Cave crickets and cave weta (Orthoptera, Rhaphidophoridae) from the southern end of the World: a molecular phylogeny test of biogeographical hypotheses

Giuliana Allegrucci, Steve A. Trewick, Angela Fortunato, Gianmaria Carchini and Valerio Sbordoni

(GA, AF, GC, VS) Dept. of Biology, University of Rome Tor Vergata Via della ricerca scientifica snc 00173 ROME – ITALY.
(SAT) Ecology Group, Institute of Natural Resources, Massey University, Palmerston North, Private Bag 11-222, New Zealand.

Abstract

In this study we reconstructed the molecular phylogeny and attempted to infer historical biogeography of a sample of cricket species, most of them cave-dwelling, belonging to the subfamily Macropathinae (Orthoptera, Rhaphidophoridae) which shows a clear Gondwanan distribution. We sequenced fragments of 4 genes (12S rRNA, 16S rRNA, 18S rRNA and 28S rRNA), for a total of 1993 bp.

We present here preliminary data based on a total of 17 species, 11 belonging to Macropathinae and representative of the main regions of Gondawanaland, 7 to Aemodogryllinae and Rhaphidophorinae from India, Bhutan, China, Philippines and the Sulawesi Islands.

The use of relaxed molecular clocks by means of Bayesian analysis allowed us to estimate the timing of the main cladogenetic events, using calibration of a molecular clock; the clock is based on the plate disjunction of Africa from South America, Australia from Zealandia (New Zealand), or Australia from Antarctica. The latter was considered at two different datings on the basis of two alternative palaeogeographic hypotheses. Node dating using separation of Africa or a model of earlier separation of Australia from Antarctica, suggests that the main cladogenetic events in the Macropathinae phylogeny could be explained by vicariance hypotheses, related to the Gondwana fragmentation. However, two other equally valid calibrations suggest that lineage formation is not consistent with vicariant processes and requires either some long-distance dispersal, or an inconceivable age of origin of this family of insects, enabling the prior existence of all lineages in Gondwanaland with subsequent regional extinction.

Key words

Gondwanaland, molecular rates, biogeography, cave crickets, Macropathinae, Rhaphidophoridae

Introduction

Is plate tectonic vicariance a convincing hypothesis for the distribution of a group of palaeoantarctic Orthoptera Grylloacridoidea? The Orthoptera appear to have originated in the Carboniferous (360-300 Mya,) (Ragge 1955, Brauckmann *et al.* 1994), prior to the breakup of Pangea and Gondwana. Within the Orthoptera, taxa belonging to the family Rhaphidophoridae are considered as phylogenetically old by virtue of their morphology and their wide, disjunct geographic distribution across the temperate areas of the Boreal and Austral hemispheres (Hubbell & Norton 1978).

Various estimates of the time of origin of the Rhaphidophoridae have been proposed, including Upper Carboniferous (Ragge 1955), Permian (Ander 1939), Triassic (Beier 1955) and Jurassic (Sharov 1971), depending on the hypothesized sequence of phyletic branching and the known ages of the fossil Ensifera considered to

be their closest relatives. Hubbell & Norton (1978) suggested that the origin of Rhaphidophoridae could not be more recent than the early Jurassic, since by the end of that epoch the separation of Gondwana had started and dispersal routes were broken.

This and other such inferences are heavily reliant on the *a priori* assumption that taxonomic diversity is explained by vicariance. However, there are few independent data to support this assumption. For instance, the only fossil record of the Rhaphidophoridae consists of three species of *Prorhaphidophora* described from Oligocene (35-25 Mya) Baltic amber (Chopard 1936), thus uninformative on the question.

Karny (1934) and Ander (1939) suggested that, on the basis of their current distribution, Rhaphidophoridae originated in the temperate areas of the southern hemisphere and spread northwards. In one scenario, an ancestral stock reached Laurasia and was the progenitor of all the northern hemisphere subfamilies.

All Rhaphidophoridae species are nocturnal and wingless, with a preference for humid habitats. Most are restricted to caves and appear to disperse little between these patchy habitats, and this lends support to the idea that they would have little potential for dispersal over sea.

At present, Rhaphidophoridae include nine subfamilies mainly distributed in the temperate regions of the northern and southern hemispheres and in the southeast Asia tropics. Among these, the Macropathinae are considered to be the sister group to all others in the family and have been referred to as "primitive" (Hubbell & Norton 1978). This subfamily comprises numerous species, belonging to about 30 genera, that together have a Gondwanan distribution in southwestern Australia, Tasmania, New Zealand and the surrounding islands, South America (Patagonia and Falkland Islands) and southern Africa (Cape Peninsula and the southern tip of South Africa), but not India, where the Rhaphidophoridae are represented by other subfamilies.

The breakup of Gondwana provides one of the most obvious test cases for vicariance biogeography. Gondwanaland started to break up around 130 Mya in early Cretaceous time into four continental blocks (South America, Africa, India-Madagascar and Australia-Antarctica-Zealandia (Veevers *et al.* 1980, Lawver *et al.* 1992, Li & Powell 2001, Trewick *et al.* 2007, Neall & Trewick 2008). India separated from Madagascar in the late Cretaceous (88–84 Mya) and began moving northward to collide with Asia at about 50 Mya. South America and Africa started to separate in the Early Cretaceous (130 Mya, Powell *et al.* 1988, Lawver *et al.* 1992) with the opening of the South Atlantic Ocean. The complete separation of Africa from South America happened during the Mid-Cretaceous (96 Mya; Powell *et al.*

Table 1. Raphidophoridae species and outgroup taxa included in this study.

Species	Collection localities	Code	GenBank accession no.
Outgroups			
Dolichopoda sbordonii	Karain Cave, Antalya, Turkey	KAR	12S:EF216936; 16S:EF216966; 18S:HM594460; 28S-D1:HM594424; 28S-D3-D5:EF216998
Troglophilus cavicola	Covoli di Velo Caves, Veneto, Northern Italy	TRO	12S:EF216946; 16S:AY793564; 18S:HM594459; 28S-D1:HM594423; 28S-D3-D5:EF217003
Euhadenoecus insolitus	Indian Grave Point Cave, The Kalb Co., TN, USA	IND	12S:EF216948; 16S:AY793563; 18S:HM594458; 28S-D1:HM594422; 28S-D3-D5:EF217005
Hadenoecus cumberlandicus	Bat Cave, Carter Cave State Park, Carter Co., KY, USA	BAT	12S:EF216947; 16S:AY793562 18S:HM594457; 28S-D1:HM594421; 28S-D3-D5:EF217004
Ingroups			
Macropathinae			
Micropathus tasmaniensis	Gross Cave (IB-144), Ida Bay, Tasmania	TAS	12S:HM594538; 16S:HM594506; 18S:HM594474; 28S-D1:HM594438; 28S-D3-D5:HM594570
	Slug Inn Cave (IB-152), Ida Bay, Tasmania	IDB	12S:HM594537; 16S:HM594505; 18S:HM594473; 28S-D1:HM594437; 28S-D3-D5:HM594569
	Junee Cave, Mt Field National Park, Hobart, Tasmania	JNC	12S:HM594539; 16S:HM594507; 18S:HM594475; 28S-D1:HM594439; 28S-D3-D5:HM594571
Pachyramma edwardsii	Mts Kaukau, Wellington, New Zealand	WEL	12S:HM594541; 16S:HM594509; 18S:HM594477; 28S-D1:HM594441; 28S-D3-D5:HM594573
	Karori, Wellington, New Zealand	CW122	12S:HM594540; 16S:HM594508; 18S:HM594476; 28S-D1:HM594440; 28S-D3-D5:HM594572
Pleioplectron simplex	Caversham, Dunedin, New Zealand	CW306	12S:HM594544; 16S:HM594512; 18S:HM594480; 28S-D1:HM594444; 28S-D3-D5:HM594576
Talitropsis sedilotti	Papaitonga, Levin, New Zealand	CW160	12S:HM594543; 16S:HM594511; 18S:HM594479; 28S-D1:HM594443; 28S-D3-D5:HM594575
Pallidoplectron turneri	Waitomo, New Zealand	GR1	12S:HM594545; 16S:HM594513; 18S:HM594481; 28S-D1:HM594445; 28S-D3-D5:HM594577
Macropathus filifer	Te Anaroa, Golden Bay, New Zealand	CW301	12S:HM594542; 16S:HM594510; 18S:HM594478; 28S-D1:HM594442; 28S-D3-D5:HM594574
Parudenus falklandicus	Falkland Islands	PAR	12S:HM594534;16S:HM594502; 18S:HM594470; 28S-D1:HM594434; 28S-D3-D5:HM594566
Heteromallus cavicola	Milodon Cave, Puerto Natales, Chile	MIL	12S:HM594531; 16S:HM594499; 18S:HM594467; 28S-D1:HM594431; 28S-D3-D5:HM594563
	Milodon Cave, Puerto Natales, Chile	MIL2	12S:HM594532; 16S:HM594500; 18S:HM594468; 28S-D1:HM594432; 28S-D3-D5:HM594564
	Milodon Cave, Puerto Natales, Chile	PAT	12S:HM594533; 16S:HM594501; 18S:HM594469; 28S-D1:HM594433; 28S-D3-D5:HM594565
Udenus w-nigrum	Mt Aymond, Pali Aike National Park, Chile	MAY	12S:HM594536; 16S:HM594504; 18S:HM594472; 28S-D1:HM594436; 28S-D3-D5:HM594568
	Riserva Nacional Cerro Castillo, La Cumbre, Argentina	CER	12S:HM594535; 16S:HM594503; 18S:HM594471; 28S-D1:HM594435; 28S-D3-D5:HM594567
Spelaeiacris tabulae	Kalk Bay Cave, Vishoek, South Africa	KAL	12S:HM594528; 16S:HM594496; 18S:HM594464; 28S-D1:HM594428; 28S-D3-D5:HM594560
	Wynberg Cave, Cape Town, South Africa	WYN	12S:HM594529 16S:HM594497; 18S:HM594465; 28S-D1:HM594429; 28S-D3-D5:HM594561
	Bat's Cave, Cape Town, South Africa	BAT	12S:HM594525; 16S:HM594493; 18S:HM594461; 28S-D1:HM594425; 28S-D3-D5:HM594557
	Boomslang Cave, Cape Town, South Africa	BOO	12S:HM594526; 16S:HM594494; 18S:HM594462; 28S-D1:HM594426; 28S-D3-D5:HM594558
	Hagmans Cave, Cape Town, South Africa	HAG	12S:HM594527 16S:HM594495; 18S:HM594463; 28S-D1:HM594427; 28S-D3-D5:HM594559
Spelaeiacris monslamiensis	Shale Peak Cave, Hex River Mountain, Ceres, South Africa.	SHA	12S:HM594530; 16S:HM594498; 18S:HM594466; 28S-D1:HM594430; 28S-D3-D5:HM594562
Aemodogryllinae			
Atachycines sp.	Cerrapunji Cave, Khasi Hills, Meghalaya, India	IND	12S:HM594556; 16S:HM594524; 18S:HM594492; 28S-D1:HM594456; 28S-D3-D5:HM594588
Diestrammena sp.	Jing Long Dong (Gold Dragon Hole), Guang-dong, QingXin County, China	CHI1	12S:HM594552; 16S:HM594520; 18S:HM594488; 28S-D1:HM594452; 28S-D3-D5:HM594584
	Guangdong, Ruyuan County, Ting Xia Wo Dang, China	CHI2	12S:HM594553; 16S:HM594521; 18S:HM5944889; 28S-D1:HM594453; 28S-D3-D5:HM594585
	Guangdong Artificial Cave, Yingde City, China	CHI3	12S:HM594554; 16S:HM594522; 18S:HM594490; 28S-D1:HM594454; 28S-D3-D5:HM594586
	Shui Chi Dong (Water Pool Cave), Bei Pan Jiang, Zhen Feng County, Guizhou Province, China	CHI4	12S: HM594555;16S:HM594523; 18S:HM594491; 28S-D1:HM594455; 28S-D3-D5:HM594587
Diestramima sp.	Dongdichu, Tashi Yangse district, Buthan	BUT1	12S:HM594550; 16S:HM594518; 18S:HM594486; 28S-D1:HM594450; 28S-D3-D5:HM594582
	Buyang-Chung Du, Tashi Yangse discrict, Bhutan	BUT2	12S:HM594551; 16S:HM594519; 18S:HM594487; 28S-D1:HM594451; 28S-D3-D5:HM594583

Table 1. Continued.

Rhaphidophorinae			
Raphidophora sp.	Gua Saripa (Saripa Cave), Bantimurung, Su-lawesi, Indonesia	CELSG	12S:HM594548; 16S:HM594516; 18S:HM594484; 28S-D1:HM594448; 28S-D3-D5:HM594580
	Gua Londa (Londa Cave), Rantepao, Sulawesi, Indonesia	CELG2	12S:HM594549; 16S:HM594517; 18S:HM594485; 28S-D1:HM594449; 28S-D3-D5:HM594581
	Urrak Cave, Mantalongon, Cebu Is, Philippines	PHI	12S:HM594546; 16S:HM594514; 18S:HM594482; 28S-D1:HM594446; 28S-D3-D5:HM594578
	Maitong Cave, Behind the Clouds, Batuan, Bohol Is, Philippines	PHI2	12S:HM594547; 16S:HM594515; 18S:HM594483; 28S-D1:HM594447; 28S-D3-D5:HM594579

1988, Lawver *et al.* 1992). Zealandia (later New Zealand), Australia, South America, and the then warm-temperate Antarctica, remained connected until late Cretaceous time: east Antarctica was closest to southern Australia and Zealandia, and southern South America was in contact with west Antarctica. Zealandia was the first to separate from Australia-Antarctica in the late Cretaceous (~83 Mya) opening the Tasman Sea (Trewick *et al.* 2007, Campbell & Hutching 2007, Graham 2008). Zealandia subsequently thinned and submerged, with marine transgression peaking in late Oligocene time, and it is uncertain how much land, if any, persisted in the vicinity of New Zealand (Trewick *et al.* 2007, Landis *et al.* 2008). New Zealand began to form in early Miocene time with the abrupt onset of plate tectonic activity, but it is possible that one or more small islands existed prior to this (McLoughlin 2001). Australia and South America remained in contact via Antarctica until final separation and formation of the Southern ocean during the late Eocene (35 Mya). Although Australia and Antarctica started to separate in the late Cretaceous (90 Mya), the process is thought to have been completed in the late Eocene (~35 Mya) (Veevers 1984, Exon *et al.* 2004). An alternative proposal for the earlier existence of a shallow marine seaway between Australia and Antarctica suggests separation by 50 Mya (Woodburne & Case 1996). Southern South America and Antarctica remained in contact through the Antarctic Peninsula until the Oligocene (35-28 Mya), when the Drake Passage opened between these continents (Lawver *et al.* 1992, Dingle & Lavelle 2000).

Meta-analyses of area cladograms (Sanmartin & Ronquist 2004) show that many animal phylogenies match the sequential break-up of Gondwana and are thus consistent with a plate tectonic vicariance hypothesis. However, there is an increasing number of southern hemisphere taxa for which divergence time estimates do not fit the temporal framework of fragmentation (Cooper *et al.* 2001, Van Bocxlaer *et al.* 2006, Raxworthy *et al.* 2002) leading to the conclusion that the distribution of these organisms resulted from overseas dispersal events (Raxworthy *et al.* 2002). In this paper we examined the historical biogeography of Macropathinae, using taxa representative of each of the regions encompassed by the subfamily. We also considered species belonging to two Asian subfamilies that are the putative sister groups to Macropathinae: Aemodogryllinae and Rhaphidophorinae from India, Bhutan, China, Philippines and the Sulawesi Islands. From the biogeographical point of view, these insects, being apterous and characterized by low vagility, could be responsive to vicariance biogeography.

We present analysis of preliminary mitochondrial and nuclear DNA data using a relaxed molecular clock approach with Bayesian analysis. The molecular clock was calibrated using the dated breakup of Gondwanaland. We considered one palaeogeographic event at time, and then assessed the extent of support for vicariance implied across the phylogeny. In this way, alternative biogeographic hypotheses (based on the available palaeogeographic data available for a given region) were analysed to determine the most plausible scenario that may explain the current distributional pattern of Macropathinae. We apply the null hypothesis that lineage formation

(phylogenetic splitting) resulted from sundering of populations by plate tectonic vicariance, and ask whether: 1) within the constraints of the available sampling, is Macropathinae tree topology consistent with the timing of Gondwanan breakup? 2) Are estimates of the age of inferred ancestors consistent with this process? 3) Do different calibrations result in agreement or do they each require a unique biogeographic hypothesis to explain the Gondwanan influence on the distribution of Macropathinae lineages?

Materials and Methods

Taxon sampling.—In this study a total of 17 species were assayed (Table 1). In particular, we considered 11 species among Macropathinae: *Spelaeiacris tabulae* and *Spelaeiacris monslamiensis* from South Africa; *Heteromallus cavicola*, *Udenus w-nigrum* and *Parudenus flaklandicus* from South America; *Micropathus tasmaniensis* from Tasmania; *Pachyramma edwardsii*, *Pleioplectron simplex*, *Tallitropsis sedilotti*, *Pallidoplectron turnerii* and *Macropathus filifer* from New Zealand.

Among Aemodogryllinae, we sampled species belonging to genera *Atachycines*, *Diestrammena* and *Diestramima* from India, China and Bhutan and among Rhaphidophorinae, species belonging to genus *Raphidophora* from the Philippines and Sulawesi Islands. As an outgroup we used representatives of other subfamilies: Nearctic Ceuthophilinae (*Hadenoecus cumberlandicus*, *Euhadenoecus insolitus*) and Palaearctic Troglophilinae (*Troglophilus cavicola*) and Dolichopodainae (*Dolichopoda sbordonii*). We analyzed more than one individual for each species where samples were available (Table 1). Our sampling included recently collected specimens as well as those from museum collections.

Laboratory procedures.—DNA was isolated from leg muscles from specimens preserved in 85-95% EtOH, following either a C-TAB extraction protocol (Doyle & Doyle 1987) or a salting-out method (Cook *et al.* in press).

Four gene fragments from each DNA sample were amplified using the polymerase chain reaction (PCR), corresponding to the genes 12S, 16S, 18S and 28S rRNA subunits. PCR and sequencing of 28S rRNA targeted domains D1 and D3-D5. Age and often suboptimal preservation of some museum samples meant that DNA was highly fragmented. So it was necessary to use reamplification with a set of nested primers to obtain complete sequences.

Initial PCR reactions used the primers 485 and 689, and 427 and 686 for amplification of the D3-D5 and D1 domains of the 28S rDNA gene (Friedrich & Tautz 1997); 8F and 9R for 18S (Giribet *et al.* 1996); 12SAi and 12SBi for 12S (Simon *et al.* 1994) and 16SAr and 16SBr for 16S rRNA (Palumbi 1996). Internal primers used in the nested-PCRs were designed from those sequences that could be obtained with a single PCR reaction with the primers above (See Table 2). Double-stranded amplifications were performed with a Perkin-Elmer-Cetus thermal cycler (PE Applied Biosystems, Foster City, CA, USA) in 25 μl of a solution containing genomic DNA, 1.5 mM $MgCl_2$, 2.5 mM of each dNTP, 0.5 μM Primer, 1 U EuroTaq

Table 2. Internal primers specifically designed for Rhaphidophoridae.

16Srha106F	5′CGGTCTGAACTCAGATCACGT3′	12Srha96F	5′AAGAGCGACGGGCGATGTGT3′
16Srha207R	5′AGATTGCGACCTCGATGTT3′	12Srha250R	5′GTTTATAATTAAGGGGAGATG3′
16Srha145F	5′CAGACTTATAATTTAAACTTC3′	12Srha158F	5′TTACTTTCAAATCCACCTTC3′
16Srha257R	5′ACCTTAGGGATAACAGCG3′	12Srha326R	5′CGCCGTCATCAGATTRTCTT3′
16Srha189F	5′AACATCGAGGTCGCAATCT3′	12Srha248F	5′AACTGCACCTTGACCTGACA3′
16Srha354R	5′TTTATTGGGGTGATARGAAG3′	12Srha378R	5′CCCATTACGATGATTCACGTT3′
16Srha244F	5′GTTATCCCTAAGGTAACTTAATC3′	12Srha307F	5′AAGARAATCTGATGACGGCG3′
16Srha438R	5′TTAGAGGACGAGAAGACCC3′	12Srha426R	5′GAAACTCAAAGAATTTGGCGG3′
16Srha409F	5′AAAGCTCTATAGGGTCTTC3′	12Srha357F	5′AACGTGAATCATCAATAACGGG3′
16Srha559R	5′GCAAAGGTAGCATAATCATTAG3′	12Srha492R	5′TAGGATTAGATACCCTATTATT3′
16Srha510F	5′CCMTTCATTCCAGTCTTCA3′	28S.H1for	5′ACCAGCTATCCTGAGGGAAAC3′
16Srha585R	5′TTRAAGGGCCGCGGTATTTA3′	28S.L1rev	5′GGGTATAGGGGCGAAAGACT3′
16Srha548F	5′TGTTACCTTTGCACGGTCAT3′	28S.L2rev	5′TAGGGGCGAAAGACTAATCG3′
16Srha651R	5′CGCCTGTTTATCAAAAACAT3′		

(EuroClone) and the buffer supplied by the manufacturer.

Standard PCR conditions included a 2-min denaturation at 94°C followed by 5 cycles at 95°C for 15s, 48°C for 45s, and 62°C for 40s, and 30 cycles at 95°C for 30s, 50°C for 30s, and 72°C for 30s, with a final extension at 72°C for 3min. The nested-PCRs included a 2-min denaturation at 94°C, followed by 30 cycles at 95°C for 30s, 50°C for 30s, and 72°C for 30s, with final extension at 72°C for 3 min. These parameters varied for each primer pair used, mainly using higher or lower annealing temperatures.

Double-stranded amplified products were checked for expected size by electrophoresis of 1/10 of the product through a 1% agarose gel. PCR fragments were purified using the ExoSAP digestion (GE Healthcare Europe, Munich, Germany), directly sequenced in both directions using the 'Big Dye Terminator Ready-reaction Kit' and resolved on ABI 3100 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA), following the manufacturer protocols. Sequence data were edited and compiled using CodonCode Aligner version 3.5.2 (CodonCode Corporation) or Sequencher ver. 4.2 sequence editor (Gene Codes Corp., Michigan).

Phylogenetic analysis.—Sequences of the four gene fragments, 12S, 16S, 18S, 28S (D1 and D3-D5 domains) were aligned separately using CLUSTALX 2.0 (Thompson *et al.* 1997) with opening gap = 10 and extended gap = 0.10. The alignments were also checked by eye. Models for DNA substitution were selected independently for each gene using MODELTEST 3.0.6 (Posada & Crandall 1998). Phylogenetic analyses were performed on the concatenated dataset, partitioned by genes. We used maximum-likelihood (ML, Felsenstein 1981), as implemented in TREEFINDER 2008 (Jobb 2004), and Bayesian estimation as implemented in MRBAYES 3.1.2 (Huelsenbeck & Ronquist 2001), using the model selected by MODELTEST. One thousand bootstrap replicates were carried out in ML analysis, while Bayesian analysis was performed using four search chains for 1,000,000 generations, sampling trees every 100 generations. The first 1,000 trees were discarded as burn-in. Parameter stability was estimated by plotting log-likelihood values against generation time, and a consensus tree with posterior probabilities was then generated.

Dating of the cladogenetic events.—A likelihood ratio test (LRT) was used to test the molecular clock hypothesis, according to Huelsenback & Crandall (1997). The LRT results suggested a heterogeneous rate variation for each gene and therefore a clocklike evolution of sequences could not be assumed. Consequently, dates of divergence were inferred using a relaxed molecular clock, following the uncorrelated relaxed lognormal clock (Drummond *et al.* 2006) as implemented in BEAST (v.1.4.7, Drummond & Rambaut 2007). This analysis was performed on the concatenated matrix, partitioned by genes. A Yule or "pure birth" prior process was applied to model speciation. The time to the most recent common ancestor (MRCA)
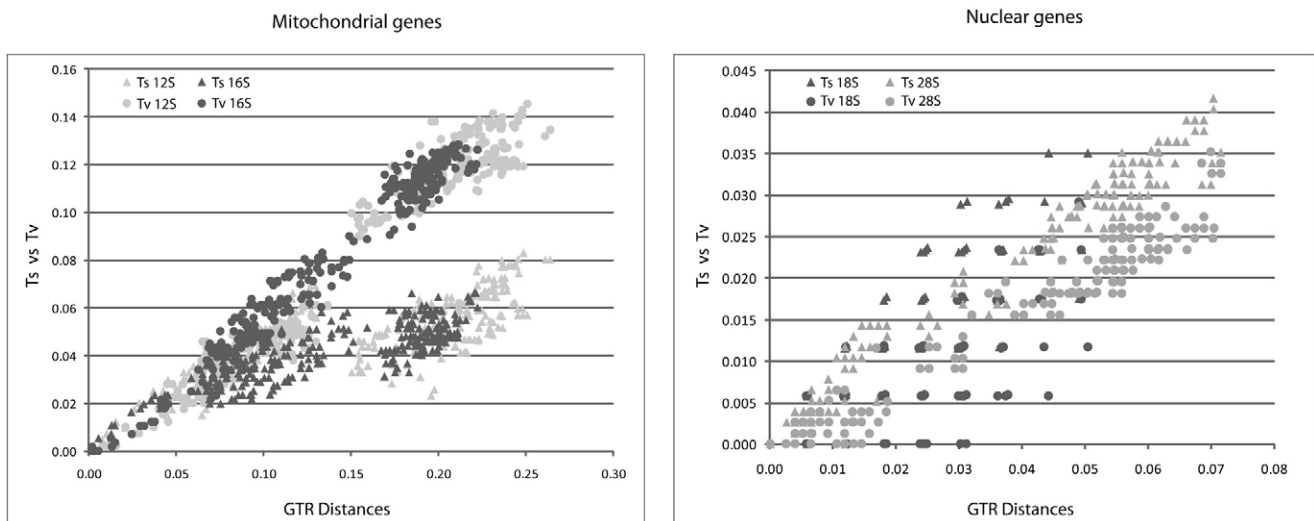

Fig. 1. Relationship between the number of transitions and/or transversions and genetic distance values (GTR distances).

between each clade was estimated under the model parameters highlighted in MODELTEST (Posada & Crandall 1998) for each gene. Two independent runs of 10 million steps were implemented with BEAST, with one million steps discarded as burn-in. Convergence to stationarity and effective sample size (ESS) of model parameters were assessed using TRACER 1.4 (Rambaut & Drummond 2003).

Current estimates of the timing of plate tectonic vicariance events were used to calibrate the molecular clock under the null hypothesis, that separation of land areas by tectonics was the cause of phylogenetic splitting in these taxa. Specifically, we considered separation of South Africa from Gondwana, separation of Australia from Zealandia and separation of Australia from Antarctica. When considering alternative palaeogeographic interpretations, these yield four potential calibrations: (i) isolation of South Africa 96-117 Ma (Powell et al. 1988, Lawver *et al.* 1992), (ii) the separation of Australia from Zealandia (New Zealand) 80-82 Ma (Li & Powell 2001), (iii) separation of Australia from Antarctica 35-40 Ma (Veevers 1984, Lawver *et al.* 1992) and (iv) separation of Australia from Antarctica 50-54 Ma (Woodburne & Case 1996). We calibrated the tree using one calibration point at a time, to assess the effect on inferred age of the other nodes, comparing this with geological age estimates.

Results

The total length of the alignment of the four gene fragments was 1993 bp, consisting of 414 bp 12SrRNA, 583 bp 16SrRNA, 173 bp 18SrRNA, and 813 bp 28SrRNA. The Ts/Tv ratio ranged from 0.17 to 5 for 12S, from 0 to 6 for 16S and 18S and from 0 to 8 for 28S. The Ts accounted for about 36% of all substitutions in mitochondrial genes and for about 63% in the nuclear ones, if outgroups are considered. Saturation was not observed, as demonstrated by the relationship between the number of Ts and Tv versus genetic distances (Fig. 1). MODELTEST indicated the general time-reversible model with among-site rate heterogeneity and a proportion of invariant sites for the mitochondrial genes and the nuclear 28S (GTR + I + Γ). GTR with the proportion of invariant sites was also indicated for 18S, but without among-site rate variation (GTR +I).

ML and Bayesian analyses gave trees of similar topology with the same nodes supported in all analyses (Fig. 2). Two principal clades can be distinguished, one including the Asian taxa and the other the palaeo-austral Macropathinae. The Asian clade consists of two clusters corresponding to the two subfamilies, Rhaphidophorinae and Aemodogryllinae. Among Macropathinae we can distinguish four well-supported clades, corresponding almost perfectly with South American, Tasmanian, New Zealand and South African taxa.
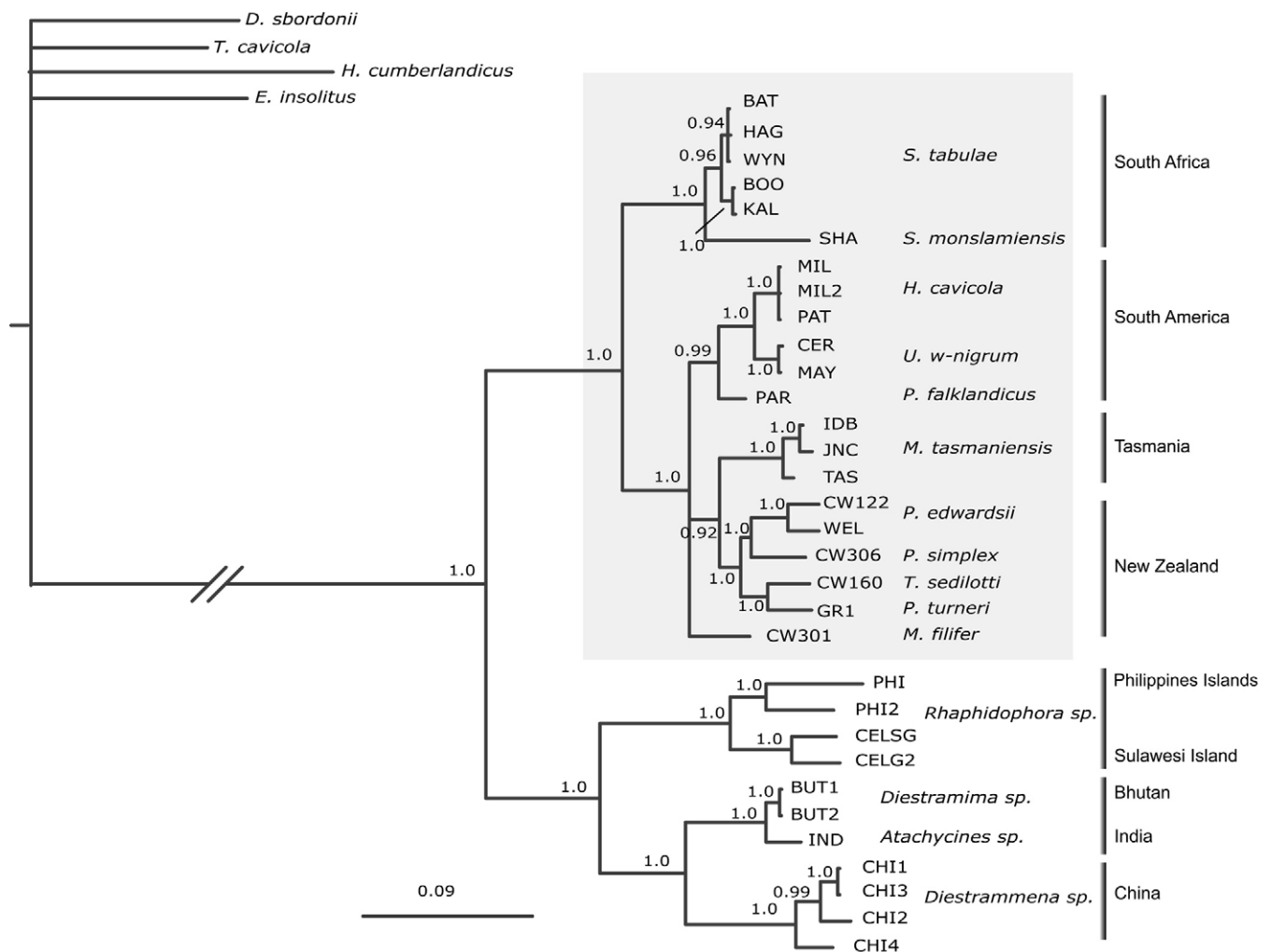

Fig. 2. Relationships among species of Rhaphidophoridae inferred by Bayesian analysis. The ingroup is indicated by the grey square. Values above the branches indicate posterior probabilities (PP) derived from Bayesian analysis.
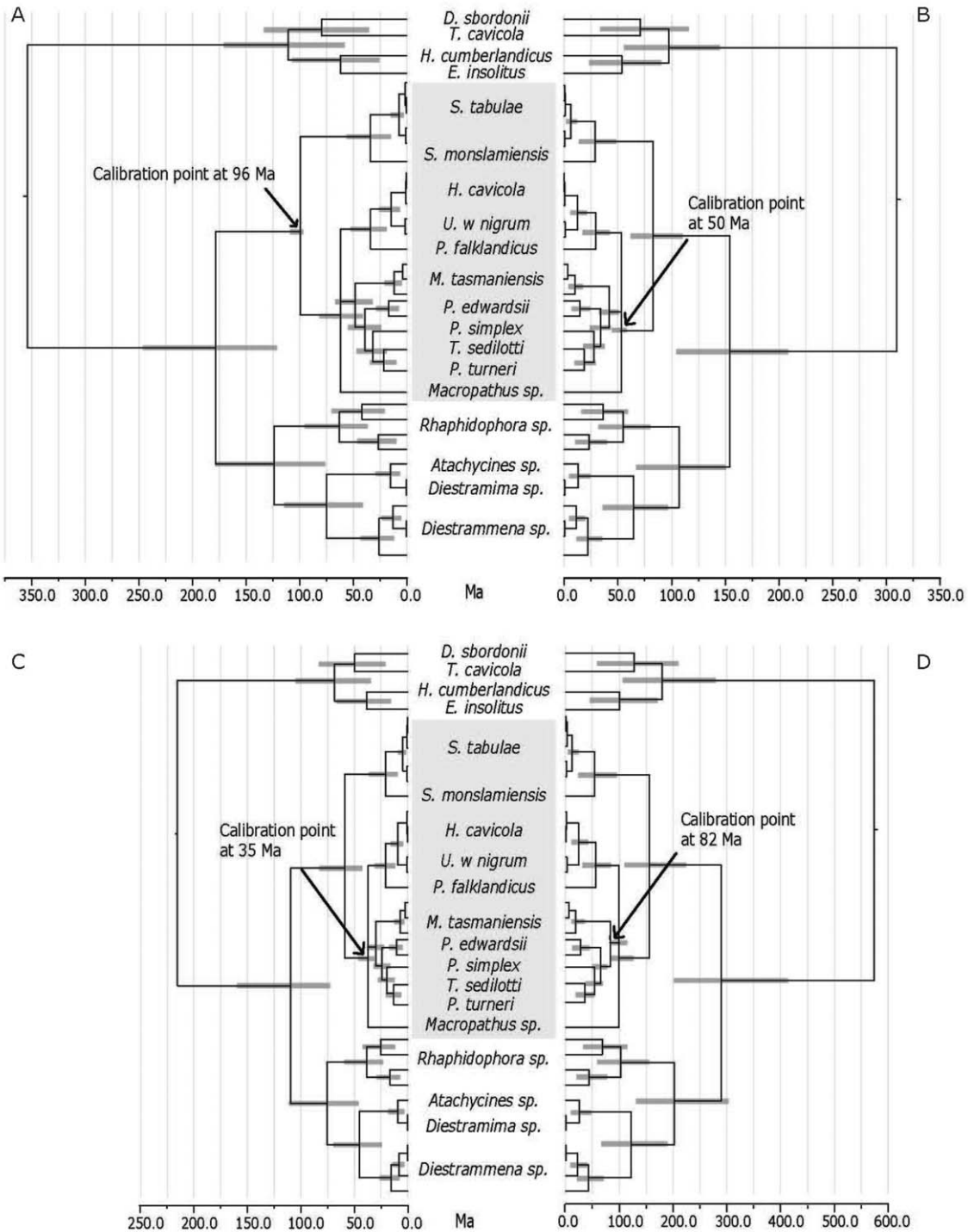
Fig. 3. Divergence times among Rhaphidophoridae species inferred by Bayesian analysis using a relaxed molecular clock and different calibration points. A) Calibration point considering the isolation of Africa at 96 Mya; B) Calibration point considering the separation of Australia from Antarctica at 50 Mya; C) Calibration point considering the separation of Australia from Antarctica at 35 Mya; D) Calibration point considering the separation of Australia from New Zealand at 80-82 Mya. Bars at the nodes represent the 95% highest posterior density (HPD) credibility interval. Ingroup is indicated by grey box.

The South African taxa were sister to the other Macropathinae sampled.

The data showed a nonclock-like behavior, with a coefficient of variation of 0.443 (95% highest posterior density, HPD: 0.222-0.688) if separation of Africa is considered. However, values for the coefficient of variation obtained when considering the other calibration

points were very similar to one another. Using dated palaeoevents for the study region allowed us to calibrate the molecular clock and to estimate the divergence times of lineages enforcing a vicariance hypothesis. Four chronograms obtained using the different calibration points show many similarities, but are not identical in inferred node ages. Calibration using the separation of Africa from South America (96 Mya) indicates that divergence times between taxa in South America and Australasia range from 36 to 73 Mya (95% HPD) with a median of 55 Mya; between Australia and New Zealand taxa range from 31 to 66 Mya (95% HPD) with a median of 48 Mya (Fig. 3A). Calibration using the separation of Australia from Antarctica at 50 Mya resulted in estimates for isolation of the South African Macropathinae lineage between 62 and 110 Mya (95% HPD) with a median of 83 Mya, while the split between New Zealand and Australian taxa is dated at 42 Mya (95% HPD: 32.5-51 Mya) (Fig. 3B). Alternatively, applying a 35 Mya separation date of Australia and Antarctica results in the inferred isolation of South African taxa at 61 Mya (95% HPD: 44-81 Mya) and the divergence of Australia and New Zealand taxa at 31 Ma (95% HPD: 26 to 35 Mya) (Fig. 3C). When we applied a calibration based on the separation of Zealandia (New Zealand) from Australia at 82 Mya, we obtained estimates for isolation of South African Macropathinae at 155 Mya (95% HPD: 107-219 Mya), and divergence of South American and Australasian taxa at 91.5 Mya (95% HPD: 82-108 Mya; Fig. 3D). This calibration also returned an estimate for the basal node in our Rhaphidophoridae phylogeny of close to 600 Mya.

Molecular substitution rates per lineage per million years were also obtained for each analysed gene. These rates are broadly consistent with estimates previously obtained for insects (Brower 1994, Friedrich & Tautz 1997) and those obtained for *Dolichopoda* species (Allegrucci *et al.* unpub.), although all our inferred substitution rates are lower (Table 3). We found that calibration with 82 Mya for a Zealandia split from Australia/Antarctica, resulted in the low-est rates, least consistent with other estimates. Calibration using a 35 Mya date for the split of Antarctica and New Zealand, yielded inferred substitution rates closest to those previously published.

Discussion

The analysis resulted in a well-resolved and generally strongly supported phylogeny. Taxa are, for the most part, clustered by geographic areas, with each main clade corresponding to a particular geographic region. Asian species are, in our analyses, sister to the palaeo-austral Macropathinae, and among the Macropathinae, the South African *S.tabulae* and *S. monslamiensis* are sister to the trans-Pacific taxa. Africa, as the first continent to separate from Gondwana, could have led to isolation of this lineage, while exchange continued between Australia and South America via Antarctica.

Thus, in terms of geographic distribution, we can say that the Macropathinae phylogeny is consistent with a vicariant history, with one exception. In our analysis of the current taxon sample and data, we find that *M. filifer* does not group with other New Zealand Macropathinae (although we also note that this is an unresolved node). This is surprising, and not as predicted from a vicariant biogeographic hypothesis. However, a similar lack of monophyly among New Zealand Orthoptera has been reported for the Anostostomatidae, in which high lineage diversity contrasts with the relatively small land area and young geological history (Pratt *et al.* 2008) and demands further study. Among the Rhaphidophoridae in our present analysis, the Asian subfamilies (Aemodogryllinae, Rhaphidophorinae) are revealed as sister to the Macropathinae. This is also consistent with plate tectonic vicariance, as India separated from other parts of Gondwana at about 130 Mya, prior to other vicariance events.

Estimating rates of molecular evolution and consequent divergence times is a critical issue and its reliability depends on the

Table 3. Substitution rates per gene / site / lineage / million years in Rhaphidophoridae species here analysed. Substitution rates are reported for each used calibration point.

Calibration point: isolation of Africa from South America (96 Myr)	Mean	St. dev. of mean	95% HPD lower	95% HPD upper
12S	1.413 E-3	2.137 E-5	9.071 E-4	2.039 E-3
16S	1.471 E-3	2.268 E-5	9.473 E-4	2.074 E-3
18S	1.274 E-4	2.495 E-6	4.776 E-5	2.217 E-4
28S	2.491 E-4	3.999 E-6	1.513 E-4	3.602 E-4
Calibration point: separation of Australia from Antarctica (35 Myr)				
12S	2.293 E-3	2.398 E-5	1.445 E-3	3.248 E-3
16S	2.373 E-3	2.505 E-5	1.509 E-3	3.274 E-3
18S	2.013 E-4	2.762 E-6	8.332 E-5	3.472 E-4
28S	4.043 E-4	4.196 E-6	2.461 E-4	5.781 E-4
Calibration point: separation of Australia from Antarctica (50 Myr)				
12S	1.609 E-3	2.096 E-5	1.069 E-3	2.207 E-3
16S	1.668 E-3	2.135 E-5	1.151 E-3	2.266 E-3
18S	1.460 E-4	2.124 E-6	6.259 E-5	2.564 E-4
28S	2.852 E-4	3.779 E-6	1.841 E-4	3.978 E-4
Calibration point: separation of Australia from New Zealand (82 Myr)				
12S	8.944 E-4	3.003 E-5	5.520 E-4	1.240 E-3
16S	9.249 E-4	3.027 E-5	5.940 E-4	1.240 E-3
18S	8.121 E-5	3.916 E-6	2.990 E-5	1.390 E-4
28S	1.578 E-4	5.692 E-6	9.920 E-5	2.190 E-4

suitability of the calibration method and the choice of calibration event(s). Here we applied a consistent approach, but varied calibration points to assess the consistency of inferred node ages. Assuming models of DNA evolution are appropriate, rate variation across the tree is accommodated, calibration points are correctly dated and assumptions about vicariant process are valid (or at least clearly stated, see null hypothesis), we can expect consistent estimates of node age among analyses if plate tectonic vicariance is the primary driver of lineage formation in this group. In the present case and following the complex geological history of Gondwanaland masses, we referred to four major geological events to calibrate our molecular clock.

We considered the separation of South America and Africa 96-117 Mya (Fig. 3A; Powell *et al.* 1988, Lawver *et al.* 1992), and it is immediately evident that the topology of our phylogeny with the first major split with the Macropathinae is consistent with this model. This analysis suggests that exchange between South America and Australasia (via Antarctica) was interrupted about 60 Mya, close to the time at which it is estimated that a shallow marine seaway formed between Antarctica and Australia (50 Mya, Woodburne & Case 1996). Inferred divergence of New Zealand and Australian Macropathinae between 31-66 Mya is younger than isolation of Zealandia from Australia. Calibration using the separation of Australia from Antarctica due to the formation of a shallow marine seaway between the two continents (50 Mya; Woodburne & Case 1996), returned divergence estimates congruent with the above, and geological estimates for isolation of Africa, but not separation of Zealandia and Australia (Fig. 3B).

Calibration using a younger, but widely cited, age for separation of Australia from Antarctica at 35 Mya yielded dates for other nodes that are too young for tectonic vicariance of taxa in other regions (Fig. 3C). Conversely, using an age of 82 Mya for the split of Zealandia (later New Zealand) from Australia/Antarctica, a date used in calibration of several bird phylogenies (see discussion in Trewick & Gibb 2010), gave inferred formation of lineage prior to tectonic events for isolation of S. America, S. Africa and Australia. Indeed, the root of the Rhapidophoridae was pushed back to nearly 600 million years (Fig. 3D).

In our calibrations the inferred age for the split of Indian (and other Asian) taxa from Macropathinae is near (Fig. 3C), or older than, the probable time of separation of India from Gondwana. This implies that either the Asian taxa are not (contrary to morphological evidence and unpublished molecular analyses) sister to Macropathinae, or the most appropriate calibration for the Macropathinae is the 35 Mya separation of Australian and S. America taxa (Fig. 3C), or that some process other than vicariance has been involved in the biogeography of the Rhaphidophoridae. We note that our other calibrations would put the root of the Rhaphidophoridae close to the hypothesized origination of the Orthoptera (*i.e.*, Carboniferous; Fig. 3A, 3B) or prior to this (Fig. 3D). As India is estimated to have started docking with Asia about 50 Ma, this route into the Asian continent would not have been available earlier, and is younger than all estimates for the age of the Asian clade.

In conclusion, we find topological support or at least agreement with a plate-tectonic vicariance process for biogeography of Macropathinae, and its putative Asian sister group. Node dating using separation of Africa or a model of earlier separation of Australia from Antarctica, are roughly in agreement and yield dates for other nodes that are closest to geological ages.

However, two other, equally valid calibrations, yield dates that are not consistent with a vicariant process for lineage formation, and require, at least some long-distance dispersal, or prior existence of all lineages in Gondwana, with subsequent regional extinction, or an implausible age of origin of this family of insects.

When we consider inferred rates of molecular substitution as well, we are forced to concede that inclusion of long-distance dispersal events (LDD) would simply reconcile phylogeny, timing and geological history. This has been the case in several studies of palaeo-Austral animals and plants (*e.g.*, birds — Trewick & Gibb 2010; plants — Meudt & Simpson 2006, Wagstaff & Hennion 2007, Knapp *et al.* 2005). However, the idea that LLD has occurred within this group is difficult to reconcile with much that is known about their ecology and population genetics (*e.g.*, Sbordoni *et al.* 1985, 1987, 1991, 2000, 2005; Carchini *et al.* 1991; Cesaroni *et al.* 1997; Allegrucci *et al.* 1987, 1992, 1997, 2005, 2009).

European and North American (Caccone & Sbordoni 1987) taxa that have been studied tend to show strong partitioning of population genetic structure and species diversity among geographic areas (Sbordoni *et al.* 1985, Allegrucci *et al.* 1997), implying little exchange. However, the reconstruction of phylogeny in genus *Dolichopoda* and the testing of different geographic hypotheses (Allegrucci *et al.*, 2005, 2009 and unpub.) would suggest that LDD could have played an important role before these crickets became isolated in caves by arid climates. Indeed, a strong phylogeographic pattern has been observed and the temporal sequence of cladogenetic events is in good agreement with the geographic distribution of *Dolichopoda* studied; this supports the inference that speciation events are strictly allopatric and mostly determined by isolation of different populations in isolated cave systems (Allegrucci *et al.* 2009 and unpub.).

Studies of some of the diverse New Zealand Rhaphidophoridae reveal more extensive gene flow among populations (Cook *et al.* 2010), and close relationships between species on different islands (Trewick 2000, Goldberg *et al.* 2008). Indeed, in the New Zealand region, several offshore islands have endemic rhaphidophorids. In New Zealand, Tasmania and Chile, Rhaphidophoridae live in temperate wet forests (SAT pers. obs), and are not restricted to cave habitats. In such environments, there is ample opportunity for movement across wide geographic scales, and in addition, occupation of holes in logs (rather than caves) is the best explanation for the colonization of islands (Trewick 2000).

Future work will use enhanced taxonomic sampling, habitat description, population genetics and phylogenetics to generate more sophisticated biogeographic and ecogeographic hypotheses to enhance our understanding of the evolutionary history of this group.

Acknowledgements

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