Molecular Ecology (2000) **9**, 269–281

Mitochondrial DNA sequences support allozyme evidence for cryptic radiation of New Zealand *Peripatoides* (Onychophora)

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Abstract

A combination of single-strand conformation polymorphism analysis (SSCP) and sequencing were used to survey cytochrome oxidase I (COI) mitochondrial DNA (mtDNA) diversity among New Zealand ovoviviparous Onychophora. Most of the sites and individuals had previously been analysed using allozyme electrophoresis. A total of 157 peripatus collected at 54 sites throughout New Zealand were screened yielding 62 different haplotypes. Comparison of 540-bp COI sequences from *Peripatoides* revealed mean among-clade genetic distances of up to 11.4% using Kimura 2-parameter (K2P) analysis or 17.5% using general time-reversible (GTR + I + Γ) analysis. Phylogenetic analysis revealed eight well-supported clades that were consistent with the allozyme analysis. Five of the six cryptic peripatus species distinguished by allozymes were confirmed by mtDNA analysis. The sixth taxon appeared to be paraphyletic, but genetic and geographical evidence suggested recent speciation. Two additional taxa were evident from the mtDNA data but neither occurred within the areas surveyed using allozymes. Among the peripatus surveyed with both mtDNA and allozymes, only one clear instance of recent introgression was evident, even though several taxa occurred in sympatry. This suggests well-developed mate recognition despite minimal morphological variation and low overall genetic diversity.

Keywords: biogeography, cytochrome oxidase I, mtDNA, New Zealand, Onychophora, peripatus

Received 11 July 1999; revision received 24 September 1999; accepted 24 September 1999

Introduction

The Onychophora are at one and the same time highly distinctive and obscure animals. Their obscurity is largely the result of rarity, behavioural crypticism and lack of study. The phylum consists of just two extant families with some hundred described species, and this number was until recently far smaller. Because of their unusual combination of traits, including a hydrolastic skeleton and clawed unjointed legs, the group is proverbially known as a 'missing link' and a 'living fossil' (e.g. Wenzel 1950; Ghiselin 1984). These descriptions are of course both semantically self-contradictory and biologically dubious. Onychophora appear to have diverged early in the arthropod radiation (Ballard *et al*. 1992; Briggs *et al*. 1992; Zrzavy *et al*. 1998) and are represented in the northern hemisphere fossil record among Cambrian marine deposits (Ramsköld 1992; Hou & Bergstrom 1995) and Tertiary amber (Poinar 1996). No fossils are known from the southern hemisphere but all extant taxa are restricted to that region, and this distribution, coupled with a presumed low vagility (Sedgwick 1908; Monge-Nájera 1995), has typically been interpreted as arising from Gondwanan vicariance (Stevens 1981; Monge-Nájera 1996).

Using molecular techniques, researchers in Australia have recently revealed a deeply structured Onychophoran fauna (Briscoe & Tait 1995). This consists of extensive sympatry of multiple genera (Sunnucks *et al.* 2000) and previously undetected morphological diversity (Reid *et al*. 1995; Reid 1996), which includes unique sperm transfer structures (Tait & Briscoe 1990).

Although they have their common origins in Gondwana, New Zealand and Australia are vastly different landmasses. New Zealand is substantially smaller, separated from Gondwana earlier (≈ 80 million years [Myr] vs. ≈ 50 Myr) and has had an exceptionally turbulent geological history, experiencing a sequence of marine inundations, volcanism and seismic activity (Cooper & Millener 1993). During the Oligocene (≈ 25 Myr), much of the landmass was totally submerged beneath the sea, and was later subjected to mountain building in the late Pliocene (≈ 5 Myr), and extensive glaciation and climate change in the Pleistocene (2 Myr). Nevertheless, New Zealand

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displays a distinctive, if depauperate, biota with many unique taxa (e.g. Moa, tuatara and Leiopelmid frogs) as well as typical southern hemisphere taxa, including Onychophora. There is also ample evidence of various taxa recently colonizing New Zealand by dispersal so that the modern assemblage has elements typical of both continental and oceanic biotas (Fleming 1979; Daugherty *et al*. 1993). Thus, the origins and phylogeographical structure of the New Zealand biota provide an interesting model system in which to explore the roles of vicariance and dispersal in the evolution of species and species assemblages.

Within New Zealand, three live-bearing (ovoviviparous) species of Onychophora can be distinguished by leg number: *Peripatoides indigo* Ruhberg (1985), *P. novaezealandiae* (Hutton 1876) and *P. suteri* (Dendy 1894), having 14, 15 and 16 pairs of legs, respectively. However, *P. novaezealandiae* consists of a species complex (Trewick 1998). Four new species (*P. morgani*, *P. aurorbis*, *P. kawekaensis* and *P. sympatrica*) were described on the basis of allozyme data, although unfortunately no morphological characters distinguishing them were apparent (Trewick 1998). The present work is a survey of mitochondrial sequence variation among the *Peripatoides* individuals used in that allozyme study, plus additional specimens extending the sample to include most regions of New Zealand. These mitochondrial DNA (mtDNA) data are here used to further explore the origins, phylogeographical structure and ecology of the endemic *Peripatoides* in light of the turbulent geological history of New Zealand.

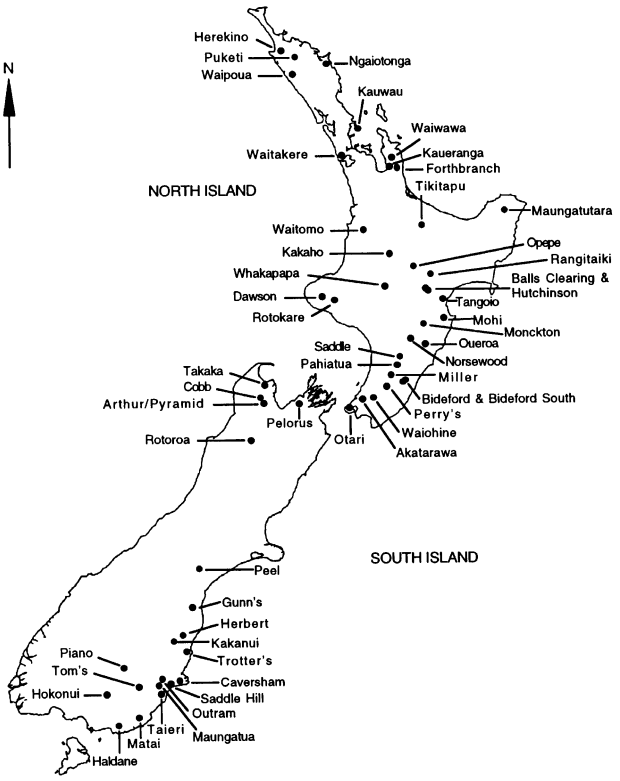

Fig. 1 Map of New Zealand showing the locations of the *Peripatoides* sampling sites.

Materials and methods

Specimens of *Peripatoides* were collected from decaying logs and other moist, dark habitats throughout South Island and North Island, New Zealand (Fig. 1, Appendix 1). Of the specimens previously studied using allozyme electrophoresis, those from Whakapapa, Takaka and Pelorus were not available for the present study, but new material was obtained from Pelorus. DNA was extracted from whole tissue derived either from frozen body sections remaining from allozyme research or, for specimens collected subsequently, from one or two legs dissected from frozen or alcohol-preserved specimens. Extractions used a simple, solvent-free proteinase K and salting-out method (Sunnucks & Hales 1996).

Peripatus individuals were screened for haplotypic variation using isotopic labelling and single-stranded conformation polymorphism analysis (SSCP) according to the method described by Trewick (1999). SSCP utilized mitochondrial primer pairs C1-J-1718: C1-N-2191, and SR-J-14233: SR-N-14588 (Simon *et al*. 1994), which amplify short fragments (< 400 bp) of the cytochrome oxidase I (COI) gene and small ribosomal subunit (12S), respectively. Haplotypes were sequenced for a longer fragment towards the 3′ end of COI using the universal primer C1-N-2195 (Simon *et al*. 1994) and either of two primers designed for use with peripatus: Perip241r and NotLEUr (Trewick 1999). Standard polymerase chain reactions (PCRs) were performed in 25-μL volumes and products were gel-purified in 2% agarose stained with ethidium bromide (see Trewick 1999). Bands of expected molecular weight (MW) were excised and the DNA extracted from the agarose using QIAquick spin columns (Qiagen). Purified DNA fragments were quantified (by eye) by comparison with a MW marker following agarose-gel electrophoresis. Cycle sequencing used Bigdye chemistry (Perkin Elmer), following the manufacturer's instructions. Sequences were aligned manually using SEQED, version 1.0.3 (ABI, PE).

Phylogenetic analyses were performed using PAUP, version 4.0 (Swofford 1998). The Mantel test was performed using GENEPOP, version 3.1b (Rousset 1997). Universal primers used were sourced from the insect mtDNA primer set (John Hobbs; UBC). Sequence data were deposited at GenBank (accession nos: AF188241–188248, 188251–188254, 188258–188262, 221447–221497) and voucher specimens at the Museum of New Zealand (MONZ) and Otago Museum (OMNZ). Outgroup sequences were obtained from individuals assignable to the egglaying species *Ooperipatellus viridimaculatus* (Dendy) and *O. nanus* Ruhberg, collected in South Island, New Zealand.

	Codon position			
	First	Second	Third	Total
A	0.285	0.197	0.418	0.300
C	0.107	0.207	0.027	0.114
G	0.272	0.151	0.036	0.153
T	0.335	0.445	0.518	0.433
A + T	0.620	0.64	0.94	0.733
Drosophila	0.524	0.537	0.891	
Greya	0.676	0.718	0.917	
Constant percentage	83.3 (150)	92.2 (166)	24.4 (44)	66.7
Variable percentage	16.7 (30)	7.8 (14)	75.6 (136)	33.3
Informative percentage	12.8 (23)	2.8 (5)	62.2 (112)	26
No. of sites	180	180	180	540

Table 1 Summary statistics for 540-bp cytochrome oxidase I (COI) sequences from *Peripatoides*

Proportions of A + T nucleotides at first, second and third codon positions in two insect taxa are given for comparison. The *Drosophila* (Diptera) data are from COI Spicer 1995) and *Greya* (Lepidoptera) from COI and COII (Brown *et al*. 1994).

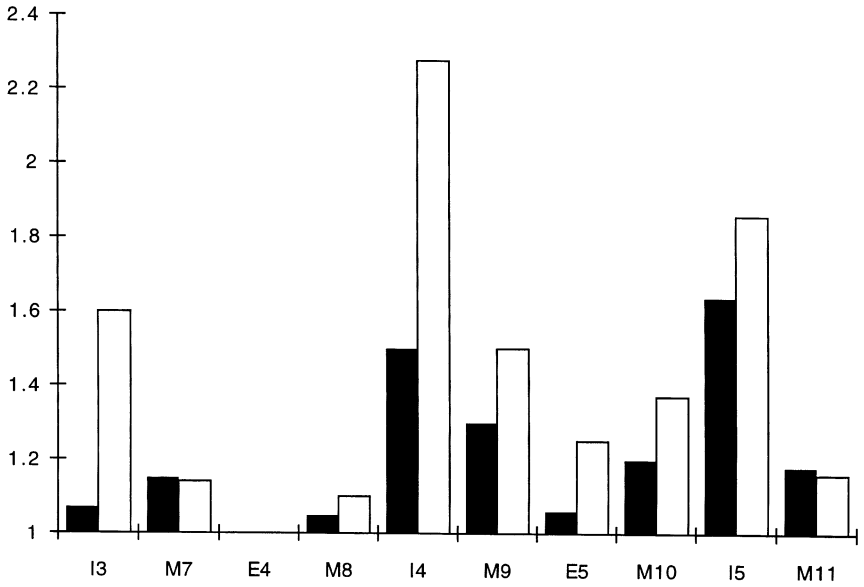

Fig. 2 Mean amino acid variability for 10 structural regions of the cytochrome oxidase I (COI) gene (Lunt *et al*. 1996) spanned by a 540-bp DNA sequence. The regions comprise membrane helices (M), external loops (E) and internal loops (I). Variability is expressed as the average number of amino acids observed per site in a given region. Open and filled bars represent data from a suite of insect taxa (Lunt *et al*. 1996) and *Peripatoides* from the present study, respectively.

Results

Sequence data

Through a combination of SSCP and sequencing, 62 COI haplotypes were identified from 157 *Peripatoides* collected at 54 sites. Aligned sequences consisted of 540 bp from the 3′ end of COI corresponding to positions 754–1293 of the insect COI sequence described by Lunt *et al*. (1996). These DNA sequences contained 140 (26%) parsimony-informative sites (149 including the outgroup). Approximately 84% of all substitutions were at third-codon positions, and 11.5% and 4.5% at first- and second-codon positions, respectively. Seventy-five per cent of third-codon positions were variable in the ingroup (Table 1). There was a transition : transversion ratio of 2.2 : 1 calculated from the neighbour–joining (NJ) tree, but this was ≈ 3 : 1 for comparisons within clades. A χ^2-test indicated no significant variation in base composition among sequences (PAUP, version 4.0). However, these sequences were AT-rich (73%), similar to a level observed for this gene in Australasian peripatus (Gleeson *et al*. 1998) but not as high as that found in some invertebrates (e.g. > 80% in beetles, Howland & Hewitt 1995). The majority of the AT bias was focused on third-codon positions, which were almost entirely A + T (94%), as in other invertebrates (Table 1). The pattern of inferred amino acid variability among structural regions of COI was also consistent with that found across a spectrum of insects (Lunt *et al*. 1996) (Fig. 2).

Table 2 Average genetic distances within and between taxa

	K2P	GTR	*O. viridimac.*	*P. suteri*	*P. aurorbis*	*P. sympat.*	*P. kaweka.*	*P. novaezea.*	*P. morgani*	'Catlins'	'Piano'
				0.01	0.04	0.09	0	0.07	0.01		
O. viridimaculatus											
P. suteri	3.1	3.6	12.4 20.8		1.75	1.23	1.25	2.66	1.72		
P. aurorbis	3.8	4.6	13.3 23.6	8.7 12.1		1.02	0.95	0.73	0.97		
P. sympatrica	2.4	2.7	12.8 20.8	7.4 9.7	11.3 16.9		0.36	0.63	0.85		
P. kawekaensis	0.8	0.4	12.6 20.5	7.9 10.5	10.5 15.9	6.5 8.9		0.44	0.48		
P. novaezealandiae	2.9	3.2	13.2 22.4	7.4 9.7	10.5 16.1	6.0 7.8	6.2 8.2		0.24		
P. morgani	1.9	4.6	13.3 21.9	7.6 10.2	10.7 16.5	6.3 8.4	6.7 7.9	3.2 4.3			
'Catlins'	2.0	2.2	14.1 24.9	9.2 11.9	7.9 10.5	9.9 13.3	9.9 13.5	9.7 12.8	9.7 13.1		
'Piano'	1.7	1.8	15.9 32.4	10.8 15.9	9.7 14.0	10.8 15.6	11.4 17.5	11.1 16.5	11.1 17.3	6.9 8.9	
'Dunedin'	2.8	3.2	14.0 28.0	9.1 12.9	8.5 11.9	10.4 15.2	10.5 15.6	11.2 17.5	11.1 16.8	7.7 13.3	9.3 9.1

Nei's D from allozyme data (Trewick 1998) is presented above the diagonal, Kimura 2-parameter (K2P) and general time-reversible (GTR + I+ Γ) mitochondrial DNA (mtDNA) cytochrome oxidase I (COI) distances are presented below the diagonal.
Within-taxon distances are given in the first row for Nei's D, and in the first and second columns for K2P and GTR + I + Γ, respectively.
O. viridimac., *Ooperipatellus viridimaculatus*; *P. sympat.*, *Peripatoides sympatrica*; *P. kaweka.*, *P. kawekaensis*; *P. novaezea.*, *P. novaezealandiae*.

Most peripatus collected from a single site shared the same COI haplotype. At all sites where sympatric species had previously been found using allozymes (Trewick 1998), more than one haplotype was detected. Individuals assigned to a particular species from allozyme data also had mtDNA haplotypes consistent with this (see phylogenetic analysis). This was true even for those rare peripatus individuals that were heterozygous or homozygous for allozyme alleles characteristic of a different species. Thus, at Balls Clearing, two *P. kawekaensis* individuals were heterozygous for an AATc allele characteristic of *P. sympatrica*, and a third *P. sympatrica* individual was homozygous for an aconitase (ACON) allele characteristic of *P. kawekaensis*. At Norsewood, two *P. morgani* had an aspartate aminotransferase (AATa) allele typical of *P. sympatrica* (Trewick 1998). In all these individuals their mtDNA haplotype was concordant with the majority of their nuclear markers.

Of the locations not surveyed for allozyme variability, only one (Piano Flat, South Island) had two haplotypes. Sequences from individuals collected at three locations in North Island (Ngaiotonga, Ball's Clearing and Opepe), which were otherwise unambiguous, contained some sites with ambiguous nucleotides. These suspect nucleotide substitutions were coded as N.

Genetic distance

Genetic distances were initially calculated using the Kimura 2-parameter (K2P) model. A NJ tree derived from these distances was then used to test for the most appropriate nucleotide substitution model by comparing likelihood scores for a suite of models: Jukes–Cantor (JC) (Jukes & Cantor 1969), K2P (Kimura 1980), Hasegawa–Kishino–Yang (HKY85) (Hasegawa *et al*. 1985) and general time-reversible (GTR) (Yang 1994) with a combination of among-site rate variation models: I (invariable sites) and Γ (gamma distribution). There was a substantial improvement in likelihood scores for models incorporating among-site rate variation, with GTR + I + Γ having a significantly better score (–lnL 3612.344) than other models. With the estimated proportion of invariant sites of 0.524, the α-shape parameter was 0.863. These parameter estimates were used to recalculate the distance matrix.

Pairwise genetic distances among the ingroup taxa (*Peripatoides*) calculated from COI sequences using K2P and GTR + I + Γ, reached a maximum of 13.4% and 22.4%, respectively. Mean distance between clades ranged from 3.2 to 11.4% using the K2P model, and 4.3 to 17.5% using the GTR + I + Γ models (Table 2). The highest within-taxon mean distance was 3.8% using the K2P model, in the *P. aurorbis* clade (maximum 6.6%). Mean genetic distances between *Ooperipatellus viridimaculatus and Peripatoides* clades ranged from 12.4 to 15.9% using the K2P model, and 20.8 to 32.4% using the GTR + I + Γ models. The greatest single distance was between *O. viridimaculatus* and a Piano peripatus (16.3% using the K2P model, 34.2% using the GTR + I + Γ models). The two outgroup *Ooperipatellus* differed by 9.7% using the K2P. The greatest linear geographical distance between sample sites with identical haplotypes was ≈ 190 km (*P. morgani*, Monckton—Tikitapu).

Phylogenetic analysis

Phylogenetic analysis of all haplotypes using NJ with K2P weighting produced the tree shown in Fig. 3.

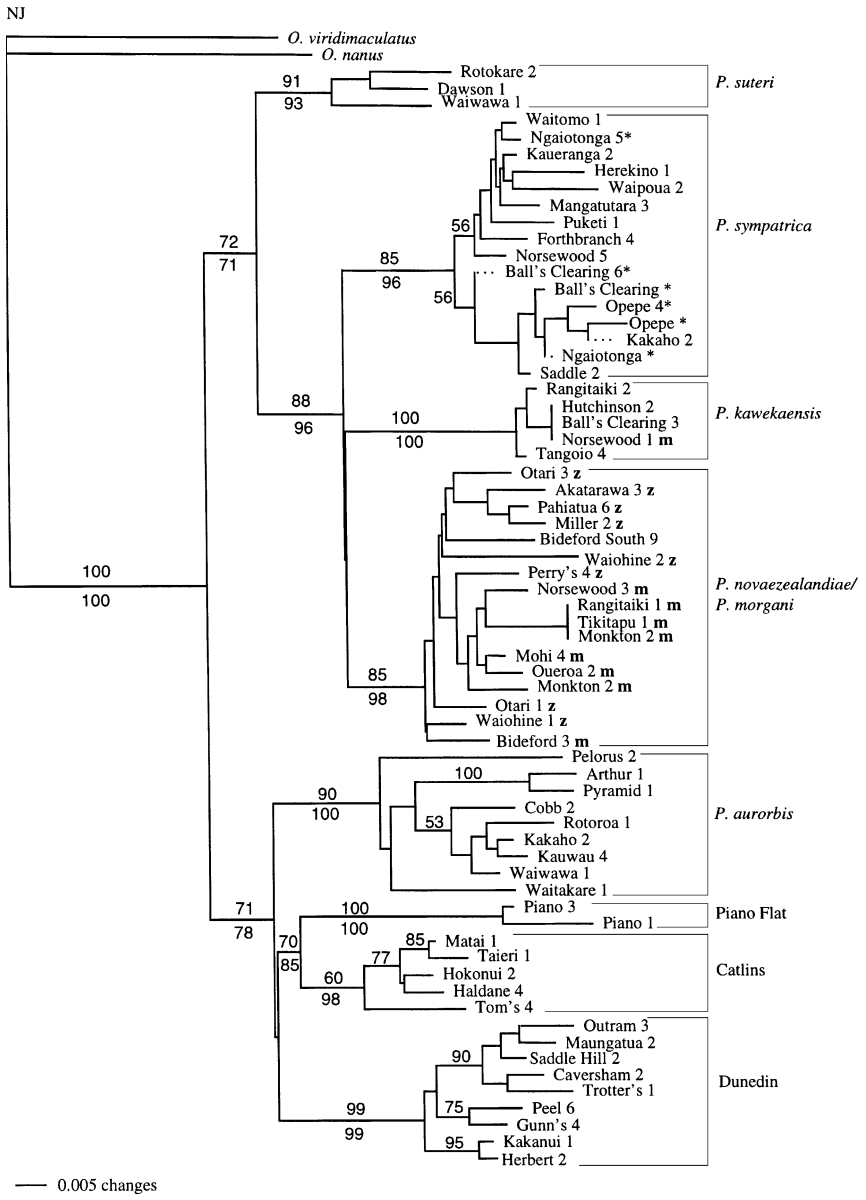

Fig. 3 Neighbour–joining (NJ) tree of mitochondrial DNA (mtDNA) cytochrome oxidase I (COI) sequences from New Zealand *Peripatoides*, with *Ooperipatellus* as outgroup. Numbers above and below the edges are maximum parsimony (MP) bootstrap values obtained from 500 replicates using: (i) the fast-stepwise addition option of PAUP, version 4.0, with equal weighting across the entire data set; and (ii) the full heuristic search option with a reduced set of operational taxonomic units (OTUs) and a transversion : transition weighting of 3 : 1. Numbers beside site labels indicate sample sizes. Other symbols indicate that only one haplotype was evident from single-strand conformation polymorphism analysis (SSCP) so that the frequency of alternatives was not known (*); and OTUs scored by allozymes as *P. novaezealandiae* (**z**) or *P. morgani* (**m**).

Maximum parsimony (MP) bootstrap support was calculated across the entire data set using the fast-search option of PAUP 4.0, with equal weighting, and this yielded a tree of similar topology to NJ. Repetition of this analysis with transversion : transition weighting 3 : 1 produced a tree with the same internal topology but poor resolution of terminal lineages within clades (bootstrap values are presented in Fig. 3). A reduced data set consisting of two to four haplotypes from each of the clades indicated by the full analysis was subjected to a full heuristic search yielding a single shortest MP tree. This was consistent with the NJ tree and each clade had equivalent or higher bootstrap support to that found by MP analysis of the full set of haplotypes (Fig. 3). A Maximum likelihood (ML) analysis of 12 taxa, incorporating the above GTR + I + Γ statistics, yielded a tree with the same internal topology as NJ and MP.

These trees showed strong support for five of the six species previously indicated by analysis of allozyme data (Fig. 4). The exception was *P. morgani*, which appeared to be paraphyletic with *P. novaezealandiae*. Altogether, eight clades were well supported by analysis of mtDNA, corresponding to five described and three undescribed species. Although the relationships among these taxa were poorly resolved, there was some bootstrap support (> 70%) for two clades consisting of several taxa:

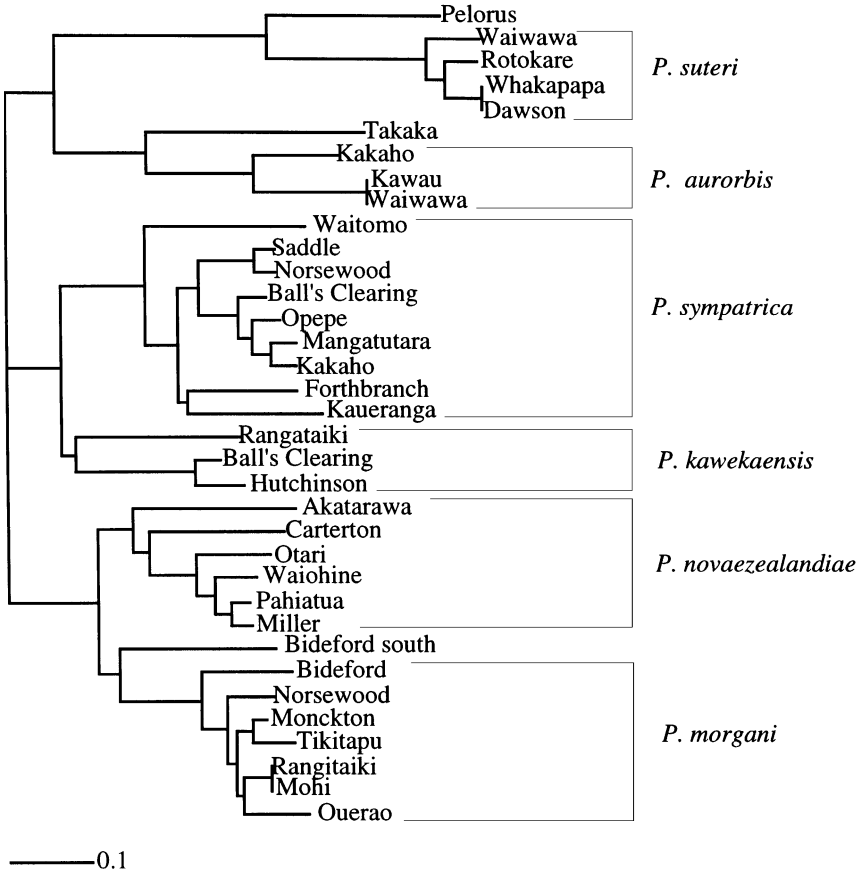

Fig. 4 Neighbour–joining (NJ) tree using the arc distance of Cavalli-Sforza & Edwards (1967) from 17 loci for 35 populations of *Peripatoides*. Species names are indicated, except for those samples of undetermined affinity which comprised single individuals and/or were basal to the principal clades (from Trewick 1998). Samples from Pelorus and Takaka, used for allozyme analysis, were not available for mitochondrial DNA (mtDNA) analysis.

P. suteri, *P. sympatrica*, *P. kawekaensis*, *P. novaezealandiae*/*P. morgani*, which are all found only in North Island, and *P. aurorbis*, 'Catlins', 'Piano' and 'Dunedin', which occur in South Island. *P. aurorbis* has populations in both main islands (Fig. 5), but mitochondrial lineages within the clade associated with northern and southern populations could not be separated phylogenetically. There was strong support (88–96%) for a clade of exclusively North Island taxa that did not include *P. suteri*.

The known range of some of the species detected with allozymes has been extended through the use of DNA sequencing, but no new taxa are evident in the regions previously surveyed using nuclear markers. Thus, northern *P. aurorbis* are shown to be present at Waitakere, and the range of the southern population extends south to Mount Arthur and Lake Rotoroa. The range of *P. sympatrica* extends into Northland (Waipoua, Ngaiotonga, Herekino and Puketi).

Although a *P. novaezealandiae*/*P. morgani* clade was evident in this analysis, the relationships among populations were not well supported (Fig. 3). MP analysis (with equal weighting) of the 17 *P. novaezealandiae*/*P. morgani* population haplotypes produced three equally short trees. These trees grouped the majority of *P. novaezealandiae* and *P. morgani* taxa into two clades that were consistent with the allozyme data (Fig. 6).

Among the peripatus surveyed with allozymes and mtDNA there was only one clear instance of gene flow (introgression) between species. One individual from Norsewood, which had nuclear markers indicative of *P. morgani*, had the same COI haplotype as *P. kawekaensis* found at Ball's Clearing and Hutchinson (Fig. 3).

Ambiguous sequence haplotypes encountered in individuals from Ngaiotonga, Ball's Clearing and Opepe clustered within a clade consistent with the allozyme species *P. sympatrica*. Detailed comparison of haplotype sequences in this clade showed that some were unambiguous and bore only one of the alternative nucleotides at the sites in question. This indicated that the ambiguous sequences could be reconciled as mixtures of alternative unambiguous haplotypes found in related individuals, and suggested heteroplasmy or nuclear integration in this clade. There was no statistical evidence of base bias or an unexpected transition/transversion ratio among these sequences, features that would otherwise provide evidence of nuclear copying. Scoring ambiguous substitutions as 'N' did not prevent resolution of a phylogeny consistent with the allozyme data.

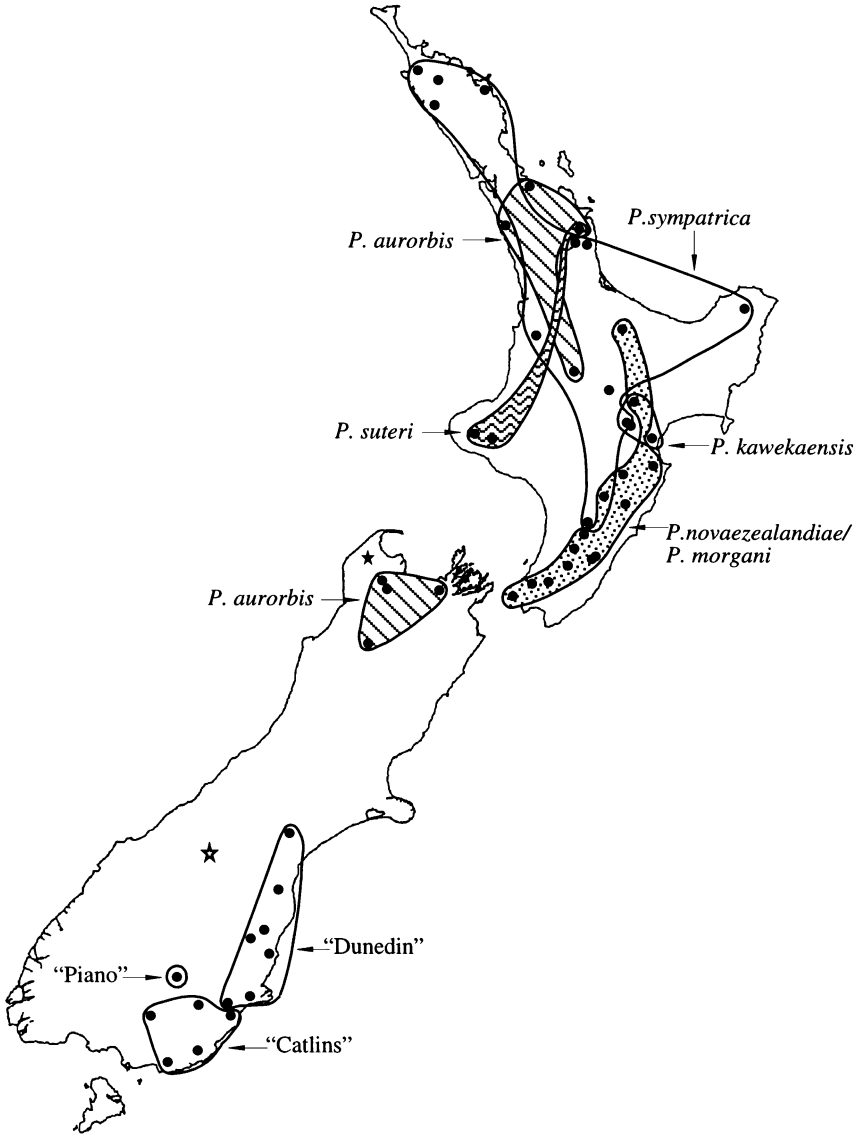

Fig. 5 Approximate geographical ranges of *Peripatoides* taxa based on phylogenetic analysis of mitochondrial DNA (mtDNA) cytochrome oxidase I (COI) sequence data. Sites from which peripatus bearing related haplotypes originate are indicated by approximate minimum area polygons drawn to avoid sites where a particular taxon has not yet been encountered. Some polygons are filled for clarity. Stars indicate the approximate location of rare peripatus taxa not included in this study; *P. indigo*, filled; undescribed species, open.

Discussion

Sequence evolution

A two-dimensional model of the insect COI gene has been proposed that consists of a number of structural regions associated with membrane-spanning helices, external loops and internal loops (Lunt *et al*. 1996). The pattern of amino acid variability among these various regions is constrained by the function of the regions. Comparison of among-region amino acid variability of insects and *Peripatoides* revealed a very similar pattern (Fig. 2). A + T composition of peripatus COI DNA sequence overall, and at third-codon positions in particular, was also similar to that found in insects (Table 1), and together these features suggest that COI composition and structure have changed little during the evolutionary history of these invertebrates.

Interspecific sequence and allozyme divergences within *Peripatoides* were higher than recorded for many insects (Fig. 7). In fact, the genetic diversity of the *Peripatoides* is more similar to that found in intergeneric studies of insects. This highlights the extreme level of morphological conservatism of the Onychophora (Ghiselin 1984) and the difficulty of predicting phylogenetic suitability of genetic markers on the basis of morphological variation. Interestingly, although there is evidence that these data from *Peripatoides* are influenced by saturation (e.g. transition : transversion ratio) and this will affect distance calibration, the phylogenetic signal appears to be relatively strong. A similar situation has been found in other studies of invertebrates using this gene (e.g. Caterino & Sperling 1999).

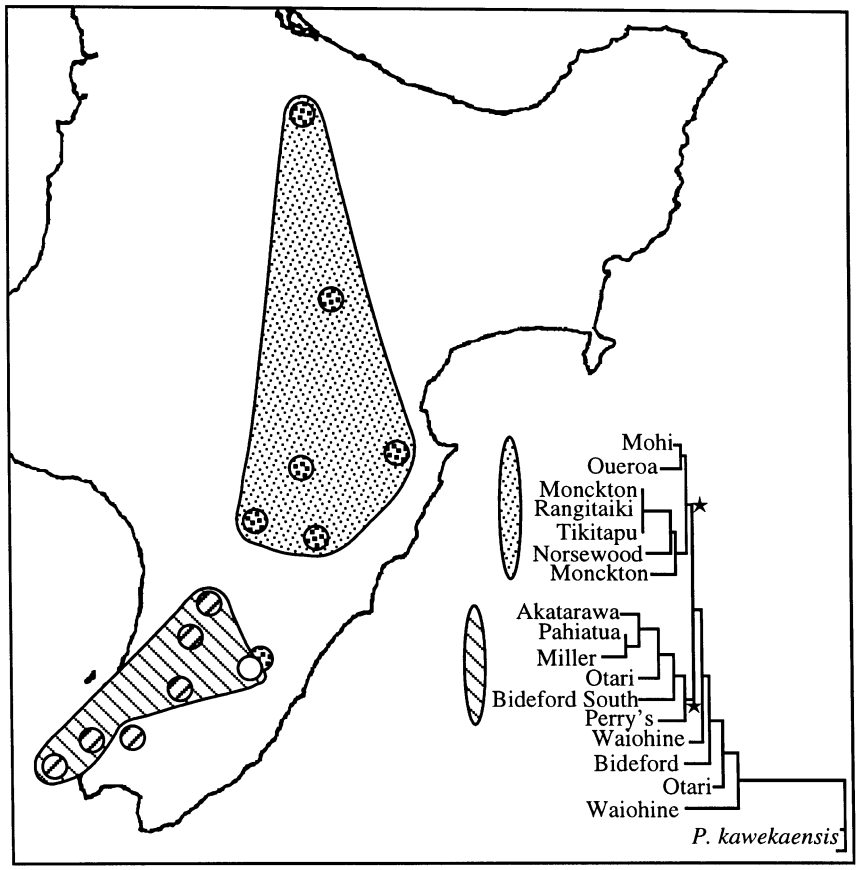

Fig. 6 Detail of North Island showing consensus support and distribution of *Peripatoides novaezealandiae*/*P. morgani* peripatus only. Small circles indicate sampling sites and allozyme genotypes: *P. morgani*, coarse stipple; *P. novaezealandiae*, coarse crosshatch; unspecified, open. Minimum area polygons indicate mitochondrial DNA (mtDNA) clustering as shown in the accompanying unweighted MP tree: *P. morgani*, fine stipple; *P. novaezealandiae*, fine crosshatch. Asterisks on the most-parsimonious (MP) tree indicate relevant nodes, supported in the three shortest trees.

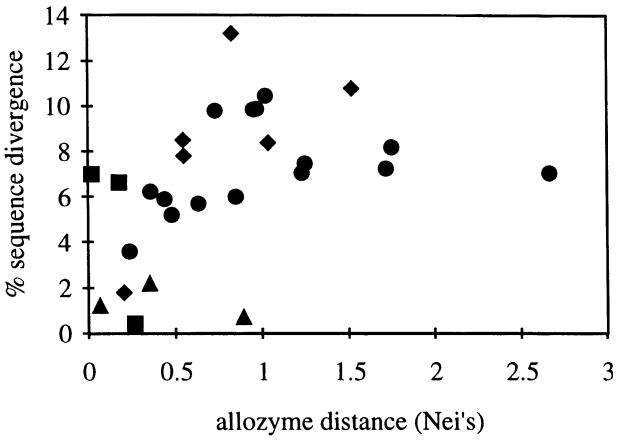

Fig. 7 Relationship between uncorrected mitochondrial DNA (mtDNA) divergence and allozyme (Nei's D) divergence from peripatus and a range of insects. *Peripatoides* (circles); *Gerris*, *Limnoporous*, *Aquarius* water striders (Sperling *et al*. 1997) (diamonds); *Anopholes* mosquitoes (triangles); *Pissodes* beetles (Langor & Sperling 1997) (squares).

Systematics

Eight distinct lineages are evident among the ingroup taxa. Five of these are consistent with the grouping previously identified by allozyme analysis, and three (Piano, Catlins, Dunedin) are based on samples from sites not included in that previous study (Trewick 1998). The combined evidence supports the notion that these latter, newly identified lineages also correspond to distinct species. The details of mtDNA diversity encountered in the Catlins and Dunedin areas have been discussed elsewhere (Trewick 1999), but in that analysis the distinctiveness of haplotypes from Piano Flat was not as apparent as it is in the present wider analysis. Further sampling at other locations in the vicinity is required to determine the distribution of this taxon.

The species *P. suteri*, which is distinctive in having 16 rather than 15 pairs of legs, is well supported by mtDNA and allozyme evidence across its range. Collection analysis indicates that the species is rare outside the Mount Taranaki area (Dawson and Rotokare), a region where no other *Peripatoides* have been reported. Four other North Island taxa form a distinct clade according to the mtDNA analyses. Of these, *P. kawekaensis* appears to have a very restricted range, in contrast to *P. sympatrica* which has the widest and most continuous distribution across central and upper North Island. The allozyme species *P. novaezealandiae* and *P. morgani* are the least well differentiated by mtDNA, but a consensus of MP mtDNA analysis, allozyme evidence and geographical distribution do support the

two groups (Fig. 6). Low mtDNA genetic distances, the existence of two lineages at some sites (Monckton, Otari, Waiohine) and absence of recent introgression, all suggest that these taxa have recently speciated or are in the process of doing so. If this is so, then minor inconsistencies between allozyme and mtDNA evidence are expected and can be explained by stochastic lineage sorting (Avise 1994).

The near absence of introgression among the peripatus surveyed with nuclear and mtDNA markers demonstrates the robustness of the described species (Fig. 3). Evidently, despite a lack of obvious morphological diversity, the *Peripatoides* do have effective species recognition systems and it is probable, given the nature of their habitat, that these involve pheromones (Eliott *et al*. 1993). In several instances of sympatry, separate species were found not only in the same patches of forest but within the same decaying log. Beyond their requirement for moist, dark conditions, with suitable invertebrate prey, the New Zealand Onychophora are tolerant of a wide range of conditions. There is no evidence for species-specific habitat requirements, with individuals of various taxa being found from sea level to an altitude of at least 1300 m, in decaying logs of native and exotic trees, in grass tussocks, in a buried rubbish dump, under stones and scree, and in forest detritus.

Biogeography

Analysis of mtDNA data supports the allozyme evidence for a species-complex within New Zealand *Peripatoides*. This diversity appears to be most pronounced in North Island where the distributions of taxa are complicated and overlapping. At this stage, the southern South Island taxa appear to be parapatric. The northern South Island taxon, *P. aurorbis*, is also present in central and northern North Island, and is therefore disjunct across Cook Strait. Two further South Island morphologically distinct species are very localized and were not available for inclusion in this analysis (see Fig. 5). Other geographical gaps in the present analysis are largely caused by the absence or scarcity of ovoviviparous peripatus in those regions (e.g. west and northeast South Island). The west coast and Alps of South Island appear to be chiefly the domain of the egglaying *Ooperipatellus*. In North Island, *P. sympatrica* is the most widespread and is represented in most instances of sympatry. The distribution of the North Island species implies high levels of sympatry, and their actual ranges will doubtless be broader than so far documented (Fig. 5). There is, however, some evidence of an east–west separation, with *P. aurorbis* and *P. suteri* in the west and *P. novaezealandiae*, *P. morgani* and *P. kawekaensis* in the east.

A Mantel test showed no significant correlation of allozyme and mtDNA genetic distances among the taxa studied using both methods (Table 2; Fig. 7). Allozyme distances (Nei's D) were very high, which suggests that divergence between the COI sequences is constrained by saturation so that the two measures cease to be correlated, or that Nei's D is not amenable to this type of calibration (Fig. 7). In a study of Jamaican peripatus, which showed low divergence, Hebert *et al*. (1991) used an allozymic clock calibration that assumed one unit of D to be equivalent to 19 Myr (Carlson *et al*. 1978; Vawter *et al*. 1980). In the present study, this would suggest divergence between the taxa with the highest Nei's D (*P. aurorbis* and *P. novaezealandiae*) of > 50 Myr. Maxson & Maxson (1979) used a rate of one D equivalent to 14 Myr, and Gardner & Thompson (1999) used the rate of Nei (1987, p. 247), equivalent to one unit of D per 5 Myr. Clearly, these various calibrations give a wide range of time estimates and may, regardless, be largely meaningless given that the high Nei's *D*-values among the *Peripatoides* appear to be derived from sorting of a small number of alternative alleles among the loci surveyed, rather than from mutations unique to particular taxa. The alternative would require the convergent evolution of alleles.

Several studies of the rate at which mtDNA evolves have yielded similar estimates for distance divergence of ≈ 2% per Myr (2%, Brown *et al*. 1979; 2.2–2.6%, Knowlton *et al*. 1993; 2.3% Brower 1994; Juan *et al*. 1995, 1996). However, analysis of shrimp taxa across the Panama isthmus has suggested that a rate nearer 1.4% may be applicable in at least some instances (Knowlton & Weigt 1998). COI sequences have proved useful for studies of recently diverged species because they rapidly accumulate phylogenetic information at that range. Furthermore, although COI sequence data tend to saturate relatively rapidly, phylogenetic information is retained at higher evolutionary levels (Lunt *et al*. 1996; Caterino & Sperling 1999). Although COI accumulates substitutions at ≈ 2% per Myr between closely related taxa, repeat substitutions cause this apparent rate to diminish markedly within divergences of less than 15% (Juan *et al*. 1995).

In the present study, *Peripatoides* clades based on COI sequence data differed by up to 11.4% using an often quoted substitution model (K2P). However, by incorporating a gamma correction for among-site rate variation and a proportion of invariant sites, the estimated maximum genetic distance is 17.5% GTR + I + Γ. These estimates imply a probable maximum time since divergence of between 5.7 and 8.75 million years ago (Ma) using a rate of 2% per Myr. Diversity within clades (highest in *P. aurorbis* 3.8% using the K2P model; 4.6% using the GTR + I + Γ model) apparently dates from ≈ 2 Myr (i.e. Pleistocene). However, a model that accommodates the effects of saturation by reducing the estimated rate of evolution over time will probably provide a more realistic indication of divergence times beyond ≈ 3 Myr (Juan *et al*.

1995; Sandoval *et al*. 1998). Thus, pairwise distances of 11.8% using the K2P model to 17.5% using the GTR + I + Γ model (the upper range in the present study of *Peripatoides*) may well indicate divergence nearer to 10 Myr (Sandoval *et al*. 1998). While these estimates have to be treated with caution, they suggest that most, if not all, of the diversity within the ovoviviparous Onychophora of New Zealand may have arisen relatively recently in the geological history of New Zealand, perhaps during the middle Miocene (15–7 Myr). In New Zealand, the late Miocene to early Pliocene period was marked by marine inundation events that formed a different configuration of islands than that of today. The North Island area, in particular, was subdivided and marine straits formed across its lower third (Stevens 1981). During the Pliocene (7–2 Myr), the South Island mountain ranges developed, and in the Pleistocene (< 2 Myr) volcanic activity formed mountains in North Island and would have been the cause of extinction events in the central region. At this time, North and South Island were a single entity, having been separated by the Cook Strait for only ≈ 16 000 years (Lewis *et al*. 1994).

Habitat fragmentation during the late Miocene may have given rise to allopatric peripatus populations, but also led to extinction of some taxa. Dispersal between the two modern islands would not have been subsequently hampered by sea until after the Pleistocene, and rapid climate fluctuations at this time could have led to population genetic structuring. The absence of deeper molecular diversity and concomitant morphological variation may be the result of high levels of extinction into the Miocene. Large-scale extinction of New Zealand biota during Oligocene marine inundation has previously been proposed (Cooper & Millener 1993). Alternatively, the molecular evidence may imply an unexpectedly recent arrival of Onychophora in New Zealand.

Analysis of COI sequence data from Australian Onychophora plus representatives from New Zealand and South America revealed a maximum genetic distance of 20.6% (Gleeson *et al*. 1998). Genetic distance between some Tasmanian and New Zealand Onychophora was as low as 10.5%. While it is apparent that these relatively low levels are to a greater or lesser extent the result of saturation, greater distances have been reported from other invertebrate taxa for the same gene (e.g. 27% in Coleoptera, Howland & Hewitt 1995; 30% in Orthoptera, S. A. Trewick, unpublished). This suggests that either the extant global Onychophoran fauna evolved far more recently than Gondwana vicariance would suggest (e.g. 140 Myr split of S. America from other continents) or that the evolutionary rate and/or capacity for change of COI in Onychophora is, despite the evidence of amino acid variability, etc., described above, very different from that in other invertebrates. Either way, the COI gene is unlikely to have the capacity to retain accurate phylogenetic information across that time frame (Howland & Hewitt 1995).

As much of New Zealands biota has traditionally been considered to be derived directly by vicariance from Gondwana (Fleming 1979; Steven 1981; Cooper & Millener 1993), evidence to the contrary is of considerable interest (e.g. Linder & Crisp 1995). Further exploration of Onychophora and other invertebrate taxa using a range of different genes may well help to resolve this enigma.

Acknowledgements

I am grateful to the Department of Conservation for the provision of relevant collecting permits. I wish to thank Mary Morgan-Richards, Jon Waters, Graham Wallis, Andrea Taylor, Paul Sunnucks, Warren Chin, Tony Harris, Ken Mason, Dave Randal, Malcolm Haddon, Scott Dunavan, Ray Forster, Lyn Forster, Sue Millar, Karen Tutt and colleagues for their contribution to the sampling. Many thanks to the members of the Evolutionary Genetics Laboratory for providing feedback on various aspects of the work, and an amiable environment. Thanks also to Paul Sunnucks and Alex Wilson who introduced me to the wonders of simple SSCP, and David Lunt who provided data on insect COI. Johns Hobbs (Nucleic Acid — Protein Service, NAPS Unit, University of British Columbia, Vancouver, BC, Canada) supplied insect mitochondrial DNA primers, taken from sequences compiled by Dr B. Crespi (Simon Fraser University, Burnaby, BC, Canada) and described on postings to bug-net@sfu.ca. This work was supported by a Foundation for Research Science and Technology fellowship (contract UOO704).

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Steve Trewick is a postdoctoral research fellow in the Evolutionary Genetics Laboratory. This study is part of a broader phylogeography programme applying molecular techniques to endemic invertebrates. The programme seeks to identify patterns and origins of diversity in New Zealand and to explore the evolutionary relationships with neighbouring biotas.

Appendix 1 Details of sampling sites: location names, latitude/longitude and other notes of interest

North Island

Ngaiotonga reserve (35°18′: 174°15′); Waipoua forest (35°38′: 173°33′); Herekino (35°09′: 173°16′); Puketi forest (35°16′: 173°40′); Waitakere forest (36°54′: 174°32′); Kawau Island [two sites separated by > 2 km] (36°30′: 174°40′); Waiwawa, Coromandel Range (36°59′: 175°37′); Kaueranga, Coromandel Range (37°06′: 175°38′); Forthbranch, Coromandel Range (37°08′: 175°44′); Waitomo Caves reserve (38°15′: 175°06′); Mangatutara, Raukumara Range (37°55′: 177°55′); Lake Tikitapu (38°11′: 176°20′); Opepe historic reserve, Taupo (38°42′: 176°10′); Rangataiki (38°59′: 175°39′); Kakaho, Pureora forest (38°33′: 175°43′); Ball's Clearing reserve (39°16′: 176°39′); Hutchinson reserve (39°16′: 176°32′); Tangoio, White Pine reserve (39°18′: 176°49′); Oueroa [farm] (40°06′: 176°41′); Mohi Bush reserve (39°35′: 177°05′); Monckton reserve (39°57′: 176°16′); Miller reserve (40°42′: 175°39′); ANZAC reserve, Norsewood (40°52′: 176°13′); Saddle Road (40°17′: 175°49′); Bideford (40°51′: 175°52′); Bideford south [paddock] (40°50′: 175°52′); Perry's Road, Carterton (41°00′: 175°35′); Pahiatua [paddock] (40°26′: 175°47′); Waiohine reserve (40°59′: 175°23′); Akatarawa (40°57′: 175°06′); Otari plant museum (41°6′: 174°45′); Waiwawa, Coromandel Range (36°59′: 175°37′); Whakapapa village (39°12′: 175°32′); Dawson Falls (39°19′: 174°06′); Lake Rotokare (39°27′: 174°24′).

South Island

Takaka scenic reserve (40°45′: 172°55′); Pelorus Bridge (41°05′: 173°30′); Cobb valley (41°05′: 172°43′); Mnt Arthur (41°11′: 172°44′); Pyramid Pk (41°11′: 172°40′); Lake Rotoroa (41°47′: 172°30′); Gunn's Bush (44°39′: 170°57′); Peel Forest (44°53′: 171°15′); Trotter's Gorge (45°24′: 170°47′); Kakanui Mnts [in scree] (45°56′: 170°28′); Caversham Valley (45°53′: 170°28′); Saddle Hill (45°54′: 169°21′); Taieri Mouth (46°03′: 170°11′); Outram (45°50′: 170°14′); Maungatua (45°53′: 170°08′); Piano Flat (45°33′: 169°01′); Tom's Creek (45°54′: 169°29′); Hokonui (46°04′: 168°50′); Matai Falls (46°30′: 169°29′); Haldane (46°34′: 169°00′).