DUNG BEETLE RADIATIONS IN MADAGASCAR

HELENA WIRTA

Department of Biological and Environmental Sciences
Faculty of Biosciences
University of Helsinki
Finland

Academic dissertation

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Supervised by: Prof. Ilkka Hanski
                     Department of Biological and Environmental Sciences
                     University of Helsinki, Finland

                     PhD Luisa Orsini
                     Department of Biology
                     Katholieke Universiteit Leuven, Belgium

Reviewed by: PhD Niklas Wahlberg
                     Department of Biology
                     University of Turku, Finland

                     Prof. Jaakko Hyvönen
                     Department of Biological and Environmental Sciences
                     University of Helsinki, Finland

Examined by: PhD Johannes Bergsten
                     Department of Entomology
                     Swedish Museum of Natural History, Sweden

Custos: Prof. Ulrika Candolin
                     Department of Biological and Environmental Sciences
                     University of Helsinki, Finland

CONTENTS

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CONTRIBUTIONS

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Manuscript preparation	HW, LO, IH	HW, IH	HW, OM	HW	IH, HW

HW: Helena Wirta
IH: Ilkka Hanski
LO: Luisa Orsini
OM: Olivier Montreuil

HV: Heidi Viljanen
PR: Pierre Rahagalala
TN: Toshka Nyman

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ABSTRACT

Speciation on islands is affected by island size and the range of habitats and resources available and often also by limited interactions with other taxa. An ancestral population may evolve into a large number of species via an adaptive radiation. In Madagascar, most groups of animals and plants have radiated on the island, having arrived via oceanic dispersal during the long isolation of Madagascar. Characteristic features of Malagasy biota are exceptionally high level of endemism, high species richness as well as lack of many higher taxa that are dominant on the African mainland.

Malagasy dung beetles are dominated by two tribes, Canthonini and Helictopleurina, with more than 250 endemic species. In this thesis I have reconstructed molecular phylogenies for the two tribes using several gene regions and different phylogenetic methods. Evolution of closely related species and among populations of the same species was examined with haplotype networks.

The Malagasy Canthonini consists of three large lineages, while Helictopleurina forms a monophyletic group. The ancestors of each of the four clades colonised Madagascar at different times during Cenozoic. The subsequent radiations differ in terms of the number of extant species (from 37 to more than 100) and the level of ecological differentiation. In addition, Onthophagini (6 species) and Scarabaeini (3) have colonised Madagascar several times, but they have not radiated and the few species have not entered forests where Canthonini and Helictopleurina mostly occur.

Among the three Canthonini radiations, speciation appears to have been mostly allopatric in the oldest and the youngest clades, while in the Epactoides clade sister species have diverged in their ecologies but have similar geographical distributions, indicating that speciation may have occurred in regional sympatry. The most likely isolating mechanisms have been rivers and forest refugia during dry and cool geological periods. Most species are generalists feeding on both carrion and dung, and competition among ecologically similar species may prevent their coexistence in the same communities. Some species have evolved to forage in the canopy and a few species have shifted to use cattle dung, a new resource in the open habitats following the introduction of cattle 1500 years ago. The latter shift has allowed species to expand their geographical ranges.

SUMMARY

Helena Wirta

1. SPECIATION AND RADIATION ON ISLANDS

Isolated islands are particularly favourable places to study lineage differentiation, because the rate of successful colonisation is low due to isolation and the evolving lineage remains within the well-defined border of the island. Islands come in various sizes, which creates dissimilar opportunities for radiation. The newly-colonised population is generally very small, and hence founder effects (only a subset of the original genetic variation present) and genetic drift (random changes in allele frequencies) play important roles in the early stages of differentiation, and small propagule sizes also shape the species composition in the island communities. Oceanic islands are initially entirely devoid of species, whereas islands that break apart from a continent usually start with a smaller or larger set of species. Especially in the first but also in the latter case colonisation by new species is facilitated by ecological release, meaning that the island is free of many species with which the focal species interacts in the source of colonisation (Emerson 2002; Paulay 1994; Whittaker & Fernández-Palacios 2007). Evolution in the absence of previous biotic interactions may lead to broadening of the niche, to loss of traits that previously helped against predators or competitors, and to generally higher densities on islands (Whittaker & Fernández-Palacios 2007 and references therein). Examples of niche widening include lizards as pollinators and dispersal agents, when birds and insects with these functions on the continent are missing (Olesen & Valido 2003). Other common trends in evolution on islands include loss of dispersal power and shifts in body size, as ecological release allows species to evolve towards the intrinsic optimum body size, away from the one affected by interspecific interactions on the mainland or by limited resources (Cody & Overton 1996; Lomolino 2005; Raia & Meiri 2006; Roff 1990; Whittaker & Fernández-Palacios 2007).

Well-known examples of the former include dodo on Mascarene islands and kiwi in New Zealand, while pygmy hippopotami in Madagascar and pygmy elephants and giant rats on Mediterranean islands are examples of major shifts in body size. Having once evolved on an island, a species is likely to remain there. Island forms and endemics are much less likely to back-colonise mainlands than vice versa, which is considered to be due to the specialisations that species have acquired while evolving on an island (Paulay 1994; Whittaker & Fernández-Palacios 2007).

The ways that new species evolve on islands can be thought to form three categories: speciations without radiation, nonadaptive radiation, and adaptive radiation (Whittaker & Fernández-Palacios 2007). In nonadaptive radiation speciation occurs primarily due to drift and genetic mechanisms in allopatric populations, but there is no substantial differentiation in ecological traits as the incipient species experience similar environmental conditions (Rundell & Price 2009; Schluter 2009). Populations may become isolated on different sides of a geographical barrier, such as rivers, mountain chains and narrow sea areas between islands, which may constitute strong enough barriers for dispersal to allow speciation in allopatry (Losos & Ricklefs 2009; Moritz et al. 2000). Retreating forest areas, forming refugia during unfavourable climatic conditions, may allow populations to evolve to separate species before the forests become reconnected (Moritz et al. 2000 and references therein). In Madagascar, where speciation mechanisms have been studied in many taxa, various modes of allopatric speciation have been proposed to explain evolution of high species richness and local endemism. Rivers appear to form dispersal barriers for many lemur species and southern tortoises, with endemic taxa evolving and occurring between river systems (Goodman &

Ganzhorn 2004; Olivieri et al. 2007; Paquette et al. 2007; Pastorini et al. 2003; Yoder et al. 2000). In other cases river catchment areas may have functioned as forest refugia, in which species would have diverged (Wilmé et al. 2006). Montane areas are also likely to have had forest refugia during cool and dry periods, and endemic taxa have evolved in these isolated forest fragments (Vences et al. 2009; Wollenberg et al. 2008).

Speciation can also be sympatric, though there is not a large number of well-documented examples (Dieckmann & Doebeli 1999; Doebeli et al. 2005; Savolainen et al. 2006; Schluter 2009). Strong diverging selection is needed when speciation occurs in sympatry, often accompanied with assortative mating, with individuals preferring to mate with the same ecotype. For divergence to hold, reproductive isolation is needed, which may occur via hybrids of the two incipient species having reduced fitness (Schluter 2009). One of the most convincing examples of speciation in sympatry is of two palm species on a small oceanic island, within which they could not have become geographically isolated, but the two forms have rather adapted to different soil types and evolved to different species with different flowering times enhancing reproductive isolation (Savolainen et al. 2006). Such speciation is also called ecological speciation, although ecological speciation can also take place in a parapatric situation, where partial allopatry strengthens the divergence. In Madagascar, adaptation to environmental (altitudinal and climatic) gradients and parapatric speciation along these gradients is likely to account for rampant speciation in many taxa, for instance in day and leaf-tailed gekkos (Raxworthy et al. 2007; Raxworthy et al. 2008).

A radiation is considered to be adaptive when new adaptations enable the species to exploit their environment and resources in a more effective way, with descendant species differing in their adaptations (Schluter 2000; Whittaker & Fernández-Palacios 2007). As the niche of a species first becomes wider, populations of the same species may start utilising somewhat different resources under dissimilar environmental conditions. These adaptive changes, coupled with allopatry, may lead to divergent natural selection with eventual split of the lineage to two, with dissimilar resource use (Losos & Ricklefs 2009). Some researchers call a

radiation adaptive only if speciation is ecological (Schluter 2000). In old radiations, it is usually impossible to tell whether speciation was adaptive or whether ecological diversification occurred following speciation due to non-ecological mechanisms in allopatry (Rundell & Price 2009). In any case, large and heterogeneous islands that lie far from the continents are especially likely to harbour adaptive radiations (Paulay 1994). Low rate of arrival of new colonists and initial lack of taxa with which the new-comer would interact facilitate diversification, as the colonisers are able to exploit novel resources (Whittaker & Fernández-Palacios 2007). In this case ecological niches are more likely to be filled by diversification rather than by continuous colonisation (Leigh et al. 2007).

2. MADAGASCAR

2.1. The island

Madagascar is the world's fourth largest island, extending 1600 km in the north-south direction. The climate varies within the island from nearly aseasonal in the north to a strongly seasonal in the south. Madagascar has a highly variable topography and a great diversity of habitats, with the tallest peak reaching 2876 meters above the sea level (de Wit 2003). Madagascar is separated from mainland Africa by the 400 km wide Mozambique Channel. The island used to be part of the supercontinent of Gondwana that existed 500-200 My ago, but it split apart from Africa about 160 My ago together with India. Madagascar moved to roughly its current place prior to the split from India 88 My ago (Briggs 2003; de Wit 2003). The modern climate in Madagascar is mostly affected by the southeastern trade winds, cyclones and the Southern Indian Drift, which all bring rains to eastern Madagascar. Heavy rain fall has created and maintains rain forests on the eastern slopes of the north-south mountain chain, whereas there are various types of drier vegetation on the high plateau and the western parts of the islands, though only remnants of the original vegetation cover remain (de Wit 2003; Green & Sussman 1990; Harper et al. 2007). The climate is tropical along the coastal areas, temperate in high-altitude inland areas and arid in the South.

Madagascar's fauna is characterised by extremely high levels of endemism as well as by lack of many faunal groups due to its prolonged and great isolation (de Wit 2003). In many taxa that are present, the numbers of species are high for the size of the island, and even then the recorded species diversities are likely to be vast underestimates, as large numbers of new species are being constantly discovered using both traditional and molecular methods (Lehtinen et al. 2007; Olson et al. 2004; Ravaoarimanana et al. 2004; Vieites et al. 2006; Wirta 2009; Yoder et al. 2000). Because of the high level of endemicity, very high species diversity in general, and the high level of threat posed to biodiversity by habitat loss, Madagascar and its neighboring islands are considered to be one of the most important, if not the most important, biodiversity hotspot (Table 1) (Myers et al. 2000). Among all the hotspot areas, Madagascar harbors an exceptional amount of phylogenetic diversity in the form of ancient endemic lineages (Sechrest et al. 2002; Spathelf & Waite 2007).

Table 1. The numbers of species and percentages of endemic species in Madagascar (www. biodiversityhotspots.org). Dung beetles include both Scarabaeinae and Aphodiidae species (Montreuil 2003-2008, Montreuil & Viljanen 2007, Rahagalala et al. 2009, Wirta & Montreuil 2009).

Taxa	Species	Percent endemism
plants	13 000	89.2
mammals	155	92.9
birds	310	58.4
reptiles	384	95.6
ambhibians	230	99.6
freswater fishes	164	59.1
dung beetles	296	96.0

2.2. Origins of the Malagasy fauna

Origins of the Malagasy fauna have been attributed either to vicariance following the break-up of the supercontinent Gondwana, vicariance following the break-up of a land bridge to Antarctica and South America ca 88 My ago, or oceanic colonisation in Cenozoic (Evans et al. 2008; Noonan & Chippindale 2006a; Yoder & Nowak 2006). The first two hypotheses cannot explain the origin of the majority of the fauna, because Madagascar was already completely isolated during the near complete faunal turnover at the Cretaceous-Tertiary boundary (Krause 2003). Furthermore, most of the modern faunal groups had not yet evolved at the time of the separation from Africa. In general, the unique combination of faunal groups and the lack of many specious groups in mainland Africa support oceanic drafting as the main mechanism of colonisation. Most well-studied groups have reached Madagascar only once and very little back-colonisation has taken place. Based on recent age estimates of various lineages, oceanic colonisation in the past 65 My has contributed most to the modern Malagasy fauna (Yoder & Nowak 2006). Rafting on a floating island, a patch of land broken of from river delta, or on floating vegetation, has probably facilitated colonisations (Zhou et al. 2006).

The faunal groups with closest relatives in South America are considered to have a vicariant origin, the ancestors having been in Madagascar prior to the submergence of the land connection to Antarctica. These groups include several freshwater fishes, boid snakes, podocnemid turtles, iguanid lizards, the elephant bird, a giant frog and extinct dinosaurs (Evans et al. 2008; Haddrath & Baker 2001; Noonan & Chippindale 2006a, 2006b; Sampson et al. 1998; Sparks 2004; Sparks & Smith 2004; Vargas-Ramirez et al. 2008). The reasoning for the vicariant origin is based on antiquity, basal position in the phylogeny for the lineage and similar age estimates among groups (Noonan & Chippindale 2006a, 2006b; Yoder & Nowak 2006).

Considering taxa that have colonised Madagascar from overseas, the colonisers have mainly arrived from Africa, as expected. Among the groups with multiple successful arrivals are colubrid snakes, hyperoliid frogs, butterflies, ants and small minnow flies (Monaghan et al. 2005; Nagy et al. 2003; Torres et al. 2001; Vences et al. 2003; Yoder & Nowak 2006). Faunal groups with a single colonisation event followed by subsequent diversification include plated lizards, day geckos,

skinks, tortoises, chameleons, swallowtail butterflies, sun birds, lemurs, tenrecs, rodents, and carnivores. The estimates of arrival vary commonly between 66 and 11.5 My ago (Austin et al. 2004; Jansa et al. 1999; Palkovacs et al. 2002; Poux et al. 2005; Raselimanana et al. 2009; Schmitz et al. 2005; Zakharov et al. 2004). Some taxa, such as *Phelsuma* day geckos, chameleons, tortoises, rodents and sun birds have dispersed further from Madagascar to some or all of the neighbouring islands (Réunion, Mauritius, Rodrigues, Seychelles, Aldabra) and even back to Africa (Austin & Arnold 2001; Austin et al. 2003; Austin et al. 2004; Palkovacs et al. 2002; Raxworthy et al. 2002).

Madagascar offers great possibilities for adaptive radiations, considering its large size and highly variable topography, vegetation and climate. Madagascar is located so far from the African mainland that colonisation events by most taxa are extremely rare, yet such colonisations are possible in the long course of time. Additionally, at the time of the K-T boundary, 65 My ago, the island is considered to have had low diversity (Krause 2003), offering free space for new colonisers to diverge in. Several endemic groups have evolved through adaptive radiations, of which the best studied ones are lemurs, tenrecs and chameleons. These radiations have produced large numbers of species, 46, 28 and 66 extant endemic ones, respectively (Goodman 2003; Henkel & Schmidt 2000; Olivieri et al. 2007; Raxworthy 2003). All the three groups are mainly forest-adapted but have variable diets. Lemurs and tenrecs have evolved various behaviours and morphological adaptations to seasonal environments (Schmid & Stephenson 2003). Tenrecs are a particularly diverse group and they exhibit specialisations and adaptations to various different life history forms and niches, such as semifossorial, arboreal and semiaquatic lifestyles (Carbutt 1999b; Olson & Goodman 2003). Most of the species in these radiations are locally endemic with high turnover in the species composition between localities (Wilmé et al. 2006). In lemurs and tenrecs geographical populations are often highly differentiated, suggesting long isolation (Craul et al. 2007; Olson & Goodman 2003), and in many cases the geographical populations are distant enough to be considered incipient species (Paquette et al. 2007; Yoder et al. 2005).

3. AIMS OF THE THESIS

The primary aim of this thesis work was to reconstruct the evolutionary history of Malagasy dung beetles. I address such questions as did the groups colonise Madagascar from overseas, how many separate colonisations and radiations have occurred, and how and when the different lineages started to diversify and speciate. In selected lineages, I examine how populations and closely related species have diverged in the past. Phylogenetic information is helpful for revising the taxonomy of Malagasy dung beetles, which is now becoming fairly well known especially through studies of Olivier Montreuil, with whom I have collaborated. I reconstructed molecular phylogenies for the two large groups of endemic dung beetles in Madagascar, the tribe Canthonini and the endemic subtribe Helictopleurina, and estimated the times of divergence of focal clades.

Since 2002, researchers and students from the Metapopulation Research Group in Helsinki, with collaborators in Madagascar, have collated much information about the distribution of species in Madagascar, and specific projects have been conducted on the ecology of particular species and communities of species (Hanski et al. 2007; Koivulehto 2004; Montreuil & Viljanen 2007; Orsini et al. 2007; Rahagalala et al. 2009; Viljanen 2004, 2009). With the help of ecological and distributional data provided by the above studies as well as using additional material collected for this study I have attempted to analyse how specific traits have evolved in the course of speciations and radiations. By studying genetic differentiation within species, I have examined how changes in the ecology have affected the distribution of species.

4. DUNG BEETLES

4.1. Systematics and ecology

Scarabaeidae is a large and very diverse beetle family with a cosmopolitan distribution. It is monophyletic, with several subfamilies (Browne & Scholtz 1995; Browne & Scholtz 1999). Scarabaeinae are generally considered as the true dung beetles, as most species in the Scarabaeinae

use dung both as larvae and adults (Browne & Scholtz 1998; Halffter & Matthews 1966) and it is strongly supported as monophyletic (Browne & Scholtz 1998, 1999; Smith et al. 2006).

There are approximately 5000 species in the subfamily, which historically have been divided into two groups, Scarabaeinae and Coprinae (or Scarabaeini and Coprini), based on their nesting behaviour (Balthasar 1963). Scarabaeinae consists of rollers, which form balls of dung and roll it away from the source of resource to be buried elsewhere to construct a nest. Coprinae includes tunnelers, which bury dung directly beneath the dung source. However, during the last two decades this division has been challenged with additional morphological and new molecular data, suggesting that different reproductive strategies have evolved and been lost several times (Monaghan et al. 2007; Ocampo & Hawks 2006; Villalba et al. 2002 and references therein). Several small, derived tribes with limited distribution, such as Onitini, Oniticellini and Scarabaeini, are monophyletic, while the large, widely distributed tribes Canthonini and Dichotomiini consist of multiple lineages (Monaghan et al. 2007; Montreuil 1998; Philips et al. 2004).

The Scarabaeinae dung beetles exhibit great diversity in morphology and nesting behaviours, which is considered to be due to their diversification to use different dung types and other food resources as well as to intense competition for these resources. In addition to using the On top of dung of various vertebrates, dung beetles in fact also feed on carrion, fungi, rotting fruit and some even prey upon living invertebrates (Cambefort 1991a; Escobar 2004; Halffter & Matthews 1966; Hanski 1989). A common feature of the food resources is that their occurrence is spatially patchy and temporally ephemeral, which, when combined with often severe resource competition, has consequences for the structure of the dung beetle community in terms of the body sizes, food choices and activity periods among coexisting species (Hanski & Cambefort 1991; Horgan 2005; Horgan & Fuentes 2005; Krell et al. 2003; Vernes et al. 2005). Dung beetles are commonly more specialised at the level of macrohabitat (forest versus open habitats) than at the level of food resources. Important factors affecting dung beetles' habitat selection are vegetation, soil type and microclimate (Hanski & Cambefort 1991). Humidity and temperature of the soil are important for egg and larval development, while humidity and temperature of the air affect the flight activity of adults. Vegetation affects both humidity and temperature as well as offers hiding places from predators and places for perching (Hanski & Cambefort 1991). Scarabaeinae dung beetles occur both in open grasslands and other such habitats, as well as in a variety of forest types, and their main distribution is in the southern hemisphere (Cambefort 1991b).

Dung beetles play an important ecological role in many ecosystems. Dung burial increases the rate of soil nutrient cycling, especially by adding nitrogen, and aerates and mixes the soil, which all facilitate plant growth (Estrada et al. 1998; Mittal 1993). Burying dung helps in controlling parasites, as it decreases the amount of dung available for such vectors of parasites that lay their eggs in the dung. Dung beetles also act as secondary seed dispersers, moving and burying seeds with the dung, and thus they facilitate germination and protect seeds from predation (Andresen 2002, 2003). Some dung beetle species pollinate carrion-mimicing flowers (Sakai & Inoue 1999).

4.2. Malagasy dung beetles

Madagascar has a rich dung beetle fauna compared to other large tropical islands (Table 2). The Malagasy Scarabaeinae fauna consists almost entirely of endemic species, which belong to four tribes. Of the endemics, there are seven genera and 191 described species and subspecies in Canthonini, two genera with 66 taxa in the subtribe Helictopleurina (Oniticellini), and one genus with three species in the tribe Scarabaeini (Lebis 1953, 1960; Montreuil 2003a, b, 2004, 2005a, b, c, d, 2006, 2007, 2008a, b, 2009, in prep.; Montreuil & Viljanen 2007; Paulian 1975, 1976, 1986). In addition, there are six Onthophagini species of which at least two are recently introduced species (Lebis 1953). Canthonini and Helictopleurina occur mostly in forests, and especially in the eastern rain forests (Fig. 1), while Scarabaeini and Onthophagini are found in open areas and in dry forests (Hanski et al. 2007; Koivulehto 2004; Rahagalala et al. 2009). Four species of Canthonini and four species of Helictopleurina use cattle dung in open areas,

together with Aphodiidae and Onthophagini species (Rahagalala et al. 2009). The shift of diet in the Canthonini and Helictopleurina species must have occurred recently, as cattle was introduced to the island only 1500 years ago (Burney et al. 2004). These species have large geographical ranges (Rahagalala et al. 2009), in contrast with most other Malagasy dung beetle species with very limited ranges (Fig. 1; Viljanen et al. 2009). Extensive forest loss and fragmentation may have already caused the extinction of many wet forest species with small ranges (Hanski et al. 2007).

Table 2. Number of dung beetle taxa (Scarabaeidae and Aphodiidae) on the largest tropical islands, their percentage endemism, and island area in square km (Hanski and Krikken 1991, Montreuil 2003-2008, Montreuil & Viljanen 2007, Wirta and Montreuil 2009).

Island	Species	Endemism	Area
New Guinea	120	83	786 000
Borneo	120	38	748 000
Madagascar	296	96	588 000
Sumatra	112	30	443 000

Canthonini are mostly nocturnal while Helictopleurina are diurnal (Viljanen et al. in prep.). The majority of Canthonini are generalists, feeding on carrion as well as dung, while dung is more used by Helictopleurina (Viljanen 2004; Viljanen et al. in prep.). Local dung beetle communities have low species richness, but turnover between localities is high, yielding a high total number of species (Viljanen et al. 2009). The numerically dominant species in local dung beetle communities are those of the genus *Nanos* (Viljanen et al. in prep.). The best-known species, *Nanos viettei*, is abundant in wet forests in southeastern Madagascar. It is a nocturnal generalist with very low fecundity (one to two offspring per season) but great longevity, as beetles may live up to two years. They are very sedentary, moving only tens of meters in up to 12 months (Viljanen 2009). Closely related species in this genus have allopatric distributional ranges, which may be due to hybridisation and low hybrid fitness (Viljanen 2009).

4.3. Sampling

In 2002-2008, a Madagascar-wide sampling program has been conducted by the Metapopulation Research Group, with the help of local personnel in parks and reserves. Sixty forest localities across Madagascar have been trapped with standard pitfall traps. Most of the trappings were conducted with fish-baited pitfall traps, with a protocol of 80 trap-days per locality (Fig. 2). More exhaustive trappings were carried out in Andasibe, Andohahelo, Anjaharibe Sud, Ambila, Makira, Manombo, Marojejy, Masoala, Nosy Mangabe and Ranomafana, using also other baits (including primate dung, meat, chicken intestines and/or rotting fruit), and placing traps at several altitudes and in different types of forest as well as in open habitats (Koivulehto 2004; Viljanen 2004; Viljanen et al. in prep.). Additionally, cattle dung pats in open habitats have been examined for dung beetles across Madagascar in eighty localities (Rahagalala et al. 2009). All distributional and altitudinal records of our trappings as well as museum specimens in Muséum National au Histoire Naturelle (MNHN) in Paris, France, have been assembled into a data base. Specimens have been preserved in 95% ethanol in the field. The species have been identified by H. W., Heidi Viljanen, Olivier Montreuil and Mirja Miinala based on comparison with type specimens and large series of specimens in MNHN.

5. PHYLOGENY RECONSTRUCTION

5.1 Phylogenetic analyses

All extant forms of life descent from a common ancestral form, and their relationships may be presented in a phylogeny. Phylogeny reconstruction is done by studying and comparing organisms and their traits, as the extant taxa carry signs of their evolution and origins in their properties. Phylogenetic relationships among a set of taxa can be reconstructed using either morphological or molecular characters or a combination of the two types of characters. Phylogenetic analyses consist of two parts, estimating the topology of the evolutionary tree and estimating the branch
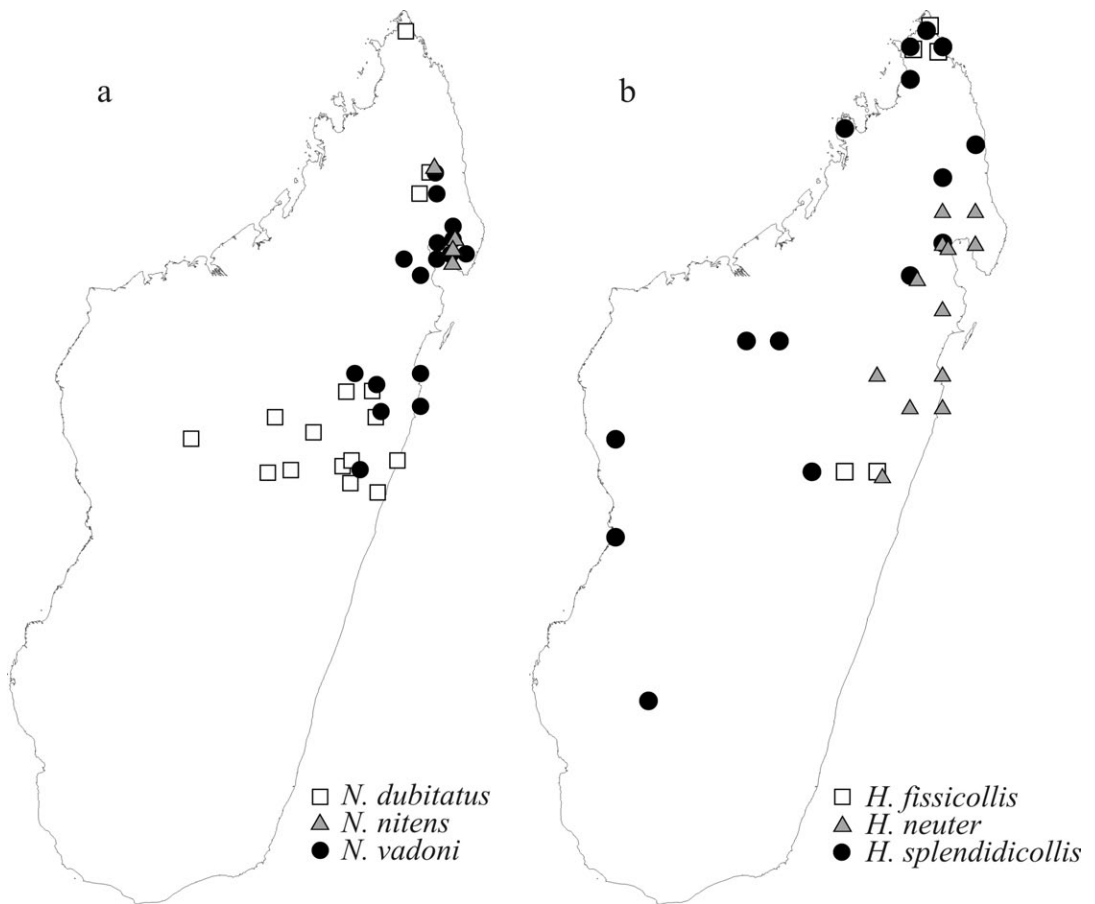
Fig. 1. Distributions of three relatively common *Nanos* (a) and *Helictopleurus* (b) species.

lengths in that tree. Commonly, the true tree is not recovered due to e.g. extinct taxa, but rather an estimation of it may be obtained (Avise 2004, Nei & Kumar 2000). A difficulty in phylogeny reconstruction is in separating the signal of shared origin from other properties, as more or less similar features may have developed several times. In DNA sequences, which consist of only four bases, saturation (multiple changes at a single site) may cover earlier changes and thus blur the signal of shared or independent origins (Nei & Kumar 2000).

The coding part of the genome typically involves some regions that are likely to be under selection and therefore may show a lower rate of change than regions that are not under selection (Avise 2004). DNA sequences with different mutation rates are commonly used to resolve phylogenetic

relationships at different taxonomic levels, with regions of higher mutation rate preferred for lower taxonomic levels. Another important factor to consider while reconstructing phylogenies is recombination. mtDNA is often preferred over nuclear DNA because it is maternally inherited and not affected by recombination (Hillis & Dixon 1991). However, there may be differences in different copies of mtDNA in the same individual (heteroplasmy) caused by either mutations or paternal leakage (Barr et al. 2005). Alignment of the DNA sequences is an important step in the analysis and a possible source of errors. Insertions and deletions within focal gene regions create difficulties in lining up bases of the same shared origin in multiple taxa. In this thesis, I have chosen a commonly used algorithm called Clustal-W (Thompson & al 1994) to align the sequences, and

Fig. 2. A pitfall trap with a bait wrapped in veil and individuals of *Nanos clypeatus* in the trap.

in cases with clearly problematic alignment I have omitted such regions from the analyses.

The most frequently used methods to reconstruct phylogenies can be classified into three groups, distance-based, parsimony and likelihood methods, of which the latter two have gained most popularity in recent years. Distance-based methods are the least used in the recent literature as they are applicable only for very simple evolutionary models and cannot handle mixed datasets (e.g. DNA and morphological data; Albert 2005; Avise 2004; Nei & Kumar 2000). In my thesis, I have used three different methods that are currently most commonly used, namely analyses based on parsimony, maximum likelihood and Bayesian inference.

Parsimony analyses, currently often coined as maximum parsimony (MP), attempt to identify a phylogenetic tree that minimises the number of evolutionary changes needed, with as few *a priori* assumptions as possible, to explain the observed variation between the terminal taxa that are studied (Albert 2005; Schuh 2000). I run MP with the program NONA (Goloboff 1999) and the interface Winclada (Nixon 1999). Maximum likelihood and Bayesian inference belong to likelihood methods which model the evolution of DNA sequences by assigning a probability to the changes in the sequence and aim to identify the most probable tree given the substitution

model and the data. A substitution model aims at correcting for differences in the rates of transitions and transversions in nucleotide sequences, which vary among gene regions (Posada & Crandall 1998). Using a substitution model alleviates the problem caused by saturation (Arbogast et al. 2002). The maximum likelihood (ML) method evaluates the likelihood that the proposed model and a phylogeny would give rise to the observed data set. Based on the model, probabilities are estimated for different kinds of mutations. The method searches for the phylogeny with the highest likelihood with standard statistical techniques (Nei & Kumar 2000). There are several freely available algorithms to infer ML-based phylogenies, but given the large datasets I have used, I preferred the web-supported program PhyML (Guindon & Gascuel 2003). In Bayesian inference of phylogenetic relationships one searches for the posterior probability distribution of trees, which is the probability distribution conditional on the data, the given model and a prior probability distribution of possible trees (Ronquist 2004). The search is conducted with a simulation technique called Markov chain Monte Carlo (MCMC) (Hastings 1970; Metropolis et al. 1953). The program MrBayes (Ronquist & Huelsenbeck 2003) was used because of its ability to handle different evolutionary models for different partitions (gene regions), thus taking into account their possible differences (Ronquist & Huelsenbeck 2003). As ML and Bayesian inferences require *a priori* assumptions on evolutionary models of sequence evolution and mutation rates, they may be expected to yield different results than the MP analysis, especially when complex or extensive phylogenies across different taxonomic levels are inferred. However, when data are robust different methods tend to return congruent results (Albert 2005), and thus finding similar topologies with different methods can be considered as strengthening the conclusions.

Assessing the reliability (robustness) of a phylogenetic tree is an important step in the analysis. For MP and ML methods jackknifing and bootstrapping are two alternative statistical methods to examine the support for the inferred phylogeny by resampling the data. Phylogenies are reconstructed for each resampled data set, and a consensus tree with support values is calculated

(Nei & Kumar 2000). In the Bayesian inference posterior probabilities show how frequently a given tree is observed among the ones sampled after the analysis has reached a stable state in the resampling process (Albert 2005; Cummings et al. 2003). A more biological test of the robustness of a phylogenetic hypothesis is to add to the analysis new data in terms of new characters and taxa. Indeed, among the common sources of error in the reconstruction of phylogenies is poor sampling of taxa, which may lead to a biased phylogeny. Limited sampling may also enhance long-branch attraction, the unreal pairing of rapidly evolving lineages (Bergsten 2005). The use of gene regions with an unsuitable level of variation may lead to lack of resolution in the phylogeny. Individual gene trees may not represent well the phylogeny of species, as single gene regions may have diverged earlier or later than when the speciation events occurred, and thus it is preferable to use of multiple gene regions of different types (e.g. nuclear and mitochondrial regions) (Avise 2004, Nei & Kumar 2000).

5.2. Divergence time estimation

Estimation of the ages of the nodes in a phylogenetic tree is based on the assumption of a molecular clock, which is calibrated with external data. However, the rate of nucleotide changes is known to vary over time, between lineages and between gene regions, and thus a universal molecular clock does not exist. The differences in rates are likely caused by differences in population size, metabolic rate, generation time, and DNA repair mechanisms. However, many of these factors are likely to be similar between closely related, similar aged taxon groups considering the same genetic region (Arbogast et al. 2002 and references therein). A molecular clock can be "relaxed" in many ways (Sanderson 2002, Yoder and Yang 2000), for instance by estimating all the parameters for each branch separately using only a given constraint such as a mean substitution rate (Drummond et al. 2006). Saturation of base changes may cause problems in calculating the acquired mutations between lineages. A model of nucleotide substitution helps in estimating the number of changes correctly (Arbogast et al. 2002).

To obtain a rate for the molecular clock, one needs external data to time at least one node on the phylogenetic tree. Fossils have been commonly used, but as the fossil records are incomplete, it is often difficult to establish the relationships between fossil and extant taxa. With a fossil, usually only a minimum age can be given, while a time interval would be more suitable for calibrating a node (Perez-Losada et al. 2004, Waters et al. 2007). Alternatively to fossils, geological events, such as formation of an island or a geographical barrier, can be used to date branching events. For a geological event often both a minimum and a maximum age may be available, but the age of the geological event may not correspond with the speciation event. For instance, a colonisation of a new island may have occurred much later than the estimated age of the island. Similarly, species may have diverged long before the establishment of a geographical barrier, and populations on the two sides of a barrier may continue to interact long after the formation of an incomplete barrier. Using multiple independent calibration points helps in minimizing such problems, but nonetheless any changes in the calibration affect the results greatly (Arbogast et al. 2002, Perez-Losada et al. 2004, Päckert et al. 2007, Waters et al. 2007). In summary, estimating the ages of the nodes in a phylogenetic tree is prone to errors and the results come with very wide 95% confidence intervals. One has to bear in mind these problems and interpret the results cautiously.

There are several methods of estimating the times of divergence based on the relaxed molecular clock. The method that I have used is based on Bayesian inference with MCMC as implemented in the program BEAST (Drummond & Rambaut 2002-2006; Drummond & Rambaut 2007). The chosen model estimates the evolutionary rate for each branch in the tree based a given mean rate of nucleotide change. This approach has been used for hundreds of data sets and its validity has been tested with simulations (Drummond et al. 2006). This method and the program that implements it are among the most popular ones in recent phylogenetic studies. As there are no fossils or suitable dated geological events for calibrating the phylogenies of Malagasy dung beetles, I have used mean rates of nucleotide substitution used for

other beetle groups, which are roughly of the same age as Malagasy beetles and for which the same gene region has been used as in the present work.

5.3. Haplotype networks

Phylogenies are useful for studying the relationships of species and higher taxa. Analyses of molecular variation at the individual and population level are often best accomplished by examining haplotype networks (Templeton et al. 1992). The program TCS collapses sequences into haplotypes and calculates a distance matrix for all pairs of haplotypes. These distances are used to construct a haplotype network, based on a parsimony criterion to represent connections with e.g. 95% similarity (Clement et al. 2000). I have constructed such networks in two chapters in this thesis to examine divergences between closely related species and populations.

6. RESULTS AND DISCUSSION

6.1. Several oceanic colonisations

Based on the present results, the endemic Malagasy dung beetles originate from several independent colonisations. The subtribe Helictopleurina has a common origin (I), but the species currently classified in Canthonini consist of three separate lineages, the ancestors of which have arrived at Madagascar at different times (II, III). Molecular phylogenies support the morphological groupings of species, suggesting that the three Canthonini lineages consist of the genera *Arachnodes* and *Epilissus*, *Apotolamprus* and *Nanos*, and *Epactoides* (II, III). The small genus *Cambefortatus* is likely to have originated from the same colonisation event as *Apotolamprus* and *Nanos*, as these species are morphologically similar (II). Additionally, the six extant species of Onthophagini have reached the island in at least three separate colonisation events (I), and the ancestors of the three endemic Scarabaeini species have colonised the island at least twice (own unpublished data).

All colonisations seem to have occurred during Cenozoic. The largest Canthonini clade, consisting of the genera *Apterepilissus*, *Arachnodes* and *Epilissus* (*Arachnodes* clade), is the oldest among

Malagasy dung beetle lineages, having shared a common ancestor 64-44 Mya (II). The subtribe Helictopleurina started to diverge in Madagascar 37-23 My ago (I), and the smaller Canthonini clades *Epactoides* and *Apotolamprus* and *Nanos* (*Nanos* clade) have diverged for 30-19 and 24-15 My. The time estimates must be considered with caution, as such estimates may not be fully comparable between studies and taxa. Nonetheless, these estimates are comparable with estimates for other Malagasy taxa and are consistent with the knowledge about dung beetles' evolution (I, II, III). The estimated times of colonisation for the Malagasy clades are too recent to support vicariance origin. The land connection with India (until 80 Mya) and the hypothesised one with Antarctica (88 Mya) (Evans et al. 2008; Noonan & Chippindale 2006a, 2006b; Yoder & Nowak 2006) could not account for the presence of these clades in Madagascar, but they must have reached the island from overseas (I, II). The most plausible origin for all colonisations is mainland Africa due to its proximity and due to phylogenetic relationships in the case of Helictopleurina (I), but other areas of origin cannot be ruled out for the Canthonini clades (II). For Helictopleurina the closest relative sampled is from Africa, while of the Canthonini clades only for *Epactoides* a close relative was detected, genus *Ochicanthon* from India (I, II).

6.2. Four different radiations

The three largest Canthonini clades as well as the Helictopleurina have radiated greatly, with numerous endemic species in Madagascar (Fig. 3, I, II, III). On the other hand, the both Scarabaeini and Onthophagini colonisations have resulted in only one or two species surviving today (I). A probable reason for the lack of radiation in these two tribes is later arrival than Canthonini and Helictopleurina in Madagascar. Having arrived at an island with a rich Scarabaeinae fauna would presumably have limited the diversification and speciation possibilities of the later arriving taxa, and in particular the latter have not been able to enter the main biome of wet forests in Madagascar. Some of the Onthophagini species are considered to be recent introductions (Lebis 1953).

The four radiations differ in age, number of descendants and in the distribution of ecological
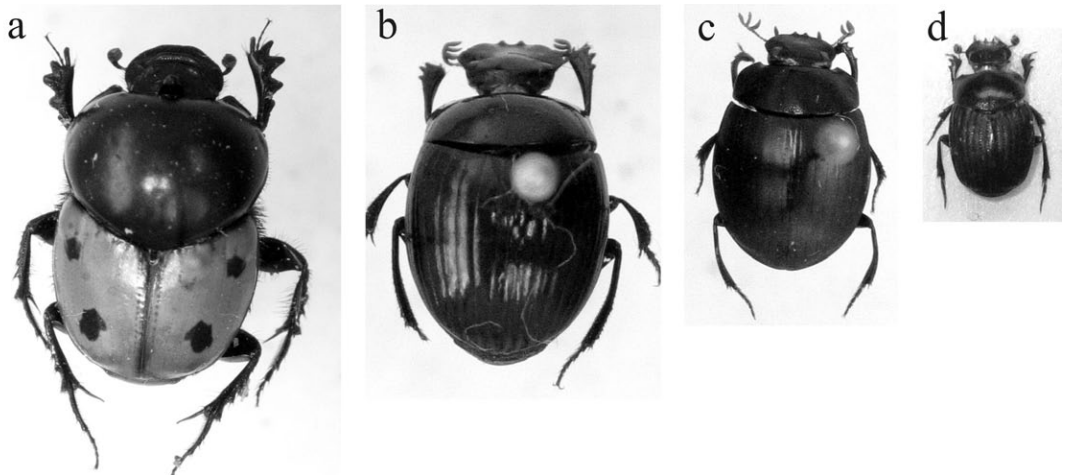
Fig. 3. Representatives of the four colonisations leading to extensive radiations: a) *Helictopleurus quadripunctatus*, b) *Epilissus apotolamproides*, c) *Nanos viettei* and d) *Epactoides frontalis*.

traits. The oldest lineage (*Arachnodes*) includes 101 extant taxa in our current species list, while Helictopleurina has 66 species and subspecies, *Nanos* has 61 and *Epactoides* 37 (I, II). Divided by their age (based on mean substitution rate of 0.012 substitutions/site/My), the smallest and the largest clade have resulted in the least species, two per a million years, while Helictopleurina has three and *Nanos* four (I, II). These differences do not need to reflect just differences in speciation rates, they may also reflect the extinction rates. Nonetheless, I suggest that the high apparent speciation rate in *Nanos* may be related to their generalist feeding habits, increasing the amount and stability of food resources (II).

The two oldest lineages, *Arachnodes* in Canthonini and Helictopleurina, resemble each other in terms of large average and great variation in body sizes, as well as the species being more specialised to use dung than species in the other clades (I, II). In Helictopleurina the basal clades use dung, while more derived ones use mostly carrion or are generalists (I). In *Arachnodes,* the basal branching order could not be resolved, but some of the dung specialists are closely related while the others are not (II). Although wet forest is the main habitat for all the four radiations, in the two oldest and largest radiations several species live in dry forests, and a few species have adapted to living in open areas using cattle dung (discussed further below). These similarities between *Arachnodes* and Helictopleurina

are likely to be due to their early arrival, as dung is the preferred as food resource (Cambefort 1991a) but is represented by a limited range in Madagascar. Large body sizes may have evolved in association with the now extinct Malagasy megafauna (II). The species in the *Nanos* clade are largely generalists and nocturnal, and they occur also to some extent in dry forests, while *Epactoides* are almost completely restricted to wet forests and many species are specialised to use either dung or carrion (II, III). Helictopleurina are diurnal, mostly active during the warmest part of the day (Viljanen et al. in prep.), while the majority of Canthonini are nocturnal. However, in each clade there are some exceptional species, increasing the width of the diel activity niche used by the clade (II).

The mode of radiation differs among the lineages, especially among the Canthonini clades, based on the most recent speciation events. Species in the two most specious clades (*Arachnodes* and *Nanos*) appear to have evolved mostly in allopatry, whereas in the *Epactoides* clade closely related species commonly have similar geographical ranges but they differ in ecological traits, suggesting that ecological speciation possibly in sympatry has contributed to lineage splitting (II). In Helictopleurina comparable analyses have not been made, but considering the feeding behaviours and body sizes, it appears that in some subclades closely related species have very similar ecologies but differ in their ranges, whereas in other clades the opposite is true (Fig. 4 in I, own

unpublished data). These results imply a role for both nonecological and ecological mechanisms of speciation, which of course could be expected a priori. In any case, and regardless of the role of nonadaptive mechanisms in radiations, it is clear that during the long history of dung beetle evolution in Madagascar, different lineages have evolved to use a range of resources available and fill the niches for dung beetles in Malagasy forests.

Only one of the four lineages consists of monophyletic genera, in fact just one genus, *Epactoides*, following the synonymisation of five small genera (III). In Helictopleurina, two genera are still recognised, although the monotypic *Heterosyphus* is placed within *Helictopleurus* both in the molecular phylogeny and morphologically (I). In the two larger Canthonini clades the genera appear polyphyletic based on the molecular phylogeny (II), but morphologically *Epilissus* and *Arachnodes* are considered to be distinct genera (Montreuil in prep.). Much taxonomic work has been conducted on Malagasy dung beetles in the past eight years (III, Montreuil 2003a, b, 2004, 2005a, b, c, d, 2006, 2007, 2008a, b, 2009, in prep.; Montreuil & Viljanen 2007), but the present molecular phylogenies suggest that there are still questions that require further work.

6.3. Speciation

The present results suggest that speciation in the four dung beetle lineages has mostly occurred in allopatry (II), which has been the conclusion also for most other Malagasy taxa (Vences et al. 2009). Many different mechanisms are likely to have contributed to the high species richness in Madagascar (Pearson & Raxworthy 2009; Raxworthy et al. 2007; Raxworthy et al. 2008; Vences et al. 2009; Wilmé et al. 2006; Wollenberg et al. 2008), but the most obvious ones for dung beetles appear to have been rivers as dispersal barriers and forest and montane refugia leading to large-scale fragmentation of species' ranges during cool and dry climatic periods (II, IV). Species in the *Arachnodes* and *Nanos* clades are very sedentary (II, Viljanen 2009), and hence relatively small barriers to dispersal could restrict gene flow (II). Changing climatic conditions in the Cenozoic (Zachos et al. 2001) could have caused the forests to periodically retreat and expand again in eastern Madagascar with highly variable topography and numerous large rivers, leading to comparable waxing and waning of populations (II, IV, Pearson & Raxworthy 2009; Vences et al. 2009; Wilmé et al. 2006; Wollenberg et al. 2008). The same forces appear to have caused divergence between populations of the *Nanos* species, which have evolved in the past seven My during which there have been strong oscillations in global climate (IV, Zachos et al. 2001). Geographic populations of the abundant species in *Nanos* are strongly diverged in the two genetic regions analysed in this study, suggesting low rate of dispersal (IV). On the other hand, and in spite of long estimated times of lineage splitting, there is evidence for genetic introgression between the two most common species, *Nanos viettei* and *N. dubitatus*, indicating that effective reproductive barriers have not yet evolved. Some geographic populations have apparently remained completely isolated for long time, indicated by strong genetical divergence (IV). Similar results have been reported for other Malagasy taxa (Guschanski et al. 2007; Hapke et al. 2005; Lehtinen et al. 2007; Olivieri et al. 2007; Olson et al. 2004; Vieites et al. 2006; Yoder et al. 2000).

Interspecific competition is likely have played a role in the radiations of Malagasy dung beetles by promoting adaptations in species and clades of species to dissimilar resources and habitats, perhaps following the secondary contact of species that have initially diverged in allopatry (Schluter 2000). Dung beetles in general are highly competitive (Hanski & Cambefort 1991; Horgan 2005; Horgan & Fuentes 2005; Krell et al. 2003; Vernes et al. 2005), which most likely contributes to small ranges and distinct altitudinal divergence of closely related species. In the *Arachnodes* and *Nanos* clades there are several very closely related, similar-sized species pairs with allopatric distributions, which occur at similar altitudes but are replaced by other congenerics at lower or higher altitudes (Viljanen et al. in prep.). The massive recent range expansions in the *Helictopleurus* and *Epilissus* species that have shifted to use cattle dung since the introduction of cattle to Madagascar further supports the notion that competition often limits the range sizes of Malagasy dung beetles (II, V, Rahagalala et al. 2009).

6.4. Ecological diversification

It is likely that the ancestral forms of Malagasy dung beetles used dung as their resource, but the species were forced to largely shift to carrion when they started to radiate in forests (I, II, III) where the range of dung resources is limited and the dung of large herbivores has been entirely lacking. Shift from dung to carrion in tropical wet forests is not restricted to Madagascar, as this shift is considered an important adaptation in the tropical forests dung beetle faunas in general (Halffter 1991), but the shift has been especially strong in Madagascar (Viljanen et al. in prep.). In both Helictopleurina and Canthonini the majority of species are generalists and able to use a variety of resources, though in both groups there are also several species specialising in primate dung (I, II). Four *Helictopleurus* and four *Epilissus* species use cattle dung in open areas and dry forests, and the most common ones of these species have exceptionally large geographical ranges (I, II, V). This resource shift is of recent origin, as native ungulates do not occur in Madagascar and cattle were introduced only 1500 years ago to the island (Burney et al. 2004). In *Helictopleurus* different processes account for the large current ranges. Two of the common cattle dung specialists have hardly any genetic variation across the entire ranges covering nearly all of the island, suggesting that the shift to cattle dung occurred within a very limited area followed by quick range expansion. In contrast, in *H. quadripunctatus* there is substantial genetic variation across the geographical range of the species, suggesting that the shift to cattle dung occurred in many populations across the past range of the species (V). The cattle dung using *Helictopleurus* are not closely related, whereas the two most common cattle dung specialising *Epilissus* species are very closely related (I, II, V).

In the genus *Arachnodes* several species have evolved to an arboreal life style (II). Elsewhere in the tropics the shift to living in forest canopy has occurred in many distinct Scarabaeinae lineages, including species in the tribe Onthophagini (Davis et al. 1997; Vulinec et al. 2007), which is closely related to Helictopleurina (II), but in Madagascar only species of the oldest Canthonini radiation (the *Arachnodes* clade) have moved to the canopy. On the other hand, within *Arachnodes* this shift appears to be relatively easy, as it has evolved several times independently. Only in one case there is indication of speciation following the shift, as three arboreal species form a monophyletic clade (II). Arboreality in dung beetles is considered to be an adaptation to reduce severe resource competition (Hanski & Cambefort 1991). In Madagascar, most arboreal species have exceptionally large geographical ranges, which may indicate relaxed competition.

In three Canthonini genera, *Apterepilissus*, *Epilissus* and *Epactoides*, some species have reduced wings or the species have completely lost their wings. In *Epactoides* the wingless species inhabit montane areas above 1300 meters asl, and the loss of wings has occurred at least twice. It is likely that the loss of wings followed the colonisation of a mountain range but before splitting into separate species, as poor dispersal capacity of the wingless species is likely to have facilitated speciation (III). For the wingless genus *Apterepilissus* with four species in the clade *Arachnodes,* distributional data are unfortunately lacking, but *Epilissus guillaumeti* with reduced wings (O. Montreuil, pers. comm. 2009) occurs between 1000-1900 meters, suggesting a similar high elevation distribution as in *Epactoides*.

7. CONCLUSIONS AND FUTURE PERSPECTIVES

The ancestors of extant Malagasy Helictopleurina and Canthonini arrived to the island from overseas in probably four separate occasions during Cenozoic. Each of these four colonisations was followed by an extensive radiation, and together the four radiations have produced the current fauna of about 250 species. The three lineages of Canthonini and the subtribe Helictopleurina have adapted to exploit a variety of resources and habitats available in Madagascar, but the vast majority of species occur in wet forests. The Helictopleurina and Canthonini appear to occupy the niches of dung beetles in forests so completely that the subsequent colonisations of Madagascar by Onthophagini and Scarabaeini have not led to radiations and none of these species have managed to enter forest habitats.

We were not able resolve the origins of the Canthonini clades, which would have been helpful for better understanding of the patterns of dispersal through time and the evolution of ecological traits. Obviously in order to address this question our sampling needs to be expanded to include a larger set of taxa from the southern continents and a larger number gene regions.

In addition to adaptive differentiation and allopatric divergence sexual selection is likely to have contributed to speciation processes in Malagasy dung beetles as evidenced by striking evolution of male genitals, which most likely contributes to reproductive isolation (Hosken & Stockley 2004; Sirot 2003; Sota & Kubota 1998; Wenninger & Averill 2006). It would be interesting to examine more closely sexual selection in Malagasy dung beetles. Further investigations of genetic differentiation of closely related species would shed light on the mechanisms that have led to the current patterns in altitudinal and geographical occurrence. A key question is whether speciation has occurred along the altitudinal gradient or whether differences in altitudinal occurrence are due to secondary contact of taxa that have diverged during periods of allopatric distributions.

8. ACKNOWLEDGEMENTS

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