New Zealand Journal of Zoology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tnzz20

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Version of record first published: 30 Mar 2010.

To cite this article: S. A. Trewick (1999): Molecular diversity of Dunedin peripatus (Onychophora: Peripatopsidae), New Zealand Journal of Zoology, 26:4, 381-393

To link to this article: http://dx.doi.org/10.1080/03014223.1999.9518201

New Zealand Journal of Zoology, 1999, Vol. 26: 381–393
0301–4223/2604–0381 $7.00/0

Molecular diversity of Dunedin peripatus (Onychophora: Peripatopsidae)

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Abstract Onychophora tend to be morphologically conservative. Several studies using molecular techniques have revealed the existence of cryptic species and population structuring. The application of allozyme electrophoresis to New Zealand peripatus that were thought to belong to a single widespread species (*Peripatoides novaezealandiae*) has revealed several undetected species, including a taxon specific to Dunedin (southern South Island, New Zealand). However, almost nothing is known about the geographic range and variability of this species, nor indeed whether it comprises one or more cryptic taxa. I used analysis of cytochrome oxidase I mtDNA sequences to explore these aspects of peripatus found in the vicinity of Dunedin. Eighteen different haplotypes were detected in 47 individuals from 21 locations. The sequence of a 540 bp COI fragment contained 64 parsimony informative sites and nucleotide diversity of up to 11% among ingroup taxa. Phylogenetic analyses, and genetic distance by geographic distance correlation, indicated probable species-level divisions within the sample. Two principal groups with a boundary on the east coast of New Zealand near the mouth of the Taieri River can be defined, and these are denoted "Dunedin" and "Catlins" peripatus. There is a third, possibly distinct lineage at Piano Flat. A rearrangement of the mitochondrial genome, relating to the position of the tLEU rna gene, was detected in these and other New Zealand peripatus and may be present in all Onychophora.

Z99006
Received 3 March 1999; accepted 4 June 1999

Keywords New Zealand; mtDNA; *Peripatoides*; invertebrate; population structure; habitat; genetics; PCR primers; mitochondrial rearrangement; cytochrome oxidase; COI; SSCP

INTRODUCTION

The living Onychophora are morphologically conservative although they can be variable in size and colour within populations. It is of no surprise therefore that early morphology-based taxonomy also tended to be conservative and to underestimate the true diversity of the group. Molecular studies and the use of electron microscopy have revealed the existence of many cryptic species and genera, most notably in Australia (Briscoe & Tait 1995; Reid 1996) although the first genetic study was on Jamaican peripatus (Hebert et al. 1991). The Australian onychophoran fauna appears to be both diverse, including some 80% of the described global fauna (Briscoe & Tait 1995; Reid 1996), and locally rich with some areas having as many as five genera (Sunnucks et al. in press). Contrary to the usual rule of conservatism, some of these taxa possess very distinctive morphology in the form of mating-related structures on the head resolved using electron microscopy (Tait & Briscoe 1990; Reid 1996).

In comparison, the onychophoran fauna of New Zealand appears to be generally less diverse and locally depauperate. Until recently, only five species of peripatus were recognised in New Zealand (Ruhberg 1985), belonging to two genera; the egglaying *Ooperipatellus* and the livebearing *Peripatoides*. Of these, *Peripatoides novaezealandiae* (Hutton) was apparently the most numerous and widespread (Ruhberg 1985). Hutton's (1876) description was based on specimens from Wellington, Nelson and Dunedin. However, analyses of allozyme data have indicated the existence of a species complex (involving at least five taxa) in North Island and a separate taxon in Dunedin (south South Island) (Tait & Briscoe 1995; Trewick 1998). While the North Island *P. novaezealandiae* species complex

has so far revealed no gross morphological differences among sister species (Trewick 1998), Tait & Briscoe (1995) noted that a combination of morphological characteristics (15 pairs of legs, midventral openings of anal glands in males, and lack of crural glands) in the Dunedin taxon distinguished it from other described genera. Allozyme data revealed no close relatives among the limited number of New Zealand and Australian peripatus studied (Tait & Briscoe 1995). A survey of Australasian Onychophora using mitochondrial DNA, that supported monophyly of the New Zealand *Peripatoides*, also included specimens from one location in Dunedin (Gleeson et al. 1998).

At present, however, Dunedin peripatus have been represented in studies only by specimens from two sites (Caversham and Leith valleys), both of which are close to the city centre (Tait & Briscoe 1995, Gleeson et al. 1998). Before any interpretation of nationwide phylogeographic patterns can be made, a more complete understanding of local diversity and geographic range is required. Any exploration of diversity is likely to raise questions of species definition, sympatry and conservation status.

There are advantages in a morphology based taxonomy (e.g., presumed ability to undertake identification in the field and without specialist equipment), but genetic approaches also have much to offer systematists, biogeographers and conservationists alike. This is because (a) information is derived, by definition, from the material that is transmitted between individuals during reproduction, and (b) these molecules evolve in a more or less predictable manner across taxa.

Perceived conflicts between genetic and morphological approaches arise because morphology has traditionally been a prerequisite of taxonomy, and at a fine scale at least, taxonomy can proceed without a phylogenetic basis. Genetic analyses assume an evolutionary process in order to interpret the historical relationships of genes and therefore their "hosts". Whilst this is in principle also true of cladistic methods, an absence of suitably variable morphological characters, or problems of homology, can be overwhelming. Many phylogenetic analyses of genetic data produce gene trees, or their approximation. It is the job of molecular biologists to infer how well these trees estimate organismal phylogeny, and an extensive array of analytical tools have been developed to do this. In a sense this is a reversal of the situation that arises from morphology-based analyses which, naturally, tend to underestimate biogenetic diversity (Avise 1994). In the study of Onychophora for instance, a group noted for their morphological conservatism, genetic methods have revealed extensive cryptic diversity (Briscoe & Tait 1995), stimulated revision of morphology based taxonomy (Reid 1996) and provided insights into the origin of diversity within, and biogeography of, species (Hebert et al. 1991; Gleeson et al. 1998).

Previous genetic studies of New Zealand Onychophora have used allozyme variation (Tait & Briscoe 1995; Trewick 1998). These nuclear loci can have many different alleles (arising from changes in the sequence of amino acids comprising a particular protein), and as such are especially useful for observing gene flow among populations of sexually reproducing organisms (Murphy et al. 1996). In cases of sympatry, the lack of exchange of alleles between sexually reproducing individuals demonstrates the presence of distinct species, even if those species are not outwardly distinguishable (King & Hanner 1998; Trewick 1998). The other most frequently used genetic marker in the 1990's, mitochondrial DNA, is however, generally non-recombining (but see Wallis 1999) and in most organisms is maternally inherited. Although this means mtDNA is not appropriate for tests of genetic exchange on its own, it is a powerful indicator of historical subdivision, and extensive datasets enable among-taxa comparisons of diversity and structure (Avise 1994). MtDNA sequence data is particularly useful for interspecific phylogenetic analysis. By using faster-evolving genes and sampling individuals throughout the range of a presumed species it is also feasible to explore intraspecific phylogeography (Riddle 1996). The use of mtDNA also has the benefit of requiring less sample tissue than allozyme electrophoresis so that the morphological integrity of specimens can more easily be maintained. This is particularly helpful in the study of small or soft bodied organisms such as peripatus.

I surveyed mtDNA sequence variation in Dunedin peripatus in order to identify their geographic range, intraspecific variability, possible existence of cryptic species, and conservation status. The Dunedin peripatus is already perceived to be of special status to the extent that New Zealand's first peripatus reserve has been gazetted for it by the city council. Whether this action is justified, or the location well chosen, has remained untested. By documenting diversity of peripatus within the Dunedin region we will be better placed to explore the benefit of such reserves, and the status of the Otago and Southland regions with respect to the wider biogeography of onychophorans in New Zealand.

METHODS

Peripatus specimens were collected in the environs of Dunedin City and up to 220 km from it (Fig. 1). This collecting area is probably close to the range limit for ovoviviparous onychophora in south-eastern South Island, which are more frequent in the north-western part of the island. Following euthanasia with ether, specimens were stored at –80°C. One or two legs were amputated from each specimen, thus leaving the animal essentially intact and available for dissection and/or long term preservation as voucher specimens. DNA was extracted from entire leg tissue using a salting-out method. Tissue was macerated and incubated with 25 µl of 10 mg/ml proteinase-K in 300 µl of TNES buffer (20 mM EDTA, 50 mM Tris, 400 mM NaCl, 0.5% SDS) at 50°C. 10% 5 M NaCl was added and the extractions shaken vigorously for 20 s followed by spinning at 14 000 rpm for 5 mins. The supernatant was removed and precipitated with an equal volume of cold 100% ethanol. DNA was collected by spinning and washed with 70% ethanol before being dried and dissolved in water.

Mitochondrial primers C1-J-1718 and C1-N-2191 (Simon et al. 1994) were used to amplify a short (~400 bp) DNA fragment toward the 5′ end of the cytochrome oxidase I gene (COI) using PCR (polymerase chain reaction). SSCP (single stranded conformation polymorphism) was used to identify peripatus individuals with variant haplotypes. PCR products were isotopically labelled by incorporation of adATP ^{33}P. 10 µl reactions (200 µm dNTPs, 2.5 mM $MgCl_2$, 0.25 U Qiagen Taq) were treated to 40 cycles of 94°C 15", 50°C 30", 72°C 1′30" with an initial denaturation of 1′ at 94°C. Following PCR, products were denatured for 5 min at 95°C in the presence of an equal volume (10 µl) of 95% formamide loading buffer. Denatured products were loaded from ice into vertical, non-denaturing polyacrylamide gels consisting of 6% 37.5:1 bis/acrylamide, 5% glycerol and 0.5x TBE. Gels were electrophoresed at 4°C for 200 watthrs at approximately 13 W. Following electrophoresis, gels were lifted on blotting paper, dried and exposed with Biomax (Kodak) autoradiography film for 24–48 h. Individuals were scored for haplotype by comparison of re-natured single-strand DNA migration patterns.

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 (see Results for details). PCR reactions were performed in 25 µl volumes and products gel-purified in 2% agarose stained with ethidium bromide. Bands of expected molecular weight were excised and the DNA extracted from the agarose using QIAquick spin columns (Qiagen). Purified DNA fragments were quantified by eye using agarose electrophoresis with a molecular weight marker. Cycle sequencing used Bigdye chemistry (Perkin Elmer) following the manufacturer's protocols. Sequences were aligned manually using SeqEd. v1.0.3 (ABI, PE). Phylogenetic analysis and Mantel tests were performed using PAUP 4.0 (Swofford 1998) and GenePop v3.1 (Raymond & Rousset 1995; Rousset 1997) respectively. Universal primers used were sourced from the insect mtDNA primer set (John Hobbs, UBC). Sequence data was deposited at Genbank (AF18841–AF18862) and voucher specimens at Otago Museum (OMNZ).

RESULTS

Sampling

All specimens of peripatus collected for this study had 15 pairs of legs, and were predominantly orange-brown in colour. Most were obtained from within decaying native timber logs (Table 1). However, there were exceptions; specimens from the Kakanui Range were found beneath stones in a damp scree among tussocks at 1300 m asl; those from Tomahawk Lagoon were found in humus/soil beneath bracken and muehlenbeckia growing at the waters edge (Ken Mason pers. comm.). Forested habitat in the central city area, Gunns Bush and Trotters Gorge consisted of regenerating broadleaf scrub with podocarps and exotics. Bush at Haldane, Hokonui and Peel consisted of podocarp forest, while Matai Falls, Taieri Mouth, Toms Creek and Piano Flat were predominantly beech (*Nothofagus*) (Table 1).

As is typical for Onychophorans, peripatus collected for this study varied considerably in size. In general the largest were mature females which ranged in size from 250–350 mg. The heaviest, found at Outram, was exceptional for NZ onychophora weighing 700 mg. Twenty-one sites in and around Dunedin were sampled (Fig. 1).

Primer design

The universal primer L2-N-3014 locates within the tLEUrna between COI and COII and successfully primes PCR in a wide spectrum of invertebrates

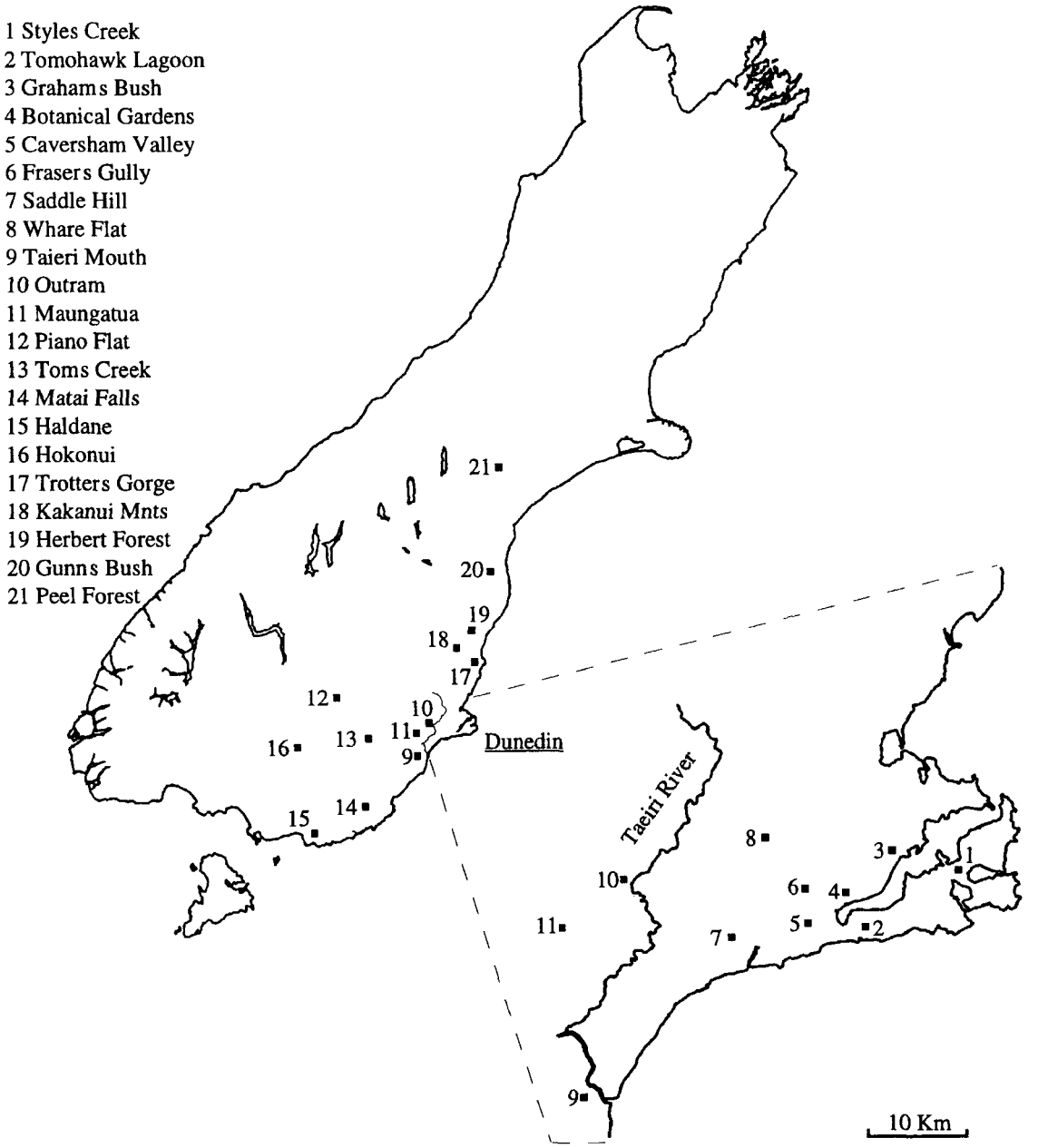

Fig. 1 Peripatus sampling sites in southeast South Island, New Zealand. Scale bar refers to inset map of Dunedin area.

(Simon et al. 1994), but it did not amplify peripatus DNA when used in conjunction with C1-J-2195 or C1-J-1718. However, a longer fragment of ~1400 bp bridging the COI-COII boundary was amplified using C1-N-2195 and C2-N-3661. I sequenced this fragment from each end and aligned these with representative COI and COII sequences from other invertebrates. The tLEUrna gene was not present between these two cytochrome oxidase genes, which indicates reorganisation of the peripatus mitochondrial genome in comparison with other invertebrates. An unusual "TA" stop codon indicates the end of the

COI gene and the COII gene is initiated with an "ATG" codon typical of many insects (Szymura et al. 1996). I designed primers in relatively conserved regions towards the 3′ end of COI in order to be able to reliably amplify a 600–800 bp fragment for sequencing, as follows:

NotLEUr ATGATCAAAAGGAGGAAT (2961), and
Perip241r TATCGTCGAGGTATTCCACT (2770).

These sequences are written 5′–3′. Both are on the minority strand, and the number in parentheses indicates the position of the 3′ end with respect to the *Drosophila yakuba* mtDNA genome (Simon et al. 1994).

Genetic diversity

The sequence of a 540 bp fragment of the mtDNA COI gene was obtained from representative peripatus (Appendix 1). Among the ingroup taxa this sequence contained 64 parsimony informative sites. Approximately 88% of substitutions were at third codon positions, 11% and 1% at first and second positions, respectively. There was a transversion:transition ratio of 1:1.5 and no significant variation in base composition among sequences (PAUP 4.0). Protein translation showed that several nucleotide substitutions were non-synonymous but amino acid substitutions did not include any aberrant stop codons (Table 2).

Eighteen unique haplotypes were obtained from 47 peripatus collected at 21 sites. Where several individuals from a particular site were analysed, all had the same haplotype except at Piano Flat, where two distinct haplotypes were present in a sample of four peripatus. Elsewhere, one haplotype was shared by nine peripatus from six sites (Botanic Garden, Caversham valley, Frasers Gully, Grahams Bush, Whare Flat and Tomohawk Lagoon), but all of these sites were within a radius of 10 km about the Dunedin city centre. Kimura 2 parameter (K2P) genetic distances between the 18 haplotypes ranged up to 11% (Piano Flat vs Outram and Trotters Gorge) (Table 2). Comparison of these animals with an ovoviviparous peripatus with 15 pairs of legs from Nelson Lakes, South Island yielded distances of 6.8–10.3%.

Phylogeny

Parsimony analysis with equal weighting yielded 28 equally short trees. A majority-rule consensus tree of these showed consistent signal for two principal clades and other nodes within these. The nodes least frequently obtained among these trees were also the least well supported in bootstrap analysis (e.g., Haldane: Hokonui and Saddle Hill; Maungatua:

Table 1 Details of peripatus sampling sites numbered according to Fig. 1.

	Location	Latitude	Longitude	Vegetation	Habitat
	Styles Creek	45° 51′	170° 37′	Podocarp regen.	decaying log
	Tomohawk Lagoon	45° 54′	170° 33′	muehlenbeckia	by water
	Grahams Bush	45° 49′	170° 35′	Podocarp regen.	decaying log
	Botanic Garden	45° 51′	170° 32′	Podocarp regen.	decaying log
	Caversham valley	45° 53′	170° 28′	Podocarp regen.	decaying log
	Frasers Gully	45° 50′	170° 29′	Podocarp regen.	decaying log
7	Saddle Hill	45° 54′	169° 21′	Podocarp regen.	decaying log
8	Whare Flat	45° 47′	170° 25′	Podocarp regen.	decaying log
9	Taieri Mouth	46° 03′	170° 11′	Beech	decaying log
10	Outram	45° 50′	170° 14′	Podocarp regen.	decaying log
11	Maungatua	45° 53′	170° 08′	Beech	decaying log
12	Piano Flat	45° 33′	169° 01′	Beech	decaying log
13	Toms Creek	45° 54′	169° 29′	Beech	decaying log
14	Matai Falls	46° 30′	169° 29′	Beech	decaying log
15	Haldane	46° 34′	169° 00′	Podocarp regen.	decaying log
16	Hokonui	46° 04′	168° 50′	Podocarp regen.	decaying log
17	Trotters Gorge	45° 24′	170° 47′	Podocarp regen.	decaying log
18	Kakanui Mnts	45° 56′	170° 28′	Native tussock	scree in tussock
20	Gunns Bush	44° 39′	170° 57′	Podocarp regen.	decaying log
21	Peel Forest	44° 53′	171° 15′	Podocarp	decaying log

Table 2 Genetic distance among ingroup and outgroup peripatus (Kimura 2 parameter below diagonal, amino acid number above diagonal) The two groups (A and B) indicated by phylogenetic analysis are bordered. Faint borders within A and B indicate distances excluding Piano Flat site (i.e., Catlins peripatus), and distances among Dunedin City populations, respectively.

Site and number		Nels	Pian	Pian	Mata	Toms	Taie	Hoko	Hald	Tomo	Styl	Outr	Sadd	Maun	Bota	Cave	Grah	Whar	Fras	Trot	Kaka	Herb	Peel	Gun
Nelson Lakes			7	7	5	6	7	5	6	4	4	5	4	4	4	4	4	4	4	4	4	5	4	4
Piano Flat	12	9.5	**A**	0	5	4	7	5	6	7	7	7	7	7	7	7	7	7	7	7	7	8	7	7
Piano Flat	12	10.3	1.7		5	4	7	5	6	7	7	7	7	7	7	7	7	7	7	7	7	8	7	7
Matai Falls	14	6.8	6.0	7.4		1	2	0	1	5	5	5	5	5	5	5	5	5	5	5	5	6	5	5
Toms Creek	13	7.2	6.2	7.7	2.7		3	1	2	6	6	6	6	6	6	6	6	6	6	6	6	7	6	6
Taieri Mouth	9	7.7	6.6	8.1	0.8	3.4		2	3	7	7	7	7	7	7	7	7	7	7	7	7	8	7	7
Hokonui	16	7.0	6.0	7.4	1.1	3.0	1.9		1	5	5	5	5	5	5	5	5	5	5	5	5	6	5	5
Haldane	15	7.2	6.0	7.4	1.1	3.0	1.9	1.1		6	6	6	6	6	6	6	6	6	6	6	6	7	6	6
Tomohawk	2	8.4	7.8	9.3	7.2	6.8	7.9	7.2	7.2	**B**	0	1	0	0	0	0	0	0	0	0	0	1	0	0
Styles Creek	1	8.6	8.0	9.5	7.4	7.0	8.1	7.4	7.4	0.2		1	0	0	0	0	0	0	0	0	0	1	0	0
Outram	10	10.1	9.5	11.0	7.6	7.6	8.3	7.8	7.6	2.3	2.5		1	1	1	1	1	1	1	1	1	2	1	1
Saddle Hill	7	9.1	8.2	9.7	7.2	6.6	7.9	7.4	7.2	1.9	2.1	2.1		0	0	0	0	0	0	0	0	1	0	0
Maungatua	11	9.5	8.9	10.3	7.2	6.8	7.9	7.4	7.2	1.9	2.1	1.5	0.9		0	0	0	0	0	0	0	1	0	0
Botanic Gdn	4	8.4	7.8	9.3	7.2	6.8	7.9	7.2	7.2	0.0	0.2	2.3	1.9	1.9		0	0	0	0	0	0	1	0	0
Caversham vall.	5	8.4	7.8	9.3	7.2	6.8	7.9	7.2	7.2	0.0	0.2	2.3	1.9	1.9	0.0		0	0	0	0	0	1	0	0
Grahams Bush	20	8.4	7.8	9.3	7.2	6.8	7.9	7.2	7.2	0.0	0.2	2.3	1.9	1.9	0.0	0.0		0	0	0	0	1	0	0
Whare Flat	8	8.4	7.8	9.3	7.2	6.8	7.9	7.2	7.2	0.0	0.2	2.3	1.9	1.9	0.0	0.0	0.0		0	0	0	1	0	0
Frasers Gully	6	8.4	7.8	9.3	7.2	6.8	7.9	7.2	7.2	0.0	0.2	2.3	1.9	1.9	0.0	0.0	0.0	0.0		0	0	1	0	0
Trotters Gorge	17	9.5	9.5	11.0	8.0	7.8	8.7	7.4	8.2	1.7	1.9	3.2	2.5	2.5	1.7	1.7	1.7	1.7	1.7		0	1	0	0
Kakanui Mnts	18	7.6	8.2	9.5	6.0	5.2	6.6	6.4	6.4	3.0	3.2	3.6	2.7	3.2	3.0	3.0	3.0	3.0	3.0	3.6		1	0	0
Herbert Forest	19	7.4	8.4	9.9	6.2	5.4	6.8	6.6	6.6	3.2	3.4	3.8	2.8	3.4	3.2	3.2	3.2	3.2	3.2	3.8	0.6		1	1
Peel Forest	21	8.6	8.6	10.1	6.2	6.8	6.2	6.4	6.2	3.6	3.8	4.0	3.2	3.6	3.6	3.6	3.6	3.6	3.6	4.2	3.0	3.2		0
Gunns Bush	20	8.2	8.4	9.9	6.4	6.4	6.8	6.2	6.4	2.7	2.8	3.4												

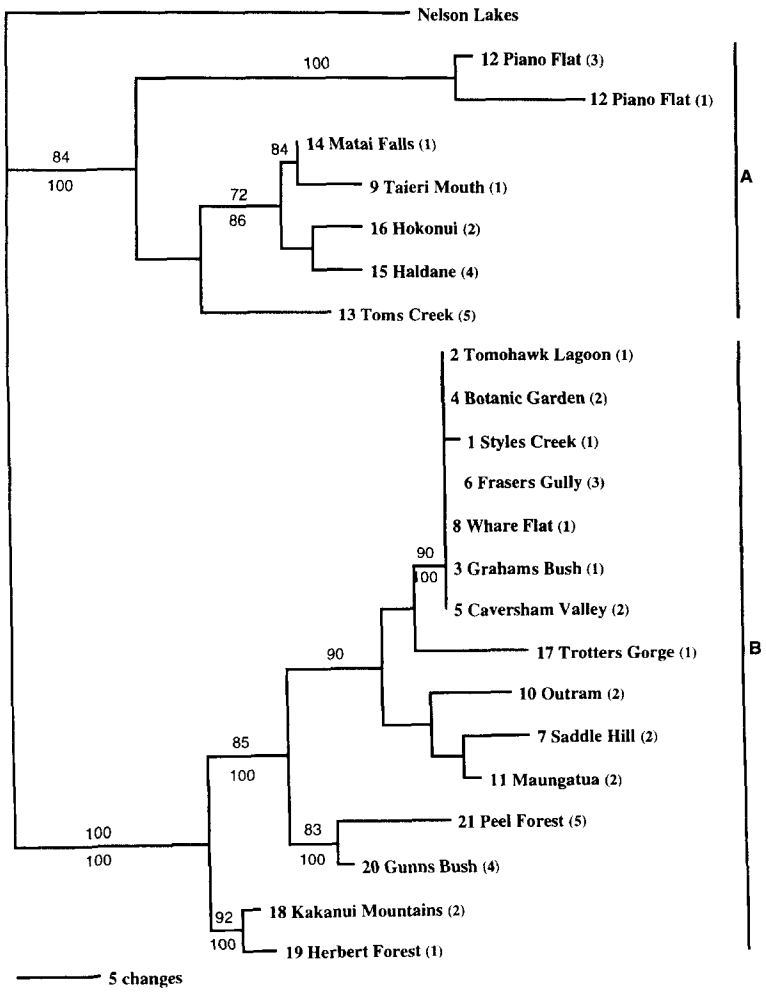

Fig. 2 Maximum-parsimony bootstrap phylogram of COI DNA sequences from peripatus. Numbers above edges are percentage support from 1000 bootstrap replications. Values below edges indicate percentage of 28 equally short MP trees having the respective node. Sample sizes are given in parentheses. Two principal clades are indicated (A and B).

Outram pairs, Fig. 2). However, neighbor-joining (Saitou & Nei 1987) using Kimura 2 Parameter weighting, and bootstrap parsimony analysis produced trees of near-identical topology (Fig. 2). The only conflict was in the Piano Flat, Toms Creek branching order. There was good support for monophyly of the Dunedin area peripatus with respect to the outgroup used. The use of transition:transversion weighting (up to 1:5) and codon position weighting did not alter the structure of MP trees.

Two clades were apparent in the ingroup. One (Fig. 2 group B) included peripatus collected around the centre of Dunedin City plus sites to the north (Kakanui mountains, Trotters Gorge, Gunns Bush, Herbert Forest and Peel Forest), and south (Saddle Hill, Maungatua and Outram) (Fig. 3). The second (Fig. 2 group A) consists of peripatus collected further south (Taieri Mouth to Haldane) and to the west (Hokonui and Piano Flat) (Fig. 3). The maximum genetic distance encountered among group B peripatus was 4% (Table 2). Within group A, however, distances reached 8.1%, but were similar (3.4%) to group B when specimens from Piano Flat were excluded. This was despite the relatively high geographic distances between peripatus from the remaining sites (Fig. 3). The two ingroup clades indicated by analysis of DNA sequence data were also evident in analysis of amino acid sequences.

Mantel tests of the correlation between genetic distance and geographic distance were significant for the data set as a whole, and for the group B peripatus ($P < 0.000$). Group A data were not significantly correlated ($P = 0.4$). Distance by distance structure

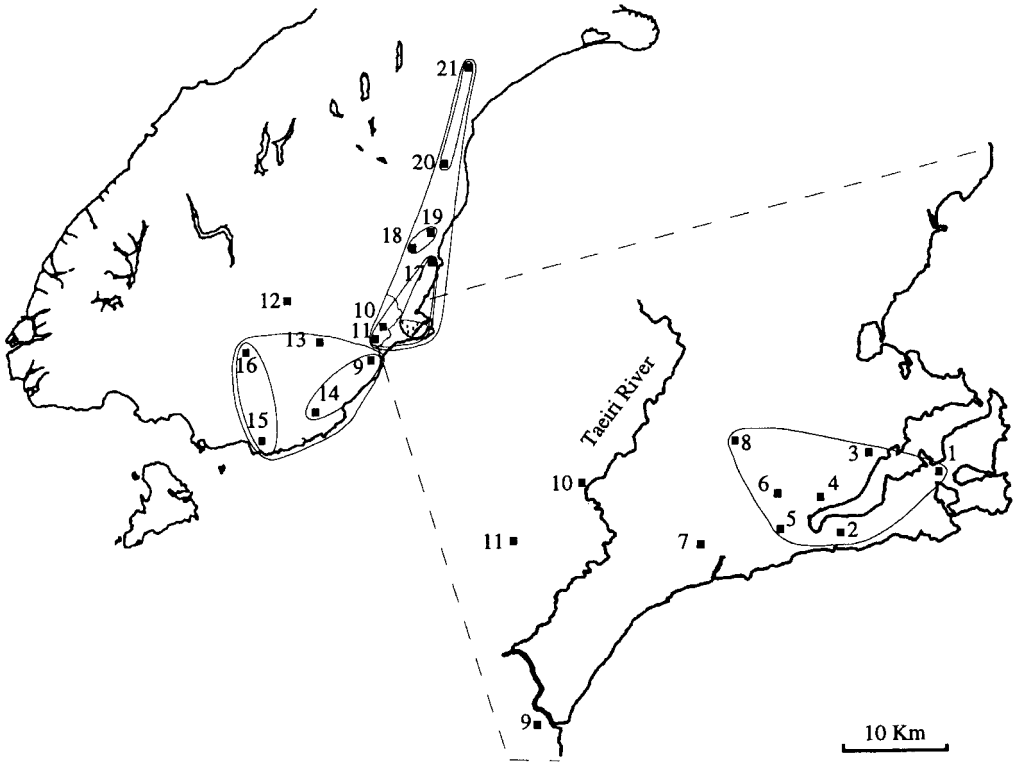

Fig. 3 Associations of peripatus populations indicated by phylogenetic analysis of COI DNA sequences.

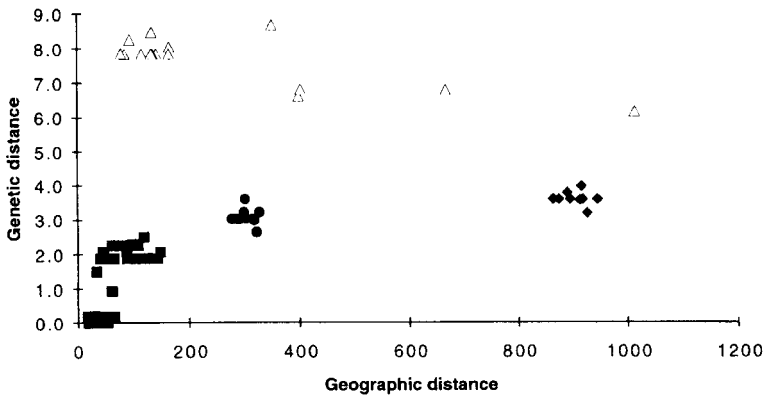

Fig. 4 Relationship of genetic distance (Kimura 2 parameter) and linear geographic distance (arbitrary units) among peripatus from the Dunedin area (group B). Markers indicate pairwise comparisons of population subsets: Within city (Botanic Gardens, Caversham Valley, Frasers Gully, Tomohawk Lagoon, Styles Creek, Grahams Bush and Whare flat)- filled squares; city vs Kakanui- filled circles; city vs Peel- filled diamonds. Group B peripatus versus Taieri Mouth (Catlins peripatus)- open triangles.

in group B was evident in the pattern of genetic similarity which had a distinct north-south orientation (Fig. 4). Geographic distances between some group A and B peripatus were shorter than many within-group distances. Distance by distance comparisons of group B peripatus with the geographically nearest representative group A peripatus (Taieri Mouth) showed high genetic distance (~ 8%) but no positive correlation with geographic distance implying distinct gene pools (Fig. 4).

DISCUSSION

All of the peripatus included in this study were consistent with Hutton's (1876) description of *Peripatoides novaezelandiae* in respect of leg number, integument colour, number of claws and distal papillae, and distribution. However, no specimens that I have dissected for this or previous studies were hermaphrodites as Hutton (1876), apparently erroneously claimed his were. The only distinction which Hutton (1876) made between *Peripatoides novaezelandiae* from different locations was to note that the largest specimens he saw came from Dunedin, and indeed one very large specimen of 700 mg was found during the present study. However, other specimens were not consistently bigger than those found elsewhere in New Zealand (author's unpubl. data). Despite apparent morphological stasis, allozyme data have shown that ovoviviparous peripatus from the Dunedin area are genetically distinct from other *P. novaezelandiae* found in north South Island and North Island, New Zealand (Tait & Briscoe 1995).

The present analysis of mtDNA sequence data confirms that peripatus in the Dunedin area are distinct from other members of the *P. novaezelandiae* complex (data not shown). It also reveals the existence of two, possibly three, distinct subgroups among peripatus of south-eastern South Island, and that these apparently have non-overlapping ranges. The coastal boundary between the two principal groups is just south of Dunedin, and the two may not be present closer together far inland owing to the arid climate of central Otago. It is possible that further sampling in the coastal area would reveal the existence of sympatric populations, as have been detected among North Island peripatus (Trewick 1998). Piano Flat appears to be occupied by a separate lineage most closely allied to the southern group, but this apparent structure may well dissolve with further sampling at intermediate locations. The presence of two monophyletic haplotypes at Piano Flat indicates isolation in this area for some considerable time and is consistent with observations on other endemic invertebrates there (e.g., spiders, Forster 1998).

Genetic distances of around 3% calculated from the mtDNA COI gene are typical for invertebrate sibling species comparisons (e.g., Sperling & Hickey 1994; Funk et al. 1995; Langor & Sperling 1997; Willet et al. 1997). Given genetic distances of up to 3.6% between populations in group B, and evidence of a significant positive correlation between genetic and geographic distances, it appears that the Dunedin peripatus conforms to a single species-level taxon. The greater genetic distances revealed by comparisons of group A and B taxa (6–11%), despite close geographic proximity of some populations from these clades (Fig. 4), supports the notion that group A represents at least one additional non-interbreeding species. Within group A, peripatus from Piano Flat may also be justifiably considered as distinct species. The fact that genetic and geographic distances within group A are not correlated also suggest absence of gene flow. With the exclusion of the Piano Flat peripatus, groups A and B have within-group genetic distances similar to one another, and similar to a study of Jamaican peripatus which found mtDNA distances between presumed sister species of 3.3% (Hebert et al. 1991). In the Jamaican example, species status was evident from the lack of gene flow encountered between sympatric peripatus using allozymes.

In the present study the two taxa sampled most extensively (other than peripatus from Piano Flat) appear to be parapatric with a mutual boundary at the east coast near the mouth of the Taieri River. These two species could be referred to as the Dunedin and Catlins peripatus (The Catlins is the area which forms a large part of the range of group B peripatus).

Within the built-up area and town-belt of Dunedin City, the peripatus show low molecular diversity, consistent with the small geographic area sampled. However, it is also possible that human activity has increased gene flow in this area. At Caversham an exceptionally large and dense peripatus population was recently discovered occupying an unnatural habitat in the form of a buried tin-can dump and piles of bricks (Harris 1991). Peripatus have also been found in the rubbish tip at an old house site on Otago Peninsula (Dean Nelson pers. comm.). The ability to exploit new anthropogenic habitats, and the survival of genetically similar peripatus in a wide range of natural habitats from near sea level (Tomohawk Lagoon) to above the tree line (Kakanui), in scrub, forest (beech and podocarp) and tussock, shows that peripatus have cosmopolitan ecological requirements. Suitability of habitat for these peripatus probably relates to the presence and continuity of prey and moist conditions rather than to particular native vegetation types. This has important conservation implications for peripatus, especially those with restricted geographic ranges. It suggests that apparently low quality and degraded habitats can have a valuable role in the maintenance of biodiversity, and

that the opportunity to rehabilitate and protect such areas should not be overlooked in conservation planning.

ACKNOWLEDGMENTS

Thanks to: Dave Randall for specimens from his Caversham garden and observations about their habitat; Mary Morgan-Richards for all kinds of assistance with field trips and the manuscript; Ray and Lyn Forster for interesting discussions and walks in their bush; Ken Mason, Tony Harris and members of the Evolutionary Genetics Laboratory for their assistance. Johns Hobbs (Nucleic Acid – Protein Service, NAPS Unit, University of British Columbia, Vancouver, B.C., Canada) supplied the set of insect mitochondrial DNA primers, drawn from sequences compiled by Dr. B. Crespi (Simon Fraser University, Burnaby, B.C., Canada) and described on postings to bug-net@sfu.ca. This work was supported by a fellowship (contract UOO704) from the Foundation for Research, Science and Technology, New Zealand

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Appendix 1 on following page

Appendix 1 Sequence data from eighteen distinct 540 bp haplotypes of a mtDNA COI gene fragment from peripatus collected in the Dunedin area, New Zealand. Dots indicate nucleotide identity with a reference sequence from a peripatus collected in the Nelson Lakes. The geographic source of peripatus bearing each haplotype is given. The Dunedin City haplotype is represented by sequence from a Caversham Valley individual.

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Nelson          AATAATTTCTCATATTATTAATCATTATAGAGGAAAAAAAGAATCTTTTGGAGCTTTAGGAATAATTTATGCTATATTGTCTATTGGATTATTAGGTTTTATTGTATGGGCTCAT
Piano Flat      T........A.....................................G.......G.....................A..A.....T........A...........A..A...
Piano Flat      T........A.....................................G.....C..G.......G.............G.....A.....T........A...........A..A...
Matai Falls     T........A.....................G.....................................GG.............A..A.....T........A...........A......
Tom's Creek     T........A.....................................C.....................G.............A..A.....T........A.........T..A......
Taieri Mouth    T........A.....................G.....................................GG.............A..A.....T........A...........A......
Hokonui         T........A.....................G.....................................GG.............A..A.....C........A...........A......
Haldane         T........A.....................G..G..................................GG.............A..A.....T........A...........A......
Tomohawk Lgn.   .........C.....................................G.........A.....................A.....T........A.........T..A..C...
Style's Creek   .........C.....................................G.........A.....................A.....T........A.........T..A..C...
Outram          .........C.....................T.....G..G.........A.........G.....................A.....T........A.........T..A..C...
Saddle Hill     ......C..C.....................T........G.........A.....................A.....T........A.........T..A..C..C
Maungatua       .........C.....................T........G.........A.........G.....................A.....T........A.........T..A..C..C
Caversham Vll   .........C.....................................G.........A.....................A.....T........A.........T..A..C...
Trotters Gge    .....................................................G.........A.....................A.....C........A.........T..A..C..C
Kakanui Mnt.    ...................................................................A.....................A.....T........A.........T..A......
Herbert Forest  ...................................................................A.....................A.....T........A.........T.......
Peel Forest     ...........................G.......................................A.....................A.....T........A.........T..A......
Gunn's Bush     ......................C.....................................A.....................A.....T........A.........T..A......

Nelson          CATATATTTACAGTTGGAATAGATGTAGACACTCGAGCATATTTTACAGCTGCAACTATGATTATTGCTGTTCCTACTGGAATTAAAATTTTTAGTTGGTTAGCAACTTTATATG
Piano Flat      ..........................T..T.....................................C.....A.....A.....................A...............
Piano Flat      ..........................T..T.....................................C.....A.....A.....................................C.......
Matai Falls     ..............C..G..........T.....................................A.........A.....A.....................A..A...............
Tom's Creek     ..........................T.....................................A.........A.....A.....................A..A...............
Taieri Mouth    ..............C..G..........T.....................................A.........A.....A.....................A..A...............
Hokonui         ..............C...........T.....................................A.........A.....A.....................A..A...............
Haldane         ..........................T.....................................A.........A.....A.....................A..A...T...........
Tomohawk Lgn.   ..........................T..A.....................A.....................A.....A.....................A..A..G..T...........
Style's Creek   ..........................T..A.....................A.....................A.....A.....................A..A..G..T...........
Outram          ..........................T..A.....................A..A..................A.....A.....................A..A.....T...........
Saddle Hill     ..........................T..A.....................A..A..................A.....A.....................A..A.....T...........
Maungatua       ..........................T..A.....................A..A..................A.....A.....................A..A.....T...........
Caversham Vll   ..........................T..A.....................A.....................A.....A.....................A..A..G..T...........
Trotters Gge    ................T.........T..A.....................A..A..................A..A..A.....................A..A..G..T...........
Kakanui Mnt.    ..........................T..A.....................A..A..................A.....A.....................A..A................
Herbert Frst.   ..........................T..A.....................A..A..................A.....A.....................A..A................
Peel Forest     ........................G..T..A.....................A..A..................A.....A.....................A..A.....T...........
Gunn's Bush     ..........................T..A.....................A..A..................A.....A.....................A..A.....T...........

Nelson          GGTCACAATTTAATTATGATTCAGCAATTTTATGAGCATTAGGTTTTATTTTTTTATTTACAATTGGTGGATTAACTGGGATTATTCTTTCTAATTCATCTTTAGATATTATTTT
Piano Flat      .A..T........A.....CC.TC.T...........T.....A.....................G..C........A...G.AT.G.....................................
Piano Flat      .A..T........A.....CC.TC.T...........T.....A.....................G..C........A...G.AT.A.....................................
Matai Falls     ....T........A......C.T.............T.....................T.....................A...G..T.A.....................................
Tom's Creek     ....T........A......C..C............T.....................T.....?.....G.....A...G.AT.A.....C.....C.............C.
Taieri Mouth    ....T........A......C.T.............T.....................T.........C.......A...G..T.A.....................................
Hokonui         .A..T........A......C.T.............T.....................T.................A...G..T.A.....................................
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