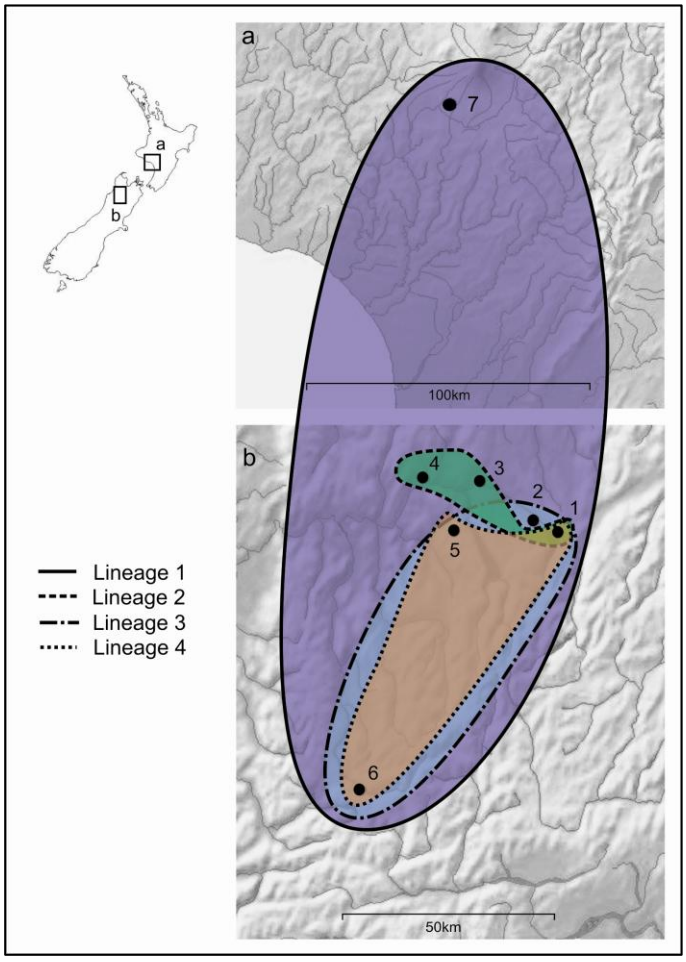


Figure 3.5. Geographical distribution of *N. forsythi* COI lineages as indicated by coloured circles encompassing sample sites – purple: Lineage 1; green: Lineage 2; blue: Lineage 3; orange: Lineage 4.

approximately 70km. This pattern implies some degree of movement of individuals across the Buller River catchment; however, the absence of Lineages 2 and 4 from Speargrass Creek and Lineages 2 and 3 from Hope and Owen Rivers suggested some restriction on dispersal.

Estimated among-lineage divergence times

Estimates of times to most recent common ancestor for the major nodes of the inferred phylogeny suggest that the initial diversification of *Naonella forsythi* began in the late Miocene with this taxon estimated to have last shared a common ancestor with the putative second stream species approximately 6.31mya (8.25-4.59mya – Table 3.3). Subsequent diversification of *N. forsythi* continued from around 5.78mya (7.44-4.27mya) just prior to the Miocene-Pliocene boundary. Diversification in *N. forsythi* continued throughout the Pliocene, culminating in the radiation of haplotypes belonging to Lineage 3 around 2.32mya (3.48-1.35mya).

Table 3.3. Time to most recent common ancestor estimated for relevant nodes in the inferred *N. forsythi* phylogram (Figure 3.4).

Node Label	Lineage/s Included	TMRCA (mya)	95% Credibility Intervals (mya)	Effective Sample Size (ESS)
A	*N. forsythi* and *N.* sp. n. 1	6.31	8.25-4.59	420.41
B	All ingroup	5.78	7.44-4.27	355.37
C	Lineage 2, 3 & 4	4.71	6.15-3.28	229.80
D	Lineage 1	4.00	5.46-2.70	371.39
E	Lineages 3 & 4	3.41	4.74-2.21	249.88
F	Lineage 2	2.99	4.37-1.77	233.50
G	Lineage 4	2.58	3.85-1.44	714.92
H	Lineage 3	2.32	3.48-1.35	242.81

The results outlined above clearly represented a problem for resolving the range and extent of genetic structure among populations of *N. forsythi*. The identification of four highly divergent, morphologically cryptic evolutionary

lineages with variable distributions resulted in the sampling design being rendered largely unbalanced. As it remains unknown whether the observed mitochondrial lineages are reproductively isolated, it was deemed prudent to analyse population structure only among sites within a single lineage. As such, the abundant and broadly distributed Lineage 1 provided sufficient geographical intensity across the study region to explore spatial phylogeographic patterns more fully.

Genetic structuring within Lineage 1

Lineage 1 comprised 107 individuals from all seven sample sites, representing 75 unique haplotypes. Tajima's D tests of neutrality were non-significant for all sites and gene diversity within sites was high (Table 3.4).

Among-site partitioning of genetic diversity

Conventional pairwise Φ_{ST} indices suggested some degree of structure among sites; however, the pattern of genetic differentiation was not obviously related to geography (Table 3.5). Amongst the South Island sites, Site 3 was significantly different to all other sites, whilst Site 5 was different to all other sites except Site 6. Significant genetic structure was observed among the relatively geographically proximate Sites 1 and 2 (<7km), yet no genetic

Table 3.4. Population genetic summary statistics for Lineage 1 of *N. forsythi*.

Sample Site	Sample size	Tajima's D	D p-value	θ_π	Gene Diversity
1	10	-1.350	0.0980	5.9430	0.9556+/-0.0594
2	18	-0.025	0.5480	13.945	0.9935+/-0.0210
3	34	-1.233	0.1000	12.815	0.9929+/-0.0090
4	12	0.267	0.6490	12.283	0.9091+/-0.0562
5	22	0.529	0.7200	1.0020	0.9307+/-0.0463
6	9	-1.498	0.0610	1.9840	0.8333+/-0.1265
7	2	0.000	1.0000	15.405	1.0000+/-0.5000

Table 3.5. Below diagonal shows conventional Φ_{ST} estimates among populations within Lineage 1 of *N. forsythi* - values in bold represent significant pairwise comparisons (P<0.05). Above diagonal shows Nei's D_A indices among populations.

	1	2	3	4	5	6
1	-	0.00461	0.01860	0.00130	0.00020	0.00011
2	**0.22770**	-	0.00248	0.00049	0.00672	0.00629
3	**0.53895**	**0.13937**	-	0.00831	0.02080	0.02146
4	0.08945	0.02524	**0.32051**	-	0.00232	0.00203
5	**0.10240**	**0.40989**	**0.64966**	**0.27173**	-	0.00010
6	0.01508	**0.29964**	**0.58613**	0.15103	0.06772	-

differentiation was observed between sites almost 80km apart, implying extensive movement among some sites, but restricted movement among others.

The haplotype network mirrored the inferred phylogeny and clearly illustrated the extremely high diversity within Lineage 1 (Figure 3.6). There was a large number of unsampled hypothetical haplotypes, implying that the current data may not be an appropriate representation of total genetic diversity within this Lineage. Only 14 haplotypes were shared by more than a single individual and of these only eight were shared among sites. Shared haplotypes were distributed across the network and comprised six internal and eight tip haplotypes; five tip haplotypes were shared among sites. The geographical distribution of haplotypes shared among sites was further resolved by mapping the frequencies of haplotypes shared among sites and demonstrated clearly several shared haplotypes distributed among geographically distant sites (Figure 3.7). For example, the internal Haplotypes 2 and 3 are shared between five and four sites, respectively. Moreover, despite being significantly different, the geographically proximate Sites 1 and 2 share three haplotypes in common (Haplotypes 1, 2 and 3), including the derived tip Haplotype 1, implying the presence of some gene flow among these sites.

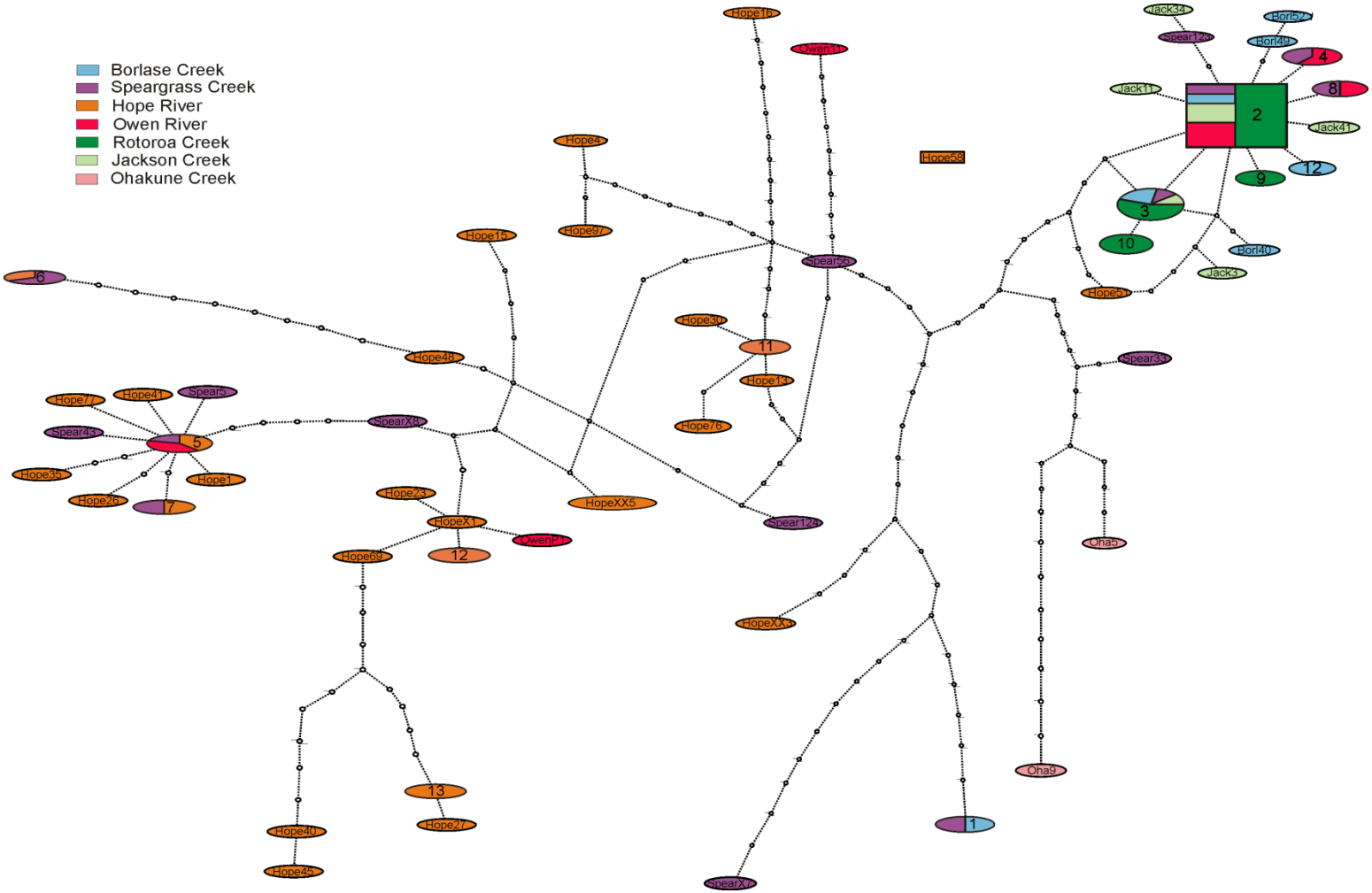


Figure 3.6. Parsimony network for COI haplotypes of Lineage 1 of *N. forsythi*. Singleton haplotype labels follow those in Figure 3.4, shared haplotypes are numbered arbitrarily and are coloured by site. Unfilled circles represent unsampled hypothetical haplotypes.

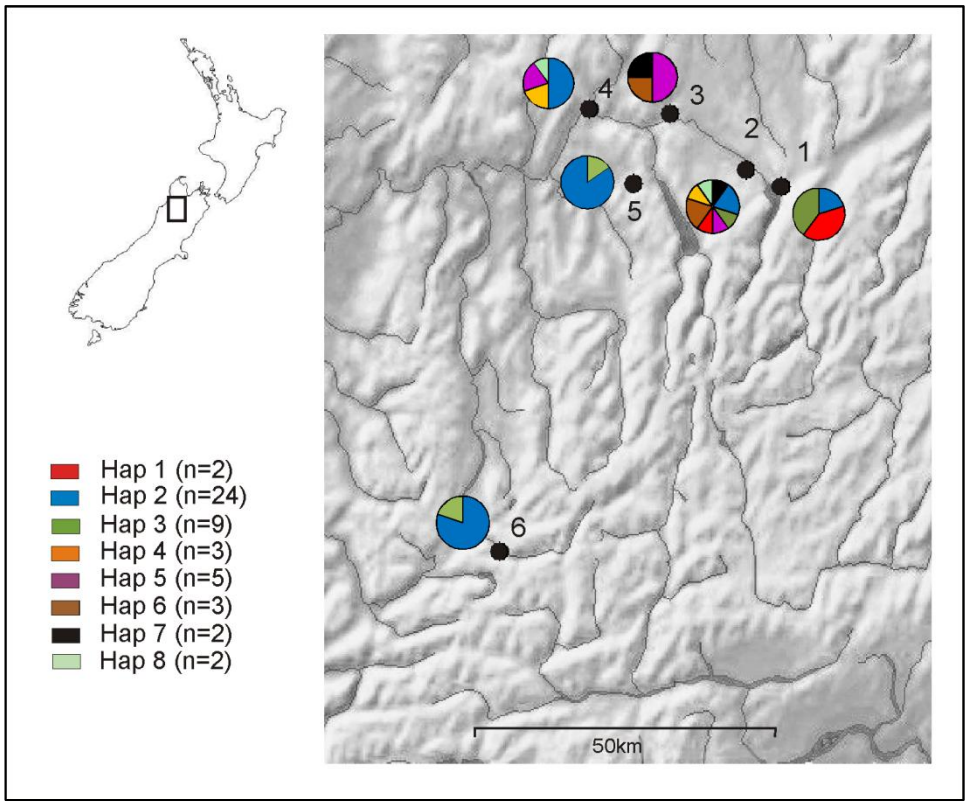


Figure 3.7. Geographical distribution of COI haplotypes belonging to Lineage 1 of *N. forsythi* that are shared among sites.

This pattern was mirrored somewhat in the unrooted neighbour-joining population tree reconstructed using Nei's D_A genetic chord distances (Figure 3.8). This suggested Sites 1, 5 and 6 to be closely related to each other, with this group more closely allied to Site 4 than Sites 2 and 3. This was perhaps driven by high within-site diversity at Sites 2 and 3 and/or by the presence of haplotypes shared between only these two sites (e.g., Haplotypes 6 and 7).

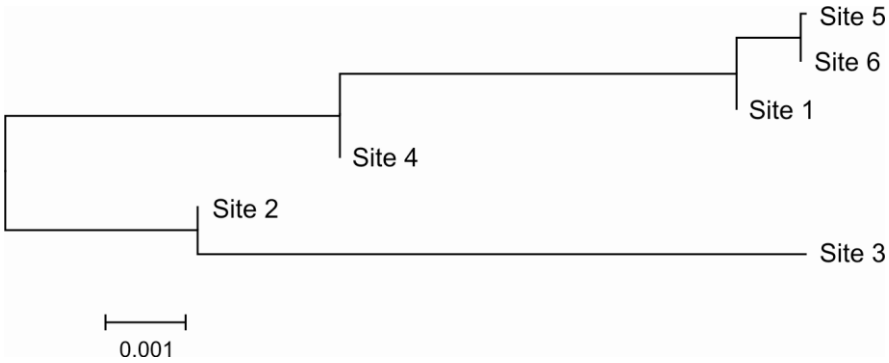


Figure 3.8. Unrooted neighbour-joining topology based on Nei's D_A genetic chord distances among sites for Lineage 1 of *N. forsythi*.

The pattern of high diversity among sites revealed by Φ_{ST} estimates was further reflected by partitioning genetic distance to stream section (Figure 3.9). All stream branches on which sites were located were attributed some genetic distance, while two of three connecting sections of the Buller River were attributed no distance. The greatest genetic distance (0.3505) was partitioned to the section of the Hope River between Site 4 and the main Buller River tributary, whilst 0.1672 was assigned to the section that separated Site 5 near Lake Rotoroa and the Buller River. Statistical support for the fit of the data to the Streamtree model was quite low (R^2 = 0.551). This may be due to the inability of the Streamtree algorithm to properly account for non-stepping stone patterns of differentiation or for highly differentiated populations, both of which can underestimate genetic distance between sites (Kalinowski *et al.*, 2008).

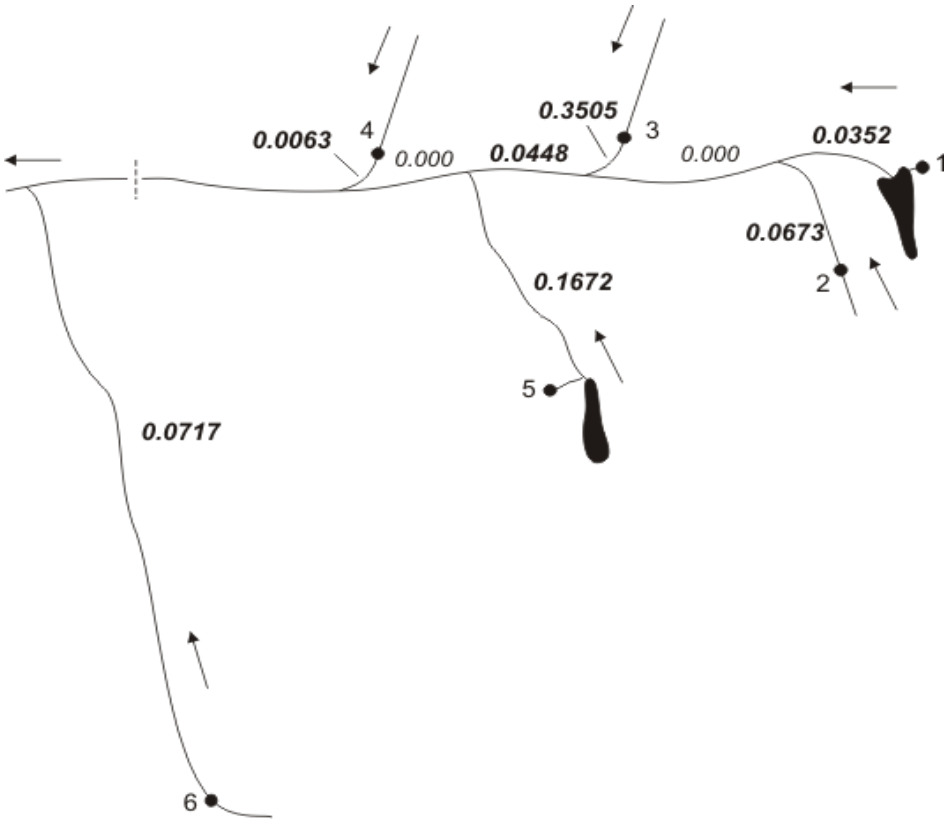


Figure 3.9. Schematic representation of the genetic distance assigned to each stream section (values in italics); i.e., genetic distance of 0.0352 assigned to the stream section between Site 1 and the point of coalescence with the stream of Site 2. Direction of stream flow is indicated by arrows.

Mantel tests of isolation by log-adjusted distance reflected the general pattern revealed by the Φ_{ST} indices in showing no correlation between geographical and genetic distance using both Euclidean distance (R = -0.218, p = 0.720) and stream distance (R = -0.227, p =0.726) (Figure 3.10). Together, these data indicate that geographical distance, even among South Island sites alone, does not play a significant role in structuring genetic diversity among sampled *N. forsythi* populations.

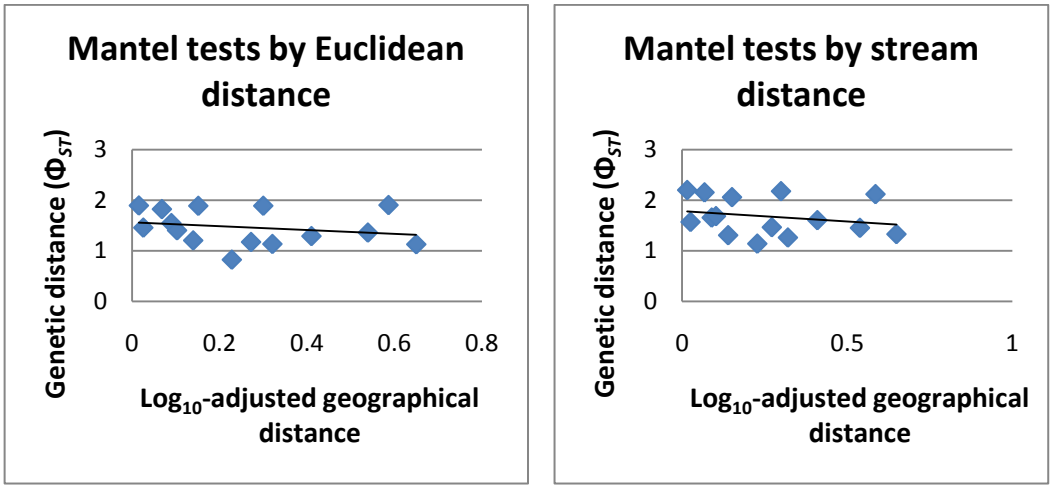


Figure 3.10. Scatterplots showing the relationship between genetic distance (Φ_{ST}) and both $\log_{10}$-adjusted Euclidean and stream distance.

Southern South America: *Ferringtonia patagonica*

Collections at eight sites on either side of the Andean mountain range in central Chile and Argentina recovered 62 individuals of *F. patagonica* (Table 3.6), representing 45 unique COI haplotypes of 734bp in length. The ratio of transitions to transversions was relatively high at 5.125 and the plot of the relationship between each substitution type and genetic distance showed evidence of a plateau in transitions as genetic distance increased (Figure 3.11). This indicates that sequences are unlikely to have reached saturation and thus the observed genetic diversity accurately represents true diversity (Arbogast *et al.*, 2002). Tajima's D tests for neutrality confirmed that sequence evolution had not been influenced by selection (D = -0.1199, p = 0.513). Assumptions of clocklike evolution were rejected (p = 0.0065) and

thus a relaxed clock model of evolution was implemented for estimating times to most recent common ancestor for relevant nodes.

Table 3.6. Geographical location of South American sample sites and numbers of *F. patagonica* sourced from each site. Elevation is given as metres above sea level (masl).

Site Name (Country)	Site Number	Latitude	Longitude	Elevation (masl)	Sample Size
Rio Palguin (CH)	1	39°27'S	71°48'W	825	2
Rio Don Baucha (CH)	2	39°23'S	71°47'W	680	13
Estero La Casilla (CH)	3	39°14'S	71°56'W	240	4
Estero Correntoso (CH)	4	39°18'S	72°04'W	215	11
Rio Chaleufu (CH)	5	40°44'S	72°18'W	465	1
Unnamed creek near Peulla (CH)	6	41°08'S	72°24'W	270	3
Arroyo Quilanlahue (ARG)	7	40°09'S	71°33'W	660	13
Arroyo Partido (ARG)	8	40°14'S	71°22'W	1185	15

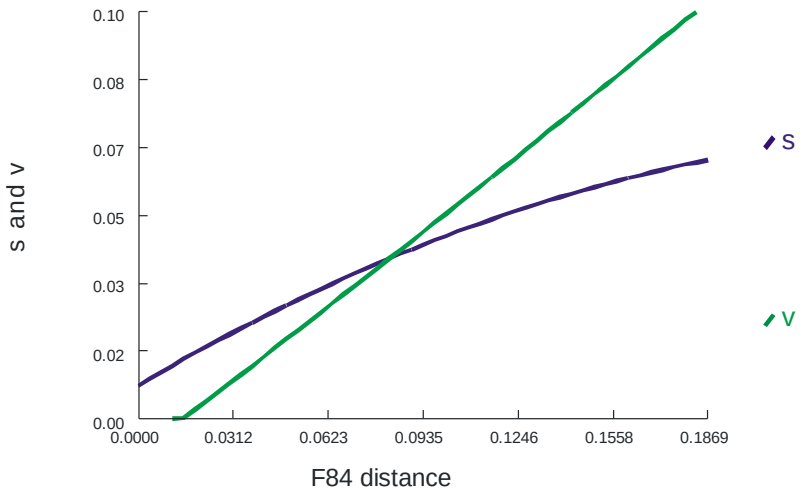


Figure 3.11. The relationship of transitions (s) and transversions (v) to genetic distance in *F. patagonica*.

Phylogenetic reconstruction

The different methods of phylogenetic reconstruction implemented produced topologies that were generally concordant, although with varying levels of resolution (Figure 3.12). Four highly divergent lineages were identified in

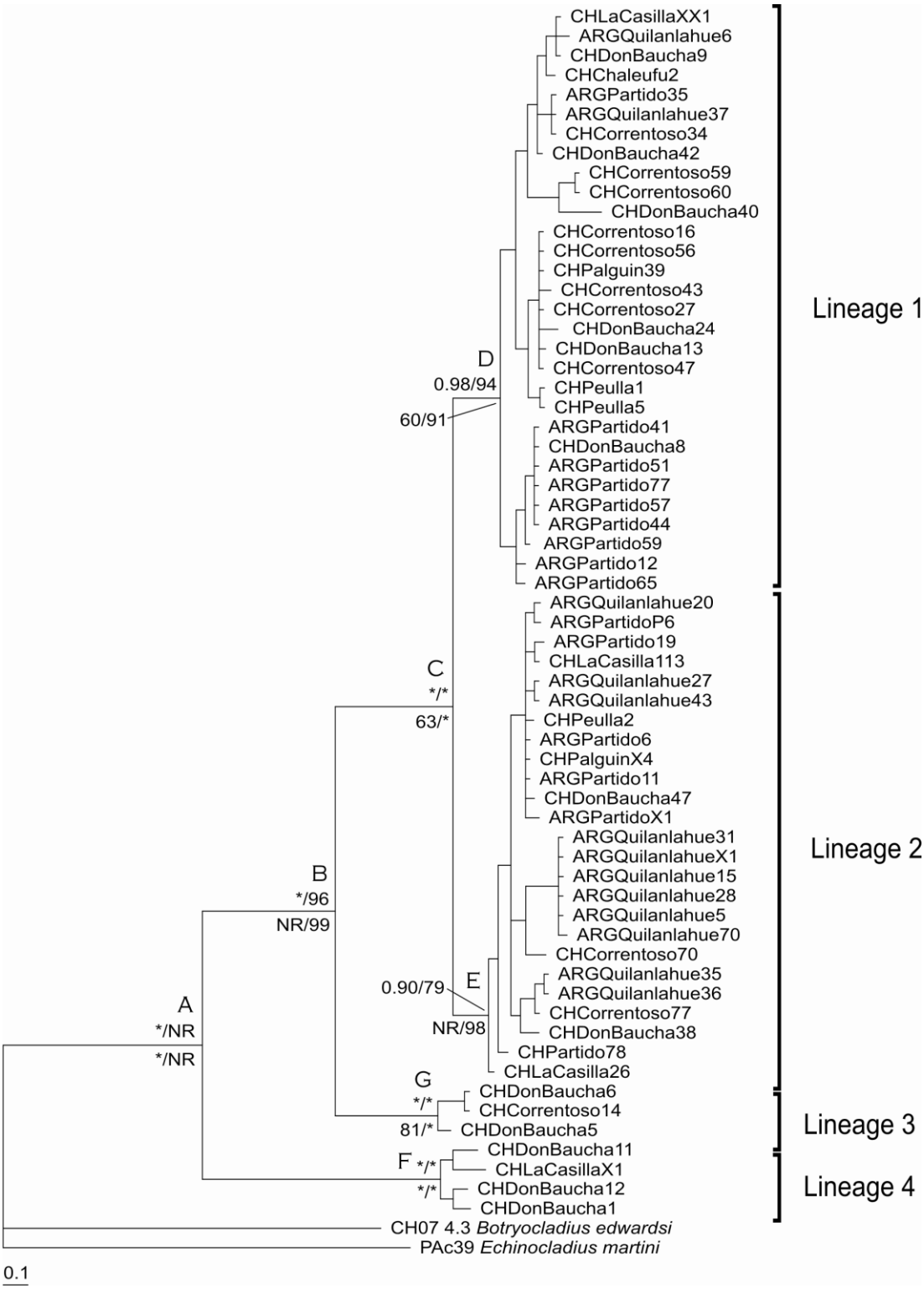


Figure 3.12. Consensus Bayesian topology for *F. patagonica* COI sequences with branch lengths representing expected substitutions per site. Node-associated values for relevant nodes are as follows: above branch – Bayesian posterior probability/MP bootstrap probability, below branch – ML bootstrap/NJ bootstrap probabilities; '*' represents posterior probabilities of 1.00 or bootstrap probabilities of 100, 'NR' indicates a node that was not resolved. Haplotypes are named by both site and molecular voucher code. Lettered nodes are those for which tmrca was estimated.

F. patagonica that were generally well-supported across methods of reconstruction, the exception being Lineage 2, which was not resolved by ML. Levels of support for nodes connecting lineages were generally high across methods, particularly the Bayesian reconstruction in which all connecting nodes possessed posterior probabilities of 1.00. One difference in tree topology was noted across the different methods of phylogenetic inference; ML placed Lineages 3 and 4 as sister groups, whereas Bayesian, MP and NJ methods all placed Lineage 4 as sister to Lineages 1+2+3, and Lineage 3 sister to 1+2. The ML bootstrap support for this anomalous grouping, however, was very low (ML: 57), whereas support for the alternative grouping was much greater (B: 1.00, MP: 96, NJ: 99). Thus, there was little discordance among phylogenies inferred from the various methods of reconstruction and node B was denoted simply as not resolved under ML in Figure 9. Corrected mean pairwise divergence among lineages ranged from 2.00% between the close sister groups Lineages 1 and 2 and 10.5% between Lineages 1 and 4, and Lineages 2 and 4 (Table 3.7). This was reflected in the shallow divergence of Lineages 1 and 2 in the inferred phylogram, compared with the much deeper divergence of Lineages 3 and 4.

Table 3.7. Corrected mean among-lineage pairwise divergence for *F. patagonica*.

	Lineage 1	**Lineage 2**	**Lineage 3**	**Lineage 4**
Lineage 1	-			
Lineage 2	2.00	-		
Lineage 3	5.80	5.70	-	
Lineage 4	10.50	10.50	9.20	-

No relationship was apparent between the distributions of the four divergent lineages revealed here and the Andean mountain range, in that sites from the western side of the Andes did not form a monophyletic group to the exclusion of sites from the east, or vice versa (Figure 3.13). As seen previously for *N. forsythi*, each lineage comprised individuals from multiple, often broadly distant, streams. Lineages 1 and 2, in particular, were widely distributed on both sides of the Andes mountain range and were recovered from eight and seven sites, respectively. Indeed, Rio Chaleufu (Site 5) was the only study

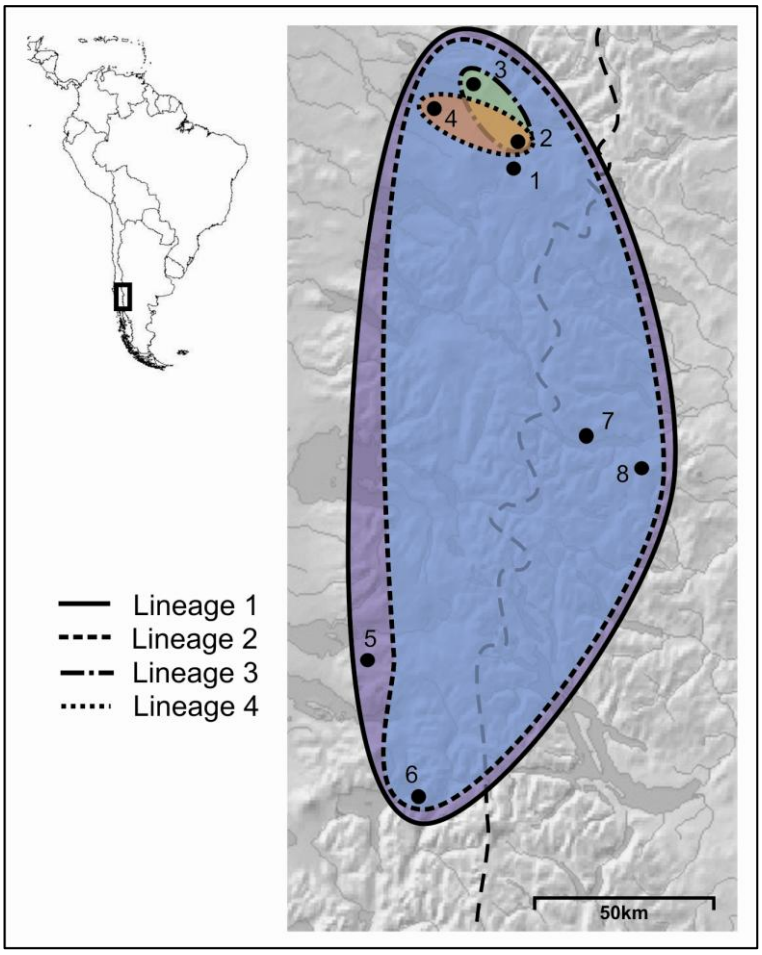


Figure 3.13. Geographical distribution of *F. patagonica* COI lineages as indicated by coloured circles encompassing sample sites – purple: Lineage 1; blue: Lineage 2; green: Lineage 3; orange: Lineage 4.

site at which Lineage 2 was absent, however, only a single individual was recovered from this site and thus the extent of diversity within this site remains unknown. Lineages 3 and 4, on the other hand, were recovered from only two sites each and were sympatric at Rio Don Baucha, but also occurred at Estero Correntoso and Estero La Casilla, respectively. Thus, whilst the distributions of these two lineages could be interpreted as somewhat restricted over the geographical range of the current study, Lineages 1 and 2 clearly had extensive distributions.

Estimated among-lineage divergence times

Times to most recent common ancestor estimated for the major nodes of the

inferred phylogeny suggest that initial diversification of *F. patagonica* began in the late Miocene, with all individuals of the species estimated to have last shared a common ancestor approximately 7.44mya (11.10-4.41mya – Table 3.8). Diversification in *F. patagonica* apparently continued throughout the late Miocene with divergence of the ancestor of Lineage 3 and the common ancestor of Lineages 1 and 2 estimated at around 6.02mya (9.20-3.37). Divergence of the ancestors of Lineages 1 and 2 occurred during the Pliocene epoch, around 4.54mya (7.41-2.30mya), prior to the radiation of haplotypes belonging to these two lineages and Lineage 4, whilst Lineage 3 radiated in the early Pleistocene, 1.78mya (4.20-0.18mya).

Table 3.8. Time to most recent common ancestor estimated for relevant nodes in the inferred *F. patagonica* phylogram.

Node Label	Lineage/s Included	TMRCA (mya)	95% Credibility Intervals (mya)	Effective Sample Size (ESS)
A	All ingroup	7.44	11.10-4.41	412.52
B	Lineages 1, 2 & 3	6.02	9.20-3.37	350.34
C	Lineages 1 & 2	4.54	7.41-2.30	231.55
D	Lineage 1	3.47	5.91-1.56	267.10
E	Lineage 2	3.45	5.92-1.48	305.97
F	Lineage 4	2.15	4.32-0.49	596.40
G	Lineage 3	1.78	4.20-0.18	1339.41

As for *N. forsythi* in New Zealand, the identification of four highly divergent, morphologically cryptic evolutionary lineages rendered the sampling design unbalanced, thus largely preventing resolution of the range and extent of phylogeographic structure among populations. Nevertheless, spatial phylogeographic patterns within individual lineages were explored among sites at which sample sizes were deemed adequate (>5).

Genetic structuring within Lineages 1 and 2

Lineage 1 comprised 30 individuals sampled from all eight sites, represented by 23 unique haplotypes. Of these, 23 individuals (15 haplotypes) were sampled from three sites where sample sizes were greater than five, two in Chile (Sites 2 and 4) and a single site in Argentina (Site 8) (Table 3.9). Tajima's D tests of neutrality were non-significant for all sites where Lineage 1 was present and gene diversity within sites was high; the latter likely related to the low sample sizes at each site. The haplotype network of this lineage showed five haplotypes that were shared by two or more individuals, four of which were shared among sites (Figures 3.14 and 3.15). Shared haplotypes were distributed across the network and the two haplotypes shared across the Andes mountain range between Sites 4, 7 and 8 and Sites 2 and 8, respectively, appeared to be tip haplotypes, suggesting recent dispersal among sites.

Table 3.9. Population genetic summary statistics for Lineages 1 and 2 of *F. patagonica*.

Lineage	Sample Site	Sample Size	Tajima's D	D p-value	θ_π	Gene Diversity
Lineage 1	1 (CH)	1	0.0000	1.0000	0.0000	1.0000+/-0.0000
	2 (CH)	6	-0.77609	0.2830	9.7893	1.0000+/-0.0962
	3 (CH)	1	0.0000	1.0000	0.0000	1.0000+/-0.0000
	4 (CH)	8	0.44274	0.6860	0.6425	0.7500+/-0.1391
	5 (CH)	1	0.0000	1.0000	0.0000	1.0000+/-0.0000
	6 (CH)	2	0.0000	1.0000	0.0000	0.0000+/-0.0000
	7 (ARG)	2	0.0000	1.0000	6.0000	1.0000+/-0.5000
	8 (ARG)	9	-1.22061	0.1270	3.3147	0.9444+/-0.7020
Lineage 2	1 (CH)	1	0.0000	1.0000	0.0000	1.0000+/-0.0000
	2 (CH)	2	0.0000	1.0000	7.0000	1.0000+/-0.5000
	3 (CH)	2	0.0000	1.0000	6.0000	1.0000+/-0.5000
	4 (CH)	2	0.0000	1.0000	8.0000	1.0000+/-0.5000
	5 (CH)	1	0.0000	1.0000	0.0000	1.0000+/-0.0000
	7 (ARG)	11	1.38108	0.9350	5.4000	0.8545+/-0.0852
	8 (ARG)	6	-1.12062	0.1850	3.2000	0.9333+/-0.1217

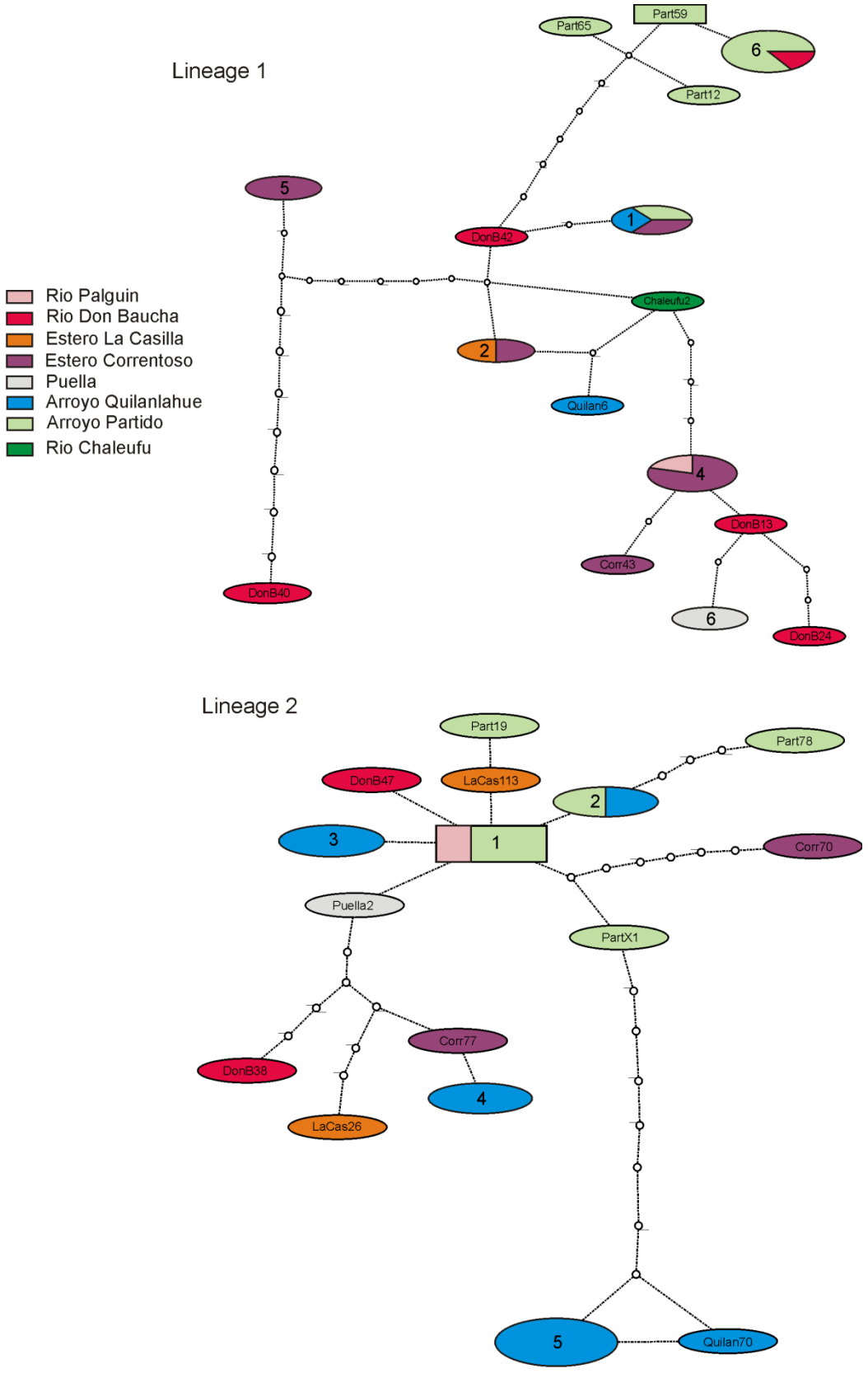


Figure 3.14. Parsimony network for COI haplotypes of Lineage 1 of *N. forsythi*. Singleton haplotype labels follow those in Figure 3.4, shared haplotypes are numbered arbitrarily and are coloured by site. Unfilled circles represent unsampled hypothetical haplotypes.

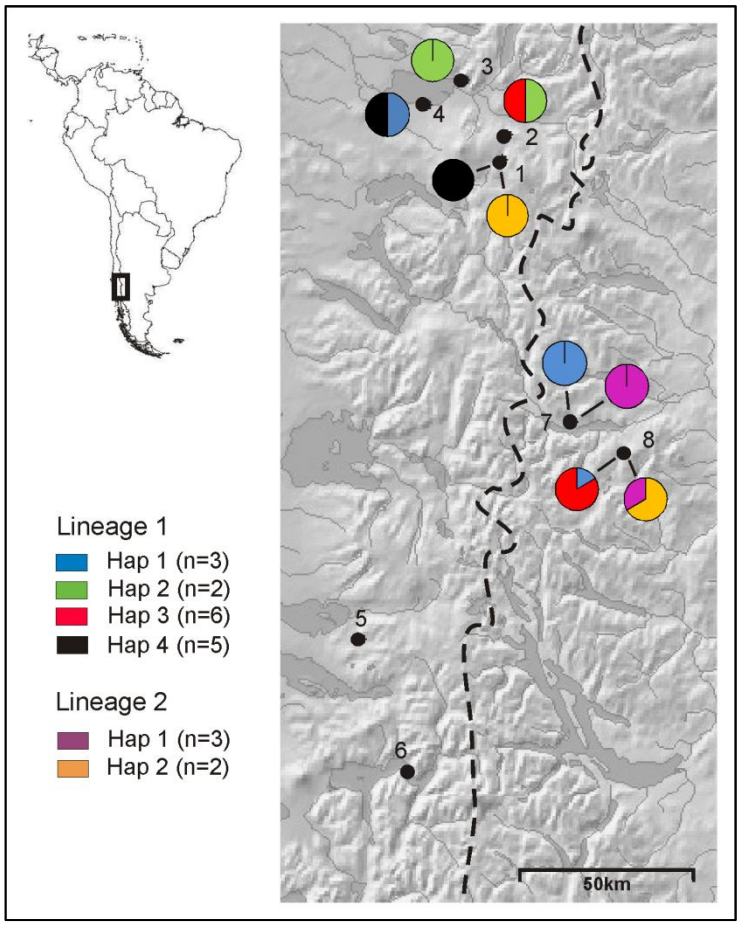


Figure 3.15. Geographical distribution of COI haplotypes shared among sites in Lineages 1 and 2 of *F. patagonica*.

Lineage 2 comprised 25 individuals from 7 sites, representing 20 unique haplotypes, of which 17 individuals (10 haplotypes) were sampled from two sites that possessed sample sizes greater than five, both of which were located in Argentina (Sites 7 and 8 - Table 3.9). Tajima's D tests of neutrality were non-significant for both sites within Lineage 2 and gene diversity within sites was high. Five haplotypes were shared by two or more individuals, three of which were confined to Site 8 and one that was shared between the geographically close Sites 7 and 8 (Figures 3.14 and 3.15). A single internal haplotype was shared across the Andes between Sites 1 and 8.

Among-site partitioning of genetic diversity

For sites within Lineage 1 that possessed sample sizes higher than five individuals (Sites 2, 4 and 8), conventional Φ_{ST} indices among populations

suggest that the Chilean Sites 2 and 4 were not significantly different to each other (Φ_{ST} = 0.03746, p = 0.24902), but that both were significantly different to the Argentine Site 8 (Site 2: Φ_{ST} = 0.32396, p = 0.00977; Site 4: Φ_{ST} = 0.55131, p = 0.00098). A Mantel test of isolation by distance showed that the large divergence among Chilean and Argentine populations did not correlate with geographical distance (R = 0.9247, p = 0.1730), despite the relatively close proximity of the Chilean sites (~23km) compared to the distance between these sites and Site 8 (Site 2: ~102km; Site 4: ~115km).

For sites within Lineage 2 that possessed sample sizes greater than five individuals (Sites 7 and 8), significant genetic differentiation was implied by conventional Φ_{ST} estimates (Φ_{ST} = 0.24272, p = 0.03809).

3.4 Discussion

The patterns of diversity presented here for *N .forsythi* and *F. patagonica* are strikingly similar and show clear patterns of high levels of genetic divergence across the study regions. In both species, and as revealed in *E. martini*, several highly divergent genetic lineages were identified that are estimated to have been extant from the early Pleistocene at the latest. Unlike *E. martini*, however, lineages within *N. forsythi* and *F. patagonica* were identified from multiple sample sites, implying no geographical restriction across the study regions; moreover, almost all sample sites possessed individuals from more than one genetic lineage. There was no distinct difference observed among sampled North and South Island populations of *N. forsythi*; North Island representatives, whilst few in number, were nested within a lineage widespread throughout the study region and appear as tip haplotypes in the parsimony network. Likewise, there was no apparent relationship between the distribution of *F. patagonica* lineages and the Andean mountain range. Within lineages for which phylogeographic structure could be tested, significant differences among populations were recorded for both taxa. There was no clear pattern of geographical genetic structure among populations

within Lineage 1 in *N. forsythi*; however, the data for *F. patagonica* suggested that populations within lineages that had diverged prior to orogenic uplift were bisected by the Andes and subsequently differentiated. Thus, the most parsimonious evolutionary explanation for both taxa appears to be one of historical population isolation leading to divergence of monophyletic mitochondrial lineages, followed by extensive post-isolation range expansion throughout the study areas but with some contemporary restrictions to gene flow driving divergence among populations within lineages. Haplotypes shared among distant sites, in the case of *N. forsythi*, or across the Andes for *F. patagonica*, thus likely represent the retention of ancestral types in the presence of restricted contemporary movement among sites.

Naonella forsythi

Congruence between the timing of divergence and glaciation events

New Zealand's South Island has experienced significant tectonic upheaval and glaciation events since the late Miocene (Walcott, 1998). Uplift of the Southern Alps began around 6mya and isolated populations of many taxa on either side, thereby restricting gene flow and driving divergence. Several glaciation events throughout this period covered the majority of the Southern Alps, rendering much of the South Island as tundra and causing retraction of the distributions of many taxa into small isolated refugia (Heads, 1998; Trewick & Wallis, 2001). Two major refuges that are considered to have remained relatively stable during glaciation events have been identified in the northwest (NSIP) and southeast (SSIP) of the South Island (Wardle, 1963; Leathwick *et al.*, 2007). Evidence from the Buller River catchment, the focus of the current study, suggested that some headwater populations may have originated in catchments on the eastern side of the Southern Alps and later colonised the Buller River catchment following river capture (Waters & Wallis, 2000).

The current data suggest that divergence in *N. forsythi* populations began in the late Miocene, coincident with the onset of major geological and climatic instability. The distributions of highly divergent mitochondrial lineages show no evidence for geographical endemicity within the Nelson Lakes section of the Buller River catchment and do not accord with the predictions of the 'Stream Hierarchy Model' (Meffe & Vrijenhoek, 1988). This implies that *N. forsythi* may have been isolated in multiple refugia throughout these periods, but there is no indication of the geographical location of such refugia. It is possible that the discrete lineages identified here arose in isolation in separate parts of the Buller River catchment, potentially in small tributaries and side branches that remained comparatively stable while the main river channel became unsuitable. In particular, sections of the catchment that do not originate on the Southern Alps (e.g., those to the north, flowing south from the mountains of the Kahurangi National Park) may have acted as refugia while headwater streams on the Alps were glaciated (Suggate & Almond, 2005). Under this hypothesis, lineages could have thus recolonised previously unsuitable habitat and expanded their distributions resulting in multiple lineages now co-existing in sympatry. This is reinforced by patterns of genetic structure revealed within Lineage 1 that suggest extensive movement over a large geographical scale. Alternatively, lineages may have colonised the Buller River catchment from neighbouring river systems, either through migration or river capture. Galaxiid fishes provide evidence for historical river capture in the Nelson Lakes region of headwater streams from east-flowing rivers, driven by differential erosion (Waters & Wallis, 2000). Feasibly, previously isolated populations of *N. forsythi* may have been incorporated into the Buller River complement of lineages through similar processes. The montane areas of the NSIP have experienced significant erosion following original uplift, some of which was glacial-driven, thus providing potential for dramatic changes to catchment boundaries over time and reflected in the general dynamism and unpredictability of New Zealand streams (Winterbourne *et al.*, 1981).

There is a considerable literature base concerning the range and extent of flight in semi-aquatic invertebrates, including chironomids (synthesised

previously in Chapter 2), which implies that some chironomid taxa are capable of dispersal over distances of up to several kilometres, but that relative vagility varies greatly among taxa. Given the relatively wide distribution of lineages among geographically distant sites, particularly in Lineage 1, it seems reasonable to infer that *N. forsythi* is capable of extensive movement among catchments. This is most likely to have occurred through headwater exchange and may be mediated further either by direct flight or passive dispersal on wind currents which are a notable climatic feature of the montane regions of New Zealand. Conversely, the steep topography that typifies the NSIP, and the Southern Alps as a whole, may consequently act as a significant physical barrier to dispersal among headwater streams of different catchments, as observed for some semi-aquatic taxa inhabiting Rocky Mountain streams in Colorado, USA (Hughes *et al.*, 1999; Hughes *et al.*, 2003). Unfortunately, however, hypotheses regarding the geographical origin of mitochondrial lineages revealed here cannot be tested formally with the current dataset and must await additional sampling of catchments in adjacent regions to acquire a more complete representation of the geographical range of the mitochondrial lineages.

Within the widely distributed Lineage 1, distinct genetic structuring is evident among some sites within the Buller River catchment, even where geographically proximate, whilst some other site pairs separated by considerable geographical distances did not differ significantly. For example, Borlase Creek is separated by ~7km straight line distance from the next closest site sampled here (Speargrass Creek) and is also isolated from the rest of the Buller River catchment by Lake Rotoiti, a potential barrier to movement. As predicted if movement among sites is restricted by the lake barrier, Borlase and Speargrass Creeks were significantly different from each other; however, Borlase Creek was not significantly diverged from two other, more distant sites within the catchment. This pattern was further exemplified by a lack of evidence for isolation by distance across the total dataset.

Taken together, these data clearly do not conform to the predictions of the ‘Stream Hierarchy Model’, and demonstrate that within a lineage genetic

structure also was not congruent simply with geography. Whilst the observed pattern did conform somewhat with that expected from largely restricted gene flow among sites with occasional adult migration, the close relationships among geographically distant Sites 1, 5 and 6 and significant genetic structure among proximate sites implied more frequent adult dispersal not confined to the stream channel. This pattern may reflect large differences in sample size among some sites, which may result in poor representation of the real diversity present within and among sites. Alternatively, input of genetic diversity into some sites through immigration from neighbouring catchments may have influenced their divergence from other sites within the catchment. To evaluate genetic structure in this region in more detail, sites in adjacent catchments need to be sampled, in combination with sites intermediate to those in the current study to encompass the within-lineage diversity more fully. Furthermore, streams in the northeast South Island, on the eastern side of the Southern Alps, should be included to test hypotheses proposed by Waters & Wallis (2000) concerning historical river capture events in the region. This will enable the genetic diversity within the Buller River catchment revealed here to be viewed in a broader regional context and may shed light on the geographical origins of observed lineages.

Ferringtonia patagonica

Congruence between the timing of divergence and glaciation events

As is the case for the South Island of New Zealand, southern South America has experienced significant tectonic uplift and extensive glaciation events since the late Oligocene that have dramatically altered regional geomorphology, climate and ecology (Lagabrielle *et al.*, 2004; Blisniuk *et al.*, 2005; Rabassa, 2008). The ongoing subduction of the Nazca plate under the South American plate has driven phases of tectonic uplift throughout the last 25 million years, the most recent of which occurred in the late Pliocene, giving rise to the Andes mountain range (Lagabrielle *et al.*, 2004). Additionally, since the late Miocene, several glaciation events have affected

much of the southern Andes from the Pacific coast eastward to the Argentine plains (Mercer, 1983), with extensive glaciation extant in this region until the start of the Holocene (~10 000ya - McCulloch *et al.*, 2000). The dramatic geological and glacial histories of southern South America have been shown to have driven population isolation and significant genetic differentiation in many taxa native to the region (e.g., Palma *et al.*, 2002; Marchelli & Gallo, 2006; Ruzzante *et al.*, 2006; Ramirez *et al.*, 2008; Victoriano *et al.*, 2008).

The data for *F. patagonica* presented here closely mirrors that recovered for *N. forsythi* in New Zealand, with diversification within *F. patagonica* suggested to have begun in the late Miocene, with all currently recognised lineages extant by the early Pleistocene. This coincides with the proposed onset of major glaciation across the region and predates the most recent and significant orogenic phase of Andean uplift. Two of the four lineages recovered here were comparatively rare, each identified from only a few individuals from two sites on the western side of the Andes, whereas the remaining two lineages were widespread across the study region and were present on either side of the Andes. This implies that Andean orogeny may not have driven diversification of the observed lineages and suggests instead that populations of *F. patagonica* may have been isolated in multiple refugia during periods of glaciation and the generally sympatric distribution of lineages across the study sites likely represents extensive post-glacial range expansion.

This pattern of strong genetic structure revealed within a taxon attributed to historical glaciations is consistent with existing evidence from other taxa in southern South America. Genetic structure in some plant and animal taxa has shown evidence for isolation in refugia that do not correspond to Andean bisection (e.g., conifers - Allnutt *et al.*, 1999; opossums - Palma *et al.*, 2002; asters - Muellner *et al.*, 2005), whilst other taxa show patterns of structure which correlate with latitude, interpreted as representing colonisation from multiple historical refugia located at glacial margins (e.g., *Nothofagus nervosa* - Marchelli & Gallo, 2006; small marsupials - Himes *et al.*, 2008; guanaco - Marin *et al.*, 2008; *Nothofagus antarctica* - Pastorino *et al.*, 2009).

Currently, it is unclear if the latter pattern can explain diversity in *F. patagonica*; determining this will require further sampling of areas to the north and south of the current study region, to understand better the geographical distribution of these lineages. Globally, glacial cycles are thought to have driven genetic diversification in a wide variety of taxa by dramatically altering the extent and composition of available habitat. Whilst much research has focused on the most recent glacial cycle at the end of the Pleistocene (reviewed in Hewitt, 2000), particularly in relation to the impact of the northern hemisphere ice caps on population structure (Knowles, 2001; Schmitt, 2007; Hewitt, 2008), earlier (Miocene-Pliocene) glacial cycles have been recognised as equally important in altering the landscape and shaping genetic structure in many southern hemisphere taxa (e.g., Martin, 1998; Moritz *et al.*, 2000; Trewick & Morgan-Richards, 2005).

Among the three sites appropriate for population level analysis where the widespread Lineage 1 was present, there was some evidence for the Andean mountain range acting as a barrier to dispersal. Fixation indices were non-significant between sites on the same side of the range, but were significant in the only two possible pairwise comparisons across the Andean range. If this lineage arose in isolation during glaciation, this may indicate some degree of population structuring driven by Andean uplift bisecting populations following re-expansion from a glacial refugium. Currently, however, this hypothesis cannot be separated from one of potential isolation by distance due to the close proximity of the two Chilean sites (Sites 2 and 4) relative to their distance from the sole Argentine site (Site 8). Seemingly in contrast, the two sites available for analysis within Lineage 2 (Sites 7 and 8), both in Argentina, were significantly different, despite being separated by a similar distance as the two Chilean sites analysed for Lineage 1. Thus both lineages exhibit some signal of contemporary restrictions to gene flow following hypothesised interglacial range expansion, however determining the driving force behind such a restriction remains difficult given the current limited dataset.

Taken together, the data presented here allow rejection of hypotheses of a clear disjunction in *F. patagonica* across the Andean barrier. Instead, several divergent lineages appear to have diversified in the late Miocene, possibly through isolation of populations in multiple refugia during glaciation. Lineages are hypothesised to have dispersed subsequently and widely to recolonise previously unsuitable areas, whilst more contemporary restrictions to gene flow may have since influenced current population structure. Nevertheless, tip haplotypes shared among sites on either side of the Andes barrier in Lineage 1 imply recent dispersal, while an internal haplotype shared across the Andes in Lineage 2 suggests historical connections among disjunct sites. Such contemporary restrictions may relate to unsuitable habitat currently persisting in areas intermediate to current sample sites, thereby interrupting dispersal among sites.

Comparison of patterns in *N. forsythi* and *F. patagonica* with those in *E. martini*

Overall, the data presented here for both *N. forsythi* and *F. patagonica* broadly contradict expectations based on data reported by Krosch *et al* (2009) for *E. martini* in eastern Australian closed forests. Whereas *E. martini* showed distinct patterns of geographical endemism across the Wet Tropics of northeast Australia, interpreted as representing historical rainforest refugia and limited range expansion post-isolation, lineage distributions for both *N. forsythi* and *F. patagonica* generally do not show geographical endemism and are often widely distributed across the study regions. Furthermore, within Lineage 1 of *N. forsythi*, there is substantial evidence for extensive gene flow among geographically distant sites (~80km). This apparent disparity in patterns amongst these closely related taxa may result from differences in both the rate and form of recovery of habitat across the three landmasses in the period since fragmentation drove initial diversification within the three taxa.

The Australian continent has undergone systemic and continued aridification initiated by northward drift following rifting from Antarctica in the Miocene and exacerbated by Plio-Pleistocene glacial cycles (Martin, 1998, 2006). The pollen record shows a dramatic and continued shift in historical vegetation composition across the distribution of *E. martini*, from favourable wet closed forest to dry open sclerophyll woodland and grassland, which began in the late Miocene and resulted in a highly fragmented mosaic of habitat suitable for *E. martini* (Truswell, 1993; Kershaw, 1994; Martin, 2006). Furthermore, the availability of permanent, flowing streams in regions intermediate to isolated refugia would have been similarly limited due to the ongoing aridification of the Australian continent, potentially further restricting movement of *E. martini* and thereby driving genetic divergence among isolated populations. Thus, the recolonisation and expansion of habitat suitable for *E. martini* has been extremely limited across much of the east coast – with some few exceptions in areas of the Wet Tropics – resulting in a historically highly fragmented distribution.

In contrast, there is no evidence for such a dramatic conversion of vegetation type in New Zealand or southern South America, implying that the floral compositions of the study regions here may have changed little (Mildenhall, 1980; Arroyo *et al.*, 1996). Moreover, Plio-Pleistocene closed forest distributions in these regions were characterised by repeated cycles of contraction into fragmented refugia during glaciation events and expansion from refugia during interglacial periods. Furthermore, the availability of permanent streams in these regions is likely to have not changed greatly in post-glacial periods. Thus, whilst distributions of plant taxa were greatly reduced and highly fragmented during New Zealand and South American glaciations, the expansion of habitat suitable for *N. forsythi* and *F. patagonica* during interglacial periods may have facilitated dispersal and recontact of divergent lineages.

Sympatry among lineages of *E. martini* occurred where lineages were argued to have been isolated historically in geographically close refugia (<20km apart), reflecting the general geographical endemism exhibited by this taxon. In contrast, the widespread geographical distributions of sympatric lineages

of *N. forsythi* (up to 80km) and *F. patagonica* (up to 150km) implies much more extensive dispersal across the study regions. Moreover, the pattern of genetic structuring within a lineage of *N. forsythi* suggests that this taxon is capable of extensive dispersal among geographically distant sites of the same catchment. Thus, in all three taxa, re-expansion from historical refugia has resulted in secondary contact among deeply divergent lineages, indicating clearly that these taxa possess some capacity for dispersal among sites, but that this may be restricted by the availability of suitable intervening habitat.

Extrapolating from this, there appears to be an overriding similarity in response to climatic upheaval and subsequent ecosystem change among these three orthoclad taxa, despite substantial differences in their individual ecology and evolutionary histories. All three taxa show patterns of several highly divergent mitochondrial lineages within a supposed single species, and the timing of divergence of these lineages is congruent across taxa with the onset of Plio-Pleistocene glaciations. Though its natural distribution extends into temperate zones, population structure in *E. martini* has currently been surveyed only from tropical Australia (but see Chapter 4), whereas both *N. forsythi* and *F. patagonica* are restricted to temperate regions. Yet regardless of these differences in landscape and life history, all three taxa appear to have been affected in similar ways by habitat fragmentation and population isolation associated either directly or indirectly with glacial cycles. This has driven the divergence of several monophyletic mitochondrial lineages within each taxon and the current distributions of these lineages appear to reflect differences in habitat recovery in post-glacial periods that facilitated significant re-expansion in *N. forsythi* and *F. patagonica* but little in *E. martini*. At a finer scale, there was some evidence for gene flow among geographically distant sites in Lineage 1 of *N. forsythi*. This contrasts with patterns of population structure from *F. patagonica* and *E. martini* that suggest movement in these taxa is more limited. These data suggest not only that orthoclad chironomids may be particularly susceptible to the reduction and fragmentation of suitable habitat, a pattern mirrored by many aquatic invertebrates globally (e.g., Taylor *et al.*, 1998; Witt & Hebert, 2000; Trewick

& Wallis, 2001; Hogg *et al.*, 2002; Hugall *et al.*, 2002; Bunje, 2005; Pauls *et al.*, 2006; Smith *et al.*, 2006a; Bell *et al.*, 2007), but also that global climate trends can affect widely separated taxa in strikingly similar ways.

Comparing and contrasting patterns of population genetic structure among related species represents the central concept underlying comparative phylogeography (Bermingham & Moritz, 1998; Taberlet, 1998; Arbogast & Kenagy, 2001). Traditionally, however, this has involved investigating co-distributed taxa that inhabit a particular geographical region in order to evaluate the relative effect of the biogeographical history of the region on the genetic structure of each taxon. This approach has been immensely powerful in demonstrating the similar effect of particular historical events on populations of co-distributed taxa. The current study has applied comparative phylogeographic theory in a novel way, instead testing and comparing the response of closely related species to historical population fragmentation in distinctly different geographical regions. Ideally, sampling of both taxa would have taken place on a similar geographical scale; however, although informed by previous studies of the general ecology and population structure of related taxa, time, access to sites and the patchy distribution of these taxa across the landscape limited both sample sizes and numbers of sites. Nevertheless, this investigation has revealed remarkably similar patterns of highly divergent, morphologically cryptic lineages within both *N. forsythi* and *F. patagonica*, which is broadly consistent with existing data for *E. martini* (Krosch *et al.*, 2009). Moreover, this study represents the first detailed phylogeographic study of any New Zealand or Neotropical chironomid species and one of only a few on any freshwater invertebrate on the South Island of New Zealand or in southern South America.

Chapter Four

Phylogeography of *Echinocladius martini* Cranston (Diptera: Chironomidae) in closed forest streams of eastern Australia

4.1 Introduction

Developing an understanding of the degree of population connectivity at a continental scale is central to a holistic study of the phylogeography of widespread fragmented populations (Taberlet, 1998). In situations where a particular taxon is widely distributed across a continent and inhabits small, isolated refuges, investigating patterns of divergence across the entire range of such taxa can permit evaluations of population ancestry, local extinctions, colonisation and divergence events (Avise *et al.*, 1987). In particular, regions for which the history of habitat change and the driving forces behind population fragmentation have been studied in depth may represent model systems for such broad scale investigations. In this way, continental scale phylogeography has been used to explore hypotheses of population connectivity in a diverse array of taxa across Europe (e.g., Taberlet & Bouvet, 1994; Dumolin-Lapegue *et al.*, 1997; Terrab *et al.*, 2008), Asia (e.g., Aizawa *et al.*, 2007; Kurachi *et al.*, 2007), Africa (e.g., Eggert *et al.*, 2002; van Hooft *et al.*, 2002), South America (e.g., Sivasundar *et al.*, 2001; Lovette, 2004) and North America (e.g., Zink, 1996; Starkey *et al.*, 2003).

Australian floral history

There are few regions in the world, however, where a continental scale approach is more relevant than in the highly fragmented rainforests of eastern Australia. Evidence from fossil pollen cores has indicated that a continuous tract of temperate rainforest of Gondwanan origin was once distributed along the entire length of the east coast (~3000km) and extended deep into what is now the arid interior of the continent (Truswell, 1993; Hill, 2004; Martin, 2006). Extant refuges that remain of these ancient forests thus represent some of the oldest temporally continuous rainforest blocks in the world. The fracturing of the Gondwanan supercontinent began approximately 180 million years ago (mya) and apparently initiated a gradual but dramatic transition in the dominant flora of the Australian continent. The uplift of the

Great Dividing Range around 90mya – generally attributed to the rifting of Zealandia (New Zealand/New Caledonia) from eastern Australia (Wellman, 1979; Ollier, 1982) – is thought to have interrupted cloud movement and, hence, rainfall patterns across much of the interior of the continent. This rain-shadow effect, in combination with the northward drift of the Australian continent, resulted in cool temperate forests that had been established at higher latitudes slowly surrendering the interior to more dry-adapted species (Hill, 2004; Martin, 2006).

At the beginning of the Cenozoic (~65mya), much of Australia possessed a warm humid climate, with seasonal rainfall present across most of the continent (Martin, 2006). By the time Australia broke from Antarctica, an event that began around 60mya and was completed by 35mya (Crook, 1981; McLoughlin, 2001), the change to an arid-dominated landscape was most likely well underway. Coupled with Australia's northward drift, alteration to ocean currents and trade wind patterns driven by the Drake Passage formation approximately 30mya (Barker & Burrell, 1977; Livermore *et al.*, 2005) may have had flow-on effects on distribution patterns of seasonal rainfall across the Australian mainland (Wilford & Brown, 1994; Hill, 2004). Forest burning resulting from lightning strike ignition became a seasonal occurrence, driving biotic evolution and further fragmenting existing blocks of temperate rainforest (Martin, 1996). The Miocene-Pliocene epochs were characterised by continued aridification across the continent, expansion of dry sclerophyll woodland and grasslands and further reduction in rainforest distributions (Martin, 1982, 1998). Evidently, a further dramatic change to a significantly drier climate occurred around 500kya that saw the ranges of many rainforest blocks retreat to isolated upland refugia, intersected by open woodland/grassland (Martin, 2006). Cycles of rainforest contraction and expansion have been catalogued throughout the Pleistocene and correlate well with glacial-interglacial periods (Kershaw, 1985, 1994). Such an ancient history of fragmentation is very likely to have had significant impacts on populations of rainforest restricted taxa in Australia.

Biogeographical barriers and restricted dispersal

Numerous studies have documented evidence of belts of dry sclerophyll woodland and grassland along the Australian east coast that appear to represent historical dispersal barriers to rainforest endemics. In the relatively understudied south-eastern forests, there is evidence that the Hunter Valley has acted as a significant biogeographical barrier to some closed forest taxa (Keast, 1961; Cracraft, 1991; Crisp *et al.*, 1995). It has been argued that the Hunter Valley represents a historical region of open woodland that intersects closed forest habitat to the north and south. There is much variation in the responses of the studied taxa to this putative gap, however, and thus its importance in driving diversification has been considered to be relatively minor (Cracraft, 1991).

The McPherson Range, which runs east-west and separates southeast Queensland forests from those of north-eastern New South Wales, is considered to have inhibited the dispersal of some bird (Keast, 1961; Ford, 1987), frog (James & Moritz, 2000), rodent (Bryant, L., pers. comm.) and lizard (Edwards & Melville, 2010) taxa. Similarly, some taxa appear to be restricted by the intersection of open woodland around the Glasshouse Mountain region of southeast Queensland (Crisp *et al.*, 1995), while others show some influence of fragmentation across an area of open woodland between Mackay and Rockhampton in mid-eastern Queensland known as the St. Lawrence Gap (Webb & Tracey, 1981; Moussalli *et al.*, 2005; Edwards & Melville, 2010). As with the Hunter Valley taxa, however, responses of rainforest endemic taxa to these putative biogeographical barriers are inconsistent, implying that either some taxa are more susceptible to fragmentation than others or that the range, extent and ‘unsuitability’ of these barriers affected taxa differently.

One of the more influential and well-documented biogeographical barriers identified on the Australian east coast is the Burdekin Gap (Keast, 1961; Galbraith, 1969). This break in rainforest habitat runs roughly east-west between Townsville to the north and Bowen to the south, correlating roughly

with the Burdekin River Valley. Significant genetic breaks across the gap, associated either with reductions in available freshwater habitats due to aridification or fragmentation of closed forest, have been reported among populations of a diverse array of taxa, including fish (McGlashan & Hughes, 2002; Wong *et al.*, 2004), vinegar flies (Kelemen & Moritz, 1999; Schiffer *et al.*, 2007), rodents (Bryant, L., pers. comm.), skinks (Moussalli *et al.*, 2005), birds (Keast, 1961; Cracraft, 1986; Ford, 1986) and frogs (James & Moritz, 2000). Studies that have attempted to date divergence events across this barrier suggest that populations on either side of the Burdekin Gap may have been isolated from each other as early as the mid-late Miocene (~15mya) (Moussalli *et al.*, 2005).

Perhaps the most studied biogeographical barrier in eastern Australia is the Black Mountain Corridor (BMC), which formed a 75km wide expanse of dry, lowland sclerophyll forest and intersected ancient rainforest assemblages in the Daintree and Atherton Tablelands (Kershaw, 1985; Bell *et al.*, 1987; Kershaw, 1994). According to palynological evidence, the BMC is thought to have arisen around 38kya, reaching its maximum extent during the last glacial maximum; only in the last 9,000 years have rainforest assemblages re-expanded into this zone (Kershaw, 1985; Nix & Switzer, 1991; Hopkins *et al.*, 1993). In contrast, phylogeographic studies of a diverse array of taxa, including lizards (e.g. Cunningham & Moritz, 1998; Moussalli *et al.*, 2005; Edwards & Melville, 2010), birds (e.g., Joseph & Moritz, 1994; Nicholls & Austin, 2005), snails (Hugall *et al.*, 2002), bettongs (Pope, 2000) and beetles (Bell *et al.*, 2007) suggest that the BMC has acted as an effective dispersal barrier for many rainforest endemic taxa, possibly since the late Pliocene (see Moritz *et al.*, 2000; Krosch *et al.*, 2009).

The current study built upon the recent work of Krosch *et al* (2009) that investigated the effect of the BMC on historical gene flow among populations of the chironomid *E. martini* in the Australian Wet Tropics. The current work aimed to resolve the evolutionary relationships among *E. martini* populations more fully by incorporating additional samples from southeast Queensland, New South Wales and Victoria into the existing dataset. Given previous data,

it was expected that additional lineages would be identified from central- and southern Australia that were geographically endemic to historical closed forest refugia. Moreover, attaining a wider coverage of the *E. martini* distribution, allowed more questions to be addressed regarding patterns of divergence and endemism along the east coast. For example, the addition of further samples from these regions was expected to help to resolve the anomalous placement of Victorian representatives within lineages from north of the BMC, as reported previously. This investigation thus provided a new level of scale for inferring relationships among populations of *E. martini* along the Australian east coast and permitted an assessment of the effect of historical biogeographical barriers other than the BMC on *E. martini* evolution.

4.2 Methods

Study sites

Larvae of *E. martini* were collected from four sites in the coastal ranges of Queensland, New South Wales and Victoria at various times between 2005 and 2009 (Figure 4.1). Site choice was based on access to suitable habitat, in concert with previous surveys of chironomid assemblages along the Australian east coast that had revealed the presence of *E. martini* at specific sites (Cranston, 2000b). Several additional locations, including several type localities, produced no *E. martini*, including streams in Finch Hatton Gorge National Park (21˚04'33"S, 148˚38'07"E) in mid-eastern Queensland, Conondale (26˚40'28"S, 152˚37'11"E) and Lamington (28˚11'46"S, 153˚10'33"E) National Parks in southeast Queensland and Brindabella National Park (35˚17'17"S, 148˚49'30"E) near Canberra. Collections in Victoria and New South Wales were carried out by Peter Cranston, while sampling of Franki's Gulch in southeast Queensland was undertaken by the author. DNA sequences from individuals collected in north Queensland were sourced from Krosch *et al* (2009) under GenBank accession numbers

EU669980-670043, with 15 additional individuals included from supplementary collections in 2008. Representatives of three closely related Orthocladiine taxa (*Cardiocladius sp., Botryocladius grapeth* and the currently undescribed genus 'FNQ2') were also included as outgroups.

Sample collection

Sample collection, preservation, identification and storage followed the process outlined in Chapter Two.

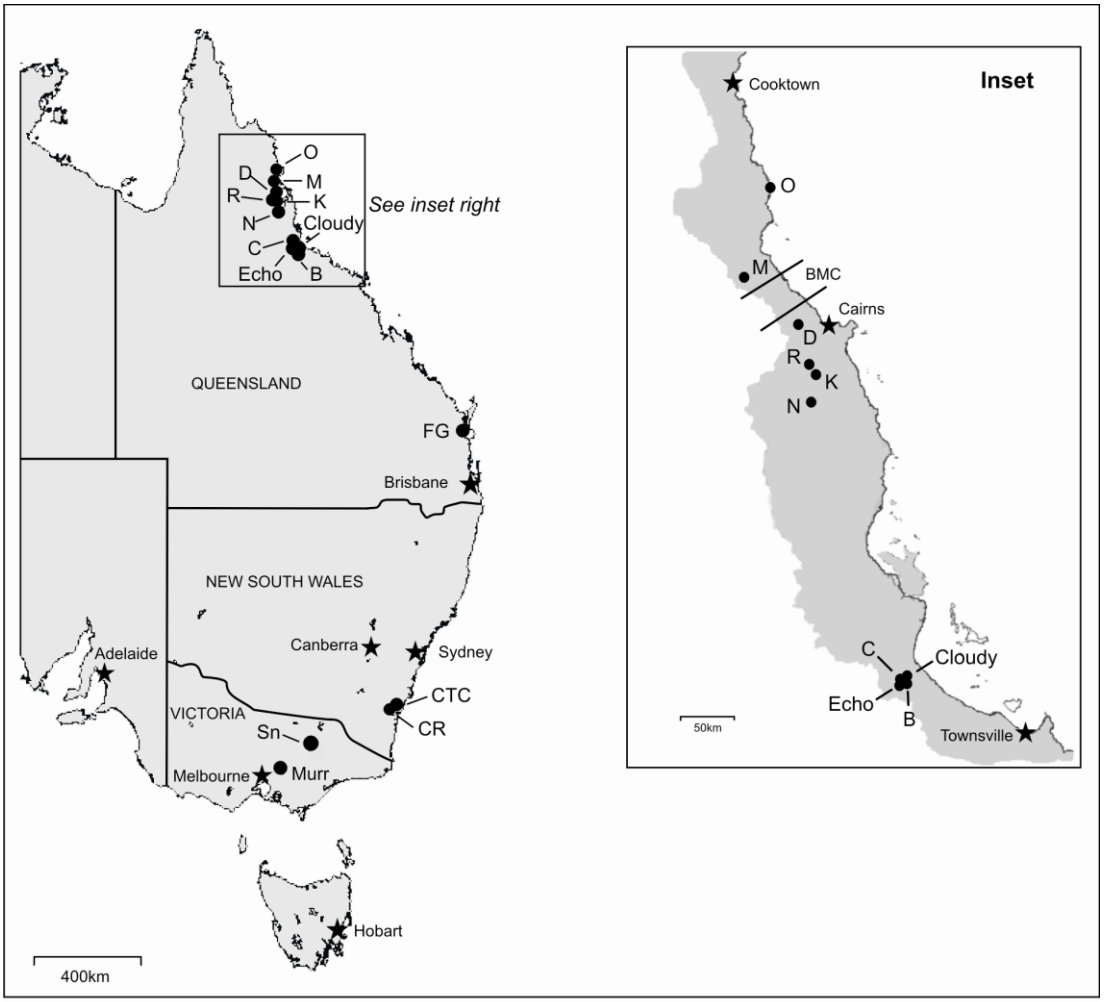


Figure 4.1. Geographical location of study sites. Inset modified with permission from Krosch, *et al* (2009). Filled circles denote sample sites; C: Camp Creek; B: Birthday Creek; Cloudy: Cloudy Creek; Echo: Echo Creek; N: Nigger Creek; K: Kauri Creek; R: Robson Creek; D: Davies Creek; M: Mary Creek; O: Oliver Creek; FG: Franki's Gulch; CTC: Cabbage Tree Creek; CR: Clyde River; Sn: Snowy Creek; Murr: Murrindindi Creek. Filled stars indicate major townships; solid lines denote state boundaries – those mentioned in the text labelled in capitals; the historical location of the Black Mountain Corridor (BMC) is shown in the inset.

Genetic procedures

Extraction of total genomic DNA and amplification, purification and sequencing of a 639bp fragment of the COI gene followed procedures outlined in Chapter Two.

Data analyses

COI sequences were aligned and edited by eye and tests for sequence saturation, Tajima's D tests of neutrality, tests of clock-like evolution and mean among-lineage corrected pairwise percent divergences were estimated using procedures outlined in Chapters Two and Three. Phylogenetic analyses followed Chapter Three and included tests of the appropriate model of nucleotide substitution, neighbour joining (NJ) (10,000 bootstraps), maximum parsimony (MP) (1,000 bootstraps), Bayesian (B) (5 million generations, 25% burnin) and maximum likelihood (ML) (1000 bootstraps) methods of phylogenetic reconstruction. Times to most recent common ancestor (tmrca) for relevant nodes were estimated using the procedure described in Chapter Three. Two runs of 40 million generations each were performed and the log files combined to produce a total run of 72 million generations after removal of burn-in. A haplotype network was constructed using the process outlined in Chapter Two with a connection limit of 90% and with the third base of each codon removed to minimise the effects of sequence saturation. This method is considered better than bifurcating trees for inferring evolutionary relationships among ancestral and derived haplotypes (Smouse, 1998; Posada & Crandall, 2001).

4.3 Results

A total of 19 *E. martini* individuals were collected from two sites in southern New South Wales (Cabbage Tree Creek and Clyde River), a single site in

Victoria (Murrindindi Creek) and a single site in southeast Queensland (Franki's Gulch), along with supplementary sampling and PCR amplification of 15 *E. martini* from Nigger, Birthday, Camp, Cloudy and Echo Creeks (Table 4.1). All 34 individuals were sequenced for the mitochondrial COI gene fragment, resulting in 30 new unique haplotypes of 639 bp in length. In combination with the dataset of 53 COI haplotypes from 96 individuals of Krosch *et al* (2009), this produced a total dataset of 130 individuals, representing 83 unique COI haplotypes.

The ratio of transitions to transversions was 4.70, with the frequency of transitions reaching a plateau as genetic distance among haplotypes increased – particularly at the third base of each codon (Figure 4.2). This could indicate possible sequence saturation (homoplasy), suggesting multiple substitutions may have occurred at a particular site, thereby potentially masking true divergence (Arbogast *et al.*, 2002). Removal of the third base of each codon is as an appropriate method used to reduce the effect of

Table 4.1. Geographical location of study sites; site codes follow Figure 4.1. Numbers of *E. martini* indicate the total from each site; values in parentheses indicate data taken from Krosch *et al* (2009) and 'N/A' denotes a site from which only outgroup taxa were collected. Elevation is given as metres above sea level (masl).

Site Name (Site code)	Latitude	Longitude	Elevation (masl)	No. of *E. martini*
Camp Ck (C)	18˚58'22"S	146˚09'54"E	850	26 (22)
Birthday Ck (B)	18˚58'49"S	146˚09'50"E	800	25 (22)
Echo Ck (Echo)	18˚59'30"S	146˚09'23"E	750	5
Cloudy Ck (Cloudy)	18˚59'84"S	146˚12'50"E	720	2
Nigger Ck (N)	17˚26'49"S	145˚28'26"E	950	12 (11)
Kauri Ck (K)	17˚08'03"S	145˚35'55"E	700	4 (4)
Robson Ck (R)	17˚06'58"S	145˚37'44"E	1000	7 (7)
Davies Ck (D)	17˚00'35"S	145˚34'56"E	520	21 (21)
Mary Ck (M)	16˚35'20"S	145˚17'50"E	950	7 (7)
Oliver Ck (O)	16˚08'30"S	145˚26'58"E	20	2 (2)
Franki's Gulch (FG)	26˚02'80"S	153˚04'26"E	65	3
Cabbage Tree Ck (CTC)	35˚34'08"S	150˚01'56"E	200	9
Clyde River (CR)	35°30'32"S	150°13'35"E	20	2
Murrindindi Ck (Murr)	37˚29'60"S	145˚33'21"E	625	7 (2)
Snowy Ck (Sn)	36°34'13"S	147°24'58"E	330	N/A

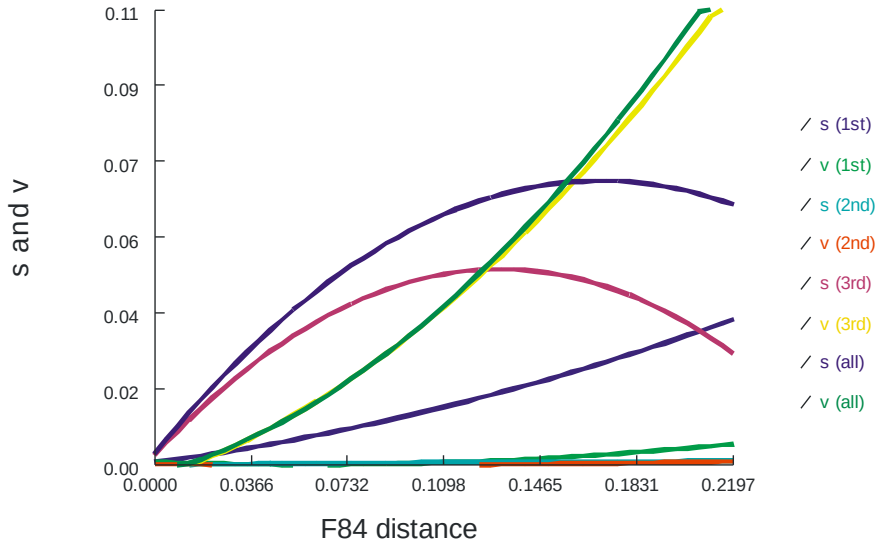


Figure 4.2. The relationship between transitions (s), transversions (v) and genetic distance for the complete COI fragment (all) and partitioned by base ($1^{st}/2^{nd}/3^{rd}$).

saturation for some subsequent analyses. Tajima's D test of neutrality was not significant (D = 0.2920, p = 0.698), suggesting that sequences had evolved neutrally. Assumptions of clocklike evolution were rejected (p = 0.0305; p<0.05) and thus a relaxed clock model was used for inferring divergence times among lineages.

Phylogenetic reconstruction

Topologies produced by the different methods of phylogenetic reconstruction were generally concordant, though levels of resolution varied among methods (Figure 4.3). The pattern of relationships among lineages generally followed that reported previously (Krosch *et al.*, 2009). Addition of individuals from the southern Wet Tropics made little difference to the overall pattern of deep genetic divergence and geographical endemism (Figure 4.4). Furthermore, the inclusion of *E. martini* populations from southern Queensland, New South Wales and Victoria strengthened the support for several relationships among lineages that previously had been poorly resolved.

The well-supported placement of the Mt. Lewis A lineage with those from the

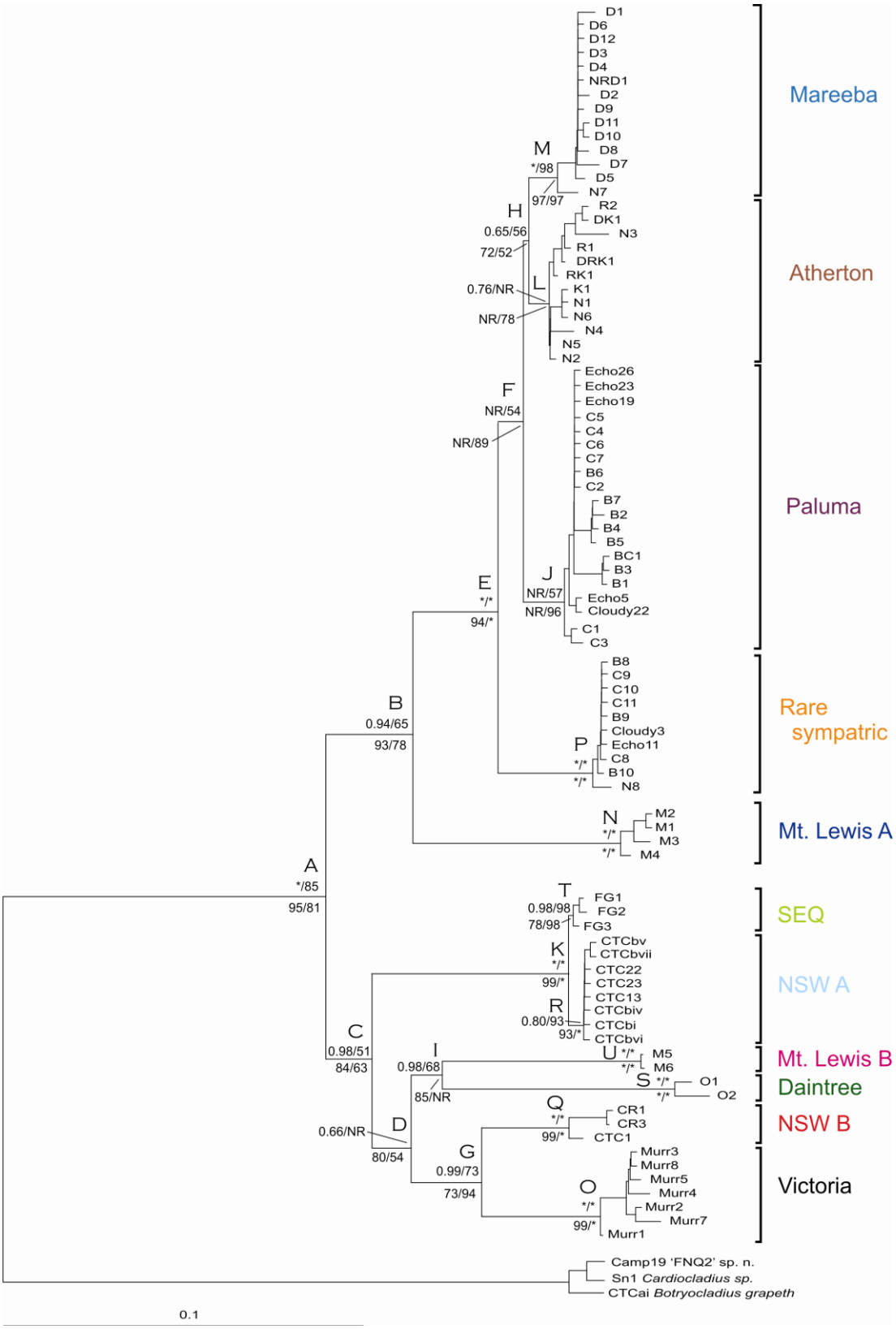


Figure 4.3. Neighbour-joining topology with branch lengths representing Hasegawa-Kishino-Yano (1985) genetic distance. Node-associated values are as follows: above branch – Bayesian posterior probability/MP bootstrap probability, below node – ML bootstrap/NJ bootstrap probabilities; '*' represents posterior probabilities of 1.00 or bootstrap probabilities of 100. Haplotype names follow Krosch *et al* (2009) for Wet Tropics haplotypes, while novel haplotypes are named by both site and molecular voucher code. Lettered nodes are those for which tmrca was estimated.

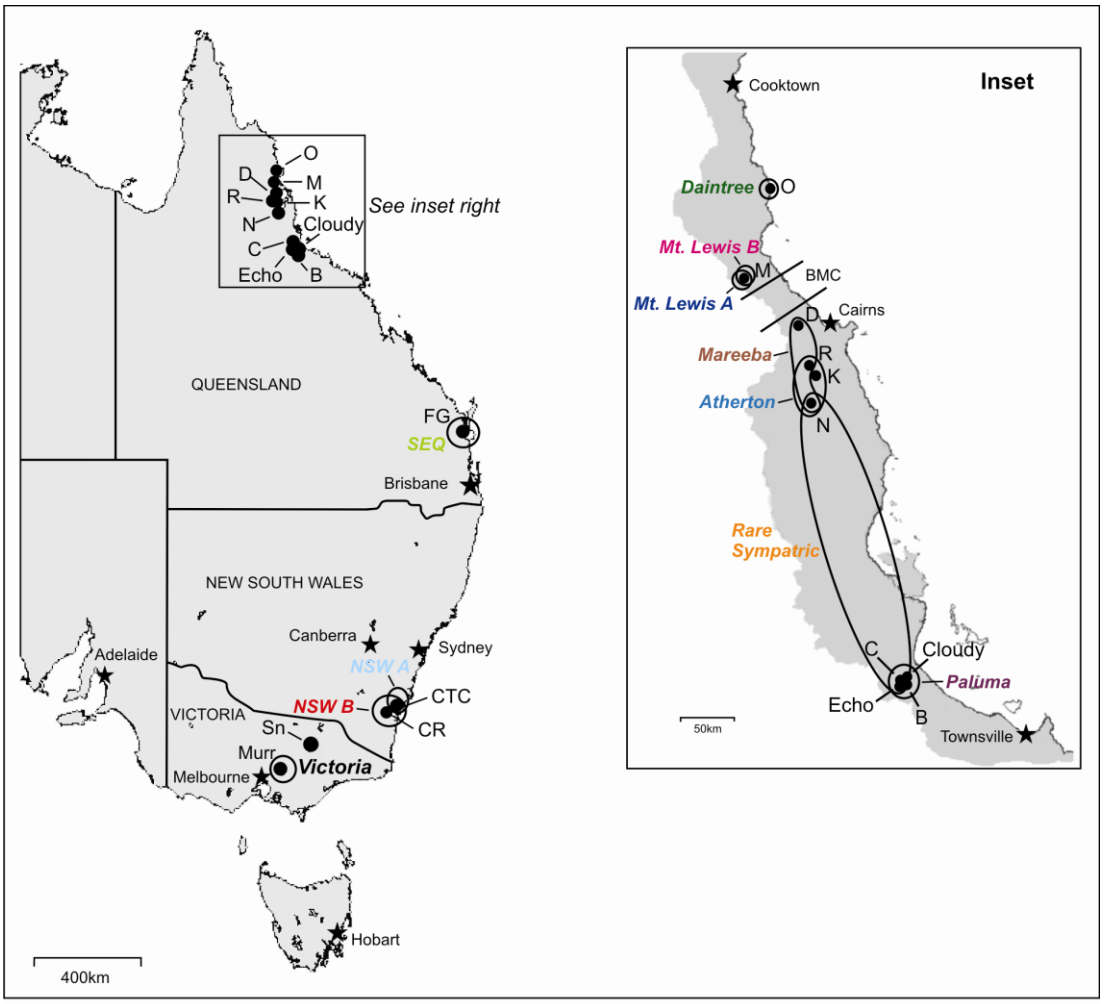


Figure 4.4. Geographical distribution of divergent COI lineages across sample sites. Site codes follow Figure 4.1; lineage names follow Figure 4.3 and are italicised in bold.

southern Wet Tropics contrasted significantly with the placement of this lineage in the earlier study (Krosch *et al.*, 2009). Previously, this lineage was located as sister to the Mt. Lewis B, Daintree and Southeast Australian lineages, albeit with very poor support (NJ: 60, MP: not resolved). Similarly, the strong support for the sister grouping of the Mt. Lewis B and Daintree lineages resolved another previously poorly supported node. This sister grouping makes geographical sense and raw sequence data showed several shared fixed differences that do appear to link the two lineages (Table 4.2), possibly reflecting common ancestry. Given the extremely long branch lengths connecting both clades, however, along with high among-lineage divergence (12.80%; Table 4.3), it is possible that this result may indicate long branch attraction. The Atherton lineage remained unresolved in both MP and ML analyses, whilst Bayesian and NJ methods showed relatively strong

support for this grouping. Furthermore, the Paluma lineage, which was reasonably well supported in the earlier study (NJ: 84, MP: 51), was unresolved in Bayesian and ML reconstructions (but MP: 57, NJ: 96), possibly due to high within-lineage diversity or the effects of homoplasy. Certainly, raw sequence data for both these lineages showed several fixed differences that clearly distinguished and were diagnostic for both lineages (Table 4.3), and were further reflected in among-lineage mean pairwise divergences (Table 4.2).

Supplementary samples from Nigger, Birthday, Camp, Cloudy and Echo Creeks formed a new lineage not previously identified. Referred to here as the 'Rare Sympatric' lineage because of the scarcity of individuals representing this lineage in the total *E. martini* sequenced for this study and in Chapter 2, this lineage was distributed broadly from Paluma to the Atherton Tableland, a distance of approximately 400km. This represents the broadest distribution identified for any *E. martini* lineage revealed so far. All five streams from which this lineage is so far known also possessed individuals from other lineages; the Atherton and Mareeba lineages in Nigger Creek and the Paluma lineage in Birthday, Camp, Cloudy and Echo Creeks. Despite this, the Rare Sympatric lineage was highly divergent (>4%) from all other Wet Tropics lineages. Given nine of the ten collected individuals of the Rare Sympatric lineage were sampled from the Paluma bioregion, this area appeared to be the centre of diversity for this lineage, with its presence on the Atherton Tableland possibly representing secondary contact via dispersal.

High levels of geographical endemicity and deep genetic divergence were also observed among sites sampled in southeast Queensland, New South Wales and Victoria. Interestingly, two sympatric highly divergent lineages (11.20%) were resolved from southern NSW (NSW A and B), which, rather than forming a monophyletic group as their geographic distributions would suggest, in fact formed a clade with the southeast Queensland and Victorian lineages, respectively. Indeed, the NSW A lineage was only 1.5% divergent from its sister lineage from Southeast Queensland, whereas the NSW B

Table 4.2. Parsimony informative sites among lineage representatives as compared to the Mareeba lineage.

	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3
	1 1 2 2 3 3 5 5 5 5 6 6 6 9 0 0 3 4 4 4 5 5 5 6 6 6 7 7 8 8 8 9 9 0 0 1 3 3 4 4 5 5 5 5 6 6 6 7 7 8 8 9 9 0 0 0 1 1 1 1 2 2 3 4 4 5 6 7 7
	6 5 8 1 2 0 6 1 4 6 7 0 3 9 2 5 5 1 4 5 3 6 9 5 8 9 4 7 3 4 9 2 5 4 7 0 5 8 0 6 5 6 8 9 1 2 4 1 3 5 8 4 7 0 6 9 2 5 8 9 4 7 6 2 5 4 6 3 5
Mareeba	A G T T T C C C C C T T T A A C T T T C G G T C G T T T G T T T T T T T C C T C T C T C T C T G T T A T G A A A T T A C T A T T A T T C T
Atherton	. A . . . T . . T . . . . A . . . C . . . . T . . . . G . A . . . C . . . . T C . . . . .
Paluma	. . . . . T . . . . . A A . . . C . . . . T . . . . G . A . . . C . T . . T . . . . .
Rare Sympatric	. A . . C T . T T . . . . G . . . . . A . . . A C . . A . . . . T . . T . . . . A . . . C . . . C T C . G . . . .
Mt. Lewis A	. A . C C T T . T . . G . T . T . . . T A A . T A . A . A . . C C G . . T . A T C . . . . A . . C A C . T . . . T . . A . G A . . .
SEQ	C T C . . T T t . . A A . C T A A C . . . C C . . C C . A C G C A . C . T T A G . . A T A T A . G t G A A T T T . C a . . . C . . A C T A
NSW A	C T C . . T T T . . A A . C T A A C . . . C C . . C C . A C G C A . C . T T A G . . A T A T A . G . G A A T T T . . . T . . . . . A C T A
Mt. Lewis B	. A . . . T T . T . . A C T T A . A C T A A . T A . . . A . A A A . . A . T A A C T A . . T A . . . . A A C T . . . T T A . . A . . C . .
Daintree	. A . . . T T A T . . A C . . . A A C T A T . T A . . C A . G A A C . C T T G . . T A . . T A . A C . A T . T T C . T T . . . A . A . . .
NSW B	T T . . . T T A T t G A C C . A A . . T A A . T A . C A A . G . a C . A T T A A . . A . . T A T A c . A t . . T . C T T C . C t G A . . A
Victoria	C C . C . T T . T T A . C C . A A A . T A . . T A . C C A . A A A C . A T T A A . T A T A T A T A . . A A G T T . C . . . . C . . A . T A

	3 3 3 3 3 4 4 4 4 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 6 6 6 6 6 6
	7 7 8 9 9 0 0 0 2 2 3 4 5 6 6 7 0 0 1 1 1 1 2 2 2 2 3 3 4 4 4 5 5 5 6 6 6 7 7 8 8 9 9 0 0 0 1 2 2
	8 9 7 0 6 0 2 5 7 9 5 1 6 5 8 1 1 8 0 4 6 9 0 2 5 8 4 7 0 3 6 0 2 8 1 7 8 0 6 5 8 1 4 0 6 9 2 4 5
Mareeba	T T T T A C T T A T T G A A A T C C T T A C T A T A T A T T T T A T C T C C T C T T A A T A T C T
Atherton	. . . . . G . . . . . A . . . . . T . . . . . T .
Paluma	. . . . . G . . . C . . . . . C C . . C . . T .
Rare Sympatric	. . . . . T . . T . . . . T . . . . . G . . . C . . . . . T . T . . . G . . . . .
Mt. Lewis A	. C A . . . . A . . . A . T G A A T A . G . . G . T . . . . A C T . T . . A . T C C . . . T C T .
SEQ	. . A C T T A A G A . . . . G . T T A . . A C . A T C . . C A . . C . C T A . T C C T G C T . . .
NSW A	. . A C T T A A G A . A . . . . T T A . . A C G . T C . . C A . . C . C T A . T C C T G C T . . .
Mt. Lewis B	. . A . T . A A . . C A T T . . A T A C T A . . . . . C . A C T A T . T A A T C C . G . . . T C
Daintree	C C . . . T A A G . . A . . . A A T A C T A . . A . C T C . A C T . . C T A C T C A . . C . C . C
NSW B	C . A . T T A A G A C A a . . . . T A C T A . . A T G . . . A C T . . . T A C T . t T . C T . T .
Victoria	. . G . T T A A G A . A T . . . T T A . . A . . . T A . . . A C C . . . T A C T . . C . C T . T .

Table 4.3. Below diagonal among-lineage corrected mean percent COI pairwise divergences.

	Mareeba	Atherton	Paluma	Rare sympatric	Mt. Lewis A	Mt. Lewis B	Daintree	Southeast QLD	NSW A	NSW B	Victoria
Mareeba	-										
Atherton	2.00	-									
Paluma	2.50	1.90	-								
Rare sympatric	5.60	4.40	4.90	-							
Mt. Lewis A	10.50	9.90	9.50	10.50	-						
Mt. Lewis B	14.20	14.10	13.20	15.10	11.90	-					
Daintree	15.90	15.50	14.20	17.30	14.40	12.80	-				
SEQ	13.50	12.90	12.80	14.90	14.60	15.40	16.00	-			
NSW A	13.30	13.10	12.70	15.10	13.80	14.40	15.20	1.50	-		
NSW B	14.10	13.40	13.10	14.50	12.10	12.00	11.30	11.00	11.20	-	
Victoria	14.20	13.60	13.90	15.40	13.10	11.80	12.60	10.20	10.20	6.70	-

lineage was 6.70% divergent from the Victorian lineage.

Perhaps of most interest was the strongly supported sister grouping of the Mt. Lewis B and Daintree lineages within the broader Victorian/NSW/SEQ group. The distribution of this group thus encompassed the northern and southern extremities of all sites sampled in this study. While the exact positions of the Mt. Lewis B and Daintree lineages within this group were not resolved using NJ methods, the other three reconstruction methods placed these lineages with good support in a sister group to the Victorian and NSW B lineages (Figure 4.3). Interestingly, the Mt. Lewis B and Daintree lineages were more divergent from each other than either was from the NSW B or Victorian lineages, and both were more divergent from the NSW A and SEQ lineages than from the NSW B and Victorian lineages (Table 4.3). Poorer resolution of relationships among these lineages in the current analysis may reflect higher within lineage variation after addition of more individuals from south of the Wet Tropics.

Estimated among-lineage divergence times

Times to most recent common ancestor estimated using BEAST were all supported by effective sample sizes greater than 300 and 95% upper and lower bounds were quite narrow, implying strong support for the current estimates (Table 4.4). The estimated node ages suggest that fragmentation of *E. martini* populations began in the late Miocene (Table 4.4 and Figure 4.5) with the split between the ancestors of the southern Wet Tropics and Mt. Lewis A lineages and those of the Mt. Lewis B, Daintree and central- and south-eastern Australian lineages occurring around 7.86mya. Diversification of the two lineage groups was estimated to have begun around 6.27mya, at which time the Mt. Lewis A lineage appears to have diverged from the common ancestor of the southern Wet Tropics lineages. Similarly, the ancestor of the SEQ+NSW A group appears to have diverged from that of the seemingly widespread ancestor of the Daintree+Mt. Lewis B+Victoria+NSW B lineage group around the same time. The bulk of

Table 4.4. Estimated time to most recent common ancestor (tmrca) for nodes labelled in Figure 4.3 and 4.5.

Node Label	Lineage/s Included	TMRCA (mya)	95% Credibility Intervals (mya)	Effective Sample Size (ESS)
A	All ingroup	7.86	10.38-5.51	387.13
B	Mareeba, Atherton, Paluma, Rare Sympatric, Mt. Lewis A	6.27	8.55-4.27	392.87
C	Southern Australia plus Mt. Lewis B and Daintree	6.26	8.61-4.08	368.39
D	Victoria, NSW B, Mt. Lewis B, Daintree	5.02	7.01-3.08	386.25
E	Mareeba, Atherton, Paluma, Rare Sympatric	4.90	6.74-3.16	527.56
F	Mareeba, Atherton, Paluma	4.15	5.81-2.61	546.59
G	Victoria, NSW B	3.58	5.43-1.91	409.72
H	Mareeba, Atherton	3.38	4.90-1.99	600.35
I	Mt. Lewis B, Daintree	3.20	4.99-1.54	295.25
J	Paluma	2.89	4.41-1.49	769.02
K	SEQ, NSW A	2.68	4.86-0.81	309.23
L	Atherton	2.39	3.74-1.15	862.38
M	Mareeba	2.36	3.73-1.18	778.23
N	Mt. Lewis A	1.94	3.84-0.43	1348.70
O	Victoria	1.65	2.92-0.61	793.48
P	Rare Sympatric	1.59	3.18-0.34	1323.66
Q	NSW B	1.27	2.63-0.18	1303.04
R	NSW A	1.19	2.44-0.23	1035.05
S	Daintree	1.06	2.31-0.10	637.06
T	SEQ	0.82	1.96-0.049	1384.30
U	Mt. Lewis B	0.63	1.70-0.021	694.71

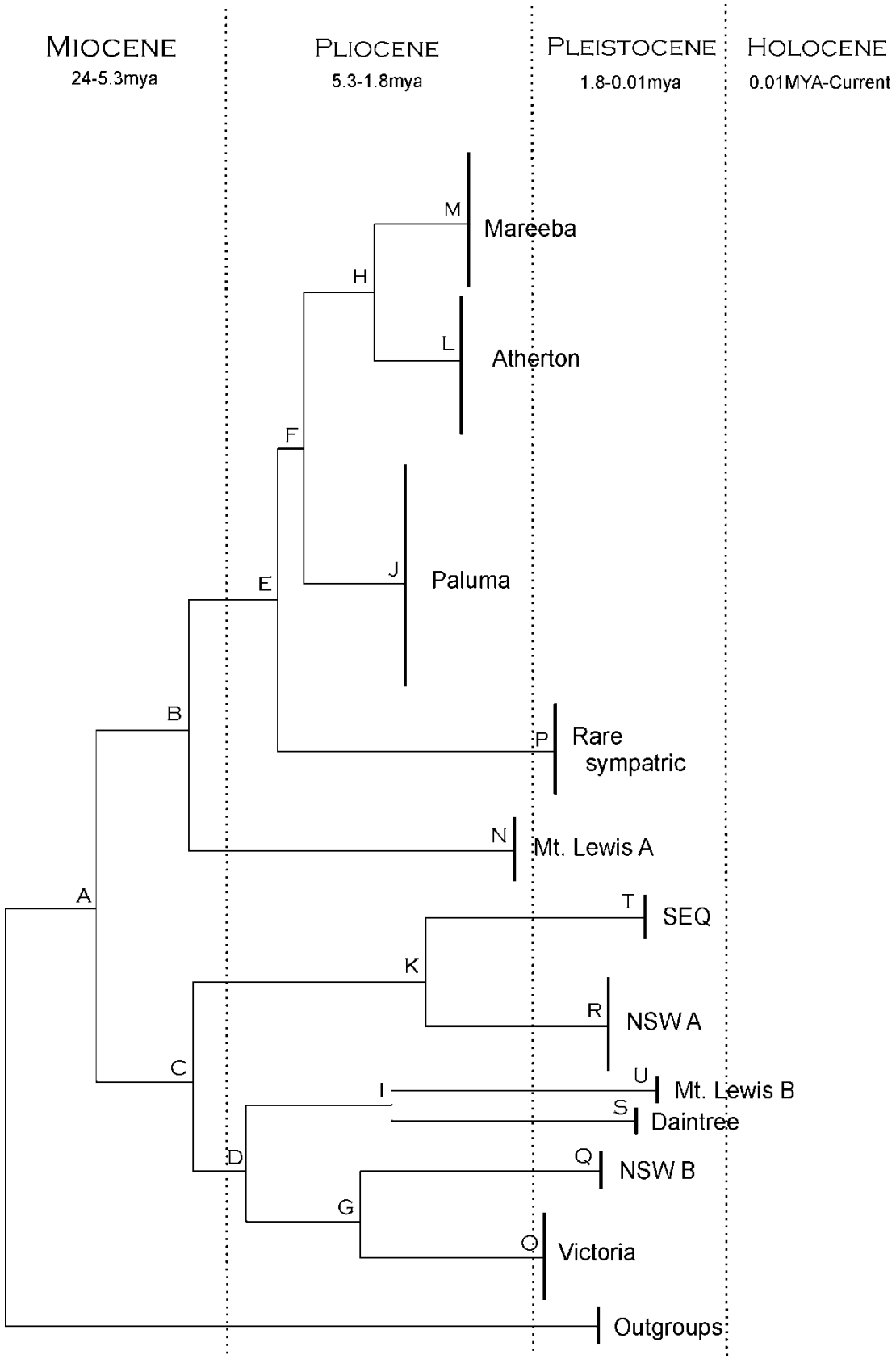


Figure 4.5. Simplified chronogram of *E. martini* lineages based on the topology presented in Figure 4.3. Branch lengths correspond to relative ages, while node and lineage labels follow that in Figure 4.3.

diversification events within and among *E. martini* populations, however, occurred during the Pliocene epoch, including the formation and diversification of the Mareeba, Atherton, Paluma and Mt. Lewis A lineages approximately 2-3mya. The remainder of the identified lineages appeared to have diversified during the early to middle Pleistocene after diverging from their sister lineages in the Pliocene.

Evolutionary relationships among haplotypes

Connections among lineages revealed in the haplotype network generally accord with, and provide additional evidence for, the pattern of relationships inferred in the phylogenetic reconstruction. The haplotype network supported the broad split between the southern Wet Tropics and Mt. Lewis A lineage group and the southern Australian, Mt. Lewis B and Daintree lineage group (Figure 4.6). The Atherton Tableland bioregion is supported as the historical centre for northern Queensland diversity in *E. martini*. The Mareeba and Paluma lineages were suggested to have derived from the Atherton lineage, while the Mt. Lewis A lineage appears to have diverged from the Mareeba lineage. The Rare Sympatric lineage similarly appears to have been derived from the Paluma lineage. The sister lineages SEQ and NSW A grouped closely together and appeared to have shared a common ancestor with the Victorian, NSW B, Mt. Lewis B and Daintree lineages. The NSW B, Mt. Lewis B and Daintree lineages appear to have been derived from the Victorian lineage. As expected the Daintree and Mt. Lewis B lineages were highly divergent from both each other and from all southern Australian lineages, though they were clearly more closely related to these than to other Wet Tropics lineages.

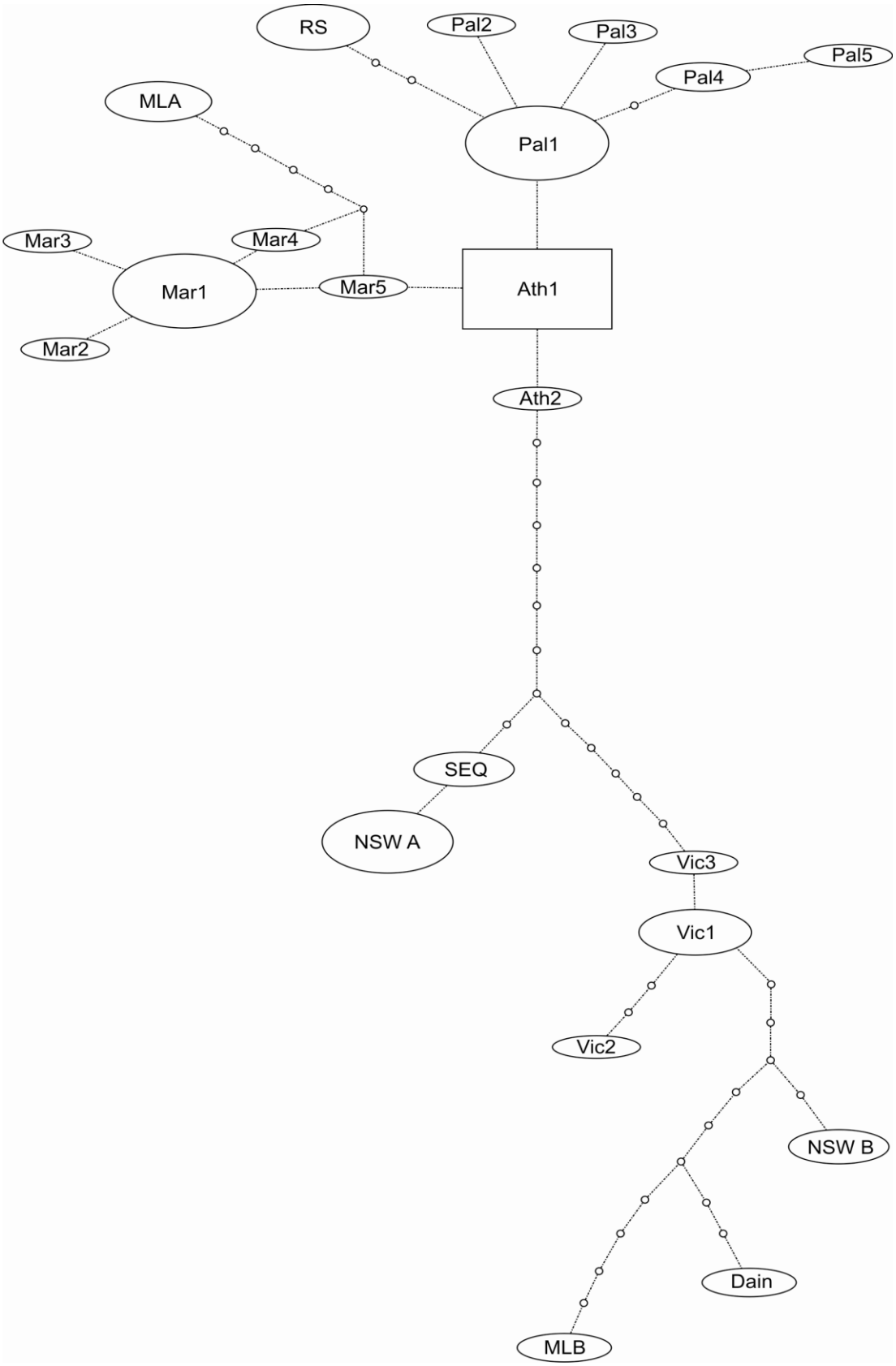


Figure 4.6. Parsimony network of *E. martini* COI haplotypes with third codon positions removed. Haplotype labels correspond to the lineage they represent; lineage names follow Figure 4.3. Hollow circles represent hypothetical unsampled haplotypes.

4.4 Discussion

The results presented here largely conformed with expectations and indicate that *E. martini* populations south of the Wet Tropics also exhibit patterns of high genetic divergence and geographical endemism. The addition of samples from southeast Queensland, NSW and Victoria identified four new highly divergent lineages, two of which occur sympatrically at a single NSW site. Supplementary sampling of Wet Tropics sites also revealed an additional lineage that occurs sympatrically with the Paluma, Atherton and Mareeba lineages. Among-lineage pairwise divergences were quite high, even among lineages that were geographically proximate. Thus, it appears that similar evolutionary processes, namely aridification driving fragmentation and contraction of suitable habitat, may have driven the diversification of southern Australian populations as has apparently occurred in the Wet Tropics. Estimation of node ages also revealed important information for understanding these processes and provided evidence for a possible sequence of evolution among the observed lineages.

Rainforest contractions driving divergence in *E. martini*

Eastern Australian coastal rainforests are believed to have been subject to ongoing contraction and isolation, driven initially by wet sclerophyll expansion since the middle Miocene, resulting in the loss of *Nothofagus* dominated forests in favour of myrtaceous forest (Martin, 1986, 1991; Hill, 2004). Conversion from widespread rainforest and wet sclerophyll forest to dry, open woodland and grassland accelerated through the late Miocene and Pliocene, particularly in central- and south-eastern Australia, and was virtually completed by around 3mya (Hekel, 1972; Martin, 1986, 1991). With the exception of a brief resurgence in river valley rainforest assemblages during the mid-late Pliocene, dry-adapted forest types have evidently dominated these areas since the late Miocene (Truswell, 1993; Martin, 2006). Rainforest distributions in north-eastern Queensland, in contrast, are thought

to have remained comparatively more stable during the Miocene than their central and southern counterparts; local extinction of *Nothofagus* and other wet rainforest plant taxa occurred much later in the fossil record (Kershaw & Sluiter, 1982). It was not until the late Pliocene to early Pleistocene that the dramatic transformation to dry woodland and grassland was observed in north-eastern pollen deposits (Kershaw, 1985; Nix & Switzer, 1991; Kershaw, 1994). Such shifts in vegetation composition are generally considered to have been gradual, driven by the opening of the Drake Passage and the subsequent formation of the circum-Antarctic current, in combination with the northward drift of the Australian continent (Martin, 1982; Hill, 2004). The pronounced reduction in rainfall across much of the continent caused by these major geological processes, along with rising temperatures as Australia drifted north towards the equator, most likely drove the aridification of the landscape and influenced subsequent contraction of wet forest assemblages.

The pattern of evolutionary relationships among *E. martini* populations inferred here, along with estimates of node ages, are congruent with current pollen evidence for the pattern of contraction and conversion of wet rainforest to dry woodland and grassland. The timing of the initial split between Wet Tropics lineages and central- and south-eastern Australian lineages support the pollen record by suggesting that populations in these regions have been evolving in isolation since the late Miocene (Hekel, 1972; Martin, 1986; Truswell, 1993). Extensive population fragmentation appears to have occurred throughout the Pliocene, driving the divergence of several geographically restricted lineages along the Australian east coast, while sympatric distributions of some lineages most likely represent secondary contact during periods of rainforest re-expansion.

Long distance dispersal

The grouping of the Mt. Lewis B and Daintree lineages sister to those from Victoria and NSW, revealed by Krosch *et al* (2009), was well-supported in the current study. Furthermore, the Mt. Lewis B and Daintree lineages were

suggested to be derived from the Victorian lineage by the haplotype network, implying a shared history. It is possible, however, that a better explanation for the close grouping of these four geographically distant lineages may be the retention of ancestral polymorphism, whereby mutations shared between the four lineages represent ancestral states with location-specific mutations elsewhere in the sequence. Certainly, the Mt. Lewis B and Daintree lineages were located on long branches in the resolved phylogram and were highly divergent from all other lineages, including Victoria and NSW B (>10%). Given that the Mt. Lewis B and Daintree lineages were also roughly as divergent from each other as they were from all other lineages, it is most likely that these lineages have evolved in allopatry, probably in refugia in the Thornton Upland region to the northwest, following simultaneous isolation around 6.27mya both from other Wet Tropics populations and from lineages further south, before re-expanding their distributions during periods of rainforest resurgence. This grouping represents the most distant geographical extreme identified here and extends for 2500km in straight line distance.

Whilst the possibility cannot be excluded, it seems highly unlikely that gene flow between populations so widely separated geographically could have persisted while populations in intermediate areas became isolated and diverged. Likewise, whilst chironomids are considered to be physiologically capable of long-distance dispersal (McLachlan, 1983, 1986), particularly through passive dispersal on wind currents (White, 1970; Oliver, 1971), it appears improbable that even a small proportion of individuals from south-eastern Australia dispersed successfully over such large distances to colonise these areas without leaving evidence of their movement in the genetic composition of sampled intermediate populations. This would likely take the form of either intermediate haplotypes or sympatry between extant lineages. Additionally, the life-span of chironomid adults is quite short, lasting only up to two weeks at most, even under laboratory conditions (Oliver, 1971, Cranston, P. S., pers. comm.), and the main activity of the adult phase is to breed and oviposit. Moreover, the pollen record does not show any evidence for a resurgence of habitat suitable for *E. martini* on a scale that would

provide a continuous corridor for movement between such geographically distant regions (Martin, 1986; Truswell, 1993). Thus, not only is there likely to be little opportunity for dispersal across distances at a continental scale, but the total data presented here indicate this is unlikely to have occurred in this instance. Furthermore, the Daintree and Mt. Lewis B lineages comprise only two individuals each and thus may not signify an accurate representation of within-lineage diversity. To illuminate the evolutionary history in this area more effectively, additional sites in the Daintree region and further north should be sampled to evaluate the genetic diversity within these areas more fully and thus provide a more complete reconstruction of relationships in this region.

Diversification of central- and south-eastern Australian populations following isolation from north-eastern Queensland is typified by regional endemism and high genetic divergence among lineages. The formation and diversification of these lineages began in the late Miocene (~6.27mya) and continued during the Pliocene. In this region, there is also evidence for secondary contact, with two highly divergent lineages (NSW A and B) identified in sympatry at Cabbage Tree Creek. Placement of the two lineages as sister groups to the SEQ and Victorian lineages, respectively, suggests that this region of southern NSW may represent a zone of overlap where historically isolated lineages from regions further north and south may have recontacted. The estimated node ages for the two lineage pairs suggests divergence around the early to middle Pliocene, a result congruent with the timing of a brief period of rainforest expansion in this region (Martin, 1986; Truswell, 1993). This may have allowed movement of northern and southern lineages through favourable habitat into NSW, prior to subsequent isolation, possibly corresponding to the hypothesised McPherson Range, Hunter Valley and eastern Victoria biogeographical barriers. This hypothesis must remain speculative at present as the low number of sample sites in coastal New South Wales unfortunately prevents further investigation of these barriers. This may be compounded further by extensive sampling in otherwise suitable habitat that has revealed no *E. martini*, implying the distribution of this taxon may be inherently scattered. Furthermore, whilst

highly divergent sympatric mitochondrial lineages may indeed represent 'cryptic species', the lack of nuclear data to test this hypothesis prevents further comment about present-day reproductive isolation among such sympatric lineages. Fixed differences at several nuclear loci observed among sympatric lineages that corroborate the pattern revealed in the mitochondrial data would provide evidence to support the lineages as being independent evolutionary units.

Populations in the southern Wet Tropics appear to have experienced a somewhat more tumultuous history than those in the Daintree and in central- and south-eastern Australia that exhibit hallmarks of long isolation allowing linear divergence whilst apparently retaining some ancestral polymorphisms. Given the pattern revealed in the network and the timing of diversification events estimated here, the southern Wet Tropics and Mt. Lewis A lineages, on the other hand, appear to have arisen after a series of dramatic range expansions over a period of around 4.5my (between ~7.86-3.38mya) and vicariance in geographically isolated refugia. The Atherton Tableland bioregion was hypothesised to constitute a historical centre for diversity in *E. martini*, with all other southern Wet Tropics lineages, as well as the Mt. Lewis A lineage, hypothesised to have been derived ultimately from populations in this region. Cycles of rainforest expansion and contraction are known to have occurred across the Wet Tropics during the Pleistocene (Hopkins *et al.*, 1993; Kershaw *et al.*, 1993) and it is reasonable to extrapolate this to earlier periods (Hugall *et al.*, 2002). This may have facilitated dispersal of previously isolated lineages through patches of suitable habitat prior to subsequent isolation in small, disconnected refugia.

The dry corridor that separates the Atherton Tableland and Daintree rainforest block known as the Black Mountain Corridor (BMC) was one of the first barriers to bisect Wet Tropics populations, as implied by the early divergence of the Mt. Lewis A lineage around 6.27mya. Following this initial split, diversification of the southern Wet Tropics lineages proceeded fairly rapidly (<1.5my) following initial isolation in refugia on the Atherton Tableland. Secondary contact observed among lineages in the Mareeba and

Atherton regions may have occurred during periods of rainforest expansion during the late-Pleistocene (Nix & Switzer, 1991; Hugall *et al.*, 2002). Whilst the diversification of southern Wet Tropics lineages predates much of the current pollen evidence for rainforest contractions and conversion to sclerophyll woodland (Nix & Switzer, 1991; Kershaw, 1994), more recent genetic data from a variety of taxa have shown that genetic divergence across this barrier appears to have occurred pre-Pleistocene (Moritz *et al.*, 2000; Hugall *et al.*, 2002).

Geographical endemism in other east coast taxa

The distinct pattern of geographical endemism driven by fragmentation of rainforest habitat along the Australian east coast is mirrored in a variety of other taxa, including several species of frogs (e.g., James & Moritz, 2000; Schauble & Moritz, 2001; Symula *et al.*, 2008), birds (e.g., Joseph *et al.*, 1995; Nicholls & Austin, 2005), lizards (e.g., Schneider & Moritz, 1999; Stuart-Fox *et al.*, 2001; O'Connor & Moritz, 2003; Chapple *et al.*, 2005), flies (Kelemen & Moritz, 1999), butterflies (Eastwood & Hughes, 2003), glow-worms (Baker *et al.*, 2008), freshwater crayfish (e.g., Ponniah & Hughes, 2004; Shull *et al.*, 2005) and snakes (Keogh *et al.*, 2003), among others. Many of these studies reported similar patterns of phylogeographic structure and timing of diversification among populations as that revealed here for *E. martini*, although the exact location of historical biogeographical barriers occasionally do vary among taxa. Broadly speaking, most taxa exhibit large phylogeographic breaks that are congruent with the BMC, the Burdekin Gap, the McPherson Range, the Hunter Valley and an area east of the Great Dividing Range near the New South Wales/Victorian border. Within these broad regions there is much variation in the patterns of genetic structure revealed among sample sites, generally relating to the specific life history traits and ecologies of each organism as well as the sampling design implemented in each study. Interestingly, despite testing phylogeographic relationships among regions in similar ways to the current study and sampling across a similar range, none of these studies show

Daintree/Thornton Upland populations to be more closely related to those from south-eastern Australia than to other Wet Tropics populations.

Even if long-distance dispersal events between such vastly distant regions as Victoria and the Daintree did not take place, there is still clear evidence for small scale dispersal among geographically proximate populations, probably during times of re-expansion of suitable habitat, resulting in secondary contact among lineages. Several such instances were evident in the data presented here, all but one of which occurred among Wet Tropics lineages, possibly reflecting the shorter distances among most of the Wet Tropics sample sites and the reconnection of historical refugia during periods of forest expansion. Similarly, sympatry of NSW A and B lineages most likely occurred during the resurgence of rainforest habitat during the middle to late-Pliocene, potentially allowing movement of populations isolated to the north and south into favourable intermediate habitat. Interestingly, however, there is anecdotal evidence from the Brindabella National Park near Canberra, which suggests that *E. martini* populations may be unable to effectively recolonise streams in the short term following local extinction of even geographically proximate sites (Cranston, P. S., pers. comm.).

Anecdotal evidence for poor recolonisation

Several streams within the National Park were surveyed annually for over a decade from the early 1990's and were known to be inhabited by *E. martini*. In 2003, the National Park and much of the surrounding area were devastated initially by severe and prolonged drought, followed by high intensity bushfires that burnt much of the surrounding forest. Surveys in the years after the fires have indicated that *E. martini* had disappeared from the streams – either directly due to the fires or because of the total drying of the streams beforehand – and were replaced by other chironomid taxa such as *Cricotopus* that are more resistant to the high nutrient load and algal growth associated with significant post-fire runoff. More recent collections in these streams have indicated that *E. martini* remains absent despite a distinct

recovery in stream water quality, thus implying that the ability of this species to recolonise streams from populations separated by unsuitable habitat may be quite poor. Alternatively, it may be that the streams themselves are yet to recover to a point at which the water quality is adequate for *E. martini* survival. Certainly, other populations of *E. martini* are known to have persisted through bushfire events in other areas when streams have continued to flow (e.g., the Cabbage Tree Creek catchment in subcoastal NSW – Cranston, P. S., pers. comm.). This suggests that the changes to stream ecosystems in the Brindabellas brought about by the 2003 fires were particularly severe. Whilst this information is important for understanding patterns of movement of *E. martini* in the short-term, it reveals the disparity in temporal scales between the anecdotal data from south-eastern Australia and the mitochondrial data of the current study, such that whilst dispersal and recolonisation might be limited in the short term, over periods of millions of years even infrequent movement among populations may be sufficient for the expansion of the geographical range of *E. martini* lineages.

Environmental tolerances

The distinct climatic unpredictability that characterises central- and south-eastern Australia is in stark contrast to the relative stability of climate experienced in the upland Wet Tropics. The vegetation types and within-stream physicochemical attributes of streams inhabited by *E. martini* in temperate Australia are vastly different to those in the dense, humid rainforests of the Wet Tropics. This is most apparent when considering the divergence from Gondwanan-type forest observed at Franki's Gulch in south-east Queensland. This stream flows through *Melaleuca sp.* and *Casuarina sp.* forest, the low and mid-storey is dominated by swordgrass (*Lomandra sp.*) and the substrate consists mostly of fine white sand typical of coastal dunes (Krosch, M. N., pers. obs.). Occurrence of *E. martini* in this habitat type, and also in a perched lake (Lake Boomanjin) on Fraser Island (Cranston, 2000b), suggest that this taxon can tolerate and thrive in a relatively wide variety habitats. Indeed, controlled laboratory testing has

revealed that *E. martini* individuals from both temperate and tropical populations can tolerate a broad temperature range, most likely a response to the stochasticity of the Australian climate (McKie *et al.*, 2004) but possibly also driven by seven million years of dramatic habitat change characterised by fragmentation and isolation. Thus, perhaps a common trait shared among *E. martini* populations is a degree of environmental flexibility, but that once conditions within a given stream become unfavourable, populations may quickly succumb to local extinction and are unlikely to be replaced easily by local dispersal. Alternatively, differences in habitat preference recorded among Wet Tropics and central- and southern Australian populations may instead reflect the status of these lineages as discrete species, whilst broad thermal tolerances may represent a shared ancestral state.

Overall, results presented here demonstrated that *E. martini* populations have been historically isolated in fragmented refugia, driving divergence of geographically endemic mitochondrial lineages. The estimated timing of the divergence and diversification of the observed lineages was roughly congruent with published fossil pollen records that suggest major fragmentation of suitable habitat along the east coast of Australia beginning in the late Miocene and extending to the mid-Pleistocene. Furthermore, there is clear evidence for secondary contact among several lineages, which most likely reflects movement through suitable habitat during periods of rainforest expansion. The close grouping of Daintree and Mt. Lewis B lineages with the NSW B and Victorian lineages is argued to be more likely due to the retention of ancestral polymorphism rather than long-distance dispersal on a continental scale. Nevertheless, rare dispersal events in the past have evidently contributed to the diverse distributional ranges of many of the observed genetic lineages. By considering all available evidence concerning the distribution and ecology of *E. martini*, a more complete understanding of the susceptibility and response to habitat change for this taxon has been formed. It is clear that whilst *E. martini* can tolerate limited fluctuation in stream environment, once conditions become unsuitable local populations are likely to succumb to extinction. Furthermore, it appears that *E. martini* is

often unable to recolonise suitable habitat in the short-term following local extinction, possibly reflecting generally poor dispersal ability.

Chapter Five

The systematics and biogeography of the Gondwanan Orthocladiinae (Diptera: Chironomidae)

5.1 Introduction

Since the time of the early biogeographers, atypical distributional patterns have been recorded among members of the southern hemisphere fauna and flora, whereby close morphological similarities are observed among taxa on different southern continents, (Hooker, 1844, 1855; Darwin, 1859; Hooker, 1860; Wallace, 1876). The development of continental drift theory – which broadly states that the distribution of continental plates across the Earth's surface is dynamic (Wegener, 1915) – and the general consensus of the order of Gondwanan continental fragmentation (Scotese *et al.*, 1988; McLoughlin, 2001), have provided a framework against which patterns of relationships among disjunct austral taxa can be tested. Developments in phylogenetic theory, molecular technologies and complex statistical estimation of divergence times among taxa have provided important novel insights into the evolutionary histories of some austral taxa and now play a central role in modern austral biogeography. The vast literature base concerning disjunct austral taxa has revealed a variety of phylogenetic patterns, many of which appear to follow the sequence of continental break-up, whereas others imply that alternative mechanisms have influenced modern distribution patterns.

Gondwanan fragmentation

Initiation of Gondwanan break-up began with rifting between the India-Madagascar block and the western side of Australia around 165mya (Scotese *et al.*, 1988). The India-Madagascar block apparently broke away from Africa soon after and drifted northeast until around 121mya, at which point it reached its current position off the coast of Mozambique and rifting of the Indian block began. South-eastern South America split from southern Africa approximately 135mya, prior to the north-eastern coast of South America rifting from the African continent around 100mya, thereby opening the Atlantic Ocean. The continental block that consisted of South America,

Australia, Zealandia (New Zealand-New Caledonia) and Antarctica remained relatively connected until around 80mya when the smaller Zealandia block split from western Antarctica and drifted northeast, a process that opened up the Tasman Sea (Sanmartin & Ronquist, 2004). This block is thought to have been submerged periodically throughout the Tertiary period, before finally splitting around 40mya (but see Ladiges & Cantrill, 2007). Australia began to separate from the eastern side of Antarctica during the late Cretaceous (~90mya), with full separation and the opening of the Southern Ocean occurring around 35mya (Veevers *et al.*, 1991). Following this, Australia drifted northward to collide with the Asian plate, leading to tectonic uplift of proto-New Guinea (McLoughlin, 2001). The final stage in the break-up of Gondwana involved rifting between South America and Antarctica around 30mya (Barker & Burrell, 1977; Lawver & Gahagan, 2003), thereby opening the Drake Passage and initiating the Circumpolar Antarctic Current and associated West Wind Drift effects (Cook & Crisp, 2005). The expected phylogenetic pattern for austral taxa according to a simple vicariance history is thus that Africa forms a sister group to New Zealand, southern South America and Australia, with New Zealand then sister to southern South America and Australia.

Phylogenetic incongruence and transoceanic dispersal

Incongruence between taxon phylogenies and the geological sequence of continental fragmentation has historically led to invocations of long-distance transoceanic dispersal post-vicariance to explain current distributions and taxonomic relationships. In particular, many plant taxa exhibit relationships and/or divergence times among the southern continents which do not correspond to continental break-up; for example, taxa in New Zealand and Australia which share a most recent common ancestor to the exclusion of the other austral landmasses are thought to have dispersed across the Tasman Sea (e.g., Linder & Crisp, 1995; Linder, 1999; McDaniel & Shaw, 2003; Cook & Crisp, 2005). Indeed, much attention has been given to the origins of the New Zealand flora which, in contrast to the once-prevalent historical

vicariance view, are increasingly argued to have arrived from Australia via west to east dispersal through West Wind Drift following re-emergence of land following Oligocene inundation of much or all of New Zealand (Cooper & Millener, 1993; Pole, 1994). There is also evidence for dispersal of some plant taxa in the opposite direction, from New Zealand to Australia (MacPhail, 1997; Swenson & Bremer, 1997). Furthermore, several studies have invoked trans-Pacific and trans-Atlantic dispersal to explain relationships among disjunct taxa that are not congruent with the geological record (e.g., Briggs, 2003; de Queiroz, 2005; Page *et al.*, 2005; Braby *et al.*, 2007; Page *et al.*, 2008; Rowe *et al.*, 2010).

In apparent contrast, phylogenetic relationships that show close concordance with the proposed geological sequence for the break-up of Gondwana can provide clear evidence for vicariance as a mechanism that drives disjunct distributions. A broad range of taxa with varying dispersal capabilities exhibit such phylogenies, including numerous groups within the Diptera (e.g., Munroe, 1974; Yeates & Irwin, 1996; Cranston & Edward, 1999) and Hymenoptera (e.g., Shaw, 1990; Gauld & Wahl, 2000), as well as the Trichopteran family Hydrobiosidae (Henderson, 1991), the butterfly genus *Ornithoptera* (Parsons, 1996), the beetle genus *Metacorneolabium* (Thayer, 1985), the bark beetle tribe Tomicini (Sequeira & Farrell, 2001) the arachnid subfamily Ballarrinae (Hunt & Cokendolpher, 1991) and the gastropod genus *Plectostylus* (Breure, 1979).

Historically, many vicariance- and dispersal-based explanations of relationships among disjunct taxa, however, have been plagued by poor scientific rigour, resulting in potentially inaccurate conclusions. Common problems have included considering the absence of a particular taxon in the fossil record of a given geographical region as absolute evidence for its primitive absence from that region, using fossils to provide absolute maximum ages for the presence of a given taxon in a region, insufficient taxonomic sampling – either because extant taxa were excluded or because of unknown extinct fossil taxa, the assumption of pre-vicariance taxon homogeneity across the Gondwanan landmass, an incomplete understanding

of the long-distance dispersal potential of the target taxon and biased or inaccurate preconceptions of the continental arrangement across Gondwana (McCarthy, 2003; Heads, 2005; Sparks & Smith, 2005; McCarthy *et al.*, 2007; Upchurch, 2008). More recently, issues regarding molecular clock calibrations and other aspects of analysis of genetic data have been acknowledged, whereby divergence time estimates may vary dramatically depending on the methods used (Hugall & Lee, 2004; Heads, 2005; Ho *et al.*, 2005). Incorporation of such errors have thus led to some poorly formed arguments for both vicariance and dispersal among disjunct austral taxa and a critical review of such cases can often reveal markedly different patterns (e.g., Hugall & Lee, 2004; Heads, 2005). Indeed, the accumulation of more molecular and fossil evidence and the development of new analytical techniques has led to revisions of inferred phylogenetic relationships, divergence time estimates and the interpretation thereof in even some 'classic' Gondwanan relict groups such as ratites (Cooper *et al.*, 2001), marsupials (e.g., Nilsson *et al.*, 2004; Beck, 2008; Meredith *et al.*, 2008), the family Araucariaceae (e.g., Gilmore & Hill, 1997; Knapp *et al.*, 2007; Liu *et al.*, 2009) and *Nothofagus* southern beeches (e.g., Linder & Crisp, 1995; Cook & Crisp, 2005). The above groups have long been considered Gondwanan relicts as they exhibit contemporary austral distributions and fossil records that imply initial divergence and radiation in the southern hemisphere prior to continental fragmentation.

The austral Chironomidae: patterns and hypotheses

Several groups of taxa that are also considered to represent 'classic' Gondwanan relicts have been proposed within the subfamilies of the Chironomidae (Diptera). The eminent systematist Lars Brundin recognised putative ancient clades within the Aphroteniinae, Podonominae and Diamesinae subfamilies which exhibited disjunct austral distributions (Brundin, 1963, 1966). Brundin used larval and pupal morphology to apply the newly developed phylogenetic theory (Hennig, 1965) and reconstructed phylogenies for the putative Gondwanan clades in the three subfamilies that

showed consistent patterns of relationships among the taxa of Australia, South America and New Zealand (Brundin, 1965, 1966). Brundin (1966) demonstrated that within each clade the New Zealand fauna always possessed a sister group in South America or South America-Australia and that there were no direct phylogenetic connections between New Zealand and Australian taxa. Brundin (1966) argued that this represented two separate connections between the southern continents, whereby New Zealand was connected to South America via west Antarctica, while Australia was connected to South America via east Antarctica. Congruence of patterns among taxa described by Brundin (1966) with the geological consensus sequence for the fragmentation of Gondwana, suggest that the inferred phylogenies for these austral taxa reflect simple vicariant evolution following Gondwanan break-up.

More recently, taxonomic descriptions and generic reappraisals of members of the more cosmopolitan Orthocladiinae subfamily have also revealed the presence of similar disjunct clades of potential Gondwanan origin. Several described genera are now considered to belong to one of these groups, including Australian *Echinocladius* (Cranston, 2000b), New Zealand *Naonella* (Boothroyd, 1994) and *Tonnoirocladius* (Cranston, 2007), South American *Ferringtonia* (Sæther & Andersen, 2010), *Limnophyes* (known from South America and now New Zealand - Sæther, 1990a; Sæther, 1990b), *Anzacladius* (from Australia and New Zealand - Cranston, 2009), *Botryocladius* (from Australia and South America - Cranston & Edward, 1999) and *Pirara* (known from Australia, New Zealand and South America - Boothroyd & Cranston, 1995). These disjunct austral taxa generally inhabit similar environments – i.e., cool, pristine, often montane streams – to the taxa investigated by Brundin (1966) and thus are considered to represent a novel Gondwanan clade within the Orthocladiinae. Beyond these preliminary suggestions, however, there has been little attention paid to resolving the phylogenetic relationships among the proposed Gondwanan genera. To date, this has been limited to postulations of similarity contained within species descriptions and morphology-based phylogenetic reconstructions based on only a subset of taxa. Thus several, often conflicting, hypotheses surround

the evolutionary affinities of the Gondwanan orthoclads. For example, the Australian endemic genus *Echinocladius* has variably been proposed to represent the sister group to the Gondwanan genus *Pirara* (Cranston, 2000b), the sister group to the non-Gondwanan genera *Paralimnophyes/Synorthocladius* (Cranston & Edward, 1999) or nested within a group of Gondwanan genera including *Naonella*, *Tonnoirocladius* and *Pirara* (Cranston, 2007). Similarly, some recent evidence suggests that *Anzacladius* and *Botryocladius* are sister taxa, with *Ferringtonia* sister to these (Sæther & Andersen, 2010), whereas previously these two genera were assumed to be not directly connected (Cranston & Edward, 1999). Furthermore, for *Botryocladius*, perhaps the most speciose of these austral orthoclad genera, preliminary morphological data suggests that this genus diversified prior to the rifting of Australia and South America from Antarctica, resulting in interspecific relationships that do not reflect monophyly on each of the two continents (Cranston & Edward, 1999). It is evident, therefore, that much confusion surrounds the patterns of phylogenetic relationships within this group of genera, but clearly the generic/species assemblages of each continent should not be expected to form monophyletic clades, thereby reflecting pre-vicariance diversification.

Accurately resolving the phylogenetic relationships among the proposed Gondwanan orthoclads is important as a comparison not only to morphology-based ideas of the evolutionary affinities of the Gondwanan orthoclad genera described above, but to the work of Brundin and the vast literature base concerning the historical effect of the fragmentation of Gondwana on austral taxa. Thus, the outcomes of the current study, which aimed to reconstruct the phylogenetic relationships of, and estimate divergence times for, the proposed Gondwanan orthoclads using molecular data, are twofold: to resolve the various evolutionary relationships among genera and to infer their biogeographical history with regard to the break-up of the supercontinent. Given patterns revealed by Brundin for other austral chironomid groups, it was expected that evolutionary relationships among the proposed Gondwanan orthoclads would follow the geological sequence of continental fragmentation, reflecting diversification largely driven by vicariance.

Moreover, testing rigorously the varied hypotheses regarding the evolutionary affinities among taxa described above was expected to contribute towards reappraisal of current taxonomic designations that are contentious. In particular, resolving the phylogenetic placement of *Echinocladius* was important for providing a more complete understanding of the evolution of this taxon in the context of the broader study. Additionally, it was expected that the intensive sampling undertaken in this study would reveal orthoclad types new to science that will require formal taxonomic description. Furthermore, as many taxonomic descriptions have been based primarily on the morphology of the pupal life stage, this study was expected to reveal life stages of described taxa, particularly larvae, for which the morphology is currently unknown. The current study also complements a broader family-level phylogenetic assessment of the Chironomidae (Cranston, P. S., pers. comm.) by providing a finer-scale evaluation of evolutionary relationships within this subset of the Orthocladiinae. Thus, understanding evolutionary relationships within and among genera will provide essential information for future phylogenetic, phylogeographic and taxonomic investigations of taxa within this group.

5.2 Methods

Target taxa and site localities

Larvae and pupae of proposed Gondwanan orthoclad genera were collected during several sampling trips between 2007 and 2009 from sites in Australia, New Zealand and southern South America, with supplementary samples provided by Prof. Peter Cranston from collections carried out between 2005 to 2010 (Figure 5.1). Representatives of the otherwise widespread and cosmopolitan genera *Parakiefferiella* sampled from Africa, Australia and South America, *Eukiefferiella* from Australia, New Zealand and North America and *Cardiocladius* from Australia and Thailand were included as

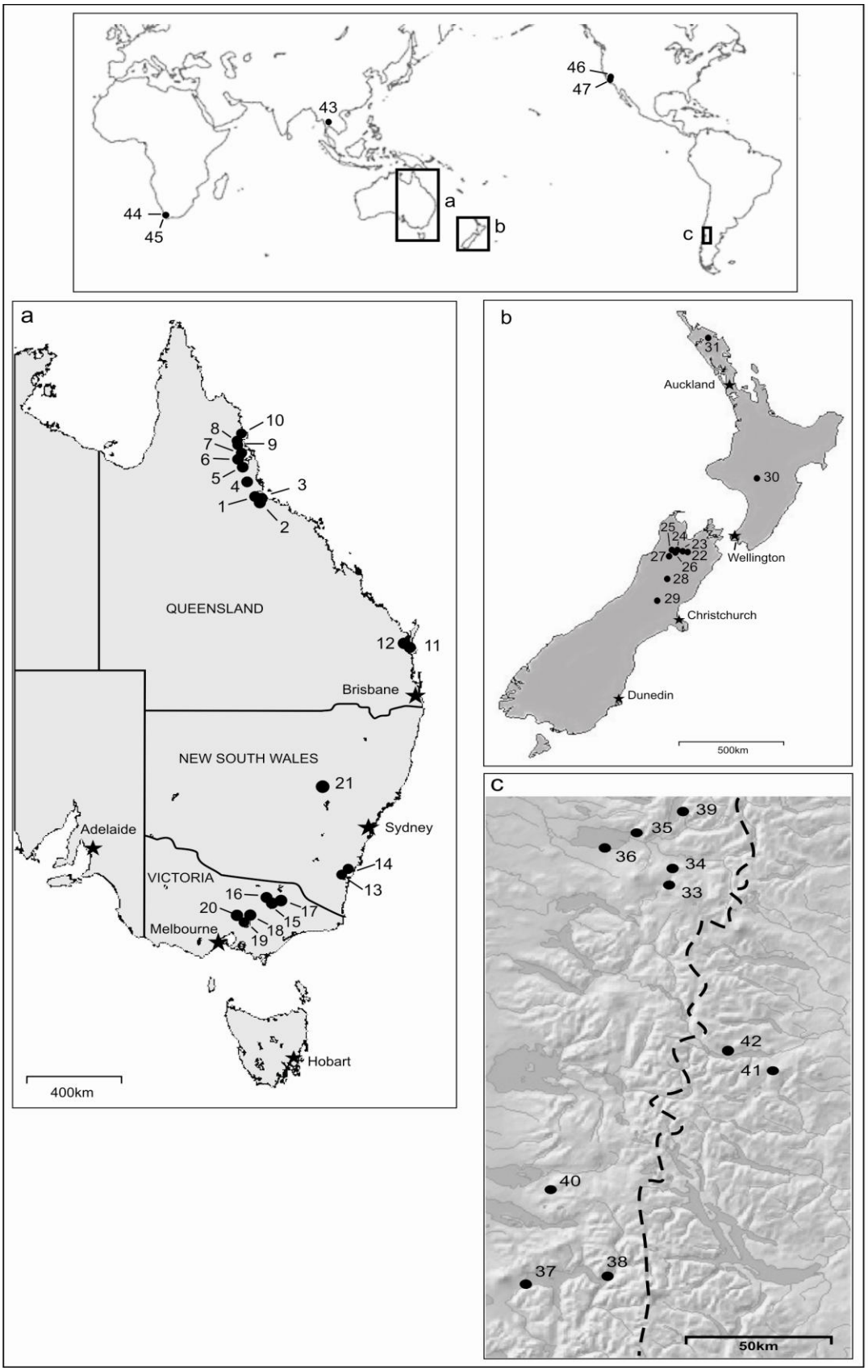


Figure 5.1. Geographical location of study sites, with study regions as boxed insets a, b and c. Sites in South Africa, Thailand and North America from which representative samples were acquired are indicated with filled circles. Site numbers correspond to those given in Supplementary Table 5.1. Filled stars in insets a and b represent Australian and New Zealand state and/or regional capital cities.

distant sister groups to the proposed Gondwanan genera. Full site details are given in Supplementary Table 5.1.

Sample collection

Sample collection, preservation, identification and storage followed the process outlined in Chapter Two, but also included the use of drift or standard sweep nets left half-submerged overnight to recover drifting pupae.

Genetic procedures

Extraction of total genomic DNA and amplification of a 732bp fragment of the COI gene followed the procedure outlined in Chapter Three. A 657bp fragment of the nuclear 28S rDNA gene and two regions (CAD1 – 753bp; CAD3 – 735bp) of the nuclear protein-coding CAD (rudimentary) gene were also amplified using published primer sequences (Table 5.1); PCR reaction

Table 5.1. Primer sequences and references for the four gene regions analysed.

Primer Name	Primer Sequence (5'-3')	Authors
F: COI-s2183 (aka Jerry)	CAA CAT TTA TTT TGA TTT TTT GG	(Simon *et al.*, 1994)
R: COI-a3014 (aka Pat)	TCC AAT GCA CTA ATC TGC CAT ATT A	(Simon *et al.*, 1994)
F: 28S-s3660	GAG AGT TMA ASA GTA CGT GAA AC	(Dowton & Austin, 1998)
R: 28S-a335	TCG GAR GGA ACC AGC TAC TA	(Whiting *et al.*, 1997)
F: CAD1 – 54F	GTN GTN TTY CAR ACN GGN ATG GT	(Moulton & Wiegmann, 2004)
R: CAD1 – 405R	GCN GTR TGY TCN GGR TGR AAY TG	(Moulton & Wiegmann, 2004)
F: CAD3 – 787F	GGD GTN ACN ACN GCN TGY TTY GAR CC	(Moulton & Wiegmann, 2004)
R: CAD3 – 1098R	TTN GGN AGY TGN CCN CCC AT	(Moulton & Wiegmann, 2004)

tube contents were the same for these regions as for COI. PCR protocols for all gene regions involved initial denaturing at 95˚C for 4 minutes and final extension at 72˚C for 3 minutes. The 28S region was amplified using a touchdown PCR cycle protocol beginning with 95˚C for 30 seconds, 58˚C for 1 minute and 72˚C for 1 minute, and reducing the annealing temperature by 2˚C every two cycles until it reached 42˚C, which was then repeated 18 times. The cycle protocol used for CAD1 was 4 cycles of 95˚C for 30 seconds, 51˚C for 30 seconds and 72˚C for 80 seconds, then 36 cycles using an annealing temperature of 45˚C. CAD3 was amplified using a cycle protocol of 4 cycles of 95˚C for 30 seconds, 51˚C for 30 seconds, 72˚C for 2 minutes, 6 cycles of 94˚C for 30 seconds, 47˚C for 1 minute, 72˚C for 2 minutes, then 36 cycles of 94˚C for 30 seconds, 42˚C for 20 seconds and 72˚C for 2.5 minutes. PCR products were purified and sequenced as outlined in Chapter Two.

Data analyses

COI sequences were aligned and edited by eye as outlined in Chapter Two, whilst 28S, CAD1 and CAD3 sequences were aligned using MUSCLE Version 3.6 (Edgar, 2004) and checked by eye in BioEdit. Heterozygous sites in nuclear sequence data were coded as ambiguous bases according to IUPAC codes. Introns in CAD sequences were removed prior to phylogenetic analysis. Tests for sequence saturation, Tajima's D tests of neutrality and tests of clock-like evolution were conducted as described in Chapters Two and Three.

As CAD1 and CAD3 form part of the same gene, these were considered to be a single locus for all following phylogenetic analyses. The most appropriate models of nucleotide substitution for each locus were determined as outlined in Chapter Three. Loci were partitioned separately in combined analyses and the most appropriate model was applied to each partition individually. Phylogenies were reconstructed using mitochondrial and nuclear data separately for the complete suite of taxa and as a partitioned dataset for

both the full suite of taxa and a reduced set of taxa containing only a few representatives of each major clade. A reduced suite of taxa was used to reduce computation time of subsequent analyses and avoid redundancy of identical sequences. Bayesian phylogenetic inference was performed under the GTR model of evolution and incorporating a gamma distribution of nucleotide frequencies, as outlined in Chapter Three. Runs of 30 million and 40 million generations (25% burnin) were conducted for the reduced and complete datasets, respectively. Maximum likelihood (1,000 bootstraps) reconstruction and estimation of a 50% majority rule consensus tree followed procedures outlined in Chapter Three.

Times to most recent common ancestor (tmrca) for relevant nodes of the concatenated dataset from the reduced suite of taxa were estimated using BEAST Version 1.4.8 (Drummond & Rambaut, 2007) under the GTR model of evolution. The Bayesian consensus topology with branch lengths rescaled in units of time in the program TreeEdit Version 1.0a10 (Rambaut & Charleston, 2001) was used as a starting tree to reduce computational time. A lognormal prior was set on the root height of the tree incorporating a zero offset of 37.5my, corresponding to the lower age bound of the oldest known fossil members of the current outgroup *Cricotopus* (Meunier, 1904). The mean was set to 121my, representing the oldest known orthoclad fossil (*Lebanorthocladius furcatus* - Veltz *et al.*, 2007), and the standard deviation set to 0.5 such that the 97.5% Highest Posterior Density (HPD) thereby encompassed the oldest known chironomid fossil (*Aenne triassica*) at 209.6my ± 1my (Krzeminski & Jarzembowski, 1999). The tree prior was set to ‘Speciation: Yule Process’ and substitution rates were allowed to vary across branches in accordance with a relaxed lognormal molecular clock prior. Four runs of 50 million generations were performed, from which 5 million generations were removed from each run prior to combining log files, producing a total run of 180 million generations.

5.3 Results

Gondwanan orthoclad genera collected from Australia were *Echinocladius*, *Botryocladius* and the previously known but currently not formally described genera 'FNQ2' and 'genus Australia' of Brundin (1966) (henceforth referred to as 'Australia' sp. n.). Members of the genera *Naonella*, *Anzacladius*, *Tonnoirocladius*, *Pirara* and the first record of *Limnophyes* were collected from New Zealand. South American members of the described genera *Ferringtonia*, *Botryocladius*, *Limnophyes* were collected, along with representatives of the putative South American *Echinocladius* and a currently unknown orthoclad genus (henceforth referred to as 'Eukiefferiella hooklets'). Members of a New Zealand taxon incorrectly assigned to the globally distributed taxonomic 'dumping ground' *Orthocladius* genus (Freeman, 1959) – referred to here as '*O.*' *pictipennis* – were included as this 'species' is hypothesised to in fact be closely related to this clade of proposed Gondwanan orthoclads (Cranston, P. S., pers. comm.). Only two specimens were obtained from Africa – one of *Tvetenia*, the other of *Parakiefferiella*, both of which represent distant sister taxa to the proposed Gondwanan clade. No putative African members of the Gondwanan clade have yet been recovered, despite extensive sampling by P. S. Cranston in South Africa annually over the last five years. Members of Australian *Pirara* and *Anzacladius* and South American *Pirara* species were sought unsuccessfully, even from type locations in southeast Queensland, New South Wales and Victoria, across several sampling attempts between 2005 and 2010.

In total, 167 individuals across 17 genera were collected and 160 were sequenced for COI, 147 for 28S, 52 for CAD1 and 58 for CAD3 (see Supplementary Table 5.1 for gene fragments sequenced per individual). There were 24 indels observed in 28S, two in CAD1 and two in CAD3 sequences. Two introns were observed in CAD1 and a single intron was observed in CAD3. Ratios of transitions to transversions were low for all fragments, suggesting only limited homoplasy, and Tajima's D tests of neutrality were non-significant for at loci (Table 5.2). The molecular clock

hypothesis was rejected for all fragments, thus a relaxed clock was implemented for estimation of divergence times.

Table 5.2. Molecular summary statistics for the four gene regions analysed. Values in bold indicate significant p-values (p<0.05).

Gene fragment	Ti:Tv	Tajima's D	Tajima's D p-value	Clocklike evolution p-value
COI	0.678	-0.11460	1.000	**<0.05**
28S	2.306	-0.06560	0.524	**<0.05**
CAD1	1.550	0.00900	1.000	**<0.05**
CAD3	1.382	-0.00657	1.000	**<0.05**

Phylogenetic reconstruction

Topologies were roughly concordant among methods of reconstruction and between the complete (Supplementary Figure 5.1) and reduced set of taxa (Figure 5.2), although ML outputs generally were less well resolved. Single gene trees and those inferred from the nuclear data alone produced good tip resolution, grouping members of each major clade together, however deeper nodes were unresolved. The partitioned dataset produced topologies that were well resolved across the tree.

Generally, members identified as belonging to a particular genus or species formed clades to the exclusion of other taxa, indicating that the genetic data reflected morphology-based taxonomic delimitation (Figure 5.2); however, there were five exceptions. First, *Ferringtonia caudicula* formed a sister group to *Anzacladius, Limnophyes* and *F. patagonica* that was highly supported by Bayesian reconstruction, but was not resolved by ML methods. Second, South American 'Echinocladius' sp. n. did not cluster with Australian *Echinocladius* but formed a sister group to *Naonella* and *Tonnoirocladius* and was nested within a group also containing '*Orthocladius*' *pictipennis* and 'Australia' sp. n.. Third, *Naonella* sp. n. 2 was supported as the sister taxon to *Tonnoirocladius*, to the exclusion of described *Naonella*, rendering this genus paraphyletic. Fourth, 'Eukiefferiella hooklets' was the sister group to the rest

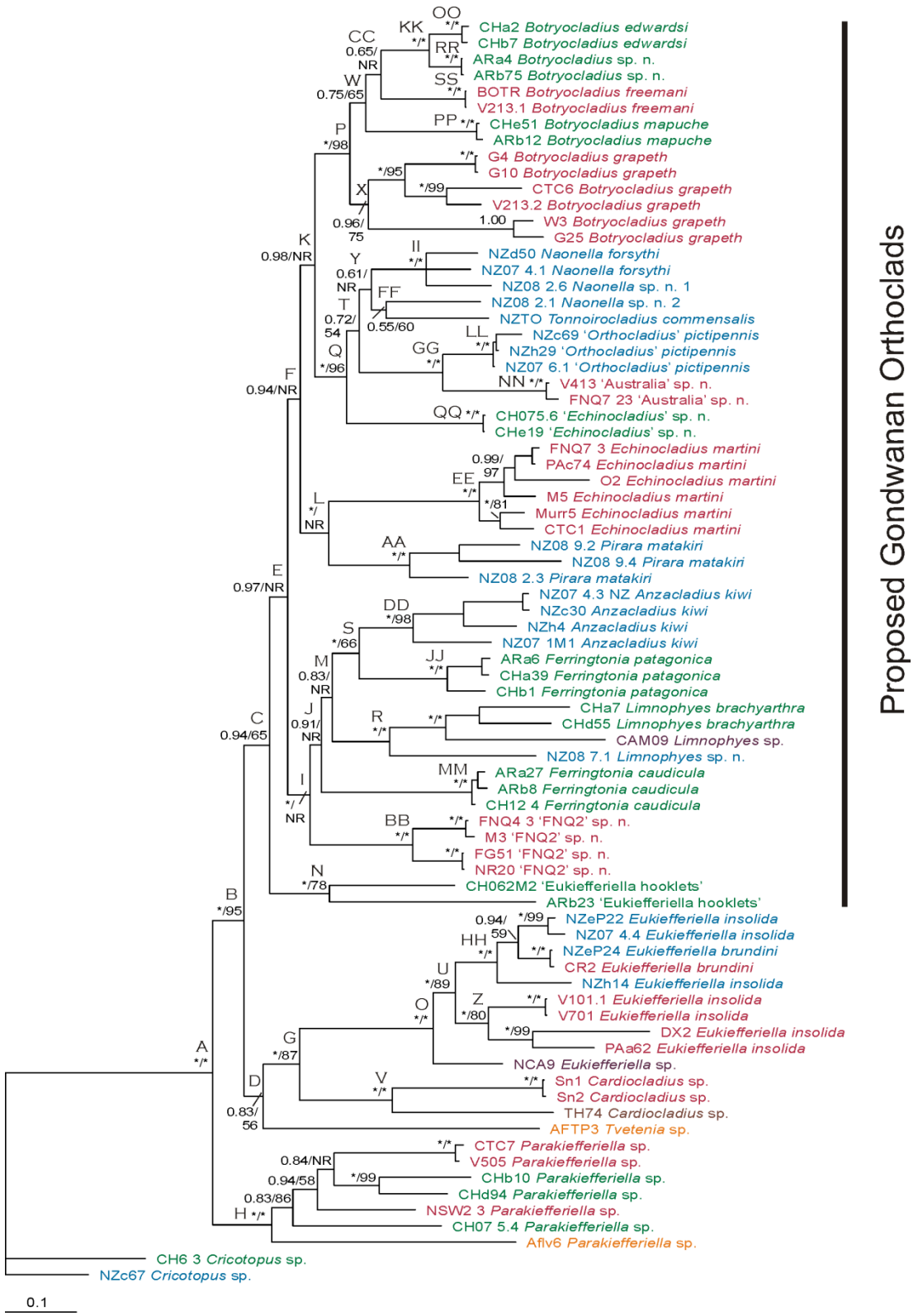


Figure 5.2. Consensus Bayesian topology for the partitioned dataset and a reduced set of taxa. Node-associated values correspond to Bayesian posterior probabilities and ML bootstrap support; 'NR' denotes nodes unresolved by ML and '*' denotes posterior probabilities of 1.00 or bootstrap support of 100. Lettered nodes are those for which tmrca was estimated. Taxon names are coloured according to their continent of origin – green: South America; blue: New Zealand; pink: Australia; orange: Africa; purple: North America; brown: Asia. Branch lengths are in expected substitutions per site.

of the proposed Gondwanan orthoclads, distant from 'true' *Eukiefferiella* which formed the expected sister group to *Cardiocladius* sp.. Finally, *Eukiefferiella insolida*, in which, instead of all members of this species forming a monophyletic group, Australian *E. insolida* forms a group to the exclusion of New Zealand *E. insolida* and *E. brundini*. Furthermore, *E. brundini* was nested within New Zealand *E. insolida*, along with an anomalous Australian *Eukiefferiella* individual, thus rendering *E. insolida* polyphyletic and implying both that the taxonomy of New Zealand *Eukiefferiella* may need revision and that recent trans-Tasman dispersal of *E. brundini* may have occurred. The first three groups mentioned above can also be considered polyphyletic with regard to the genera with which they were originally aligned, thereby implying taxonomic revision is required, likely as new genera in their own right. Additionally, the placement of '*Orthocladius*' *pictipennis* – which if truly a member of the northern hemisphere genus *Orthocladius* should be highly divergent from these proposed Gondwanan taxa – nested within the Gondwanan clade accorded with anecdotal data that supports this designation and implied that the taxonomic assignment of this taxon may require revision.

Biogeographical patterns

Overall, the inferred topology did not show monophyly among the continents, as all taxa from an individual continent did not share a most recent common ancestor to the exclusion of taxa from other continents. The placement of African *Tvetenia* and *Parakiefferiella*, North American *Eukiefferiella* and *Limnophyes* and south-east Asian *Cardiocladius* as divergent sister taxa to the remainder of their respective clades generally accorded with predictions from generic and species descriptions which imply that these groups were already diverse at the initiation of Gondwanan break-up and hence probably represent the result of much earlier radiations in the northern hemisphere. Relationships among genera in some cases followed those previously postulated in the literature. For example, known *Naonella* and *Tonnoirocladius* are shown here to be sister taxa, in agreement with

Cranston (2007); *Pirara* was the sister group to *Echinocladius*, as predicted by Cranston (2000b); the placement of *Eukiefferiella, Cardiocladius* and *Tvetenia* accorded with Sæther & Halvorsen (1981); and, despite the obvious intrageneric diversity of *Botryocladius*, all species of this genus shared a most recent common ancestor with respect to all other genera and the observed pattern of continental non-monophyly follows that proposed by Cranston & Edward (1999). In contrast, Sæther & Anderson (2010) suggested *Anzacladius* and *Botryocladius* were sister taxa, with *Ferringtonia* sister to them: the phylogeny presented here showed not only that *Ferringtonia* as a genus was polyphyletic, but that *F. patagonica* was sister to *Anzacladius*, whilst *Botryocladius* formed the sister group to a clade containing *Naonella, Tonnoirocladius*, '*Orthocladius*' *pictipennis*, 'Australia' sp. n. and South American '*Echinocladius*' sp. n.. It must be mentioned here that the New Zealand genus *Kaniwhaniwhanus* had originally been included in this analysis, as Cranston (2007) had predicted that it was the sister taxon of *Pirara*, however preliminary analysis showed *Kaniwhaniwhanus* to be a deeply divergent sister to the entire ingroup (data not shown). A recent unpublished phylogeny for the entire Chironomidae suggested that the placement of *Kaniwhaniwhanus* is at the base of the subfamily and thus inappropriate for inclusion here (Cranston, P. S., pers. comm.).

The placement of several formally undescribed taxa considered part of the Gondwanan element of the Orthocladiinae have now been resolved. Newly discovered morphotypes identified as belonging to *Naonella* (*N.* sp. n. 1 and 2), *Botryocladius* (*B.* sp. n.) and *Limnophyes* (*L.* sp. n. – the first record of *Limnophyes* in New Zealand) generally were nested within these genera, however *Naonella* sp. n. 2 instead shared a most recent common ancestor with *Tonnoirocladius*. Similarly, two South American taxa '*Echinocladius*' sp. n. and 'Eukiefferiella hooklets' did not share most recent common ancestors with the genera to which they had been assigned originally. The taxon 'Australia' sp. n. (Cranston, 2000a), for which there was no specific hypothesis for evolutionary affinity, was well supported as an Australian sister group of '*O.*' *pictipennis* from New Zealand. In similar fashion, 'FNQ2' sp. n. was shown to be sister to a clade containing *Ferringtonia*, *Anzacladius* and

Limnophyes. Additionally, members of *Pirara* and 'FNQ2' sp. n. were recovered from novel localities and habitats: *Pirara matakiri*, considered a stream-dweller, was sampled from Lake Grasmere in New Zealand, and the undescribed taxon 'FNQ2' sp. n. thought to be restricted to upland streams in Far North Queensland – from which the taxon name derives – was also collected from lowland southeast Queensland streams.

Several novel associations between larval and pupal stages were also made that will assist taxonomic revisions of this group (Supplementary Table 5.1 and Figure 5.1). This is crucial for the accurate identification of the immature stages because morphological descriptions are heavily weighted towards pupal and adult characters. For example, whilst none of the South American *Botryocladius* species have been described previously from larvae (Cranston & Edward, 1999), here associations were made between larvae and pupae of *B. edwardsi* and *B. mapuche*. Similar associations were made also for '*O.*' *pictipennis*, '*Echinocladius*' sp. n., and *Ferringtonia*, whilst morphological associations between life stages were confirmed or extended for *N. forsythi*, *E. martini*, *A. kiwi*, *P. matakiri*, *Parakiefferiella sp.* and New Zealand *Eukiefferiella*.

Estimated times to most recent common ancestor

Estimates of times to most recent common ancestor (tmrca) all possessed effective sample sizes greater than 200, indicating some degree of support for the estimates (Drummond & Rambaut, 2007); however the 95% upper and lower bounds were generally quite broad, potentially compromising the accuracy of the estimates (Table 5.3). Nevertheless, the total ingroup was estimated to have last shared a common ancestor around 72mya (43-108mya), whilst this proposed Gondwanan element of the Orthocladiinae is approximately 60my old (35-91my). The two included African representatives apparently last shared common ancestors with their respective sister groups around 57mya (33-88mya) for *Tvetenia* and the ancestor of *Cardiocladius* and *Eukiefferiella*, and around 52mya (82-27mya) for disjunct members of

Table 5.3. Times to most recent common ancestor estimated using BEAST. Genus names have been shortened for brevity where they appear in groups such that, for example, *Para* = *Parakiefferiella*.

Taxa included	Node Label	Mean (mya)	95% Credibility Intervals (mya)	Effective Sample Size (ESS)
Ingroup	A	72.32	42.91-108.11	632.81
Ingroup minus *Para*	B	65.77	39.02-99.50	611.17
All 'Gondwanan' taxa	C	60.04	35.05-91.39	596.01
Euk + *Cardio* + *Tvet*	D	57.42	32.90-88.10	612.04
All Gondwanans minus 'Euk hooklets'	E	55.63	32.01-84.59	571.93
Echino + *Pir* + *Botryo* + *Nao* + *Tonn* + '*Ortho*' + 'Aust' sp. n. + '*Echino*' sp. n.	F	52.94	30.32-80.90	557.51
Euk + *Cardio*	G	52.90	29.61-81.29	578.1
Parakiefferiella	H	51.69	27.10-81.86	558.55
Ferr + 'FNQ2' sp. n. + *Anza* + *Limn*	I	51.62	29.33-79.24	514.67
Ferr + *Anza* + *Limn*	J	48.00	26.74-73.77	483.17
Nao + *Botryo* + '*Ortho*'	K	47.78	27.10-73.93	546.39
Echino + *Pir*	L	47.42	26.77-73.23	555.39
F. patagonica + *Anza* + *Limn*	M	45.31	25.60-70.18	462.33
'Eukiefferiella hooklets'	N	43.03	20.31-69.68	874.87
Eukiefferiella	O	42.68	22.14-66.89	393.96
Botryocladius	P	42.19	22.81-65.31	452.76
Nao + *Tonn* + '*Ortho*' + '*Echino*' sp. n.	Q	41.84	22.70-65.93	501.52
Limnophyes	R	41.3	22.34-64.48	429.64
F. patagonica + *Anza*	S	39.81	21.03-62.05	466.1
Nao + *Tonn* + '*Ortho*' + 'Aust' sp. n.	T	38.72	20.38-61.15	466.73
Australian + New Zealand *Euk*	U	38.58	19.44-61.52	352.28
Cardiocladius	V	38.43	17.29-62.66	519.05
B. edwardsi + *B. mapuche* + *B. freemani* + *B.* sp. n.	W	35.34	17.78-56.52	506.66
B. grapeth	X	34.78	18.15-55.69	599.77
Nao + *Tonn*	Y	33.48	17.33-53.47	552.01
Australian *Eukiefferiella*	Z	31.50	14.45-51.98	316.91
Pirara	AA	31.29	14.32-51.88	611.1
'FNQ2' sp. n.	BB	31.23	10.95-54.60	446.87
B. edwardsi + *B.* sp. n. + *B. freemani*	CC	30.14	14.26-49.04	512.61
Anzacladius	DD	29.01	12.96-47.37	460.75
Echinocladius	EE	28.59	11.62-47.68	305.64
Tonn + *N.* sp. n. 2	FF	28.07	12.88-46.48	591.84
'*Ortho*' + 'Aust' sp. n.	GG	26.86	9.69-47.10	261.24

Table 5.3 (continued).

Taxa included	Node Label	Mean (mya)	95% Upper and Lower Bounds (mya)	Effective Sample Size (ESS)
New Zealand *Eukiefferiella*	HH	26.27	10.64-44.13	392.25
Naonella	II	21.28	9.13-35.74	451.1
F. patagonica	JJ	18.61	5.75-33.51	406.72
B. edwardsi + *B.* sp. n.	KK	17.77	6.42-61.16	421.13
'*O.*' *pictipennis*	LL	4.15	1.62-7.33	304.69
F. caudicula	MM	3.21	1.22-5.78	989.63
'Australia' sp. n.	NN	2.35	0.60-4.58	644.32
B. edwardsi	OO	2.12	0.48-4.22	2015.65
B. mapuche	PP	1.15	0.20-2.53	2251.1
'*Echinocladius*' sp. n.	QQ	0.37	0.050-0.83	1394.56
B. sp. n.	RR	0.23	0.0013-0.61	2117.72
B. freemani	SS	0.14	0.0013-0.39	1555.24

the widespread genus *Parakiefferiella*, though more complete taxonomic coverage of these groups could reveal them to be much more ancient. Similarly, northern and southern hemisphere members of the cosmopolitan genera *Cardiocladius* and *Eukiefferiella* appear to have last shared common ancestors approximately 38mya (17-63mya) and 43mya (22-67mya), respectively, but again additional sampling may show these taxa to be much older. Described genera within the Gondwanan clade range in age from 42my (23-65my) for the trans-Pacific genus *Botryocladius* to 21my old (9-26my) for the New Zealand genus *Naonella*, whilst undescribed taxa range in age from 43my (20-70my) for trans-Andean 'Eukiefferiella hooklets' to 0.37my old (0.05-0.83my) for '*Echinocladius*' sp. n. from Chile. Trans-Tasman sister group relationships range in age from approximately 47my (27-73my) between *Echinocladius* and *Pirara* to 27my old (10-47my) between '*O.*' *pictipennis* and 'Australia' sp. n.. Trans-Pacific relationships range in age from 41my (22-64my) between New Zealand and New World members of the genus *Limnophyes* to 30my old (14-49my) between the Australian taxon *B. freemani* and the ancestor of the South American taxa *B. edwardsi* and *B.* sp. n.. Interestingly, the tmrca estimates for the genera

Echinocladius, *Naonella* and *Ferringtonia* presented here differ greatly from those estimated in Chapters Three and Four, with mean ages ranging from 11-20my older. This is most likely due to the implementation of different modes of calibration and/or the different representation of taxa. Whereas in previous chapters a molecular rate calibration was used to estimate divergences among lineages within a species, inherently wide bounds on the root height of the phylogeny based on fossil evidence were used here for estimates among higher taxonomic groups. Both these factors are likely to contribute to differences in tmrca estimates and it is unclear which set of estimates could be regarded as more accurate. There is, however, no evidence to suggest that either is definitely incorrect and as such each warrant further investigation and discussion on their own merits.

5.4 Discussion

The phylogeny resolved in the current analysis clearly demonstrates that, as expected, the proposed Gondwanan Orthocladiinae assemblages of each southern continent do not form monophyletic groups with respect to continental distribution. Instead, there was a complex pattern of relationships among the disjunct austral taxa which overall did not support a simple model of vicariant evolution as the sole process that has driven diversification in this group. The phylogenetic placements of African, southeast Asian and North American representatives accorded somewhat with expectations of simple vicariance or secondary dispersal. The complex pattern of relationships among Australian, New Zealand and Patagonian taxa, however, did not follow the predicted pattern of relationships resolved for austral taxa in other chironomid subfamilies (Brundin, 1966), and instead accorded more closely with a recent global phylogenetic reconstruction of the Podonominae (Cranston *et al.*, 2010). Furthermore, estimates of times to most recent common ancestor suggest that divergence in this group – assuming a homogeneous distribution across Gondwana and with particular reference to the New Zealand taxa – may not have begun until well after the initiation of

continental fragmentation. *Echinocladius* was resolved as the sister group to New Zealand *Pirara* (as predicted by Cranston, 2000b), with the genera estimated to have last shared a common ancestor around 47mya (27-73mya), suggesting that trans-Tasman dispersal has been important in the evolution of these two taxa. It seems likely, therefore, that contrary to previous ideas, the evolutionary history of this group has been characterised by several transoceanic, particularly trans-Tasman, dispersal events.

Comparison of the timing of continental fragmentation and estimated divergence times

The expected phylogenetic pattern according to a simple model of vicariant evolution that follows the geological sequence of Gondwanan fragmentation and Brundin's (1966) hypothesis, was that Africa formed a sister group to New Zealand, southern South America and Australia, with New Zealand then sister to southern South America and Australia. Moreover, Brundin (1966) predicted that there were no direct phylogenetic connections between Australian and New Zealand taxa, reflecting the early separation of the two landmasses. By extension, the geological timeline for the break-up of Gondwana predicts that any New Zealand clade and its sister group should have shared a common ancestor approximately 80mya and any clade of Australian and South American taxa around 30mya. In contrast, the phylogeny inferred here shows three sister group relationships between New Zealand and Australian taxa – *Pirara* and *Echinocladius*, '*O.*' *pictipennis* and 'Australia' sp. n., and New Zealand and Australian *Eukiefferiella*, including one apparent very recent arrival of *Eukiefferiella brundini* from New Zealand into south-eastern Australia. Furthermore, all trans-Tasman relationships were estimated to have shared recent common ancestors within the last 47my, post-dating the proposed rifting of Zealandia from Gondwana by around 33my. Similarly, the three trans-Pacific phylogenetic sister groups associating New Zealand and South American taxa (*Naonella/ Tonnoirocladius* and 'Echinocladius' sp. n., *Anzacladius* and *Ferringtonia* and New Zealand and New World *Limnophyes*) apparently shared common

ancestors within the last 41my. Moreover, both African taxa were estimated to have shared a most recent common ancestor with the remainder of their respective clades more than 50my after the proposed rifting of Africa from South America. Direct phylogenetic connections between Australian and South American taxa were recorded only within *Botryocladius*, and the divergence of the Australian species *B. grapeth* from the ancestor of the rest of the genus around 35mya, and that of Australian *B. freemani* from South American *B. edwardsi* and *B.* sp. n. around 30mya, both coincided roughly with the opening of the Drake Passage. Taken together, phylogenetic relationships among taxa inferred here were not congruent with expected patterns, nor with predicted divergence times according to the current geological consensus for Gondwanan break-up, implying instead that the evolutionary history of this group cannot be explained by a simple vicariance hypothesis.

Sister group relationships incongruent with a simple vicariance hypothesis

Historically, incongruence between taxon phylogenies and the geological sequence of continental fragmentation generally has been interpreted as evidence for long-distance transoceanic dispersal post-vicariance. Existing molecular dating evidence also suggests that post-vicariance radiation among the Gondwanan continents has been important in driving diversification in a variety of taxa (e.g., Foighil *et al.*, 1999; Raxworthy *et al.*, 2002; Briggs, 2003; Cook & Crisp, 2005; Page *et al.*, 2005; Barker *et al.*, 2007; Braby *et al.*, 2007; Beck, 2008; Rowe *et al.*, 2010). Moreover, there is abundant meteorological and oceanographic evidence for so-called West Wind Drift – east-flowing ocean and wind currents across the southern oceans – that provides a potential mechanism for the passive dispersal of taxa among separated continents (e.g., Tomlinson, 1973; Close *et al.*, 1978; Chiswell *et al.*, 2003). The pattern of phylogenetic relationships inferred here suggests that at least three post-vicariance dispersal events have occurred between New Zealand and South America and at least three between New

Zealand and Australia, including an apparently quite recent dispersal of *Eukiefferiella brundini* most likely from New Zealand to Australia, thereby suggesting these chironomids are not only capable of extensive long-distance transoceanic dispersal, but that this has occurred several times throughout their evolution. There are three plausible mechanisms for such dispersal: aerial dispersal by active flight, passive transport on wind currents or carriage in the gut passage of migratory birds.

Long-distance dispersal and chironomid flight

As discussed earlier, there is little definitive data surrounding the dispersal capabilities of chironomids, though overall active flight appears limited to less than 10km (McLachlan, 1983, 1986; McLachlan & Neems, 1996) and is in any case restricted by the duration of the adult life stage (generally only a few days - Oliver, 1971; Huryn & Wallace, 2000). Anecdotal data, however, implies that chironomids may be suited for long-distance passive transport on wind currents, based on the ability of some species to colonise new habitats rapidly after their formation (Oliver, 1971) and the recovery of chironomids both at significant altitude (up to 600masl - White, 1970) and large distances from the nearest landmass (often several hundred kilometres - Holzapfel & Harrell, 1968). As far as is currently known, however, passive dispersal of chironomids in this manner is not directed at suitable habitat and relies on wind currents persisting for the full extent of the ocean barrier in order for chironomids to disperse successfully. If chironomids were able to gain sufficient altitude, it is possible that the prevailing westerly winds at high latitudes would be capable of transporting individuals across the Tasman Sea (Tomlinson, 1973), or in the alternate direction on less frequent anti-cyclone frontal systems (Wardle, 1963). Certainly other insect taxa, particularly migratory Lepidoptera, have been shown to utilise such wind currents to aid their long-distance dispersal both across the Tasman Sea (e.g., Fox, 1978; Early *et al.*, 1995) and elsewhere (e.g., Drake & Farrow, 1988; Gatehouse, 1997; Chapman *et al.*, 2010). An interesting alternative dispersal mechanism is based on evidence that has demonstrated the survival and viability of

chironomid larvae in the gut passage of migratory birds (Green & Sanchez, 2006). Whilst this data relates to European sea birds that feed in saltpans – a very different environment to that inhabited by the proposed Gondwanan orthoclads – if extrapolated to the southern hemisphere fauna it provides an alternative potential mode of dispersal among widely separated continents. Furthermore, passive dispersal in the gut of migratory birds may also provide a mechanism by which chironomids may have dispersed passively from New Zealand to Australia, against the prevailing westerly winds.

Inadequacies in taxonomic sampling and calibration of molecular phylogenies

While the results presented here clearly demonstrate that several transoceanic dispersal events may have occurred among the southern continents, thereby driving diversification of this group, several issues in the interpretation and dating of molecular phylogenies need to be considered. Most significant to the accurate interpretation of the inferred phylogeny is appropriate taxonomic sampling; all taxa relevant to the investigation should be included in the analysis to ensure that inferred evolutionary relationships reflect 'reality'. The current study unfortunately lacks representatives from two southern landmasses of two known taxa – *Pirara* from South America and Australia and *Anzacladius* from Australia – despite several sampling attempts at type localities. Furthermore, given the overall lack of knowledge about the taxonomy and evolution of southern hemisphere Orthocladiinae, along with the discovery of at least one new taxon ('Eukiefferiella hooklets') and the recovery of members of known taxa in new locations (for example, *L.* sp. n. from New Zealand) during sampling for this study, it is likely that there remains much unknown diversity, both extant and extinct. The omission of such taxa may dramatically alter resolved phylogenetic relationships, thereby leading to inaccurate inferences about the evolutionary history of this group (Goldberg *et al.*, 2008). For example, if taxa have diversified under a simple model of vicariant evolution, expected phylogenetic relationships would follow the geological sequence of Gondwanan fragmentation such that Australian

taxa would form the sister group to South American taxa and New Zealand taxa sister to these. Thus, the omission of the South American sister of an Australian taxon may result in the Australian taxon being incorrectly inferred to have shared a most recent common ancestor with the New Zealand sister of the South American+ Australian taxa. This pattern obviously does not follow expectations based on the geological record and would likely be incorrectly interpreted as representing transoceanic dispersal between New Zealand and Australia.

Additionally, the use of fossil evidence to calibrate molecular trees can introduce significant error into estimates of divergence times if not incorporated appropriately (e.g., Heads, 2005; Ho *et al.*, 2008). In addition to the intrinsic error in the estimation of the age of fossils, errors in calibration of molecular data have typically related to the inappropriate consideration of the age of a given fossil as the maximum node age for the particular clade to which the fossil belongs, restricting the age of the clade to be no older than the age of the fossil. Instead, fossil ages should be considered as minimum node ages, in that the clade must be at least as old as the fossil. In the current study, fossil evidence was used to constrain the root height of the tree in order to calibrate the internal nodes and thus estimate times to most recent common ancestor for relevant clades. Unfortunately, limited fossil evidence even for the root height of the tree resulted necessarily in the application of wide prior bounds, in order to encompass an appropriate and biologically meaningful prior distribution. The lack of internal calibration points reflects the paucity both of austral insects fossilised in amber – the best source of identifiable fossil taxa – and of fossil representatives of the proposed Gondwanan orthoclad group as a whole – perhaps subsequently due to the lack of synapomorphies for the Orthocladiinae, despite molecular monophyly, thereby hindering their identification as fossils (Cranston, P. S., pers. comm.). Moreover, the use of only a root height prior may bias estimates of divergence times by acting as either too strong or too weak a prior (Heads, 2005; Ho *et al.*, 2008). Although Effective Sample Sizes (ESS) always exceeded 200, as advised by the BEAST software authors to be an indication of good support for the parameter estimate (Drummond &

Rambaut, 2007), the broad 95% upper and lower bounds suggest that the root height prior may be providing too little information for the accurate estimation of divergence times.
Supporting this, the dates estimated here were substantially younger across the phylogram than those inferred in a study that used a dataset which encompassed the entire Chironomidae family and, most importantly, incorporated internal calibration points that lie much deeper in the phylogeny than the proposed Gondwanan orthoclad clade (Cranston, P. S., pers. comm.). This study estimated this group of Gondwanan orthoclads to have shared a most recent common ancestor around 110mya, which, although the 95% credibility intervals overlap, predates the current study estimate by around 50my. It is feasible, therefore, that if internal calibration points could be incorporated into the current dataset, estimates of times to most recent common ancestor may change greatly. Moreover, if all nodes in such an analysis were estimated to be of ages similar to that inferred in the family-level study (Cranston, P. S. pers. comm.), a simple model of vicariant evolution may be a more plausible explanation for many of the observed patterns of relationships. Coupled with more appropriate taxonomic sampling to include known taxa omitted in the current study, incorporating internal calibration points would provide a more accurate reconstruction of the evolutionary relationships of this group. Taken together, these issues represent significant concerns for the interpretation of the current phylogeny, yet both remain only speculation at present, pending further sampling and discovery of fossil Gondwanan orthoclads.

Nevertheless, even given these potential sources of error, the data as it stands currently suggests at least two examples of possible transoceanic dispersal events identified here – both of which involve trans-Tasman movement – that would not be greatly changed by such revised estimates of divergence times. The group comprising '*Orthocladius*' *pictipennis* and 'Australia' sp. n., estimated currently to have last shared a common ancestor approximately 27mya (10-47mya), still would not predate the rifting of New Zealand from Gondwana if the estimate was increased in line with the timing suggested by the family-level study (Cranston, P. S., pers. comm.) –

although feasibly the 95% credibility intervals may then encompass the rifting event. More importantly, the apparently recent arrival of New Zealand *Eukiefferiella brundini* in Australia provides clear evidence of dispersal across the Tasman Sea that would not fit with a simple model of vicariant evolution even if estimated divergence times were increased. Thus, regardless of the uncertainty surrounding estimates of times to most recent common ancestor for groups within the proposed Gondwanan orthoclads, the overall pattern of phylogenetic relationships still provides evidence against Brundin's (1966) simple model of vicariant evolution, and implies that the evolutionary history of this group involves a complex interaction between vicariance and long-distance dispersal.

Novel phylogenetic placements and life stage associations

The pattern of evolutionary relationships inferred here revealed several novel relationships that imply that the taxonomy and systematics of some taxa may require formal revision. Most pertinent is the polyphyly revealed for the two described species of *Ferringtonia*. The phylogenetic placement of *F. caudicula* as sister to the group comprising *Limnophyes*, *Anzacladius* and *F. patagonica* implied that the two described *Ferringtonia* species may instead represent distinct genera. Certainly, there seems no evidence from morphology to support the expansion of the *Ferringtonia* genus to include *Limnophyes* and *Anzacladius* (Cranston, P. S., pers. comm.).

Similarly, trans-Tasman *Eukiefferiella insolida* was also polyphyletic with regard to the New Zealand taxon *E. brundini*, implying either that the two may actually be members of the same species or, more likely, that Australian and New Zealand *Eukiefferiella* are more diverse than was previously thought. There is substantial morphological evidence from all life stages which suggests *E. brundini* is distinct from *E. insolida* (Boothroyd & Cranston, 1995), thereby precluding the two taxa from being combined under a single species name and suggesting that a revision of those specimens currently identified as New Zealand *E. insolida* clearly is required. Given that the name

E. insolida was first used to describe Australian members of the species (Freeman, 1961) and, with the exception of the recent disperser sampled from the Clyde River in New South Wales and nested within *E. brundini*, *Eukiefferiella* appears to exhibit a distinct disjunction across the Tasman Sea, it would thus be appropriate for the New Zealand members of *E. insolida* to be assigned two new species names, following formal corroboration with morphological data. Furthermore, the divergence within Australian *E. insolida* that is correlated with geography may represent the distinction between two distinct taxa and this also warrants further investigation.

Several novel taxa assigned originally to a particular described genus were shown to be most closely related to other taxa within the proposed Gondwanan clade. Of the two novel members of the New Zealand genus *Naonella*, one shared a recent common ancestor with the remainder of the genus, whereas the second (*N.* sp. n. 2) was the sister group to *Tonnoirocladius commensalis*, rendering *Naonella* paraphyletic. A possible explanation is that *N*. sp. n. 2 may not be a member of *Naonella* and is instead a second species of *Tonnoirocladius*. More likely, however, is that the unusual larval life history of *T. commensalis*, which live commensally on the underside of net-winged midge larvae (Diptera: Blephariceridae - Cranston, 2007), may have driven morphological anagenesis in the larva that has rendered it distinct from *Naonella*. Thus, whilst morphological characteristics alone suggest the two genera are distinct, patterns in molecular data reflect their common ancestry and imply that *T. commensalis* should be revised as a new species of *Naonella*.

Both '*Echinocladius*' sp. n. and 'Eukiefferiella hooklets' from South America clearly represent novel taxa that do not form monophyletic groups with the described genera to which they were assigned and hence will require revision, potentially as genera in their own right. Furthermore, the long branches that link the two representatives of 'Eukiefferiella hooklets' suggest that these taxa may represent distinct species. Similarly, the novel taxon *Botryocladius* sp. n. appeared to be distinct from other *Botryocladius* species and may represent either a described South American species known only

from pupal morphology for which associations between known and unknown life stages have not yet been made (e.g., *B. glacialis* or *B. tronador* - Cranston & Edward, 1999), or an entirely new species. This awaits further sampling of other South American *Botryocladius* to more accurately confirm the identity of this unknown taxon. Additionally, *B. grapeth* exhibited high levels of intraspecific diversity that could not be explained by geographical separation, possibly indicating the presence of sympatric cryptic species. This pattern was also observed within the genera *Pirara*, *Limnophyes* and *Anzacladius*, thereby implying that these taxa may require sampling and analysis at a finer scale to more accurately resolve the taxonomic boundaries of the currently described species.

Overall, the results presented here represent the first attempt to use molecular data to test Brundin's (1966) predicted patterns of trans-Antarctic relationships in Orthocladiine chironomids. The inferred phylogeny clearly contradicted predictions of evolutionary relationships among disjunct austral orthoclad taxa and implied that this group possesses a much more complex history than can be explained by simple vicariance alone. Additionally, estimates of relevant node ages implied that the diversification of this group largely post-dates the fragmentation of the Gondwanan supercontinent, though several potential sources of error associated with node calibration and taxonomic sampling have been identified.

This notwithstanding, taken together these data imply that the current distributions and evolutionary affinities of the proposed Gondwanan orthoclads have been shaped not only by continental fragmentation but also by relatively extensive transoceanic dispersal among the major southern landmasses. This pattern accords with much of the current evidence from proposed Gondwanan taxa that has suggested some combination of vicariance and dispersal in driving diversification in most ancient austral taxa (see Sanmartin *et al.*, 2006; Upchurch, 2008). Similar patterns of complex relationships among the southern continents are thus expected for 'Gondwanan' elements of other chironomid subfamilies, including the Diamesinae, Aphroteniinae and Podonominae investigated using

morphological data by Brundin (1966). Indeed, a recent molecular phylogeny for the Podonominae suggested austral members of this subfamily may also possess a complex evolutionary history shaped in varying parts by both continental fragmentation and transoceanic dispersal events (Cranston *et al.*, 2010). The sampling regime implemented here is the most intensive yet performed for austral members of the Orthocladiinae and unsurprisingly has revealed both novel taxa and phylogenetic relationships within and among described genera. Moreover, important associations between described mature and unknown immature life stages were resolved for several taxa. The results of this study will inform taxonomic revisions of members of the proposed Gondwanan orthoclads and provide molecular support for the description and taxonomic placement of novel taxa and unknown life stages.

Supplementary Table 5.1. Geographical location of sample sites and gene regions sequenced per individual in the complete dataset.

Taxon Name	Molecular Voucher	Country	Site/Region	Site Number	Latitude	Longitude	Life Stage	Gene regions sequenced			
								CO1	28S	CAD1	CAD3
Naonella forsythi	NZ08 2.5	New Zealand	Ohakune, Manawatu-Wanganui	30	39˚24.00'S	175˚27.00'E	P	✓			
Naonella forsythi	NZc33	New Zealand	Speargrass Creek, Nelson Lakes	23	41˚46.50'S	172˚46.30'E	L	✓	✓		
Naonella forsythi	NZ08 2.9	New Zealand	Ohakune, North Island	30	39˚24.00'S	175˚27.00'E	P	✓			
Naonella forsythi	NZf99	New Zealand	Rotoroa, Nelson Lakes	26	41˚48.11'S	172˚32.31'E	L	✓	✓		
Naonella forsythi	NZ08 9.5	New Zealand	Hope River, Nelson Lakes	24	41˚41.41'S	172˚37.02'E	P	✓			
Naonella forsythi	NZ07 4.1	New Zealand	Hope River, Nelson Lakes	24	41˚41.41'S	172˚37.02'E	P	✓	✓	✓	✓
Naonella forsythi	NZ NA1	New Zealand	Puketi, Northland	31	35˚12.41'S	173˚47.37'E	L	✓			
Naonella forsythi	NZa72	New Zealand	Borlase Creek, Nelson Lakes	22	41˚48.24S	172˚50.45'E	L	✓	✓		
Naonella forsythi	NZ08 8.2	New Zealand	Speargrass Creek, Nelson Lakes	23	41˚46.50'S	172˚46.30'E	P	✓	✓		
Naonella forsythi	NZh30	New Zealand	Jackson Creek, Lewis Pass	28	42˚22.17'S	172˚16.01'E	L	✓	✓		
Naonella forsythi	NZ08 7.3	New Zealand	Borlase Creek, Nelson Lakes	22	41˚48.24S	172˚50.45'E	P	✓			
Naonella forsythi	NZd50	New Zealand	Hope River, Nelson Lakes	24	41˚41.41'S	172˚37.02'E	L	✓	✓		✓
Naonella forsythi	NZeP3	New Zealand	Owen River, Nelson Lakes	25	41˚41.13'S	172˚27.09'E	P	✓	✓		
Naonella forsythi	NZg1	New Zealand	Campbell Creek, Lewis Pass	27	41˚48.00'S	172˚13.00'E	L		✓		
Naonella sp. n. 1	NZ08 2.6	New Zealand	Ohakune, Manawatu-Wanganui	30	39˚24.00'S	175˚27.00'E	P	✓	✓	✓	✓
Naonella sp. n. 2	NZ08 2.1	New Zealand	Ohakune, Manawatu-Wanganui	30	39˚24.00'S	175˚27.00'E	P	✓	✓		✓
Tonnoirocladius commensalis	NZTO	New Zealand	Blackadder Creek, Canterbury	32	42˚16.00'S	172˚11.00'E	L	✓	✓	✓	✓
'*Orthocladius*' pictipennis	NZa37	New Zealand	Borlase Creek, Nelson Lakes	22	41˚48.24S	172˚50.45'E	L	✓	✓		
'*Orthocladius*' *pictipennis*	NZ08 7.8	New Zealand	Borlase Creek, Nelson Lakes	22	41˚48.24S	172˚50.45'E	P	✓			✓
'*Orthocladius*' *pictipennis*	NZ07 6.1	New Zealand	Rotoroa, Nelson Lakes	26	41˚48.11'S	172˚32.31'E	P	✓	✓	✓	
'*Orthocladius*' *pictipennis*	NZh29	New Zealand	Jackson Creek, Lewis Pass	28	42˚22.17'S	172˚16.01'E	L	✓	✓		✓

Supplementary Table 5.1 continued.

Taxon Name	Molecular Voucher	Country	Site/Region	Site Number	Latitude	Longitude	Life Stage	Gene regions sequenced			
								CO1	28S	CAD1	CAD3
'*Orthocladius*' *pictipennis*	NZf8	New Zealand	Rotoroa, Nelson Lakes	26	41˚48.11'S	172˚32.31'E	L	✓	✓		
'*Orthocladius*' *pictipennis*	NZc69	New Zealand	Speargrass Creek, Nelson Lakes	23	41˚46.50'S	172˚46.30'E	L	✓	✓		
Echinocladius martini	FNQ4 7	Australia	Nigger Creek, Atherton Tableland NP	5	17˚26.49'S	145˚28.26'E	L	✓			
Echinocladius martini	FNQ7 3	Australia	Davies Creek, Davies Creek NP	7	17˚00.35'S	145˚34.56'E	L	✓	✓	✓	✓
Echinocladius martini	D5	Australia	Davies Creek, Davies Creek NP	7	17˚00.35'S	145˚34.56'E	L	✓	✓		
Echinocladius martini	PAc74	Australia	Little Birthday Creek, Paluma NP	2	18˚58.22'S	146˚09.52'E	L	✓	✓	✓	✓
Echinocladius martini	DK1	Australia	Davies Creek, Davies Creek NP	7	17˚00.35'S	145˚34.56'E	L	✓	✓		
Echinocladius martini	PAj3	Australia	Cloudy Creek, Paluma NP	3	18˚59.50'S	146˚12.30'E	L	✓	✓		
Echinocladius martini	O2	Australia	Oliver Creek, Daintree NP	10	16˚08.30'S	145˚26.58'E	L	✓	✓		
Echinocladius martini	M5	Australia	Mary Creek, Mt. Lewis NP	9	16˚35.20'S	145˚17.50'E	L	✓	✓	✓	✓
Echinocladius martini	M1	Australia	Mary Creek, Mt. Lewis NP	9	16˚35.20'S	145˚17.50'E	L	✓	✓		
Echinocladius martini	CR1	Australia	Clyde River, southern NSW	13	35°30.23'S	150°13.26'E	L	✓	✓		
Echinocladius martini	CTC1	Australia	Cabbage Tree Creek, Currowan SF	12	35˚34.00'S	150˚02.00'E	P	✓	✓		
Echinocladius martini	Murr5	Australia	Murrindindi Creek, Talangi NP	19	37˚29.00'S	145˚33.00'E	L	✓	✓	✓	✓
Echinocladius martini	FG1	Australia	Franki's Gulch, Cooloola NP	11	26˚02.50'S	153˚04.44'E	L	✓	✓		
'Echinocladius' sp. n.	CH07 5.6	Chile	Estero Correntoso, Lakes District	36	39˚18.00'S	72˚04.00'W	P	✓	✓	✓	✓
'Echinocladius' sp. n.	CHe22	Chile	Estero Correntoso, Lakes District	36	39˚18.00'S	72˚04.00'W	L	✓	✓		✓
'Echinocladius' sp. n.	CH07 4.2	Chile	Estero La Casilla, Lakes District	35	39˚14.00'S	71˚56.00'W	P	✓	✓		
'Echinocladius' sp. n.	CH06 2M1	Chile	Rio Palguin, Lakes District	33	39˚26.13'S	71˚47.25'W	P	✓	✓		
'Echinocladius' sp. n.	CHe19	Chile	Estero Correntoso, Lakes District	36	39˚18.00'S	72˚04.00'W	L	✓	✓		✓

Supplementary Table 5.1 continued.

Taxon Name	Molecular Voucher	Country	Site/Region	Site Number	Latitude	Longitude	Life Stage	Gene regions sequenced			
								CO1	28S	CAD1	CAD3
'Echinocladius' sp. n.	CH07 5.7	Chile	Estero Correntoso, Lakes District	36	39˚18.00'S	72˚04.00'W	P	✓	✓		
Botryocladius edwardsi	CH07 4.3	Chile	Estero La Casilla, Lakes District	35	39˚14.00'S	71˚56.00'W	P	✓	✓		
Botryocladius edwardsi	ARb25	Argentina	Arroyo Quilanlahue, Neuquen Province	42	40˚09.00'S	71˚33.00'W	L	✓	✓		
Botryocladius edwardsi	CHa2	Chile	Rio Palguin, Lakes District	33	39˚26.13'S	71˚47.25'W	L	✓	✓	✓	✓
Botryocladius edwardsi	CH07 5M3P	Chile	Estero Correntoso, Lakes District	36	39˚18.00'S	72˚04.00'W	P	✓	✓		
Botryocladius edwardsi	CHe37	Chile	Estero Correntoso, Lakes District	36	39˚18.00'S	72˚04.00'W	L	✓	✓		
Botryocladius edwardsi	ARG072M2	Argentina	Arroyo Quilanlahue, Neuquen Province	42	40˚09.00'S	71˚33.00'W	P		✓		
Botryocladius edwardsi	CHb7	Chile	Rio Don Baucha, Lakes District	34	39˚23.33'S	71˚47.11'W	L	✓	✓		
Botryocladius sp. n.	ARa4	Argentina	Arroyo Partido, Neuquen Province	41	40˚14.00'S	71˚22.00'W	L	✓	✓	✓	✓
Botryocladius sp. n.	ARb75	Argentina	Arroyo Quilanlahue, Neuquen Province	42	40˚09.00'S	71˚33.00'W	L	✓	✓		
Botryocladius freemani	V216	Australia	Murrindindi Creek, Talangi SF	19	37˚29.00'S	145˚33.00'E	L	✓	✓		
Botryocladius freemani	V213.1	Australia	Murrindindi Creek, Talangi SF	19	37˚29.00'S	145˚33.00'E	L	✓	✓	✓	
Botryocladius freemani	BOTR	Australia	Murrindindi Creek, Talangi SF	19	37˚29.00'S	145˚33.00'E	L	✓	✓	✓	✓
Botryocladius mapuche	CHa23	Chile	Rio Palguin, Lakes District	33	39˚26.13'S	71˚47.25'W	L	✓	✓		
Botryocladius mapuche	ARb12	Argentina	Arroyo Quilanlahue, Neuquen Province	42	40˚09.00'S	71˚33.00'W	L	✓	✓	✓	✓
Botryocladius mapuche	CH07 5.5	Chile	Estero Correntoso, Lakes District	36	39˚18.00'S	72˚04.00'W	P	✓	✓		
Botryocladius mapuche	CHe51	Chile	Estero Correntoso, Lakes District	36	39˚18.00'S	72˚04.00'W	L	✓	✓		
Botryocladius grapeth	V213.2	Australia	Murrindindi Creek, Talangi SF	19	37˚29.00'S	145˚33.00'E	L	✓	✓		✓
Botryocladius grapeth	V213.0	Australia	Murrindindi Creek, Talangi SF	19	37˚29.00'S	145˚33.00'E	L	✓	✓		✓
Botryocladius grapeth	FG7	Australia	Franki's Gulch, Cooloola NP	11	26˚02.50'S	153˚04.44'E	L	✓	✓		
Botryocladius grapeth	V302.2	Australia	Falls Creek, Talangi SF	18	37˚24.00'S	145˚33.00'E	L	✓	✓		
Botryocladius grapeth	CTC6	Australia	Cabbage Tree Creek, Currowan SF	13	35˚34.00'S	150˚02.00'E	L	✓	✓		

Supplementary Table 5.1 continued.

Taxon Name	Molecular Voucher	Country	Site/Region	Site Number	Latitude	Longitude	Life Stage	Gene regions sequenced			
								CO1	28S	CAD1	CAD3
Botryocladius grapeth	G4	Australia	Goddard Creek, Kirrama NP	4	18˚13.10'S	145˚48.50'E	L	✓	✓		
Botryocladius grapeth	G10	Australia	Goddard Creek, Kirrama NP	4	18˚13.10'S	145˚48.50'E	L	✓	✓		
Botryocladius grapeth	G25	Australia	Goddard Creek, Kirrama NP	4	18˚13.10'S	145˚48.50'E	L	✓	✓	✓	
Botryocladius grapeth	W3	Australia	Windmill Creek, Mt. Lewis NP	8	16˚34.40'S	145˚16.20'E	L	✓	✓		✓
Botryocladius grapeth	W4	Australia	Windmill Creek, Mt. Lewis NP	8	16˚34.40'S	145˚16.20'E	L	✓			✓
Pirara matakiri	NZ08 8.10	New Zealand	Speargrass Creek, Nelson Lakes	23	41˚46.50'S	172˚46.30'E	L	✓		✓	✓
Pirara matakiri	NZc68	New Zealand	Speargrass Creek, Nelson Lakes	23	41˚46.50'S	172˚46.30'E	L	✓			
Pirara matakiri	NZ08 9.2	New Zealand	Hope River, Nelson Lakes	24	41˚41.41'S	172˚37.02'E	P	✓	✓		
Pirara matakiri	NZ10a1	New Zealand	Lake Grasmere, Canterbury	29	43˚03.57'S	171˚46.22'E	P	✓	✓	✓	✓
Pirara matakiri	NZ10a10	New Zealand	Lake Grasmere, Canterbury	29	43˚03.57'S	171˚46.22'E	L	✓	✓	✓	✓
Pirara matakiri	NZ08 9.4	New Zealand	Hope River, Nelson Lakes	24	41˚41.41'S	172˚37.02'E	P	✓	✓	✓	✓
Pirara matakiri	NZ08 2.3	New Zealand	Ohakune, Manawatu-Wanganui	30	39˚24.00'S	175˚27.00'E	P	✓	✓		
Ferringtonia patagonica	ARa6	Argentina	Arroyo Partido, Neuquen Province	41	40˚14.00'S	71˚22.00'W	L	✓	✓	✓	
Ferringtonia patagonica	ARG07 1.1	Argentina	Arroyo Partido, Neuquen Province	41	40˚14.00'S	71˚22.00'W	P	✓	✓	✓	
Ferringtonia patagonica	CH12 2	Chile	Peulla, Lakes District	38	41˚05.12'S	72˚01.16'W	L	✓	✓		
Ferringtonia patagonica	CH2 4	Chile	Rio Palguin, Lakes District	33	39˚26.13'S	71˚47.25'W	L	✓	✓	✓	
Ferringtonia patagonica	ARG07 2M1	Argentina	Arroyo Quilanlahue, Neuquen Province	42	40˚09.00'S	71˚33.00'W	P	✓	✓		
Ferringtonia patagonica	ARb5	Argentina	Arroyo Quilanlahue, Neuquen Province	42	40˚09.00'S	71˚33.00'W	L	✓	✓		
Ferringtonia patagonica	CHd26	Chile	Estero La Casilla, Lakes District	35	39˚14.00'S	71˚56.00'W	L	✓	✓		
Ferringtonia patagonica	CHa39	Chile	Rio Palguin, Lakes District	33	39˚26.13'S	71˚47.25'W	L	✓	✓	✓	
Ferringtonia patagonica	CHe47	Chile	Estero Correntoso, Lakes District	36	39˚18.00'S	72˚04.00'W	L	✓	✓		

Supplementary Table 5.1 continued.

Taxon Name	Molecular Voucher	Country	Site/Region	Site Number	Latitude	Longitude	Life Stage	Gene regions sequenced			
								CO1	28S	CAD1	CAD3
Ferringtonia patagonica	CH12 1	Chile	Peulla, Lakes District	38	41˚05.12'S	72˚01.16'W	P	✓	✓		
Ferringtonia patagonica	CH07 17M2	Chile	Rio Chaleufu, Lakes District	40	40˚44.00'S	72˚18.00'W	P	✓	✓		
Ferringtonia patagonica	CHe14	Chile	Estero Correntoso, Lakes District	36	39˚18.00'S	72˚04.00'W	L	✓	✓		
Ferringtonia patagonica	CHb1	Chile	Rio Don Baucha, Lakes District	34	39˚23.33'S	71˚47.11'W	L	✓	✓	✓	
Ferringtonia patagonica	CH5 1	Chile	Estero La Casilla, Lakes District	35	39˚14.00'S	71˚56.00'W	L	✓	✓		
Ferringtonia caudicula	CHd14	Chile	Estero La Casilla, Lakes District	35	39˚14.00'S	71˚56.00'W	L	✓	✓		
Ferringtonia caudicula	ARb8	Argentina	Arroyo Quilanlahue, Neuquen Province	42	40˚09.00'S	71˚33.00'W	L	✓	✓		
Ferringtonia caudicula	CH07 4.5	Chile	Estero La Casilla, Lakes District	35	39˚14.00'S	71˚56.00'W	P	✓	✓		
Ferringtonia caudicula	CH07 5.2	Chile	Estero Correntoso, Lakes District	36	39˚18.00'S	72˚04.00'W	P	✓	✓		
Ferringtonia caudicula	CHe5	Chile	Estero Correntoso, Lakes District	36	39˚18.00'S	72˚04.00'W	L	✓	✓		
Ferringtonia caudicula	ARa27	Argentina	Arroyo Partido, Neuquen Province	41	40˚14.00'S	71˚22.00'W	L	✓	✓	✓	✓
Ferringtonia caudicula	CHb18	Chile	Rio Don Baucha, Lakes District	34	39˚23.33'S	71˚47.11'W	L	✓	✓		
Ferringtonia caudicula	CH12 4	Chile	Peulla, Lakes District	38	41˚05.12'S	72˚01.16'W	P	✓	✓		✓
Anzacladius kiwi	NZa42	New Zealand	Borlase Creek, Nelson Lakes	22	41˚48.24S	172˚50.45'E	L	✓	✓		✓
Anzacladius kiwi	NZ08 8.1	New Zealand	Speargrass Creek, Nelson Lakes	23	41˚46.50'S	172˚46.30'E	P	✓	✓		
Anzacladius kiwi	NZ07 1	New Zealand	Speargrass Creek, Nelson Lakes	23	41˚46.50'S	172˚46.30'E	P	✓	✓	✓	✓
Anzacladius kiwi	NZf22	New Zealand	Rotoroa, Nelson Lakes	26	41˚48.11'S	172˚32.31'E	L	✓	✓		
Anzacladius kiwi	NZ07 4.3	New Zealand	Hope River, Nelson Lakes	24	41˚41.41'S	172˚37.02'E	P	✓	✓	✓	✓
Anzacladius kiwi	NZc30	New Zealand	Speargrass Creek, Nelson Lakes	23	41˚46.50'S	172˚46.30'E	L	✓	✓		
Anzacladius kiwi	NZ08 7.4	New Zealand	Borlase Creek, Nelson Lakes	22	41˚48.24S	172˚50.45'E	P	✓			
Anzacladius kiwi	NZg3	New Zealand	Campbell Creek, Lewis Pass	27	41˚48.00'S	172˚13.00'E	L	✓	✓		
Anzacladius kiwi	NZh4	New Zealand	Jackson Creek, Lewis Pass	28	42˚22.17'S	172˚16.01'E	L	✓	✓	✓	✓

Supplementary Table 5.1 continued.

Taxon Name	Molecular Voucher	Country	Site/Region	Site Number	Latitude	Longitude	Life Stage	Gene regions sequenced			
								CO1	28S	CAD1	CAD3
Anzacladius kiwi	NZ07 1M1	New Zealand	Speargrass Creek, Nelson Lakes	23	41˚46.50'S	172˚46.30'E	P	✓	✓		
Limnophyes brachyarthra	CHb14	Chile	Rio Don Baucha, Lakes District	34	39˚23.33'S	71˚47.11'W	L	✓	✓		
Limnophyes brachyarthra	CHd55	Chile	Estero La Casilla, Lakes District	35	39˚14.00'S	71˚56.00'W	L	✓	✓	✓	✓
Limnophyes brachyarthra	CHe54	Chile	Estero Correntoso, Lakes District	36	39˚18.00'S	72˚04.00'W	L	✓	✓		
Limnophyes brachyarthra	CH13 4	Chile	Peulla, Lakes District	38	41˚05.12'S	72˚01.16'W	P	✓	✓		✓
Limnophyes brachyarthra	ARa70	Argentina	Arroyo Partido, Neuquen Province	41	40˚14.00'S	71˚22.00'W	L	✓	✓		
Limnophyes brachyarthra	CHa7	Chile	Rio Palguin, Lakes District	33	39˚26.13'S	71˚47.25'W	L	✓	✓		✓
Limnophyes brachyarthra	ARaP8	Argentina	Arroyo Partido, Neuquen Province	41	40˚14.00'S	71˚22.00'W	P	✓			
Limnophyes sp.	CAM09	USA	Mailliard Redwoods SFR, California	46	38˚54.22'N	123˚19.36'W	L	✓	✓	✓	✓
Limnophyes sp. n.	NZ08 7.1	New Zealand	Borlase Creek, Nelson Lakes	22	41˚48.24S	172˚50.45'E	P	✓	✓	✓	
'FNQ2' sp. n.	M3	Australia	Mary Creek, Mt. Lewis NP	9	16˚35.20'S	145˚17.50'E	L	✓	✓	✓	✓
'FNQ2' sp. n.	C19	Australia	Little Birthday Creek, Paluma NP	2	18˚58.22'S	146˚09.52'E	L	✓	✓		
'FNQ2' sp. n.	G13	Australia	Goddard Creek, Kirrama NP	4	18˚13.10'S	145˚48.50'E	L	✓	✓		
'FNQ2' sp. n.	FNQ4 3	Australia	Nigger Creek, Atherton Tableland NP	5	17˚26.49'S	145˚28.26'E	L	✓	✓	✓	
'FNQ2' sp. n.	FG51	Australia	Franki's Gulch, Cooloola NP	11	26˚02.50'S	153˚04.44'E	L	✓	✓	✓	
'FNQ2' sp. n.	NR20	Australia	Noosa River, Cooloola NP	12	26˚03.28'S	153˚00.23'E	L	✓	✓		
'Eukiefferiella hooklets'	CH06 2M2	Chile	Rio Palguin, Lakes District	33	39˚26.13'S	71˚47.25'W	P	✓	✓		✓
'Eukiefferiella hooklets'	ARb23	Argentina	Arroyo Quilanlahue, Neuquen Province	42	40˚09.00'S	71˚33.00'W	L	✓	✓	✓	✓
Eukiefferiella brundini	NZd38	New Zealand	Hope River, Nelson Lakes	24	41˚41.41'S	172˚37.02'E	L	✓	✓		
Eukiefferiella brundini	NZ08 8.9	New Zealand	Speargrass Creek, Nelson Lakes	23	41˚46.50'S	172˚46.30'E	L	✓			
Eukiefferiella brundini	NZeP24	New Zealand	Owen River, Nelson Lakes	25	41˚41.13'S	172˚27.09'E	P	✓	✓	✓	✓

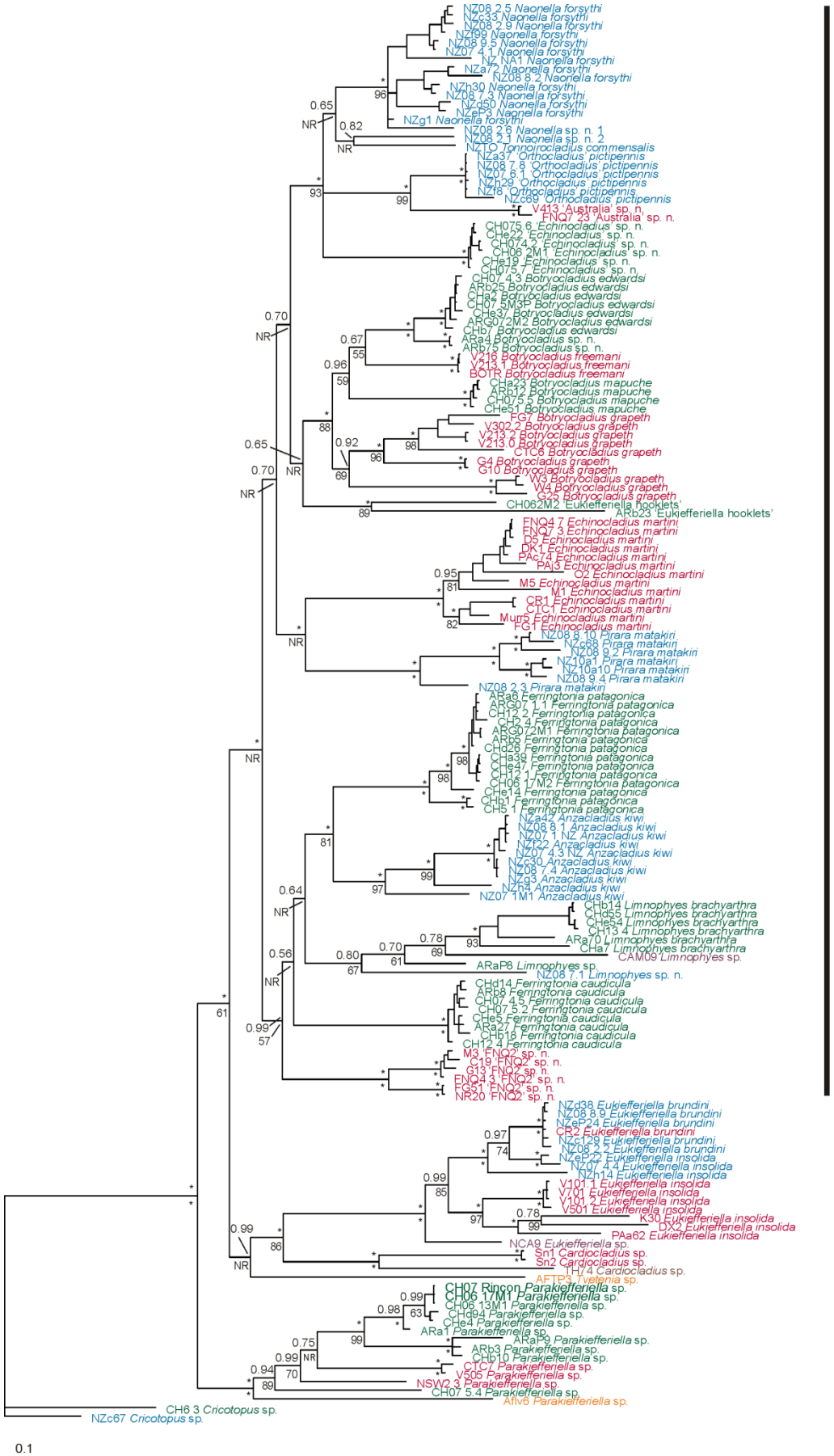
Supplementary Table 5.1 continued.

Taxon Name	Molecular Voucher	Country	Site/Region	Site Number	Latitude	Longitude	Life Stage	Gene regions sequenced			
								CO1	28S	CAD1	CAD3
Eukiefferiella brundini	NZc129	New Zealand	Speargrass Creek, Nelson Lakes	23	41˚46.50'S	172˚46.30'E	L	✓	✓		
Eukiefferiella brundini	NZ08 2.2	New Zealand	Ohakune, Manawatu-Wanganui	30	39˚24.00'S	175˚27.00'E	P	✓			
Eukiefferiella insolida	NZeP22	New Zealand	Owen River, Nelson Lakes	25	41˚41.13'S	172˚27.09'E	P	✓	✓	✓	✓
Eukiefferiella insolida	NZ07 4.4	New Zealand	Hope River, Nelson Lakes	24	41˚41.41'S	172˚37.02'E	P	✓		✓	
Eukiefferiella insolida	NZh14	New Zealand	Jackson Creek, Lewis Pass	28	42˚22.17'S	172˚16.01'E	L	✓			
Eukiefferiella insolida	V101.1	Australia	Sylvia Creek, Talangi SF	20	37˚31.00'S	145˚03.00'E	L	✓	✓		
Eukiefferiella insolida	V701	Australia	Lords Creek, Mitta Mitta	16	36˚35.00'S	146˚19.00'E	L	✓	✓	✓	✓
Eukiefferiella insolida	V501	Australia	Buckland River, Buckland SF	15	36˚47.00'S	146˚50.00'E	L	✓	✓		
Eukiefferiella insolida	V101.2	Australia	Sylvia Creek, Talangi SF	20	37˚31.00'S	145˚03.00'E	L	✓			
Eukiefferiella insolida	K30	Australia	Kauri Creek, Atherton NP	6	17˚08.03'S	145˚35.55'E	L	✓	✓		
Eukiefferiella insolida	DX2	Australia	Davies Creek, Davies Creek NP	7	17˚00.35'S	145˚34.56'E	L	✓	✓		
Eukiefferiella insolida	PAa62	Australia	Birthday Creek, Paluma NP	1	18˚58.49'S	146˚10.03'E	L	✓	✓	✓	✓
Eukiefferiella sp.	CR2	Australia	Clyde River, southern NSW	14	35°30.23'S	150°13.26'E	L	✓	✓		
Eukiefferiella sp.	NCA9	USA	Castle Lake, Siskiyou Co., California	47	41˚13.00'N	122˚23.00'W	L	✓	✓	✓	✓
Cardiocladius sp.	Sn1	Australia	Snowy Creek, Mitta Mitta	17	36°34.13'S	147°24.58'E	L	✓	✓		
Cardiocladius sp.	Sn2	Australia	Snowy Creek, Mitta Mitta	17	36°34.13'S	147°24.58'E	L	✓	✓	✓	✓
Cardiocladius sp.	TH74	Thailand	Phrae, Wieng Ko Sai NP	43	17˚58.00'N	99˚35.00'E	L	✓	✓	✓	✓
'Australia' sp. n.	FNQ7 23	Australia	Davies Creek, Davies Creek NP	7	17˚00.35'S	145˚34.56'E	L	✓	✓	✓	✓
'Australia' sp. n.	V413	Australia	Buckland River, Buckland SF	15	36˚47.00'S	146˚50.00'E	L	✓	✓	✓	✓
Tvetenia sp.	AFTP3	Africa	Gifberg Pass, Western Cape, SA	45	31˚46.00'S	18˚46.00'E	L	✓	✓	✓	✓
Parakiefferiella sp.	CH07 Rincon	Chile	Estero el Rincon, Lakes District	39	39˚08.00'S	71˚43.00'W	P	✓	✓		
Parakiefferiella sp.	CH06 17M1	Chile	Rio Chaleufu, Lakes District	40	40˚44.00'S	72˚18.00'W	P	✓			
Parakiefferiella sp.	CH06 13M1	Chile	Peulla, Lakes District	38	41˚05.12'S	72˚01.16'W	P	✓			

Supplementary Table 5.1 continued.

Taxon Name	Molecular Voucher	Country	Site/Region	Site Number	Latitude	Longitude	Life Stage	Gene regions sequenced			
								CO1	28S	CAD1	CAD3
Parakiefferiella sp.	CHd94	Chile	Estero La Casilla, Lakes District	35	39˚14.00'S	71˚56.00'W	L	✓	✓	✓	✓
Parakiefferiella sp.	CHe4	Chile	Estero Correntoso, Lakes District	36	39˚18.00'S	72˚04.00'W	L	✓	✓		
Parakiefferiella sp.	ARa1	Argentina	Arroyo Partido, Neuquen Province	41	40˚14.00'S	71˚22.00'W	L		✓		
Parakiefferiella sp.	ARaP9	Argentina	Arroyo Partido, Neuquen Province	41	40˚14.00'S	71˚22.00'W	P	✓			
Parakiefferiella sp.	ARb3	Argentina	Arroyo Quilanlahue, Neuquen Province	42	40˚09.00'S	71˚33.00'W	L		✓		
Parakiefferiella sp.	CHb10	Chile	Rio Don Baucha, Lakes District	34	39˚23.33'S	71˚47.11'W	L	✓	✓		
Parakiefferiella sp.	CTC7	Australia	Cabbage Tree Creek, Currowan SF	13	35˚34.00'S	150˚02.00'E	L	✓	✓		
Parakiefferiella sp.	V505	Australia	Buckland River, Buckland SF	15	36˚47.00'S	146˚50.00'E	L	✓	✓	✓	✓
Parakiefferiella sp.	NSW2 3	Australia	Castlereaigh River, Warrumbungle NP	21	31˚16.22'S	149˚11.28'E	L	✓	✓	✓	✓
Parakiefferiella sp.	CH07 5.4	Chile	Estero Correntoso, Lakes District	36	39˚18.00'S	72˚04.00'W	P	✓	✓		
Parakiefferiella sp.	Aflv6	Africa	Jonkershoek, Western Cape, SA	44	34˚00.21'S	18˚59.40'E	L	✓	✓	✓	✓
Cricotopus sp.	CH6 3	Chile	Petrohue, Lakes District	37	41˚08.38'S	72˚24.07'W	P	✓	✓	✓	✓
Cricotopus sp.	NZc67	New Zealand	Speargrass Creek, Nelson Lakes	21	41˚46.50'S	172˚46.30'E	L	✓	✓		

Supplementary Figure 5.1 (next page). Consensus Bayesian topology based on the partitioned dataset and the complete set of taxa. Node-associated support values represent Bayesian posterior probabilities above branches and maximum likelihood bootstrap support below branches; 'NR' denotes nodes that were not resolved by ML and '*' denotes posterior probabilities of 1.00 or bootstrap support of 100. Taxa are coloured according to their continent of origin – green: South America; blue: New Zealand; pink: Australia; orange: Africa; purple; North America; brown: Asia. Branch lengths are in expected substitutions per site.


NZ08 2.5 Naonella forsythi
NZc33 Naonella forsythi
NZ08 2.9 Naonella forsythi
NZ99 Naonella forsythi
NZ08 9.5 Naonella forsythi
NZ07 4.1 Naonella forsythi
Nz NA1 Naonella forsythi
NZa72 Naonella forsythi
NZ08 8.2 Naonella forsythi
NZh30 Naonella forsythi
NZ08 7.3 Naonella forsythi
NZd50 Naonella forsythi
NZeP3 Naonella forsythi
NZg1 Naonella forsythi
NZ08 2.6 Naonella sp. n. 1
NZ08 2.1 Naonella sp. n. 2
NZTO Tonnoirocladius commensalis
NZa37 'Orthocladius' pictipennis
NZ08 7.8 'Orthocladius' pictipennis
NZ07 6.1 'Orthocladius' pictipennis
NZh29 'Orthocladius' pictipennis
NZf8 'Orthocladius' pictipennis
NZc68 'Orthocladius' pictipennis
V413 'Australia' sp. n.
FNQ7 23 'Australia' sp. n.
CH075.6 'Echinocladius' sp. n.
CHe22 'Echinocladius' sp. n.
CH074.2 'Echinocladius' sp. n.
CH06 2M1 'Echinocladius' sp. n.
CHe19 'Echinocladius' sp. n.
CH075.7 'Echinocladius' sp. n.
CH07 4.3 Botryocladius edwardsi
ARb25 Botryocladius edwardsi
CHa2 Botryocladius edwardsi
CH07 5M3P Botryocladius edwardsi
CHe37 Botryocladius edwardsi
ARG07.2M2 Botryocladius edwardsi
CHb7 Botryocladius edwardsi
ARa4 Botryocladius sp. n.
ARb75 Botryocladius sp. n.
V218 Botryocladius freemani
V213.1 Botryocladius freemani
BOTR Botryocladius freemani
CHa23 Botryocladius mapuche
ARb12 Botryocladius mapuche
CH07.5.5 Botryocladius mapuche
CHe51 Botryocladius mapuche
FG2 Botryocladius grapeth
V302.2 Botryocladius grapeth
V213.2 Botryocladius grapeth
V213.0 Botryocladius grapeth
CTC6 Botryocladius grapeth
G4 Botryocladius grapeth
G10 Botryocladius grapeth
W3 Botryocladius grapeth
W4 Botryocladius grapeth
G25 Botryocladius grapeth
CH062M2 'Eukiefferiella hooklets'
ARb23 'Eukiefferiella hooklets'
FNQ4 7 Echinocladius martini
FNQ7 3 Echinocladius martini
D5 Echinocladius martini
DK1 Echinocladius martini
PAc74 Echinocladius martini
PAj3 Echinocladius martini
O2 Echinocladius martini
M5 Echinocladius martini
M1 Echinocladius martini
CB1 Echinocladius martini
CTC1 Echinocladius martini
Murrs Echinocladius martini
FG1 Echinocladius martini
NZ08 8.10 Pirara matakiri
NZc68 Pirara matakiri
NZ08 9.2 Pirara matakiri
NZ10a1 Pirara matakiri
NZ10a10 Pirara matakiri
NZ08 9.4 Pirara matakiri
NZ08 2.3 Pirara matakiri
ARa6 Ferringtonia patagonica
ARG07.1.1 Ferringtonia patagonica
CH12.2 Ferringtonia patagonica
CH2.4 Ferringtonia patagonica
ARG07.2M1 Ferringtonia patagonica
ARb6 Ferringtonia patagonica
CHd26 Ferringtonia patagonica
CHa39 Ferringtonia patagonica
CHe47 Ferringtonia patagonica
CH12.1 Ferringtonia patagonica
CH06 17M2 Ferringtonia patagonica
CHe14 Ferringtonia patagonica
CHb1 Ferringtonia patagonica
CH5.1 Ferringtonia patagonica
NZa42 Anzacladius kiwi
NZ08 8.1 Anzacladius kiwi
NZ07.1 NZ Anzacladius kiwi
NZf22 Anzacladius kiwi
NZ07 4.3 NZ Anzacladius kiwi
NZc30 Anzacladius kiwi
NZ08 7.4 Anzacladius kiwi
NZa3 Anzacladius kiwi
NZh4 Anzacladius kiwi
NZ07 1M1 Anzacladius kiwi
CHb14 Limnophyes brachyarthra
CHd55 Limnophyes brachyarthra
CHe54 Limnophyes brachyarthra
CH13.4 Limnophyes brachyarthra
ARa70 Limnophyes brachyarthra
CHa7 Limnophyes brachyarthra
CAM09 Limnophyes sp.
ARaP8 Limnophyes sp.
NZ08 7.1 Limnophyes sp. n.
CHd14 Ferringtonia caudicula
ARb8 Ferringtonia caudicula
CH07 4.5 Ferringtonia caudicula
CH07 5.2 Ferringtonia caudicula
CHe5 Ferringtonia caudicula
ARa27 Ferringtonia caudicula
CHb18 Ferringtonia caudicula
CH12.4 Ferringtonia caudicula
M3 FNQ2 sp. n.
G19 FNQ2 sp. n.
G13 FNQ2 sp. n.
FNQ4 3 FNQ2 sp. n.
FG51 FNQ2 sp. n.
NR20 FNQ2 sp. n.
NZd38 Eukiefferiella brundini
NZ08 8.9 Eukiefferiella brundini
NZeP24 Eukiefferiella brundini
CR2 Eukiefferiella brundini
NZc129 Eukiefferiella brundini
NZ08 2.2 Eukiefferiella brundini
NZeP22 Eukiefferiella insolida
NZ07 4.4 Eukiefferiella insolida
NZh14 Eukiefferiella insolida
V101.1 Eukiefferiella insolida
V701 Eukiefferiella insolida
V101.2 Eukiefferiella insolida
V501 Eukiefferiella insolida
K30 Eukiefferiella insolida
DX2 Eukiefferiella insolida
PAa62 Eukiefferiella insolida
NCA9 Eukiefferiella sp.
Sn1 Cardocladius sp.
Sn2 Cardocladius sp.
TH7.4 Cardocladius sp.
AFTP3 Tvetenia sp.
CH07 Rincon Parakiefferiella sp.
CH06 1.1M1 Parakiefferiella sp.
CH06 13M1 Parakiefferiella sp.
CHd94 Parakiefferiella sp.
CHe4 Parakiefferiella sp.
ARa1 Parakiefferiella sp.
ARaP9 Parakiefferiella sp.
ARb3 Parakiefferiella sp.
CHb10 Parakiefferiella sp.
C1C7 Parakiefferiella sp.
V505 Parakiefferiella sp.
NSW2.3 Parakiefferiella sp.
CH07 5.4 Parakiefferiella sp.
Ativ6 Parakiefferiella sp.
CH6 3 Cricotopus sp.
NZc67 Cricotopus sp.
0.1
Proposed Gondwanan Orthoclads

Chapter Six

General Discussion

The overall objective of the current study was to undertake a holistic investigation of the evolutionary history of *Echinocladius martini* and its close relatives. This included examining contemporary dispersal patterns in *E. martini* within and among streams in north-eastern Australia, an assessment of regional phylogeographic patterns in its close relatives *Naonella forsythi* in New Zealand and *Ferringtonia patagonica* in southern South America, a continental-scale evaluation of phylogeographic structure in *E. martini* in eastern Australia, and reconstruction of deeper phylogenetic relationships among these and other closely allied orthoclad taxa. The results presented here demonstrate that the evolutionary history of this group of proposed Gondwanan orthoclad taxa has been characterised by ongoing habitat fragmentation and population isolation on varying spatial and temporal scales, ranging from the break-up of ancient supercontinents to the isolation of proximate populations due to stream hierarchy effects. The interruptions to migration, colonisation and gene flow driven by such fragmentation have clearly contributed to the development and maintenance of much of the diversity in this group. That said, the current study also provides evidence for occasional dispersal of these chironomids at varying geographical scales, ranging from transoceanic dispersal among separate continents to movement within and among streams of the same catchment.

6.1 The roles of population fragmentation and dispersal in the overall evolution of *Echinocladius* and its relatives

Historically, chironomids have been considered to be relatively resistant to the effects of habitat change compared with many other freshwater invertebrate taxa (McKie *et al.*, 2004). This is thought to be moderated by an inferred capacity for extensive dispersal based on evidence regarding particular physiological traits, their recovery both up to several hundred metres above sea level and several hundred kilometres from the nearest landmass, and their ability to colonise new habitat rapidly (Holzapfel & Harrell, 1968; White, 1970; Oliver, 1971). As such, chironomids have long been considered colloquially to be ‘aerial plankton’. In contrast, assessments

of genetic structure among populations of some chironomid taxa have suggested that dispersal may be considerably more restricted and that population fragmentation has in fact played a major role in the evolution of diversity in this group (Martin *et al.*, 2002; Werle, 2005; Krosch *et al.*, 2009; Kaiser *et al.*, 2010). Moreover, previous reconstructions of evolutionary relationships among Gondwanan continents have suggested that the evolution of disjunct austral chironomid taxa has been influenced largely by vicariance (Brundin, 1966). As such, patterns identified here were expected to show that the evolution of these proposed Gondwanan orthoclad taxa has been characterised either by extensive dispersal on all geographical scales or, alternatively, by several distinct phases of population fragmentation that have influenced the diversity in this group at all taxonomic scales.

Utilising a multi-tiered approach to investigating the evolutionary relationships within and among *Echinocladius* and its relatives has demonstrated that population fragmentation at several levels of evolutionary scale has indeed been important in driving diversification of this group. Isolation of taxa following continental break-up was shown to have influenced diversification among some members of the group. Once taxa were isolated on separate continents they evidently diverged in isolation, resulting in largely regionally-discrete faunas.

As continents drifted further apart following tectonic rifting, previously continuous habitat was fragmented through orogenic uplift, glaciation events and/or aridification. Regardless of the landmass they inhabited, these events appear to have impacted chironomid populations in similar ways. Large populations that were previously connected were subsequently isolated in smaller refugia and taxa were apparently unable to cross intervening unsuitable habitat, thereby likely restricting gene flow and driving further diversification.

Within stable refugia, movement of chironomids also appears highly restricted, with little gene flow evident even among geographically proximate streams within the same catchment. This implies generally strong natal site

fidelity and dispersal limited largely to within the stream channel, possibly due to a combination of high riparian density, and the effects of habitat stability and large population size that may mitigate required movement among streams.

Despite this, evidence for several dispersal events among geographically distant localities was observed both at all levels of evolutionary scale investigated here and for several different taxa. Within a rainforest refuge, occasional flight by *E. martini* adults was suggested to have occurred among streams in a system where dispersal was otherwise restricted to the stream channel. Although the question of whether dispersal of *E. martini* adults occurs via upstream flight following downstream larval drift or flight over land remains unresolved, these data provide clear evidence for occasional passive and active dispersal events in both life stages.

At a regional scale, evidence for sympatry of divergent lineages implied extensive dispersal among historical refugia. Moreover, gene flow within a lineage was inferred among *N. forsythi* populations that were located up to 75km apart. This appears to reflect areas where the distribution of favourable habitat re-expanded during periods of milder climate following initial fragmentation, thus apparently facilitating dispersal across previously unsuitable regions. In some cases, this resulted in secondary contact with neighbouring populations and in others it promoted gene flow among geographically distant populations. Dispersal among isolated refuges is likely to have been rare and anecdotal evidence suggests that this is perpetuated by a low short-term recolonisation potential. Thus, if a population inhabiting an isolated refuge succumbs to local extinction, it may not be readily replaced even by conspecifics from neighbouring refugia.

Several transoceanic dispersal events were inferred among the southern continents, as implied by close sister group relationships and estimated times to most recent common ancestor among disjunct austral taxa that did not fit a pattern of vicariant evolution. This implies that the current distributions and evolutionary affinities of the proposed Gondwanan orthoclads may have been

influenced not only by continental fragmentation but also by relatively extensive transoceanic dispersal among the major southern landmasses. This accords with much of the current evidence that implies some combination of vicariance and dispersal in driving diversification in many disjunct austral taxa (Sanmartin & Ronquist, 2004; McCarthy *et al.*, 2007; Upchurch, 2008).

Taken together, the results of the current study suggest that whilst the chironomid taxa investigated here may not disperse as readily as previously thought, they are not wholly restricted and are able to disperse large distances both over land and across oceans. This result contrasts with initial expectations that either extensive dispersal would occur regularly over large geographical distances or that gene flow would be highly restricted, leading to distinct philopatry of isolated populations. The pattern of evolutionary relationships within and among taxa revealed here instead demonstrates that the overall evolution of this group is characterised by a complex relationship between population fragmentation and dispersal.

6.2 Congruence of the responses of different orthoclad taxa to past global climatic fluctuations

The three distinct austral landmasses examined here have responded to global climate fluctuations in broadly similar ways, in that glacial cycles have dramatically influenced the distribution of closed forest habitats in all three regions. The apparent disparity in phylogeographic patterns among the three taxa studied here may thus reflect differences in both the rate and form of recovery of favourable habitat among the three landmasses in the period since fragmentation drove initial diversification within the three taxa. In Australia, late Miocene climate change drove the intersection and fragmentation of wet closed forest across the east coast by dry open sclerophyll woodland and grassland, resulting in the isolation of several small refugia. Recolonisation and expansion of this habitat has been extremely limited, resulting in historically highly fragmented rainforest distributions. In

contrast, there is no evidence for similarly dramatic conversions of vegetation type in New Zealand or southern South America. Moreover, changes to closed forest distributions in these regions have instead been characterised by repeated cycles of contraction and expansion. While *E. martini* in Australia may have therefore remained isolated in small refugia intersected by areas of unsuitable habitat, both *N. forsythi* and *F. patagonica* were perhaps able to re-expand from their respective refugia as intervening habitat became suitable, facilitating movement among disjunct populations.

Importantly, regardless of these differences in landscape and life history traits, all three taxa have clearly been dramatically affected by habitat fragmentation and population isolation driven by late Miocene to Pliocene climatic upheavals and associated impacts on ecosystems. Moreover, this suggests that historical fluctuations in global climate have independently impacted in similar ways on these closely related taxa, despite being distributed on different landmasses. Thus, a certain susceptibility to the effects of habitat fragmentation and population isolation appears to be a shared attribute of these taxa and may reflect a life history trait common to the broader group of proposed Gondwanan orthoclads. It is likely, therefore, that other taxa related to the three orthoclads considered here would exhibit similar patterns both of high levels of genetic divergence within a currently-recognised single species and of gene flow and dispersal among populations and historical refugia that reflect the availability of suitable habitat in intervening regions.

Furthermore, these data may contribute more generally to the broader field of comparative phylogeography. Many phylogeographic studies extrapolate patterns observed in a given taxon to postulate that these patterns may also be exhibited by other close relatives of that taxon. By demonstrating here that closely related orthoclad taxa distributed on separate austral continents have been impacted by historical habitat change in similar ways, these data may thus act to partially validate these common assumptions. This may also provide a platform on which future studies may build to investigate the parallel effects of historical ecosystem changes in other taxa.

6.3 Towards a holistic understanding of Gondwanan orthoclad evolution

This study represents one of few concerted attempts at reconstructing the evolutionary history of a given taxon and its relatives over multiple evolutionary scales. Overall, the evolution of this group of disjunct austral chironomid taxa appears to have been characterised by a complex interaction between the impacts of habitat fragmentation and population isolation and long-distance dispersal at varying spatio-temporal scales. The interruptions to regular effective dispersal, colonisation and gene flow driven by population fragmentation has clearly contributed to the development and maintenance of much of the diversity in this group. Nevertheless, long-distance, if rare, dispersal events at all levels of evolutionary scale have also been important in influencing diversification of this group, both by promoting divergence via the isolation of dispersing taxa on disjunct continents, or by promoting infrequent gene flow among geographically distant populations. The results presented here may provide possible insights into the relative susceptibility of chironomids to future population fragmentation and highlight the ability of molecular studies to identify patterns of dispersal over long evolutionary time frames. For example, anecdotal data that suggests local extinction and lack of recolonisation of *E. martini* in the short-term implies, if considered exclusively, that dispersal is highly restricted in this taxon. Whereas, regional phylogeographic structure for *E. martini* clearly demonstrates that, over longer evolutionary time frames, comparatively extensive movement has occurred where facilitated by the presence of intervening suitable habitat. This implies that while short-term recolonisation of habitat by chironomids may be limited, over time and if conditions are favourable, populations may be re-established via repeated chance migration events. This demonstrates that incorporating a more historical perspective may result in different interpretations regarding the ability of chironomids to disperse not just among streams or among regions, but also among disjunct continents.

The outcomes of the current study also serve to highlight how investigating evolutionary relationships at multiple levels of scale can reveal patterns that may otherwise remain cryptic and can provide a more holistic understanding of organismal evolution. For example, were only a fine-scale investigation of *E. martini* dispersal within and among streams of the same catchment undertaken, much evidence for the movement of this taxon among catchments would be overlooked, potentially leading to interpretations of dispersal ability that do not fully reflect actual patterns. Similarly, were only the population structure of individual taxa within the proposed Gondwanan group investigated, repeated transoceanic dispersal events, and hence the propensity for these chironomids to cross large ocean gaps, revealed by a broader phylogenetic reconstruction may be overlooked. By collating information from several levels of evolutionary scale, and hence, several different points in the evolution of a given taxon, more questions can be addressed regarding the evolution of the taxon and its relatives.

For example, the huge variety of inferred dispersal patterns and susceptibility to population fragmentation described for freshwater invertebrate taxa as a whole implies that their responses to historical habitat change have been equally diverse. Many studies that investigate only a single facet of the evolution of a given freshwater invertebrate taxon may therefore overlook important events that have contributed to the total history of the taxon. Thus, investigating patterns of relationships at multiple levels of evolutionary scale can provide a more complete overview of the patterns and processes that influence the evolution of other freshwater invertebrates. It is likely that many, if not most, other freshwater invertebrate taxa would share similarly complex histories to that identified here for austral chironomids. The combined effects of historical population fragmentation and long-distance dispersal are thus expected to have played significant roles in influencing diversity in other freshwater invertebrates.

6.4 Taxonomic reappraisals and future directions

Several novel phylogenetic relationships revealed here suggest that the taxonomy and systematics of some of the taxa studied may require formal revision. The genus *Ferringtonia* was clearly polyphyletic, with the genera *Limnophyes* and *Anzacladius* nested within the *Ferringtonia* clade, implying instead that the two described species *F. patagonica* and *F. caudicula* may represent distinct genera. Similarly, trans-Tasman *Eukiefferiella insolida* was also polyphyletic with regard to the New Zealand taxon *Eu. brundini* and, given the substantial morphological divergence in all life stages that suggests that *Eu. brundini* is distinct from *Eu. insolida* (Boothroyd & Cranston, 1995), a revision of taxa currently identified as New Zealand *Eu. insolida* is therefore clearly required.

Several novel taxa originally assigned to a particular described genus were shown to be most closely related to other taxa within the proposed Gondwanan clade. One of the two novel members of the New Zealand genus *Naonella* identified here (*N.* sp. n. 2) was sister to *Tonnoirocladius commensalis*, rendering *Naonella* paraphyletic and implying that *T. commensalis* may instead represent a species of *Naonella*. 'Echinocladius' sp. n. and 'Eukiefferiella hooklets' from South America both represented novel taxa that did not form monophyletic groups with the described genera to which they were assigned originally and hence require revision, likely as genera in their own right. Similarly, the novel taxon *Botryocladius* sp. n. appeared to be distinct from other *Botryocladius* species and may represent either a described South American species known only from pupal morphology for which associations between known and unknown life stages have not yet been made (e.g., *B. glacialis* or *B. tronador* - Cranston & Edward, 1999), or an entirely new species. Additionally, the genera *Pirara*, *Limnophyes* and *Anzacladius* and the species *B. grapeth* exhibited high levels of intraspecific diversity that could not be explained by geographical separation, possibly indicating the presence of sympatric cryptic species and implying that these taxa require analysis at a finer scale to resolve taxonomic boundaries more accurately. Moreover, important associations between

either described mature and immature stages, or described mature and unknown immature life stages were resolved for several taxa, including *Ferringtonia*, New Zealand *Eukiefferiella*, *Parakiefferiella sp.*, *N. forsythi*, *E. martini*, *A. kiwi*, *P. matakiri*, *B. edwardsi*, *B. mapuche*, '*O.*' *pictipennis* and 'Echinocladius' sp. n.. All novel associations among life stages will require morphological characterisation and formal descriptions to complement molecular data presented here.

Overall, the investigation of within and among stream gene flow in *E. martini* in the Paluma region represents the most geographically intensive survey of population genetic structure in chironomids conducted to date. Future investigations should aim to acquire additional samples from sites currently under-represented and additional sites from neighbouring catchments within the same habitat patch to compare patterns of genetic structure and test for gene flow among catchments. Specifically, higher sample size at the site downstream of the Birthday Creek Falls would allow better tests of the influence of the falls on gene flow along Birthday Creek and additional sites along Echo Creek and further downstream of the Confluence site would provide greater resolution for determining the extent of movement among streams. Furthermore, sites from headwater streams of the Crystal Creek catchment arise in close proximity to those of the Birthday Creek/Running River catchment and are contained within the same continuous rainforest patch. Thus, more extensive sampling of the Crystal Creek streams, where possible given the difficulty of accessing these streams due to dense rainforest, would provide an important comparison of genetic structure among neighbouring catchments within the same habitat. Genetic structure among *E. martini* populations inhabiting more open forested temperate regions of Australia could be investigated using a similar sampling design to this study to allow comparisons between temperate and tropical regions of the influence of riparian vegetation density and relative habitat stochasticity on movement of *E. martini* among streams.

Fast-evolving and bi-parentally inherited nuclear microsatellite markers could be incorporated in future studies to compare and contrast patterns of genetic

structure with those from maternally-inherited mitochondrial data presented here and to test hypotheses about more recent, small scale movement within and among streams. Such markers are useful for testing hypotheses of sex-biased dispersal as they provide information about male dispersal patterns; this is important as dispersal of many freshwater taxa is thought to be dominated by female movement whilst males are often highly philopatric. Thus, if dispersal by males is more restricted than females, nuclear microsatellite data may reveal patterns of even greater genetic structuring among streams than the mitochondrial data. Alternatively, if males are dispersing more widely compared with females, nuclear data may show evidence for panmixia among streams.

The comparative phylogeographic investigation of *N. forsythi* and *F. patagonica* presented here represents the first detailed analysis of population genetic structure of New Zealand and Neotropical chironomids. This study also represents a novel application of comparative phylogeographic theory to investigate population structure among closely related species that inhabit discrete landmasses. Future investigations of phylogeographic structure in these taxa should aim to expand the sampling design to encompass the total distribution of each taxon more fully. Attaining additional samples from surrounding regions in both New Zealand and Patagonia may help to identify the location of historical refugia and would allow more accurate assessments of gene flow across known mountain barriers. This would enable more rigorous tests of hypotheses about the evolutionary processes that drive diversification in New Zealand and Patagonian chironomids and would thereby provide a more holistic perspective of the evolution of these taxa. Patterns of relationships should also be assessed at appropriate nuclear loci to investigate the possibility that observed sympatric lineages may represent cryptic species, as fixed differences at nuclear loci can provide valuable evidence for the current reproductive status of sympatric lineages. This is applicable to both *N. forsythi* and *F. patagonica*, as both taxa may represent cryptic species complexes. Ideally, morphological characters that differentiate each lineage should be identified, as this would facilitate targeted sampling

and provide further evidence for the separate evolutionary trajectories of the currently identified lineages.

Future assessments of within lineage population structure for *F. patagonica* should aim to acquire larger sample sizes from the current sites and from additional sites to the north and south, on both sides of the Andes, to encompass the geographical distribution of these cryptic lineages more completely. This would enable more rigorous tests of the relevance of isolation by distance among sites and potential disjunctions across the Andean mountain range, thereby informing more comprehensive inferences of population structure in this region and allowing diversity in this taxon to be viewed in a broader regional context. Incorporation of fast-evolving, bi-parentally inherited nuclear markers such as microsatellites to test hypotheses about sex-biased dispersal and more recent movement among sites for both taxa would allow comparison with patterns of genetic structure among sites based solely on maternally-inherited genes presented here, as discussed above.

Future studies of *E. martini* phylogeographic structure along the Australian east coast should include additional sample sites from throughout central- and south-eastern Australia, as well as from Tasmania and areas further north of the Daintree/Thornton Uplands to illuminate the patterns of relationships among regions more fully. This would also allow the close grouping of lineages from north of the BMC with those from NSW and Victoria to be tested more rigorously, with higher sample size and a more complete representation of the extant diversity within *E. martini*. Appropriate nuclear markers could be incorporated to evaluate current reproductive status of observed sympatric lineages and, hence, whether these lineages may represent cryptic species, as discussed above. Specifically, this may help to resolve the status of the co-distributed Atherton, Mareeba, Paluma and Rare Sympatric lineages in the southern Wet Tropics, the Mt. Lewis A and B lineages in the northern Wet Tropics and the NSW A and B lineages in New South Wales.

The resolution of evolutionary relationships among the proposed Gondwanan orthoclads represents the first detailed molecular phylogenetic reconstruction for this group and only the second to test austral patterns identified by Brundin (1966). Moreover, the sampling regime implemented in the current study is the most intensive yet performed for austral members of the Orthocladiinae and unsurprisingly has revealed both novel taxa and phylogenetic relationships within and among described genera. Future studies of the Gondwanan orthoclads would benefit from more complete taxonomic sampling, particularly of *Pirara* and *Anzacladius*, and a broader coverage of potential African taxa to resolve evolutionary relationships among genera more fully. Furthermore, the incorporation of more appropriate methods of molecular calibration, particularly including internal calibration points when and if they become available, may allow more accurate estimates of times to most recent common ancestor. This would act as an important comparison to the current study and would provide important data about whether current patterns are reflective of the actual evolutionary history of the group.

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Appendix One

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Deeply divergent mitochondrial lineages reveal patterns of local endemism in chironomids of the Australian Wet Tropics

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Abstract

The Wet Tropics bioregion of north-eastern Australia has been subject to extensive fluctuations in climate throughout the late Pliocene and Pleistocene. Cycles of rainforest contraction and expansion of dry sclerophyll forest associated with such climatic fluctuations are postulated to have played a major role in driving geographical endemism in terrestrial rainforest taxa. Consequences for the distributions of aquatic organisms, however, are poorly understood. The Australian non-biting midge species *Echinocladius martini* Cranston (Diptera: Chironomidae), although restricted to cool, well-forested freshwater streams, has been considered to be able to disperse among populations located in isolated rainforest pockets during periods of sclerophyllous forest expansion, potentially limiting the effect of climatic fluctuations on patterns of endemism. In this study, mitochondrial COI and 16S data were analysed for *E. martini* collected from eight sites spanning the Wet Tropics bioregion to assess the scale and extent of phylogeographic structure. Analyses of genetic structure revealed several highly divergent cryptic lineages with restricted geographical distributions. Within one of the identified lineages, strong genetic structure implied that dispersal among proximate (<1km apart) streams was extremely restricted. The results suggest that vicariant processes, most likely due to the systemic drying of the Australian continent during the Plio-Pleistocene, may have fragmented historical *E. martini* populations and, hence, promoted divergence in allopatry.

Introduction

The idea that habitat fragmentation can have detrimental effects on the biotic assemblages of a given region hearkens back to the very beginnings of island biogeography, equilibrium theory and species turnover (MacArthur & Wilson, 1967; Simberloff, 1974). Fragmentation of large continuous habitats into several small refugia is thought to interrupt colonisation and gene flow among populations in some taxa, potentially resulting in inbreeding and, in the worst case, extinction. A positive impact of habitat fragmentation is that it can potentially drive genetic divergence and speciation among isolated populations. This process is considered to represent an important component for the development and maintenance of biodiversity and endemism throughout the world (Moritz *et al.*, 2000).

The effect of habitat fragmentation on regional biotas has been particularly well documented in the Wet Tropics of north-eastern Australia. The fragmentation of the Gondwanan supercontinent some 130 million years ago (mya) (Scotese *et al.*, 1999) and, in particular, the separation of the Australian continent from Antarctica (~23mya) (Barker & Burrell, 1977; Scotese *et al.*, 1999) likely contributed to major climate change and biotic evolution. More recent pollen records track a major transition from widespread rainforest to dry sclerophyll forest containing predominantly eucalypt species, coinciding with glaciations elsewhere, beginning around

150,000 years ago (150kya) and reaching the maximum extent around 38 – 26kya (Kershaw, 1985; Nix & Switzer, 1991; Hopkins *et al.*, 1993; Kershaw, 1994).

Changes in the vegetation of the Wet Tropics include the intermittent bisection of two extant rainforest refugia from around 38kya by expansions of sclerophyllous vegetation; an area named the Black Mountain Corridor (BMC) (Bell *et al.*, 1987). Indeed, extensive rainforest vegetation has returned to much of this region only during the last 9kya (Nix & Switzer, 1991; Hugall *et al.*, 2002). Phylogeographic studies of terrestrial organisms such as lizards, birds and beetles suggest that the BMC has acted as an effective dispersal barrier for many rainforest endemic taxa during the late Pleistocene (see Moritz *et al.*, 2000). Similar scenarios of habitat fragmentation are postulated to have influenced the present-day distributions of taxa elsewhere in Australia (e.g., McGuigan *et al.*, 1998; James & Moritz, 2000; Baker *et al.*, 2003; Chapple *et al.*, 2005) and throughout the world (Templeton *et al.*, 1990; e.g., Hogg *et al.*, 1999; Waters *et al.*, 2000; Baguette *et al.*, 2003).

Globally, aquatic invertebrates such as snails (Bunje, 2005), amphipods (Hogg *et al.*, 2006), diving beetles (Ribera & Vogler, 2004), mayflies (Smith & Collier, 2001; Smith *et al.*, 2006a) and caddisflies (Pauls *et al.*, 2006) appear to have responded similarly to habitat fragmentation. In contrast, some taxa appear to be too dispersive to show such historical structuring, as with the lentic phantom midge *Chaoborus flavicans* (Berendonk & Spitze, 2006) and some terrestrial species of *Drosophila* (Kelemen & Moritz, 1999; Schiffer *et al.*, 2007). As members of the Family Chironomidae (Diptera) are generally considered to be highly dispersive, we investigated the extent to which habitat fragmentation has affected population connectivity in the widely-distributed chironomid species *Echinocladius martini* (Cranston) across the Wet Tropics region of north-eastern Australia.

The choice of species was based on several criteria: (i) published morphological, and unpublished molecular data, which suggest that this species belongs to a lineage that originated early, since relatives (*Botryocladius, Naonella, Pirara*, etc.) are present on other Gondwanan landmasses (e.g., New Zealand, South America) (Boothroyd, 1994; Boothroyd & Cranston, 1995; Cranston & Edward, 1999; Cranston, 2000b); (ii) the species' ecophysiological tolerances had been studied (McKie *et al.*, 2004; McKie *et al.*, 2005), allowing targeting of sample sites and in-stream microhabitat; and (iii) the basic distribution of the species across the eastern coast of Australia was well known. Previously, several genera closely related to *Echinocladius* were believed to be restricted to cool, montane regions of south-eastern Australia (Freeman, 1961; Brundin, 1967). More recently, however, surveys in the Wet Tropics have shown the group to be present consistently in shaded but comparatively warm streams (Cranston, 2000b; McKie *et al.*, 2005). *Echinocladius martini* occurs widely along the eastern seaboard, from Tasmania to the Daintree region of north-eastern Queensland; a distance of around 4000km. Taken together, these findings

informed McKie *et al*'s (2004, 2005) hypotheses that individuals of such a widely-distributed species may possess broad environmental tolerances that compensate for the stochasticity of the Australian climate, while their widespread distributions can be partly attributed to the high vagility believed to characterise chironomid adults.

Few studies, however, have attempted to investigate how far chironomids disperse as adults. Studies have considered relative adult longevity [up to several weeks in some species (Oliver, 1971)], characteristics of wing size and shape, and flight muscle ratio (McLachlan, 1983, 1986a) as suitable traits for prolonged flight. Anecdotal evidence also suggests that these taxa may disperse extensively on wind currents (White, 1970; Oliver, 1971). In contrast, there is evidence to suggest that lateral (among-stream) dispersal by adult chironomids is restricted by the density of riparian vegetation (Delettre & Morvan, 2000). Furthermore, the only phylogeographic study of Australian chironomids conducted to date revealed extremely restricted contemporary gene flow among isolated populations of the evolutionarily distant genus *Austrochlus* (as *Archaeochlus* in Martin, *et al.*, 2002b). Thus, although chironomids may be physiologically capable of extensive flight, many extrinsic factors may limit actual dispersal.

The present study aimed, therefore, to evaluate the extent to which historical habitat fragmentation has affected Wet Tropics populations of *E. martini* by determining the range and extent of mitochondrial phylogeographic structuring among populations across the region. To provide information at a continental scale, representatives from south-eastern Australia were also acquired. The main hypothesis tested here was as follows: that extensive dispersal has countered any effect of historical changes to rainforest boundaries and, over time, resulted in panmixia among *E. martini* populations across the Wet Tropics region of north-eastern Queensland.

Methods

Study sites

Site selection was based on previously identified locations where *E. martini* was abundant in the Wet Tropics (McKie *et al.*, 2005). The focus of the sampling effort was populations within extant patches of rainforest that are, or have been, intersected by unfavourable habitat. Individuals of *E. martini* were sought from 13 streams across the World Heritage listed Wet Tropics region of north-eastern Queensland. Only eight sites, however, yielded *E. martini* larvae (Figure 1). Representative samples were also acquired from one additional site in south-eastern Australia (Murrindindi Creek, Victoria – not shown in Figure 1 for reasons of clarity) for comparisons at the broadest geographical scale. Where possible, streams both within and among several discrete catchments were sampled to provide higher resolution of the geographical extent of genetic structure present (Table 1).

Sample collection

Sampling took place during September 2005, involving both kick sampling and removal of entire leaf packs from the stream. The contents were strained through a series of sieves to remove large organic matter while retaining chironomid larvae. Samples were transferred immediately to 90% ethanol for long-term storage before transport to Queensland University of Technology, Brisbane, Australia. Preserved heads of individual larvae were slide mounted using Hoyers' mountant (van der Meer, 1977) and species identification subsequently confirmed via examination of head capsule morphology under 400X magnification. Body sections were stored at room temperature in 70% ethanol prior to use in genomic DNA extractions.

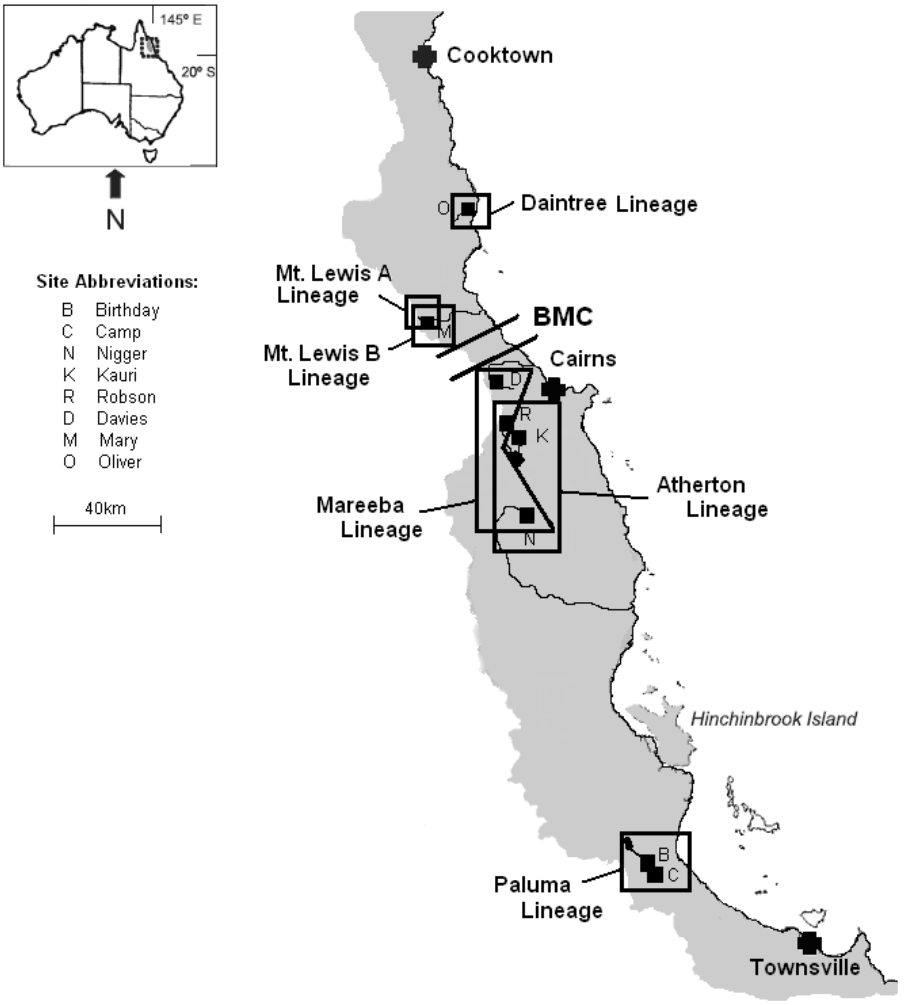

Figure 1. Location of sample sites. Site localities are indicated by black squares, while the boxed areas illustrate the site/s at which each highly divergent COI lineage was sampled. The shaded area depicts the extent of rainforest vegetation ~7000 years BP (Nix & Switzer, 1991). The historical location of the BMC is depicted by two diagonal lines. Adapted from McKie, *et al* (2005).

Genetic procedures

Total genomic DNA was extracted from larval tissue using the Qiagen DNeasy® extraction kit, following the manufacturers' guidelines. A 639 bp fragment of the Cytochrome *c* oxidase subunit I (COI) gene was amplified using universal invertebrate COI primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). The COI gene was used for this analysis as it is fast evolving and thus is considered to be an optimal marker for intraspecific population analysis (Avise, 1986; Moriyama & Powell, 1997). Each reaction tube contained 2 µL of template DNA from extractions, 0.5 µL of each primer (10pmol/µL), 2.5 µL of 10X polymerase buffer, 2.0 µL of 25 mM $MgCl_2$, 1.0 µL of 10 mM dNTP's, 0.5 µL of 99% dimethyl sulphoxide (DMSO), 0.2 µL of 5U/µL *Taq* polymerase and were adjusted to a final volume of 25 µL with dH_2O. The PCR cycle protocol involved initial denaturing at 94˚C for 4 minutes followed by 10 cycles of 94˚C for 30 seconds, 40˚C for 30 seconds and 72˚C for 40 seconds. This was then followed by 25 cycles of 94˚C for 30 seconds, 55˚C for 30 seconds and 72˚C for 40 seconds, before final extension at 72˚C for 10 minutes. Total PCR product was then purified using a High Pure PCR Product Purification Kit (Roche) following manufacturer's guidelines.

In addition, a 523 base pair fragment of the 16S rRNA gene was also amplified from a subset of samples to provide greater resolution of more basal nodes. The same reaction mix was used as for COI above. Primers used were universal 16S primers 16S71 and 1472 (Crandall & Fitzpatrick Jr., 1996). The cycle protocol involved initial denaturation at 94˚C for 1 minute, followed by 35 cycles of 94˚C for 30 seconds, 50˚C for 30 seconds and 72˚C for 30 seconds, prior to final extension at 72˚C for 7 minutes. The total PCR product was purified as above.

Purified PCR product of both mitochondrial fragments was amplified using a standard sequencing protocol. A standard isopropanol precipitation protocol was then used to clean the product prior to sequencing at the Australian Genome Research Facility at the University of Queensland (St. Lucia, Australia) for analysis on a 3730xl sequencing platform. All sequences were deposited in GenBank (Accession No. EU669980 – EU670043).

Data analyses

COI sequences were aligned and edited by eye using BioEdit (Hall, 1999). Tests for sequence saturation (an indicator of homoplasy) were conducted by calculating the mean ratio of transitions to transversions in MEGA Version 3.1 (Kumar *et al.*, 2004). Tajima's D test of neutrality was performed in DNAsp Version 4.0 (Rozas *et al.*, 2003) to determine if sequences were evolving neutrally. Clock-like evolution was assessed in Tree-Puzzle Version 5.2 (Schmidt *et al.*, 2002) under a Hasegawa-Kishino-Yano (HKY) model of sequence evolution, which was determined to be the most appropriate model available in this software package for the dataset.

The model of nucleotide substitution that best fitted the COI dataset was determined in Modeltest Version 3.7 (Posada & Crandall, 1998) and was used for all subsequent phylogenetic reconstructions. Neighbour joining (NJ) (10,000 bootstraps) and maximum parsimony (MP) (1,000 bootstraps) methods were used for the COI dataset in PAUP* Version 4.0. Individuals of two more distantly related Orthocladiine genera (*Cardiocladius* and *Botryocladius*), collected from southeast Australia were used as outgroups to root the tree. To aid analyses of divergence among haplotype groups, any groupings observed in the phylogeny were designated a 'lineage' based on two criteria. First, the groups must possess bootstrap support values greater than 50. Second, mean pairwise sequence divergence among groups must be 2.5% or more. Rudimentary divergence times among lineages were estimated from mean pairwise divergence using the chironomid-specific mitochondrial divergence rate of 1.5% per million years (Martin *et al.*, 2002a) and a generation time of 12 generations per year (P. Cranston, pers. comm.).

A COI haplotype network was constructed using a method of statistical parsimony in TCS Version 1.21 (Clement *et al.*, 2000) with a connection limit of 95%. This method is considered better than bifurcating trees for inferring evolutionary relationships among ancestral and derived haplotypes (Posada & Crandall, 2001). Where sample size allowed, population genetic structure within lineages was inferred from conventional among-site F_{ST} values ($P<0.05$) and exact tests of genetic differentiation based on haplotype frequencies ($P<0.05$) in Arlequin Version 2.0 (Excoffier *et al.*, 2005). Gene diversity and the mean number of pairwise differences between haplotypes within each site was also calculated using Arlequin. Divergence times among populations within a lineage were estimated in the manner described above.

Representative mitochondrial 16S sequences were aligned using the ClustalW facility in BioEdit. The mean ratio of transitions to transversions was calculated in MEGA Version 3.1. As different regions of the mitochondrial genome evolve at different rates, analysing genetic structure at multiple regions has been shown to provide much greater resolution of phylogenetic relationships than single fragments (Moritz *et al.*, 1987). The 16S sequences were thus concatenated to their corresponding COI sequence to form a combined dataset. A partition homogeneity test was carried out on the combined COI/16S dataset in PAUP* 4.0b10 (Swofford, 2001) to determine if the two datasets produced similar phylogenetic signals. Maximum likelihood (ML) (100 bootstraps) and MP (10,000 bootstraps) methods were also used in PAUP* 4.0b10 for both the 16S dataset alone, as well as the combined COI/16S dataset.

Results

COI Dataset

In total, 96 *E. martini* individuals from the Wet Tropics and two individuals from south-eastern Australia were sequenced for the COI gene

fragment (Table 1). From these sequences, 51 unique haplotypes of 639 bp in length were obtained. The mean ratio of transitions to transversions was 4.05:1, which suggested that multiple substitutions may have occurred at some sites and hence the true divergence among individuals may have been underestimated (Arbogast *et al.*, 2002). This was taken into consideration by using the most appropriate model of evolution available that incorporates sequence saturation (i.e., HKY) for both tests of clocklike evolution and divergence time estimates (Arbogast *et al.*, 2002). Tajima's D test of neutrality was not significant (D = -1.2216, P>0.10), indicating that the sequences evolved neutrally, thereby validating the use of phylogenetic analyses (Tajima, 1989). Modeltest indicated that the General Time Reversible model, that estimates the proportion of invariant sites and a gamma distribution (GTR +I +γ), was the model of nucleotide substitution that best fitted the data.

Table 1. Geographical location of study sites.

Site Name (Site Code)	Longitude (E)	Latitude (S)	Catchment	Elevation (masl)	Sample size
Birthday (B)	146˚10.03	18˚58.49	Burdekin	800	22
Camp (C)	146˚09.54	18˚58.22	Burdekin	850	22
Nigger (N)	145˚28.26	17˚26.49	Wild	950	11
Kauri (K)	145˚35.55	17˚08.03	Barron	700	4
Robson (R)	145˚37.44	17˚06.58	Barron	1000	7
Davies (D)	145˚34.56	17˚00.35	Barron	520	21
Mary (M)	145˚17.50	16˚35.20	Mitchell	950	7
Oliver (O)	145˚26.70	16˚08.30	Noah Ck	20	2
Murrindindi (Murr)	145˚33.21	37˚29.06	Murrindindi	Unknown	2

In the phylogeny, major haplotype groupings were concordant for both NJ and MP methods of tree reconstruction (Figure 2). There appeared to be a generally well-supported relationship between haplotype grouping and geographical location, in that haplotypes from a given geographical region generally grouped more closely together in the tree. Six major divergent lineages were identified, four of which represented the Paluma, Atherton, Mareeba and Daintree regions, while two lineages occurred in the Mt. Lewis region. The phylogeny suggested that bisection of continuous habitat by the BMC preceded fragmentation of refugia on either side. Mean average pairwise sequence divergence among lineages was 2.87% to the south of the BMC, 12.3% to the north and 12.23% among lineages on either side (Table 2). Levels of divergence observed among lineages separated by large geographical distances (e.g., Paluma-Daintree) were similar to those located more proximately (e.g., Mt. Lewis A-Mareeba) and even within the same stream (e.g., Mt. Lewis A-Mt. Lewis B).

The full COI dataset failed to conform to clocklike evolution (0.0285; P<0.05), but when each lineage was tested separately [an approach advocated by Arbogast *et al*., (2002)] only the Atherton lineage failed to conform (0.0278; P<0.05). This suggested that only sequences within the Atherton lineage were not evolving in a clock-like manner, possibly as a result of high sequence saturation. As non-clocklike evolution can lead to

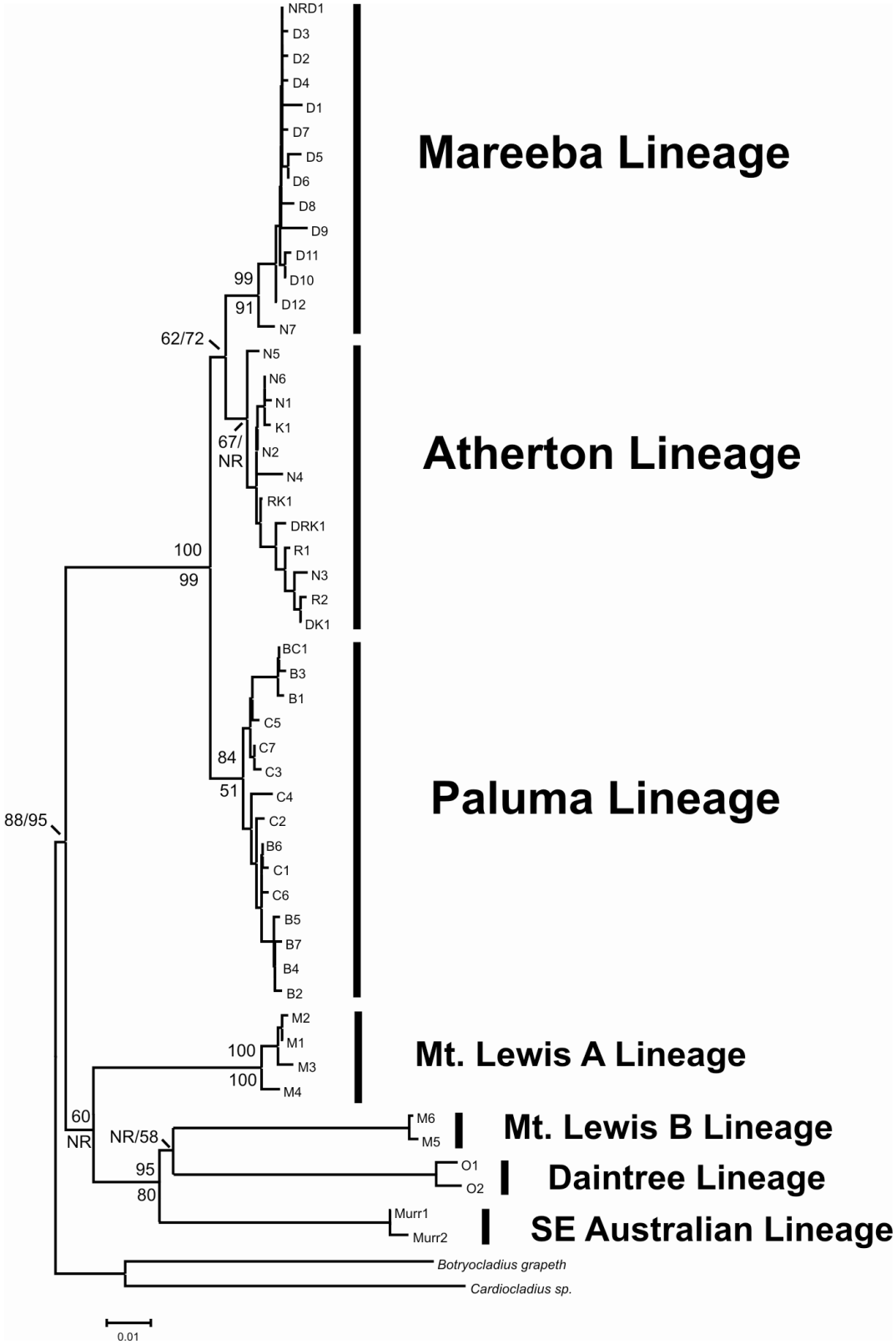

Figure 2. Inferred phylogram based on COI data. Bootstrap values for the neighbour joining method are shown above nodes and maximum parsimony bootstrap values below nodes. Branches that were collapsed under either method are designated "not resolved" (NR). Haplotypes are lettered according to the sample site/s at which they occurred and numbered in the order they were sequenced. Branch lengths indicate percent difference among haplotypes.

inaccurate estimates of divergence times (Arbogast *et al.*, 2002), all such pairwise estimates involving the Atherton lineage were subsequently omitted.

As the Atherton lineage could not be used for among lineage divergence time estimates only the split between the Paluma-Mareeba lineages (178kya – Table 2) could be calculated for lineages on the southern side of the BMC. Divergence time estimates among lineages occurring to the north of the BMC ranged from 628kya (Mt. Lewis A-Mt. Lewis B) to 750kya (Mt. Lewis A-Daintree), while divergence times across the BMC ranged from 533kya (Paluma-Mt. Lewis A) to 800kya (Daintree-Mareeba). Both southeast Australian *E. martini* haplotypes were highly divergent from all Wet Tropics lineages (11.30-13.10%) with divergence between the two regions estimated to have occurred 628-723kya.

The haplotype network separated haplotypes into groups identical to the lineages produced in the phylogeny (Figure 3). All lineages appeared to be geographically restricted and only five haplotypes were shared among sites. Four of the five shared haplotypes were internal to the network, which, under coalescent theory (Kingman, 1982), indicated retention of an ancestral type (Castelloe & Templeton, 1994; Clement *et al.*, 2000). The striking pattern of high haplotype diversity within lineages and geographical isolation among lineages, suggested low ongoing gene flow among sites, with each lineage confined to a narrow geographical distribution. Some secondary contact was suggested among lineages, however, with both Atherton and Mareeba lineages sampled from Robson, Nigger and Davies Creeks, and two highly divergent lineages both sampled from Mary Creek (Mt. Lewis A and B).

Table 2. Below diagonal are among-lineage mean percent pairwise COI sequence divergences. Above diagonal are estimated among-lineage divergence times in thousands of years, using a rate of 1.5% per million years and a generation time of 12 generations per year. Lineages omitted due to non-clocklike evolution are designated 'OM'.

	Paluma	**Atherton**	**Mareeba**	**Mt. Lewis A**	**Mt. Lewis B**	**Daintree**	**Southeast Australia**
Paluma	-	OM	178	533	694	734	722
Atherton	2.70	-	OM	OM	OM	OM	OM
Mareeba	3.20	2.70	-	566	722	800	723
Mt. Lewis A	9.60	9.80	10.20	-	628	750	678
Mt. Lewis B	12.50	13.10	13.00	11.30	-	672	628
Daintree	13.30	14.20	14.40	13.50	12.10	-	672
Southeast Australia	13.0	12.70	13.10	12.20	11.30	12.10	-

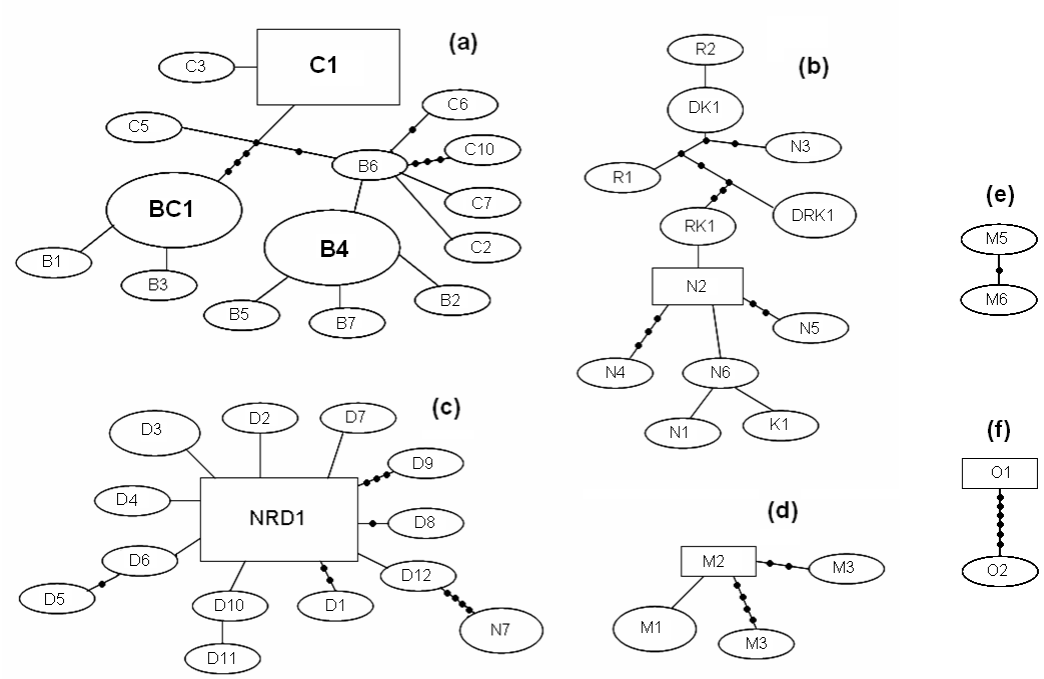

Figure 3. COI haplotype network of the six lineages produced in TCS (95% connection limit). (a) Paluma lineage, (b) Atherton lineage, (c) Mareeba lineage, (d) Mt. Lewis A lineage, (e) Mt. Lewis B lineage and (f) Daintree lineage. Size of node indicates haplotype frequency. Rectangular nodes indicate ancestral haplotypes. Haplotype codes are as per Figure 2.

16S dataset

Random samples of individuals from each discrete COI lineage were also sequenced for the mitochondrial 16S region. Eleven unique 16S haplotypes of 523bp in length were obtained from a total of eleven sampled individuals. There were 14 indels. The transition to transversion ratio was 0.713:1, strikingly lower than that observed for the COI fragment and potentially indicating that the 16S fragment was not affected by sequence saturation. Uncorrected pairwise sequence divergence ranged from 0.3% between individuals from the Paluma and Atherton lineages, to 9.5% between Mt. Lewis A and Mt. Lewis B haplotypes. The 16S tree corroborated the COI phylogeny in identifying all lineages (data not shown), although members of the Daintree lineage failed to amplify for 16S. Nevertheless, the concordance between the two datasets suggested that the COI data were not affected by the presence of nuclear mitochondrial DNA sequences (NUMTS).

Combined 16S/COI dataset

The partition homogeneity test was not significant (P = 0.09) indicating that the COI and 16S datasets provided similar phylogenetic signals, allowing representative haplotypes to be concatenated and analysed as a single 1162 bp mtDNA dataset under the same model of sequence evolution as COI. The tree topology for the combined dataset corroborated the phylogenies for each individual gene region (Figure 4). Nodes generally possessed greater support than for the 16S and COI fragments alone,

although this could also be an effect of reducing the amount of within-lineage variation. Nevertheless, this analysis provided better resolution of the phylogenetic relationships at deeper nodes.

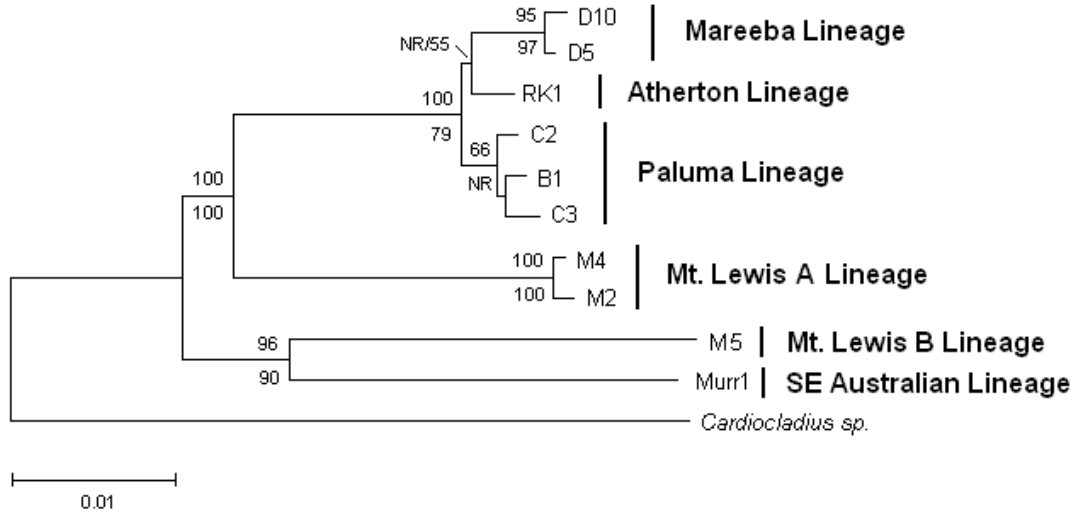

Figure 4. Inferred phylogram based on combined COI/16S representative samples. Bootstrap values for the maximum parsimony method are shown above the nodes and maximum likelihood bootstrap values below the nodes. Haplotypes are named according to their COI haplotype. Branches that were collapsed under either method are designated "not resolved" (NR). Branch lengths indicate percent difference among haplotypes.

The results of the above analyses clearly presented a problem for determining the detailed range and extent of phylogeographic structuring among populations of *E. martini* in the Wet Tropics. The recovery of deep phylogenetic divergence and a suite of inferred cryptic evolutionary lineages rendered the sampling design unbalanced. Furthermore, the lack of geographical intensity within any given lineage precluded formal phylogeographic analyses by methods such as Analysis of Molecular Variance (AMOVA) (Excoffier *et al.*, 1992) or testing of spatial patterns such as isolation by distance (Slatkin, 1993). Nonetheless, the genetic data between population pairs within the Paluma lineage provided important baseline information about relative gene flow and dispersal within *E. martini* populations and an opportunity to test our hypothesis of panmixia formally.

Paluma Lineage

The Paluma lineage contained 15 unique haplotypes in 43 individuals from two sample sites (Camp and Birthday Creeks) (Fig. 4a). Gene diversity was 0.6762 +/- 0.1110 at Camp Creek and 0.7576 +/- 0.0659 at Birthday Creek. The mean number of pairwise differences (π) among haplotypes at each site was 4.619 +/- 2.356 at Birthday Creek and 2.876 +/- 1.575 at Camp Creek. Despite only 600m separation between the two sites, only a single ancestral haplotype was shared. Mean pairwise sequence divergence between the two sites was 1.10%. Conventional F_{ST} and exact tests both indicated highly significant genetic structuring between the two sites [F_{ST}: 0.29503 (P = 0.00000); Exact test: P = 0.00000 +/- 0.0000]. The estimated

divergence time between the two sites was 61kya. These data strongly suggest historical population isolation of the two sites with retention of an ancestral haplotype and restricted contemporary gene flow.

Discussion

Our results showed unexpectedly high genetic divergence among *E. martini* individuals sampled at both the broad geographic scale (North Queensland versus south-eastern Australia) as well as among some populations within the Wet Tropics region, indicating the presence of several cryptic evolutionary lineages. Even at the smallest spatial scale (<1km), within the Paluma lineage, the data revealed strong genetic structuring. Overall, therefore, our data suggest extensive historical isolation and minimal dispersal among sampled *E. martini* populations. Thus, we reject our hypothesis that *E. martini* are panmictic and that their extensive dispersal has negated the effects of habitat fragmentation on population connectivity. Instead, our data suggests that historical processes involving changes in climate and riparian vegetation composition have resulted in numerous evolutionarily divergent cryptic lineages with only limited geographical distributions.

Kershaw (1994) suggested that rainforest contractions in the Wet Tropics, driven by past climatic changes associated with glacial-interglacial cycles, may have occurred from around 150kya. Additionally, palynological evidence from sites in New South Wales, which possessed similar rainforest assemblages during the Tertiary, suggests extensive rainforest contractions occurred from around 5mya, with complete conversion to woodland/grassland by the start of the Pleistocene (~1.8mya) (Martin, 1986). It is conceivable, therefore, that the Wet Tropics also experienced extensive changes to rainforest boundaries from the late Pliocene onwards. Indeed, the existing genetic evidence for several rainforest endemic taxa in the Wet Tropics has suggested that divergence among lineages within species occurred mainly during the late Pliocene to middle Pleistocene (~1–5mya) (see Moritz *et al.*, 2000). Without exception, these studies invoke population isolation resulting from cycles of rainforest contractions during the late Tertiary and early Quaternary as the main driving force behind the observed deep genetic divergences.

The *E. martini* phylogeny inferred here offers a plausible hypothesis for the sequence of evolution among the observed lineages. Wet Tropics lineages appear to have diverged basally across the BMC, suggesting that bisection of this region by the BMC led to the initial fragmentation of *E. martini* populations in the Wet Tropics. Sister groupings among divergent lineages on either side of the BMC also suggest extensive habitat fragmentation to have occurred on both sides following initial bisection. This pattern concords generally with existing evidence not only for the study region (Williams & Pearson, 1997; Moritz *et al.*, 2000) but also for aquatic invertebrate species elsewhere (e.g., Baker *et al.*, 2003; Smith *et al.*, 2006a), implying that chironomid populations were similarly affected by habitat

changes brought on by glacial-interglacial cycles. The curious sister grouping of the Daintree-Mt. Lewis B lineages with the southeast Australian lineage may have arisen through the sharing of ancestral fixed differences among the two regions. *Echinocladius martini* populations may have thus been isolated in rainforest pockets and have since evolved in allopatry, giving rise to the observed genetically distinct, geographically restricted lineages.

The results presented here also suggest that secondary contact may have occurred between the currently sympatric Atherton-Mareeba and Mt. Lewis A-Mt. Lewis B lineages. In each instance, the most parsimonious explanation is that expansion of rainforest boundaries around 8,000 years ago (Nix & Switzer, 1991; Hugall *et al.*, 2002) allowed historically isolated, geographically proximate populations to reconnect. While highly divergent sympatric lineages may indeed constitute 'cryptic species' – a pattern not uncommon in invertebrates (e.g., Jackson & Resh, 1998; Witt & Hebert, 2000; Baker *et al.*, 2004; Herbert *et al.*, 2004) – the lack of supporting nuclear sequence or allozyme data prevents us commenting on present-day reproductive isolation among these lineages.

Nevertheless, historical population isolation may have exposed separated populations to different environmental conditions and local selection pressures, potentially driving genetic divergence. Although individuals of *E. martini* appear to possess broad thermal tolerances (McKie *et al.*, 2004), these may have been selected for in response to the stochasticity of the Australian climate. Thus, while variations in stream thermal regimes may not have restricted dispersal among populations, subtle differences in other microhabitat parameters, both within and among streams, may have restricted effective dispersal (or establishment) among populations. Alternatively, priority effects (whereby the existing population has competitive dominance over colonizing individuals) may prevent new genotypes being incorporated into the existing populations (De Meester *et al.*, 2002). Furthermore, even if discrete lineages were not reproductively isolated prior to recontact, they could have developed such attributes in sympatry as a result of asynchronous mating times (Oliver, 1971; Pinder, 1986). This is speculation at present, however; formal testing awaits further sampling with more strategic collection of larvae in association with substrate type.

While divergent lineages presently occurring in sympatry do suggest some historical dispersal events, within the Paluma lineage we found striking evidence for very restricted dispersal between sites less than one kilometre apart. Although our data were limited to a single pair of sites and are of relevance only at the local scale, these findings concord with the only previous phylogeographic study of chironomids (Martin *et al.*, 2002b). These results also directly contrast both with data on chironomid flight physiology (McLachlan, 1983, 1986a) and global evidence that suggests generally high dispersal potential in many semi-aquatic insects (Kovats *et al.*, 1996; Bunn & Hughes, 1997; Kelly *et al.*, 2001; Smith & Collier, 2001; Baker *et al.*, 2003; Berendonk & Spitze, 2006). It has been shown that the density of riparian vegetation can impede dispersal among streams in some chironomid species

(Delettre & Morvan, 2000). Thus, despite the close proximity of sample sites within the Paluma lineage in the present study, the intervening riparian zone of dense tropical rainforest may act as an effective overland barrier to adult dispersal. Thus, rather than dispersing widely, individuals of *E. martini* may tend to both reproduce and oviposit within or near to their natal stream, with little movement occurring among streams. This pattern concords with numerous studies that have indicated similar patterns of restricted dispersal to be common in other dipteran groups (e.g., Williams & Hynes, 1976; Brittain & Eikeland, 1988; Wishart & Hughes, 2003; Hurtado *et al.*, 2004; Finn & Adler, 2006).

Overall, this study has demonstrated that analysing molecular data can elucidate patterns of diversity among populations of a supposed single species that may otherwise remain undetected. Furthermore, habitat fragmentation appears to have isolated *E. martini* populations into separate refugia, driving genetic divergence in allopatry. Restricted dispersal among streams within refugia suggests that the potential for chironomid populations to relocate and colonise nearby suitable habitat in response to habitat fragmentation is likely to be similarly restricted.

Future investigations of *E. martini* dispersal patterns within lineages will aim to incorporate fast evolving nuclear markers like microsatellites along with acquiring larger sample sizes from each site. Also, a hierarchical sampling design that encompasses several sites along the same stream and multiple streams per catchment, will allow more fine-scale resolution of the geographical range and extent of effective dispersal of discrete evolutionary lineages.

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