

Support for vicariant origins of the New Zealand Onychophora

Julia Allwood^{1,2}, Dianne Gleeson¹, Georg Mayer^{3,4}, Savel Daniels⁵,
Jacqueline R. Beggs² and Thomas R. Buckley^{1*}

¹Landcare Research, Private Bag 92170, Auckland, New Zealand, ²School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand, ³Department of Anatomy and Cell Biology, University of Melbourne, Melbourne, Vic. 3010, Australia, ⁴Department of Genetics, Friedrich-Schiller-Universität Jena, Philosophenweg 12, D-07743 Jena, Germany, ⁵Department of Botany & Zoology, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa

ABSTRACT

Aim The distribution of Onychophora across the southern continents has long been considered the result of vicariance events. However, it has recently been hypothesized that New Zealand was completely inundated during the late Oligocene (25–22 Ma) and therefore that the entire biota is the result of long-distance dispersal. We tested this assumption using phylogenetic and molecular dating of DNA sequence data from Onychophora.

Location New Zealand, Australia, South Africa, Chile (South America).

Methods We obtained DNA sequence data from the nuclear genes 28S and 18S rRNA to reconstruct relationships among species of Peripatopsidae (Onychophora). We performed molecular dating under a Bayesian relaxed clock model with a range of prior distributions using the rifting of South America and South Africa as a calibration.

Results Our phylogenetic trees revealed that the New Zealand genera *Ooperipatellus* and *Peripatoides*, together with selected Australian genera (*Euperipatoides*, *Phallocephale* and an undescribed genus from Tasmania), form a monophyletic group that is the sister group to genera from Chile (*Metaperipatus*) and South Africa (*Peripatopsis* and *Opisthopatus*). The relaxed clock dating analyses yielded mean divergence times from 71.3 to 78.9 Ma for the split of the New Zealand *Peripatoides* from their Australian sister taxa. The 0.95 Bayesian posterior intervals were very broad and ranged from 24.5 to 137.6 Ma depending on the prior assumptions. The mean divergence of the New Zealand species of *Ooperipatellus* from the Australian species *Ooperipatellus insignis* was estimated at between 39.9 and 46.2 Ma, with posterior intervals ranging from 9.5 to 91.6 Ma.

Main conclusions The age of *Peripatoides* is consistent with long-term survival in New Zealand and implies that New Zealand was not completely submerged during the Oligocene. *Ooperipatellus* is less informative on the question of continuous land in the New Zealand region because we cannot exclude a post-Oligocene divergence. The great age of *Peripatoides* is consistent with a vicariant origin of this genus resulting from the rifting of New Zealand from the eastern margin of Gondwana and supports the assumptions of previous authors who considered the Onychophora to be a relict component of the New Zealand biota.

Keywords

Biogeography, dispersal, Gondwana, molecular clock, Oligocene Drowning, Peripatopsidae, peripatus, velvet worms, vicariance.

*Correspondence: Thomas R. Buckley, Landcare Research, Private Bag 92170, Auckland, New Zealand.
E-mail: buckleyt@landcareresearch.co.nz

INTRODUCTION

The relative contributions of vicariance and dispersal in the assembly of the New Zealand biota have been debated for many years (e.g. Darlington, 1965; Gaskin, 1970; Raven & Axelrod, 1972; Craw, 1978; Fleming, 1979; Pole, 1994, 2001; Lee *et al.*, 2001; McGlone, 2005; Waters & Craw, 2006; Trewick *et al.*, 2007; Landis *et al.*, 2008; Heads, 2009; Michaux, 2009; Wallis & Trewick, 2009). It is generally accepted that New Zealand began to split away from the remnants of Gondwana c. 80 million years ago (Ma), and by 55 Ma the modern Tasman Sea was fully formed (Gaina *et al.*, 1998; Li & Powell, 2001; McLoughlin, 2001). The Tasman Sea opened first in the south and then progressively northwards, and direct connections with Australia were soon severed. There is, however, growing evidence for land connections or at least island arc formation between New Zealand and New Caledonia sometime in the Tertiary, and it is unclear exactly how long the New Zealand landmass has been isolated for (e.g. Herzer *et al.*, 1997; Ladiges & Cantrill, 2007; Schellart *et al.*, 2009). During the early Tertiary, New Zealand was relatively tectonically inactive and the land slowly subsided and reduced in elevation and area (Fleming, 1979). By the late Oligocene (25–22 Ma) the land had reduced to a very small proportion of its current size (Fleming, 1979; Cooper & Cooper, 1995). Some early molecular phylogenetic studies showed an increase in speciation rate following the Oligocene, and this was attributed to a mass extinction event caused by the reduction in land area, which is referred to as the ‘Oligocene Drowning’ (Cooper & Cooper, 1995). However, some authors have suggested that New Zealand was completely submerged during the late Oligocene and that the entire modern biota is the result of long-distance dispersal (Pole, 1994; Waters & Craw, 2006; Trewick *et al.*, 2007; Landis *et al.*, 2008). These arguments have been in part inspired by the growing number of molecular dating studies that have inferred long-distance dispersal as the mechanism by which many lineages, in particular plants, arrived in New Zealand (e.g. Brown *et al.*, 1999; Winkworth *et al.*, 2002; Arensburger *et al.*, 2004; Bunce *et al.*, 2005; Perrie & Brownsey, 2007; Smith *et al.*, 2007). However, there are a number of lineages in New Zealand for which molecular dating studies have shown ages greater than the Oligocene and which are, therefore, more consistent with vicariance (e.g. Barker *et al.*, 2007; Knapp *et al.*, 2007; Roelants *et al.*, 2007), although extinction has been invoked to discount these examples (e.g. Waters & Craw, 2006).

Sanmartín & Ronquist (2004) showed a higher degree of congruence between organismal phylogenies and area phylogenies for Southern Hemisphere animals than plants. Therefore, the inferred recent age of many plant lineages in New Zealand may not be representative of the biota as a whole. This is an important point because the biota is dominated by hyperdiverse groups such as invertebrates for which there are very few phylogenetic studies with divergence dates estimated using slowly evolving nuclear genes and methods that do not assume rate constancy through time (e.g. Arensburger *et al.*, 2004;

Pratt *et al.*, 2008; Buckley *et al.*, 2009). In order to build a more accurate picture of the biogeographic history of the New Zealand biota and assess the relative roles of vicariance and dispersal, further phylogenetic studies on invertebrates are required.

Onychophora (velvet worms) are excellent candidates for a study on the vicariant origins of New Zealand fauna for three reasons. First, Onychophora have a poor tolerance of salt water and are prone to desiccation, thus being restricted mainly to microhabitats characterized by high moisture levels (e.g. Bursell & Ewer, 1950; Monge-Nájera *et al.*, 1993). These habitats often include those within or under rotting logs, under stones and within decomposing vegetation (Watt, 1961; Monge-Nájera, 1995; Gleeson, 1996; Woodman *et al.*, 2007). This environmental sensitivity, especially to sea water (Monge-Nájera *et al.*, 1993), has led to the traditional assumption that Onychophora are poor dispersers (Brinck, 1957; Monge-Nájera, 1995). Second, they are known to be an ancient group, with fossil evidence suggesting either Early Cambrian (Krumbiegel *et al.*, 1980) or Late Carboniferous (Thompson & Jones, 1980; Heyler & Poplin, 1988) origins, indicating that they may have been widespread on Pangea and later on Gondwana prior to the separation of the modern continents. Third, among the extant Onychophora, two families with distinct geographic distributions, the Peripatidae and the Peripatopsidae, are present. The Peripatidae are restricted to the Neotropics, the Antilles, West Africa and areas of Asia including Borneo, Malaysia and Indonesia (Newlands & Ruhberg, 1978; Storch & Ruhberg, 1993). The Peripatopsidae display a classic Gondwanan distribution, being present in Australia, New Guinea, Chile, South Africa and New Zealand (Ruhberg, 1985). The Peripatopsidae are not known from the remaining Gondwanan remnants of New Caledonia, Madagascar, India and Antarctica (Newlands & Ruhberg, 1978; Monge-Nájera, 1995). For the above reasons, it has often been suggested that the Onychophora have been distributed primarily by vicariance (Brues, 1923; Peck, 1975; Ruhberg, 1985). However, dispersal by means of rafting within vegetation or logs has also been suggested (Monge-Nájera, 1995).

The New Zealand Onychophora species are currently placed within the genera *Peripatoides* Pocock, 1894 and *Ooperipatellus* Ruhberg, 1985 (Fig. 1a,b), the latter being found also in Australia (Ruhberg, 1985; Gleeson, 1996; Gleeson *et al.*, 1998; Reid, 1996). Although the name *Ooperipatellus insignis* (Dendy, 1890) has been applied to populations from both New Zealand and Australia, previous authors have shown that they are almost certainly not conspecific (Tait & Briscoe, 1995; Reid, 1996; Gleeson *et al.*, 1998). There are currently nine species described from New Zealand (Hutton, 1876; Dendy, 1894; Gleeson, 1996; Reid, 1996; Trewick, 1998), and further undescribed species are known (Tait & Briscoe, 1995; Gleeson, 1996; Trewick, 2000). Reid (1996) and Gleeson *et al.* (1998) showed that the New Zealand fauna was not monophyletic, which is consistent with the current division into two genera. Gleeson *et al.* (1998) did not include taxa from South Africa or Chile, and the age of the New Zealand taxa was not explicitly



Figure 1 Representatives of both genera of New Zealand Onychophora. (a) *Peripatoides* sp., an undescribed ovoviviparous species from Dunedin (South Island). Specimen length is about 75 mm. (b) *Ooperipatellus nanus*, a small oviparous species with 13 pairs of legs, the lowest number of leg pairs known among Onychophora. The specimen photographed was found in Cheviot Hills (Takitimu Mountains, South Island). Specimen length is about 10 mm.

estimated because the phylogenetic signal from the gene selected (mitochondrial cytochrome *c* oxidase subunit I) was probably saturated. We have used sequence data from the slowly evolving nuclear genes 18S rRNA and 28S rRNA to reconstruct relationships among Peripatopsidae genera from all major landmasses from which they are known, with the exception of New Guinea. We used a biogeographic calibration that is independent of the geological history of New Zealand to estimate the age of the New Zealand Onychophora and the impact of the Oligocene Drowning.

MATERIALS AND METHODS

Taxon sampling

Species of *Peripatoides* and *Ooperipatellus* were sampled from 17 locations across New Zealand (Table 1). These samples were

selected to maximize the taxonomic and phylogenetic diversity within the fauna, as guided by unpublished phylogeographic data. We also included available species from mainland Australia, Tasmania, Chile and South Africa (Table 1). An undescribed species from Tasmania, 'Tasmania sp. 2' (Reid, 1996, p. 900), was included because of its putative close phylogenetic relationship to New Zealand species (Reid, 1996, fig. 28). Some of the New Zealand Onychophora species described by previous authors do not have modern taxonomic descriptions and in some cases type material is absent, so specimens are very difficult to identify (e.g. Hutton, 1876; Pocock, 1894; Bouvier, 1907; Watt, 1961; Ruhberg, 1985; Trewick, 1998, 1999, 2000). Trewick (1998) described four species of *Peripatoides* from New Zealand based largely on allozyme data, but did not include an identification key. Furthermore, none of these descriptions listed diagnostic morphological characters, making identification problematic, especially given the sympatric nature of some taxa in New Zealand and the presence of undescribed species (Gleeson, 1996). Therefore, we have listed all specimens of *Peripatoides* as '*Peripatoides* spp.', pending further taxonomic work. We were able to identify *Ooperipatellus nanus* Ruhberg, 1985 and *Ooperipatellus viridimaculatus* (Dendy, 1900) using morphological characters described by Ruhberg (1985); however, this genus also includes undescribed species, one of which is included in our study and listed as *Ooperipatellus* sp. (Table 1).

DNA sequencing

DNA extractions from tissue samples were performed using an AquaPure Genomic DNA Isolation Kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. A region of the 28S gene was amplified using the primer pair C1 and D2 (Jamieson *et al.*, 2002). The 18S gene was amplified using the primers 18SperF (this study) and A1984 (Vawter, 1991). The primer sequences are: C1, ACCCGCTGAATTTAAGCAT; D2, TCCGTGTTTCAAGACGG; A1984, TCCCTGGTTGATCCTGCCAGTA; 18SperF, GACAAATCGCTCCACCAACT. Polymerase chain reactions (PCRs) were performed on a GeneAmp PCR System 9700 Thermocycler (Applied Biosystems, Foster City, CA, USA). Reagent volumes were 25 μ L and comprised 1 μ L of DNA template, 2.5 μ L of FastStart *Taq* DNA Polymerase PCR Buffer (10 \times concentration), 1 μ L MgCl₂ (25 mM), 2.5 μ L dNTP (2 mM), 1 μ L of each primer (10 μ M) and 0.3 μ L of FastStart *Taq* DNA Polymerase (Roche, Basel, Switzerland). The PCR thermal cycling conditions included a 5-min denaturation at 95 $^{\circ}$ C, followed by 35 cycles of denaturation for 1 min at 94 $^{\circ}$ C, 1-min annealing at 60 $^{\circ}$ C, followed by 1.5 min at 72 $^{\circ}$ C. The reactions were completed with an extension period of 10 min at 72 $^{\circ}$ C. PCR products were purified using a High Pure PCR Product Purification Kit (Roche) and sequenced using BIGDYETM TERMINATOR 3.1 (Applied Biosystems) following the manufacturers' protocols. Sequences were electrophoresed using an Applied Biosystems 3100-Avant Automated Sequencer. Sequences were assembled and manually edited using SEQUENCHER 4.6 (Gene Codes,

Table 1 Sample locality data for Onychophora species used in this study, with their GenBank accession numbers. New Zealand locality codes follow Crosby et al. (1998). DNA extraction codes are followed by New Zealand Arthropod Collection (NZAC) accession numbers in parentheses. GenBank numbers are given for each individual and gene region, and missing data are indicated with a '-'.[†]

Country/Taxon	Collection locality	Locality code	GenBank numbers (28S/18S)
New Zealand			
<i>Peripatoides</i> sp.	Te Pahi, ND, New Zealand (34°28' S, 172°45' E)	P30 (NZAC03005978)	GQ911170/GQ911193
<i>Peripatoides</i> sp.	Mangawhai, AK, New Zealand (36°8' S, 174°37' E)	P8 (NZAC03005926)	GQ911177/GQ911199
<i>Peripatoides</i> sp.	Bethells Beach, AK, New Zealand (36°53' S, 174°26' E)	P3 (NZAC03005937)	GQ911169/GQ911192
<i>Peripatoides</i> sp.	Mount Eden, AK, New Zealand (36°52' S, 174°45' E)	P9 (NZAC03005915)	GQ911178/-
<i>Peripatoides</i> sp.	Shine Falls, HB, New Zealand (39°6' S, 176°51' E)	P13 (NZAC03005940)	GQ911156/GQ911182
<i>Peripatoides</i> sp.	Bellbird Bush, HB, New Zealand (39°7' S, 176°49' E)	P2 (NZAC03005946)	GQ911161/GQ911185
<i>Peripatoides</i> sp.	Ohinetonga, TO, New Zealand (38°59' S, 175°23' E)	P14 (NZAC03005989)	GQ911157/GQ911183
<i>Peripatoides</i> sp.	Cobb Ridge, NN, New Zealand (41°6' S, 172°41' E)	P29 (NZAC03005993)	GQ911168/GQ911191
<i>Peripatoides</i> sp.	Mount Arthur, NN, New Zealand (41°11' S, 172°42' E)	P31 (NZAC03005902)	GQ911171/GQ911194
<i>Peripatoides</i> sp.	Dalgety Range, SC, New Zealand (44°12' S, 170°36' E)	P6 (NZAC03005966)	GQ911175/GQ911197
<i>Peripatoides</i> sp.	Albury Range, SC, New Zealand (44°5' S, 170°41' E)	P7 (NZAC03005975)	GQ911176/GQ911198
<i>Peripatoides</i> sp.	Titan Rocks, CO, New Zealand (45°32' S, 168°58' E)	P5 (NZAC03005933)	GQ911174/GQ911196
<i>Peripatoides</i> sp.	Table Hill, SL, New Zealand (46°29' S, 169°29' E)	P4 (NZAC03005896)	GQ911173/GQ911195
<i>Ooperipatellus nanus</i> Ruhberg, 1985	Gordons Knob, NN, New Zealand (41°46' S, 173°36' E)	P1 (NZAC03005944)	GQ911153/GQ911180
<i>Ooperipatellus viridimaculatus</i> (Dendy, 1900)	Cobden Beach, BR, New Zealand (42°25' S, 171°12' E)	P28 (NZAC03005918)	GQ911167/GQ911190
<i>Ooperipatellus viridimaculatus</i> (Dendy, 1900)	Boyd Creek, OL, New Zealand (45°8' S, 167°56' E)	P10 (NZAC03005889)	GQ911154/-
<i>Ooperipatellus</i> sp. Australia	Longwood Range, SL, New Zealand (46°13' S, 167°51' E)	P11 (NZAC03005996)	GQ911155/GQ911181
'Tasmania' sp. 2	King William Range, Tasmania, Australia (42°12' S, 146°07' E)	TAS (NZAC03005908)	
<i>Ooperipatellus insignis</i> (Dendy, 1890)	Mount Macedon, Macedon Regional Park, Victoria, Australia (37°23' S, 144°34' E) (type locality)	P17 (NZAC03005887)	GQ911158/-
<i>Euperipatoides rowelli</i> Reid, 1996	Tallaganda State Forest, New South Wales, Australia (35°26' S, 149°33' E) (type locality)	P20 (NZAC03006004)	GQ911162/GQ911186
<i>Phallocephale tallagandensis</i> Reid, 1996	Anembo, Tallaganda State Forest, New South Wales, Australia (35°50' S, 149°28' E) (close to type locality)	P32 (NZAC03006007)	GQ911172/-
Chile			
<i>Metaperipatus blatinvillei</i> (Gervais, 1837)	Lago Tinquillo, IX Region de la Araucania, Chile (39°09' S, 71°42' W)	P19 (NZAC03005945)	GQ911160/-
<i>Metaperipatus inae</i> Mayer, 2007	Contulmo, VIII Region del Biobio, Chile (38°01' S, 73°11' W) (type locality)	P18 (NZAC03006011)	GQ911159/GQ911184
South Africa			
<i>Peripatopsis mosleyi</i> (Wood-Mason, 1879)	Keiskamma area, Eastern Cape, South Africa (32°36' S, 27°14' E)	P26 (NZAC03006006)	GQ911166/GQ911189
<i>Peripatopsis clavigera</i> Purcell, 1899	Knysna, Western Cape, South Africa (34°01' S, 23°11' E)	P22 (NZAC03005981)	GQ911163/-
<i>Peripatopsis sedgwicki</i> Purcell, 1899	Knysna, Western Cape, South Africa (34°01' S, 23°11' E)	P23 (NZAC03005939)	GQ911164/GQ911187
<i>Opisthopatus cinctipes</i> Purcell, 1899	Nkandla Forest, KwaZulu-Natal, South Africa (28°44' S, 31°08' E)	P25 (NZAC03005999)	GQ911165/GQ911188

Ann Arbor, MI, USA) and then exported into CLUSTALX 1.83 (Thompson *et al.*, 1997) for alignment using the default parameter settings (extension penalties 0.2, gap opening 10). Alignments for 18S required manual editing, which was completed using BIOEDIT (Hall, 1999). Once the final alignment was obtained, we ran it through GBLOCKS (Castresana, 2000; Talavera & Castresana, 2007) to identify regions of the alignment that had a large number of contiguous conserved positions of a minimum length. We used the GBLOCKS server and implemented the 'options for a less stringent selection'. All DNA sequences have been submitted to GenBank (Table 1).

Phylogenetic analyses

We used the Akaike information criterion (AIC, Akaike, 1973) to select the best-fit substitution model for the concatenated alignment. The program MODELTEST 3.6 (Posada & Crandall, 1998) was used in conjunction with PAUP*4.0b10 (Swofford, 1998) to calculate AIC values. Phylogenetic analyses were performed in a Bayesian framework using the program BEAST 1.4.8 (Drummond & Rambaut, 2007). A log-normally distributed relaxed clock was used to estimate divergence times and phylogenetic relationships (Drummond *et al.*, 2006). We used the following prior distributions: GTR relative rate matrix (Jeffrey's prior), α -shape parameter for the gamma distribution of among-site rate variation (exponential, mean = 1), proportion of invariable sites (uniform = 0–1), mean rate (exponential = 1.0), coefficient of variation (exponential = 1.0), covariance (exponential = 1.0), Yule birth rate (Jeffrey's), Birth–Death process (Jeffrey's). A thinning interval of 1000 was used for sampling from the Markov chain Monte Carlo. We used TRACER 1.4 (Rambaut & Drummond, 2007) to monitor the convergence of all parameters from the phylogenetic model, to ensure that all effective sample sizes were > 200 and to select appropriate burn-in sizes. Each analysis was run four times for 20 million cycles and then concatenated for calculation of marginal distributions.

We also performed bootstrap analyses under maximum likelihood and parsimony. The parsimony analyses were conducted in PAUP*, with 1000 pseudoreplicates with 100 stepwise addition trees and tree bisection–reconnection branch swapping. The maximum likelihood analyses were performed in RAxML 7.0.4 (Stamatakis, 2006). We used a partitioned model in which the 28S and 18S genes were assigned separate GTR+ Γ models. We used the hill-climbing algorithm invoked with the '-f d' command and 1000 pseudoreplicates in the bootstrapping.

Divergence time estimation

We used the divergence of the South African *Peripatopsis* Pocock, 1894 and the Chilean *Metaperipatus* Clark, 1913 as a calibration point in the tree. South America and South Africa separated in a south to north direction, and there is some ambiguity as to when exactly this process initiated (McLoughlin, 2001; Eagles, 2007; Torsvik *et al.*, 2009). The oldest sea-

floor anomalies are estimated to be 135 Ma, indicating that rifting was underway by this time (McLoughlin, 2001), and by 105 Ma all connections are assumed to have been severed (McLoughlin, 2001; Torsvik *et al.*, 2009), although slivers of continental material between northern South America and Africa may have been present as late as 100 Ma (Eagles, 2007). Because South American *Metaperipatus* and South African *Peripatopsis* form a monophyletic group, albeit with low support (see Results), we used a divergence date of between 135 and 105 Ma for these two genera. This assumption was expressed using two different priors. For the first prior we used a normal distribution centred on 120 Ma with a standard deviation of 5 Myr. This distribution had almost all of its mass in the range 105–135 Ma; however it includes the possibility that divergence occurred outside these dates. Second, we used a uniform prior between 105 and 135 Ma. According to this last prior, the divergence of *Metaperipatus* and *Peripatopsis* might have occurred at any point between 105 and 135 Ma with equal probability. The comparison of the normal and uniform priors is critical because it is known that priors with hard bounds (e.g. uniform) can have detrimental effects on Bayesian inference in some cases (e.g. Drummond *et al.*, 2006; Yang & Rannala, 2006; Sanders & Lee, 2007; Ho & Phillips, 2009). Because the prior distribution on tree shape can affect various aspects of Bayesian inference, including divergence times (Welch *et al.*, 2005; Drummond *et al.*, 2006; Ho & Phillips, 2009), we also analysed the data under both Yule (Yule, 1924) and Birth–Death (Nee *et al.*, 1994; Yang & Rannala, 1997) priors.

We shifted the calibration deeper in the tree to the common ancestor of all the sampled South African and Chilean genera to assess the sensitivity of the results to the phylogenetic placement of the calibration. This analysis was performed using the Yule prior with a uniform calibration (105–135 Ma). We also performed a cross-validation analysis, in which we posed the question that if both the New Zealand genera and the South America/South Africa split are the result of vicariance then placing a vicariance prior on the New Zealand/Australia split should yield a date estimate on the South Africa/South America split that is consistent with the age of rifting of these two continents (105–135 Ma). This analysis was performed by placing a prior on the divergence of *Peripatoides* from its Australian sister taxa between 80 and 90 Ma, which encompasses the range of dates for the initiation of sea-floor spreading between New Zealand and Australia (e.g. McLoughlin, 2001). This analysis was performed with a uniform prior (80–90 Ma) under the Yule speciation prior and all other settings as above.

RESULTS

Patterns of DNA sequence variation

We obtained 27 partial 28S sequences from the 28S gene, and 20 partial sequences from the 18S gene. The 28S and 18S alignments were 773 and 1719 base pairs long, respectively. Of

the 27 individuals sampled, 13 had some missing data. Once the poorly aligned regions were excluded using the criteria from GBLOCKS there were 1973 sites, including 337 parsimony-informative sites and 481 varied sites. The best-fit model under the AIC for the concatenated data set was the GTR+I+ Γ model with the following parameter values: base frequencies (A = 0.2071, C = 0.2925, G = 0.2902, T = 0.2102), Q-matrix (r_{AC} = 1.3022, r_{AG} = 4.1139, r_{AT} = 1.8824, r_{CG} = 0.5174, r_{CT} = 5.3045, r_{GT} = 1.000), proportion of invariable sites (0.6664) and α -shape parameter for among-site rate variation (0.5925).

Phylogenetic relationships among Peripatopsidae genera

The log-normally distributed clock model placed the root of the Peripatopsidae between the Chilean and South African genera on one side and the Australian and New Zealand genera

on the other. This root position was supported by a posterior probability of 100%. Within the Chilean and South African clade, the South African genera were paraphyletic, with *Metaperipatus* (Clark, 1913) from Chile grouping with *Peripatopsis* from South Africa to the exclusion of *Opisthopatus* (Purcell, 1899) from South Africa. This arrangement was not well supported, with only the parsimony analysis giving a nodal support value > 50% (Fig. 2). The New Zealand and Australian clade was supported as monophyletic, with a posterior probability of 99% and parsimony and likelihood bootstraps of 100% (Fig. 2). Within this clade the two New Zealand genera did not form a monophyletic group. The New Zealand and Australian *Ooperipatellus* formed a clade with a posterior probability of 84% but with bootstrap values lower than 50% (Fig. 2). Within the *Ooperipatellus* clade, the New Zealand species formed a monophyletic group with variable support (Fig. 2). The New Zealand genus *Peripatooides* formed a clade with the Australian genera *Euperipatooides* Ruhberg,

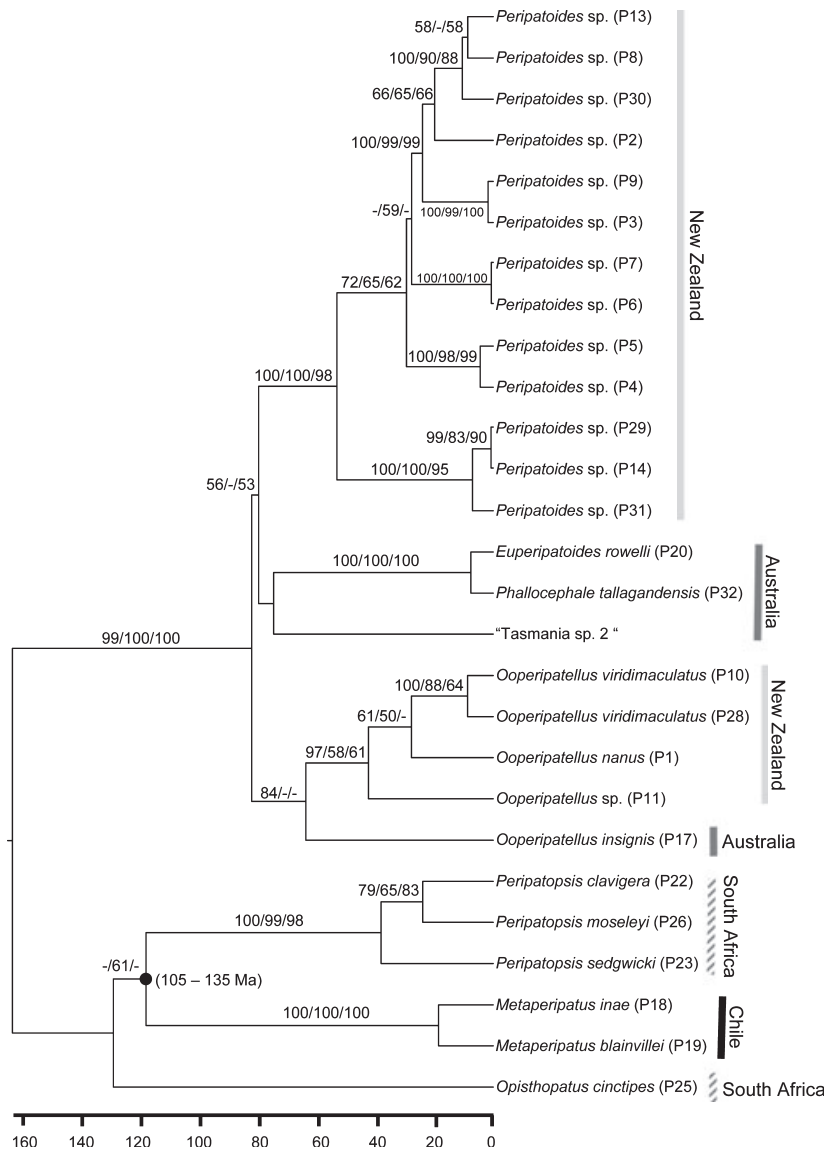


Figure 2 Bayesian phylogenetic reconstruction using 18S and 28S genes of Onychophora (Peripatopsidae) under the GTR+I+ Γ model with the Yule speciation prior and normal calibration. Branch lengths are drawn proportional to time and the scale bar is measured in Ma. Numbers above branches are posterior probabilities followed by maximum likelihood bootstraps and then parsimony bootstraps, and support values lower than 50% were omitted. The calibration point is indicated with a filled circle.

1985, *Phallocephale* Reid, 1996 and ‘Tasmania’ sp. 2, but this was weakly supported (Fig. 2). Relationships among *Peripatooides*, *Euperipatooides*, *Phallocephale* and ‘Tasmania’ sp. 2 were not well resolved, but *Euperipatooides* and *Phallocephale* did form a well-supported monophyletic group, with all support values 100% (Fig. 2).

Divergence time analyses

The four combinations of speciation priors and calibrations gave very similar divergence time estimates (Table 2). The root of the Peripatopsidae was estimated to range from 173.8 Ma (105.0–271.8 Ma, Table 2) under the Yule speciation prior with a uniform calibration to 181.8 Ma (108.0–295.0 Ma, Table 2) under the Birth–Death speciation prior and normal calibration. The radiation of the New Zealand and Australian genera probably occurred in the Cretaceous, with mean estimates ranging from 85.3 Ma (31.8–152.6 Ma, Table 2) under the Birth–Death speciation prior with uniform calibration to 93.9 Ma (39.3–162.7 Ma, Table 2) under the Yule speciation prior with a normal calibration. The *Ooperipatellus* species from Australia and New Zealand were estimated to have diverged from one another 39.9 Ma (9.8–81.7 Ma, Table 2) to 46.2 Ma (12.6–91.6 Ma) under the Birth–Death speciation prior with uniform calibration and the Yule speciation prior with normal calibration, respectively. All of the *Ooperipatellus* divergences overlapped with both the Oligocene Drowning and the rifting of New Zealand from Australia.

The New Zealand species of *Peripatooides* diverged from the Australian *Euperipatooides*, *Phallocephale* and ‘Tasmania’ sp. 2 at 71.3 Ma (26.6–128.5 Ma, Table 2) under the Birth–Death speciation prior with uniform calibration, or 78.9 Ma (33.8–137.6 Ma, Table 2) under Yule speciation prior with a normal calibration. The estimates of divergence times of *Peripatooides* from its Australian sister taxa all pre-dated the peak of the Oligocene Drowning, which dates back to 25–22 Ma (Landis *et al.*, 2008), with the single exception of the Birth–Death speciation prior with the normal calibration. The extreme lower 0.95 posterior interval of this prior and calibration combination overlapped the peak of the drowning by 0.5 Myr.

The analysis, in which we placed a uniform prior on the deeper node uniting *Metaperipatus*, *Opisthopatus* and *Peripat-*

opsis, yielded a date for the divergence of *Peripatooides* from *Euperipatooides*, *Phallocephale* and ‘Tasmania’ sp. 2 at 70.5 Ma (124.7–28.7 Ma). The divergence of the New Zealand and Australia *Ooperipatellus* was dated at 41.4 Ma (81.9–11.4 Ma). These dates are all slightly younger than the first calibration we used, but the *Peripatooides* divergence still pre-dates the Oligocene Drowning.

When we placed a calibration of 80–90 Ma on the separation of *Peripatooides* from its Australian sister taxa, we obtained a divergence time of 101.9 Ma (38.5–198.8 Ma) for the divergence of *Metaperipatus* from *Peripatopsis*. This estimate overlapped with the previously assumed calibration for *Metaperipatus* and *Peripatopsis* of 135–105 Ma.

DISCUSSION

Origins of the New Zealand Onychophora

Our results show that the New Zealand and Australian Onychophora form a monophyletic clade and are the sister group to South African *Peripatopsis* and *Opisthopatus* and Chilean *Metaperipatus*. The two South African genera are not sister taxa, which is similar to results obtained by Reid (1996) and Daniels *et al.* (2009). The biogeographic pattern of South Africa grouping with South America to the exclusion of New Zealand and Australia does not perfectly match the accepted pattern of Gondwana breakup. Although Africa and South America separated from Australia in the Cretaceous (Upchurch, 2008), biotic connections between South America and Australia were possible via Antarctica until well into the Tertiary (Lawver & Gahagan, 1998; Livermore *et al.*, 2005; Upchurch, 2008; Lagabrielle *et al.*, 2009; Svenson & Whiting, 2009). These potential biotic connections could have been severed either by the formation of the Southern Ocean between Australia and Antarctica and the Drake Passage, or by glaciation in Antarctica, which began close to the Eocene–Oligocene boundary (Barker & Thomas, 2004). The lack of any close phylogenetic relationship between the Onychophora of Australia and South America indicates that this land connection did not allow the exchange of Onychophora species, possibly owing to inhospitable conditions across the Patagonia/Antarctica connection during the Cretaceous

Table 2 Divergence times (Ma) of Onychophora species estimated with a Bayesian relaxed clock with different prior distributions.

	Yule speciation prior		Birth–Death speciation prior	
	Uniform calibration	Normal calibration	Uniform calibration	Normal calibration
NZ versus Australian <i>Ooperipatellus</i>	45.8 (12.4–90.8)	46.2 (12.6–91.6)	39.9 (9.8–81.7)	40.4 (9.5–82.2)
Radiation of NZ <i>Ooperipatellus</i>	25.8 (6.1–52.8)	26.0 (5.7–52.5)	21.7 (4.8–44.9)	22.2 (4.9–46.2)
<i>Peripatooides</i> versus <i>Euperipatooides</i> + <i>Phallocephale</i> + ‘Tasmania’ sp. 2	78.1 (32.3–136.7)	78.9 (33.8–137.6)	71.3 (26.6–128.5)	72.5 (24.5–130.3)
Radiation of NZ <i>Peripatooides</i>	49.5 (19.5–87.6)	50.5 (19.5–89.9)	43.6 (15.5–80.1)	44.3 (15.9–81.5)
New Zealand and Australian genera	92.3 (38.6–159.6)	93.9 (39.3–162.7)	85.3 (31.8–152.6)	87.2 (30.9–156.3)
Root height	173.8 (105.0–271.8)	175.9 (108.4–275.4)	178.6 (105.0–288.5)	181.8 (108.0–295.0)

(Upchurch, 2008). The inferred relationship between South Africa and South America instead indicates that the separation of these two continents from East Gondwana and then their own rifting beginning 135 Ma was the event that led to the basal splits in the Peripatopsidae.

Our dating analyses indicate that the New Zealand taxon *Peripatoides* probably diverged from its Australian sister taxa in the late Cretaceous, with the upper and lower posterior intervals in the range 24.5–137.6 Ma. Although the posterior distributions on our divergence dates are very broad, possibly because there is only a single calibration point (Kishino *et al.*, 2001), they do allow us to assess key dates in the geological history of New Zealand. The rifting of New Zealand from Australia is thought to have begun *c.* 80 Ma (McLoughlin, 2001; Mortimer, 2008) in the south of what is now the Tasman Sea, and the mean posterior divergence dates of *Peripatoides* correspond well with this event. Therefore, it is likely that the rifting of New Zealand from Australia caused the split of *Peripatoides* from its Australian sister taxa.

The mean divergence time of Australian and New Zealand *Ooperipatellus* is more recent than that of *Peripatoides*, with all of the mean estimates falling in the range 39.9–46.2 Ma. However, the posterior intervals do encompass the date at which the rifting of New Zealand from Australia occurred, and, therefore, we cannot exclude a vicariant origin, as favoured for *Peripatoides*. Although dispersal can be invoked to explain this shallower date for *Ooperipatellus*, geological reconstructions suggest that this might not be necessary. It is currently unknown exactly when land connections between New Zealand, New Caledonia and other landmasses to the north-east of New Caledonia were severed (Ladiges & Cantrill, 2007). However, recent authors have found evidence for island arcs and emergent land between New Zealand and New Caledonia during the Tertiary (Herzer *et al.*, 1997; Schellart *et al.*, 2009), and Ladiges & Cantrill (2007) argued for land connections between Australia and New Caledonia during the Tertiary. If *Ooperipatellus* did indeed diverge from Australian relatives after the rifting of New Zealand from Australia then this genus may have migrated from Australia to New Zealand via a landmass such as New Caledonia. This hypothesis would require us to invoke an extinction event in New Caledonia, as Onychophora are not known from this area. Given that New Caledonia was originally part of the Australian plate margin in the Cretaceous, an extinction event must be invoked in any case to explain this apparent absence.

Calibration assumptions and testing hypotheses on vicariance

Selecting a calibration point that matches the true age of lineage divergence is absolutely essential for obtaining accurate divergence time estimates (e.g. Thorne *et al.*, 1998; Bromham & Penny, 2003; Heads, 2005; Ho *et al.*, 2008; Ho & Phillips, 2009). We have assessed the robustness of our divergence dates by using different prior distributions on the calibration point and the tree shape. Although these different priors give slightly

different divergence time estimates, they do not significantly alter our conclusions. We also have used slowly evolving nuclear genes, which are more suitable for testing hypotheses on Mesozoic age divergences than rapidly evolving mitochondrial genes. Despite this, our dates are conditional on a good fit of the relaxed clock (Aris-Brosou & Yang, 2002; Lepage *et al.*, 2007) and nucleotide substitution models (e.g. Arbogast *et al.*, 2002).

Our analyses explicitly assume that the divergence of *Metaperipatus* and *Peripatopsis* resulted from the rifting apart of South America and Africa. The paraphyletic nature of the South African Peripatopsidae raises the possibility that *Metaperipatus* formed from a long-distance dispersal event from South Africa to South America. As with any dispersal hypothesis, we cannot directly exclude this possibility, but we consider it unlikely. The assumption that both the *Metaperipatus* and *Peripatopsis* split and the *Peripatoides* and Australian genera split are the result of vicariance predicts that, if we constrain the *Peripatoides* and Australian genera node at the age of the rifting of New Zealand from Australia, the corresponding divergence of *Metaperipatus* and *Peripatopsis* will match the age of the breakup of South America and Africa. This prediction is indeed confirmed by our analyses, because placing a calibration of 80–90 Ma for the split between *Peripatoides* and its Australian sister taxa leads to an estimate of 101.88 Ma (38.5–198.77 Ma) for *Metaperipatus* and *Peripatopsis*. This posterior distribution encompasses the timing of the rifting of South America from Africa (105–135 Ma; McLoughlin, 2001). Therefore, the relative temporal spacing in the tree of the divergences of *Peripatoides* and its Australian relatives and *Metaperipatus* and *Peripatopsis* is consistent with vicariance in both cases.

A further possibility is that *Metaperipatus* and *Peripatopsis* diverged before the split between South America and Africa. In this event, then, we would expect our dates to be underestimated. This of course would strengthen the argument that the New Zealand genera are the result of vicariance because the dates we estimated for the New Zealand and Australian divergence would be correspondingly underestimated.

Another assumption of our analysis and interpretation of inferred dates is that the age of divergence of extant taxa from different landmasses reflects the age of the lineages from those landmasses. Waters & Craw (2006) pointed out that extinction of sister taxa can lead to overestimates of the age of a lineage in a geographic area. Extinction of a more closely related lineage in Australia than those sampled could have led to inflation in age of the New Zealand Onychophora lineages. However, without fossils, hypotheses on extinction are *ad hoc*, unable to be tested and can be used to explain almost any biogeographic pattern. For these reasons, while acknowledging the potentially confounding effects of extinction, we have no evidence that this process has misled our biogeographic interpretations. We also note that although the use of biogeographic calibrations is open to criticism, fossils also have a number of weaknesses, including problems with assignment to extant lineages, the fact that fossils indicate minimum divergence times only, and

uncertainties in dating strata (Bromham & Penny, 2003; Magallón, 2004; Heads, 2005; Gandolfo *et al.*, 2008).

New Zealand Onychophora and the Oligocene Drowning

Most of our dating analyses yielded divergence time estimates in which the lower limits of the Bayesian posterior intervals for the divergence of *Peripatoides* exclude the late Oligocene. The Birth–Death speciation prior with normal calibration had a lower posterior interval that only just overlapped with the upper limit of the Oligocene Drowning. However, this divergence date estimate does not overlap with the emergence of land after the drowning (< 22 Ma, Landis *et al.*, 2008), which is the predicted divergence time, if *Peripatoides* colonized New Zealand by long-distance dispersal after the re-emergence of land. Therefore, our analyses allow us to exclude an arrival of *Peripatoides* in New Zealand after the Oligocene Drowning. The age of New Zealand *Ooperipatellus* is somewhat more recent, and under all prior assumptions the lower divergence time limits cross into the Miocene. Despite the ancient posterior mean age of *Ooperipatellus*, we cannot exclude a post-Oligocene arrival in New Zealand. The result from *Peripatoides* implies that there has been emergent land in the New Zealand region since rifting from the margin of Gondwana during the Late Cretaceous. The New Zealand Onychophora, therefore, add to the growing list of taxa with molecular dates that pre-date the Oligocene Drowning. These taxa include *Toronia* and *Knightia* (Proteaceae, Barker *et al.*, 2007), *Agathis* (Araucariaceae, Knapp *et al.*, 2007), ratite birds (Cooper *et al.*, 2001; Haddrath & Baker, 2001), *Sphenodon* (e.g. Rest *et al.*, 2003) and *Leiopelma*, tailed frogs (Roelants *et al.*, 2007). All of the above studies use calibrations that are independent of the date for the rifting of New Zealand from Australia and, therefore, avoid the circularity outlined by Waters & Craw (2006).

Waters & Craw (2006) and Landis *et al.* (2008) listed some of the above examples and argued that their apparent antiquity was the result of the extinction of sister taxa in other landmasses. However, as this list grows with the addition of taxa such as *Peripatoides*, these *ad hoc* arguments become less tenable. There are also phylogenetic studies that have shown New Zealand clades to be sister groups to widespread clades that are strongly suspected or known to be ancient (e.g. Barker *et al.*, 2001; Ericson *et al.*, 2002; de Kloet & de Kloet, 2005; Leschen, 2005; Wright *et al.*, 2008; Boyer & Giribet, 2009) or in which New Zealand clades fall in a phylogenetic position that matches the breakup sequence of Gondwana (e.g. Pollock, 1995). Furthermore, there are the well-known cases of higher-level taxa that are found only in New Zealand (e.g. Leschen *et al.*, 2003, 2005; Driskell *et al.*, 2007), or of post-Oligocene fossils belonging to lineages not found elsewhere (e.g. Worthy *et al.*, 2006). Although we do not doubt the importance of dispersal in the assembly of the New Zealand biota (e.g. Fleming, 1979; Pole, 2001; Winkworth *et al.*, 2002), given the mixture of recent and ancient lineages, New Zealand fits the

definition of a ‘fragment island’ (*sensu* Gillespie & Roderick, 2002). Many more phylogenetic studies on the hyper-diverse invertebrate fauna need to be completed before statements about what proportion of the New Zealand biota is the result of dispersal versus vicariance can be made with any certainty.

CONCLUSIONS

The two New Zealand Onychophora genera, *Peripatoides* and *Ooperipatellus*, were found to fall within separate clades, both related to Australian genera. Divergence time estimates between *Peripatoides* and Australian sister taxa suggest divergence at the time New Zealand rifted from Australia in the late Cretaceous. The age of this divergence is older than the Oligocene Drowning, which allows us to exclude the possibility that New Zealand was completely submerged during the late Oligocene. The divergence of New Zealand and Australian *Ooperipatellus* may have occurred somewhat later, and we cannot exclude a post-Oligocene age. At least the New Zealand *Peripatoides* can be considered an ancient and relict component of the New Zealand biota, as has long been maintained by previous authors (e.g. Watt, 1961; Fleming, 1979; Stevens, 1980).

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BIOSKETCH

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