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DNA Barcoding is not enough: mismatch of taxonomy and genealogy in New Zealand grasshoppers (Orthoptera: Acrididae).

Steven A. Trewick*

Allan Wilson Center for Molecular Ecology and Evolution, Massey University, Private Bag 11-222, Palmerston North, New Zealand

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Abstract

DNA barcoding has been touted as a program that will efficiently and relatively cheaply inform on biological diversity; yet many exemplars purporting to demonstrate the efficacy of the method have been undertaken by its principal proponents. Critics of DNA barcoding identify insufficient within-taxon sampling coupled with the knowledge that levels of haplotypic paraphyly are rather high as key reasons to be sceptical of the value of an exclusively DNA-based taxonomic. Here I applied a DNA barcoding approach using mtDNA sequences from the cytochrome oxidase I gene to examine diversity in a group of endemic New Zealand grasshoppers belonging to the genus *Sigaus*. The mtDNA data revealed high genetic distances among individuals of a single morpho-species, but this diversity was geographically partitioned. Phylogenetic analysis supported at least four haplogroups within one species (*Sigaus australis*) but paraphyly of this species with respect to several others. In some instances two morphologically and ecologically distinct species shared identical mtDNA haplotypes. The mismatch of genealogy and taxonomy revealed in the *Sigaus australis* complex indicates that, if used in isolation, DNA barcoding data can be highly misleading about biodiversity. Furthermore, failure to take into account evidence from natural history and morphology when utilizing DNA barcoding will tend to conceal the underlying evolutionary processes associated with speciation.

On the face of it barcoding is an attractive proposition (Dasmahapatra and Mallet, 2006). While obviously not a panacea it offers an opportunity for biologists to rapidly and simply obtain a measure of biodiversity. In much the same way that the application of phylogeographic approaches preceded the defining terminology (Avise, 2000), DNA barcoding-like methods had long been in use before the introduction of a popularizing jargon (Hebert et al., 2003). However, the proposal for a DNA barcoding program has sparked intense debate driven by claims about the likely efficiency and applicability of the approach (e.g., Wheeler, 2004; Will and Rubinoff, 2004; Ebach and Holdrege, 2005; Rubinoff et al., 2006). Some advocates of barcoding are seen as arguing for a replacement of traditional taxonomy (e.g., Tautz et al., 2003), while others argue that replacement of methods is not the goal (Schindel and Miller, 2005). Perhaps, the most disappointing product of the DNA barcoding program is that it has reopened the wounds of earlier molecules versus morphology debates for which some reconciliation had been achieved (Hillis, 1987). The application of polymerase chain reaction (PCR) and universal mitochondrial primers revolutionized studies of biodiversity at the species level and above in the 1980s and 1990s (Wilson et al., 1985; Saiki et al., 1988). There is no doubt that mtDNA haplotype data have provided a powerful means to explore evolutionary history (genealogy) of species, informing on a wide range of evolutionary and ecological issues and revealing hitherto unrecognized (cryptic) diversity (Avise, 2004). It has also revealed that genealogical paraphyly is rather common in many groups of closely related animals (e.g., the well-known brown bear/polar bear relationship; Talbot and Shields, 1996). However, the extent of paraphyly and frequency of haplotype/lineage sharing among sister species is not known (Moritz and Cicero,

*Corresponding author:
E-mail address: s.trewick@massey.ac.nz

2004; but see Funk and Omland, 2003). While there is often a good match between recognized (or recognizable) morphological/behavioral distinctions that form the basis of traditional taxonomy and molecular phylogenies, this is not always the case. The disparity between gene trees and species trees is well recognized (e.g., Doyle, 1997) and has fueled a rapid development in coalescent theory and analysis (Wakeley, 2004). The championing of barcoding as an alternative to traditional taxonomy, perhaps inevitably led to a polarized and often unnecessarily acrimonious debate about the relative utility and suitability of these approaches. As with arguments about species concepts, there is no single solution to the classification of biodiversity. Indeed, despite claims of success with barcoding, prominent examples actually draw heavily on existing detailed observation on ecology, morphology and behavior painstakingly documented by ecologists and taxonomists (e.g., Hebert et al., 2004a). The difficulties of matching the products of evolution (as we observe them now) within a categorical system remain, hence the attraction of molecular phylogenetic approaches that potentially include information about evolutionary history. But, the difficulty of dealing with species-level distinctions existed before the introduction of molecular methods, with exponents of traditional taxonomic methods debating, for example, the meaning and necessity for subspecies classification (Wilson and Brown, 1953; Mallet, 2001). Some feel that the existence of subtly different forms that are separated spatially should be recognized and recorded as subspecies (or other categorizations below the species level), others contend that such variants should be subsumed within a species if insufficient evidence exists to raise them as species in their own right. Idiosyncrasies in the manner in which individual taxonomists interpret fine-scale differences means there is often only approximate equivalence of species-level classification among groups.

DNA-based phylogenetic approaches that can potentially provide a measure of (neutral) within-species genetic diversity, distinction among "species" and an evolutionary hypotheses for deeper-level systematic relationships offer information that traditional taxonomy may not. But, this potential is not best realized when used in isolation. Molecular systematics approaches (including those incarnated as barcoding) offer the most exciting and challenging opportunities when integrated with traditional taxonomy, studies of behavior, ecology, development, molecular biology, experimental manipulation, etc. Although testing of systematic hypotheses often results in satisfying congruence of evidence (e.g., divisions indicated by morphology are mirrored by groupings in molecular phylogenies) it is when contradictions are encountered that our assumptions about evolution are best challenged. Understanding these anomalies should not be about resolving differences of opinion but about exploring the intricacies of evolution.

New Zealand arid-alpine grasshoppers

To explore the usefulness of the approach in a New Zealand setting where it is increasingly apparent through molecular studies that much diversity is relatively young (Trewick et al., 2007), I applied a barcoding approach to the genealogy of a group of endemic grasshoppers (Orthoptera, Acrididae). The work was motivated by a need to identify appropriate methods to assess diversity for conservation planning and management. Most of New Zealand's endemic acridid grasshoppers occupy alpine or subalpine habitats in South Island. All species of the four endemic genera are restricted to native herbfields, grasslands, or scrub/grasslands. They are generally referred to as alpine grasshoppers, although some species/populations have ranges that extend below the alpine zone as judged by the maximum altitude at which native trees are replaced by a distinct montane flora. The modern tree line in New Zealand is typically about 1300 m a.s.l. but native grasslands now extend below this in some areas due to forest clearance by humans (McGlone, 2001; Wardle, 2001). The majority of grasshopper species occur across several mountain ranges (Bigelow, 1967) that form the extensive Southern Alps. Four genera are described (Bigelow, 1967), but taxonomic diversity is predominantly in two (*Brachaspis* and *Sigaus*). The present research focuses on the genus *Sigaus* and within that the *Sigaus australis* complex, which appears to consist of one common and widespread species plus a number of localized taxa that are broadly sympatric or parapatric with it (Morris, 2002, 2003a,b). Can a DNA barcoding approach succeed in recognizing and delimiting New Zealand's endemic grasshopper fauna? To approach this question I obtained mtDNA sequence data of the type used in barcoding studies (Hebert et al., 2003; Hebert and Gregory, 2005), and attempted to characterize its taxonomic information using single-stranded confirmation polymorphism (SSCP), phylogenetic analysis and estimation of genetic distance.

Taxonomy and ecological background

The genus *Sigaus* Hutton (Orthoptera: Acrididae) is endemic to New Zealand (Hutton, 1897, 1898). All species are flightless as are all members of the four endemic New Zealand grasshopper genera. All but one species of the endemic genera occur in South Island, New Zealand (Fig. 1). *Sigaus australis* (Hutton) is a relatively large grasshopper (adult females $\approx$26 mm) found through the upland regions of central and southern South Island (Fig. 1). It occurs predominantly between about 1000 m and 1800 m a.s.l., but has been

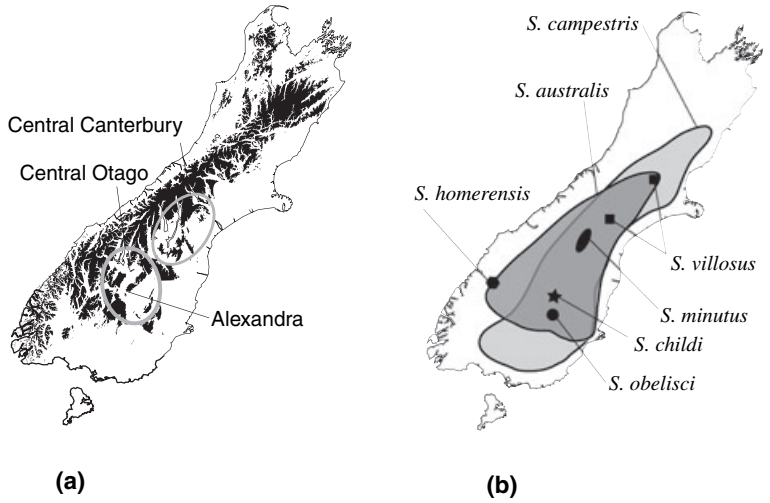

Fig. 1. Maps showing: (a) distribution of mountain ranges in New Zealand (areas above 1000 m a.s.l.); (b) approximate ranges/locations of *Sigaus* species in South Island.

recorded locally below 1000 m where suitable native grassland habitat is present (Bigelow, 1967). Whether all or any individuals found at lower altitudes are resident or are seasonal altitude migrants is not known. A related species, *S. campestris* (Hutton) has a wide range in South Island. It is broadly sympatric with *S. australis* but these species exhibit some degree of altitudinal separation with *S. campestris* tending to lower altitudes. Morris (2002) distinguishes two species complexes within *Sigaus*; the *S. campestris* group that have a sinuous caudal margin on the pronotum (*S. campestris*, *S. minutus*, *S. childi*) and the *S. australis* group that have a smooth margin to the pronotum (*S. australis*, *S. obelisci* and several undescribed species). *Sigaus obelisci* is restricted to one mountain range (Old Man Ra.) in the southern part of the *S. australis* distribution. Two recently described species *S. homorensis* Morris and *S. takahe* Morris have narrow geographic ranges in the mountains of Fiordland (south-west South Island) (Morris, 2003a). The large and distinctive species *S. villosus* (Salmon) is restricted to a few mountain ranges within the spatial ranges of *S. australis* and *S. campestris*. *Sigaus villosus* is, however, isolated by altitude as it occupies the highest elevations of the New Zealand grasshoppers (> 2000 m a.s.l.). A number of low-altitude species *S. minutus* Bigelow, *S. childi* Jamieson, *Sigaus* species A (Morris, 2002) are rare and restricted to semiarid habitats of central Canterbury and around the town of Alexandra in central Otago, respectively, also within the spatial ranges of *S. australis* and *S. campestris* (Fig. 1). A prominent feature of *S. childi* compared with other New Zealand grasshoppers is the extent to which cryptic color and patterning is developed. Unlike the more widespread sister species (*S. campestris* and *S. australis*—in more typical montane habitat) that are boldly patterned and color polymorphic within locations, *S. childi* individuals are strikingly camouflaged to the rocky substrate upon which they are found. Jamieson (1999a) records color patterns ranging from gray to brown and some individuals with bright green and black patterns matching the tumbling lichen (*Chondropsis semiviridis*) that grows on rocks in their habitat. Color morphs of *S. childi sensu* Jamieson (1999a), tend to be specific to particular locations and substrate types. A number of other species within this genus, most with limited distributions, have not yet been formally described (Morris, 2002, 2003b).

Methods

Field sampling and molecular methods

Grasshoppers were collected by hand during their active summer season (November–March). Species identification followed Bigelow (1967) and Jamieson (1999a). Identification of *S. childi* individuals was confirmed by a taxonomist specializing in the group (Simon Morris) who also provided specimens from his collection. For the target taxa, 160 individuals and 26 populations were sampled across their known ranges (Table 1). This scheme allowed the best opportunity to assess interpopulation and intraspecific haplotype diversity.

In most cases grasshoppers were preserved by freezing or in 95% ethanol. In these instances muscle tissue was dissected from hind femora and DNA extracted using a salting-out method (Sunnucks and Hales, 1996; Trewick

Table 1
Sampling locations with longitude and latitude references, and sample sizes

Location	Latitude	Longitude	Altitude (m a.s.l.)	n	Species
Alexandra-Conroy Dam	45 16	109 19	284	2	*S. australis*
Alexandra-Little Valley Road	45 16	169 25	424	3	*S. australis*
Craigeburn	43 17	171 41	1400	1	*S. australis*
Crawford Hills	45 13	109 35	520	2	*S. australis*
Danseys Pass	44 57	170 22	930	17	*S. australis*
Dunstan Ra.	44 57	109 29	1000	1	*S. australis*
Earl Mountains	44 45	107 59	1050	3	*S. homerensis*
Flagstaff Hill	45 49	170 27	650	8	*S. australis*
Fog Peak/Tariesse	43 10	171 44	140	3	*S. australis*
Harris Saddle	44 43	108 10	1250	9	*S. australis*
Kakanui	44 10	170 27	1200	1	*S. australis*
Lirrdis Pass	44 35	109 38	550	2	*S. australis*
Mt Dobson	43 50	170 40	1690	12	*S. australis*
Mt John	43 58	170 27	1000	1	*S. australis*
Mt Stott	44 59	168 56	1360	5	*S. australis*
Mt St Bathans	44 43	168 46	2000	9	*S. australis*
Mt Sutton	44 13	169 46	1500	4	*S. australis*
Old Woman Ra.	45 10	109 04	1200	5	*S. australis*
Remarkables	45 01	108 47	1600	5	*S. australis*
Rob Roy	44 20	109 441	1000	2	*S. australis*
Rock & Pillar Ra.	45 25	170 04	1400	15	*S. australis*
Rocky Tao	44 40	170 18	1900	5	*S. australis*
Sealy Tarns	43 43	170 04	1200	7	*S. australis*
Flagstaff Hill	45 49	170 27	680	1	*S. campestris*
Old Man Ra.	45 19	109 12	1200	1	*S. campestris*
Alexandra-Graweyara Gully	45 15	169 23	200	4	*S. childi*
Alexandra-Little Valley Road	45 10	109 25	420	3	*S. childi*
Alexandra- Little Valley Road	40 10	170 25	421	1	*S.* ''undescribed''
Alexanara-Earnscleugh	45 14	109 20	160	3	*S. childi*
Alexanara-Earnscleugh	40 14	170 20	160	1	*S.* species A
Tekapo	44 00	170 24	800	1	*S. minutus*
Old Man Ra.	45 19	109 12	1600	13	*S. obelisci*
Mt Dobson	43 56	170 39	2000	1	*S. vilosus*
Total				164	

and Morgan-Richards, 2005). For specimens preserved for more than 1 year (including pinned museum material), DNA was extracted using incubation at 55 °C with Proteinase K and a CTAB buffer (2% hexadecyltrimethylammonium bromide, 100 mmol/L Tris–HCl pH 8.0, 1.4 mol/L NaCl, 20 mmol/L EDTA), followed by a combined phenol/chloroform/isoamyl alcohol (25 : 24 : 1) cleanup.

Molecular analysis used DNA sequences obtained with primers that target two regions of the mitochondrial genome: cytochrome oxidase I gene (COI) and small ribosomal subunit III (12S) and large ribosomal subunit (16S). The COI fragment targeted with these primers has been effectively applied to intra- and interspecific studies of a wide range of invertebrate taxa (Lunt et al., 1996), including orthopterans (e.g., Funk et al., 1995; Szymura et al., 1996; Zhang and Hewitt, 1996; Trewick et al., 2000). Similarly, the 12S–16S gene fragment has also previously proved informative in species-level studies of orthopterans (e.g., Shaw, 1996; Dopman et al., 2002).

Single-stranded conformational polymorphism

In order to survey a relatively large number of individuals without sequencing all I used SSCP. This has previously proved effective for identifying haplotypes within New Zealand Orthoptera (Trewick et al., 2000; Trewick, 2001a). For this purpose the primers SR-J-14233 and SR-N-14588 (Simon et al., 1994) were used to amplify a ≈380 bp fragment of the 3′ end of 12S rRNA. PCR products were labeled with a radio isotope by incorporation of αdATP ^{33}P. Ten microliter reactions (200 μM dNTPs, 2.5 mM $MgCl_2$, 0.25 U Qiagen Taq) were treated to 40 cycles of 94 °C for 15 s, 50 °C for 30 s, 72 °C for 90 s with an initial denaturation of 94 °C for 60 s. Amplification products were denatured for 5 min at 95 °C in the presence of an equal volume (10 μL) of 95% formamide loading buffer. These were loaded from ice on to vertical, non-denaturing polyacrylamide gels consisting of 6% 37.5 : 1 bis/acrylamide, 5% glycerol and 0.5 × TBE. Gels were electrophoresed at 4 °C for 200 W/h at approximately 13 W and then

lifted on blotting paper, dried and exposed with Biomax (Kodak) film for 24–48 h. Individuals were scored for haplotype by comparison of re-natured single-strand DNA migration patterns (Sunnucks et al., 2000).

MtDNA sequence data

Representatives of each haplotype resolved by SSCP were subjected to further PCR to amplify and sequence a larger fragment comprising the 3′ end of the 12S rRNA, the tRNA valine and the 5′ end of the 16S rRNA using primers LR-J-13417 and SR-N-14588 (Simon et al., 1994). The 12S–16S fragment of at least one individual of each population presenting a particular SSCP pattern was sequenced to confirm homology. A portion of the 3′ end of COI was amplified and sequenced using primers C1-N-2195 and C1-J-3014 (Simon et al., 1994).

PCR reactions for sequencing were performed in 25 μL volumes using the same conditions as for SSCP. Products were purified using High Pure purification columns (Roche). Cycle sequencing used Perkin Elmer Bigdye chemistry following the manufacturer's protocols and analyzed on a Prism 377 DNA sequencer (Applied Biosystems, Inc., Foster City, CA, USA).

Sequences were checked against the ABI trace file and aligned manually using SeqEd v1.0.3 (Applied Biosystems, Inc.), Sequencher v4.1 (Applied Biosystems, Inc.) and SeAL v2.0 (Rambaut, 1996).

Phylogenetic analysis

Distance estimation and phylogenetic analyses using maximum parsimony (MP), neighbor-joining (NJ) and maximum likelihood (ML) approaches were performed using PAUP*4.0b10 (Swofford, 2002). Character evolution was assessed using MacClade version 3.07 (Maddison and Maddison, 1997). When applied to the COI and 12S–16S data sets, the χ^2-test of base composition implemented by PAUP*4.0b10 (Swofford, 2002) revealed no significant heterogeneity among sequence haplotypes, for all sites and for parsimony informative sites only. MP analysis used unweighted data, a range of transition/transversion weightings (1 : 2, 1 : 5, 1 : 10), or down-weighting of third codon positions for the COI data. NJ analyses were performed with distance optimization using several evolutionary models, including the ML model as determined below. For ML analyses, permutations of alternative nucleotide substitution and among-site rate variation models (I-invariable sites, and Γ-gamma distribution) were first assessed by comparing likelihood scores for a suite of 56 models. Model-test version 3.06 (Posada and Crandall, 1998) was used to perform a hierarchical choice test (using χ^2) based on log likelihood scores, with the model having the highest significant fit to the data being applied in subsequent phylogenetic analysis using ML. Trees for barcoding inference were constructed using distance criteria with K2P genetic distances and the NJ algorithm.

Results

Haplotype diversity

A total of 164 *Sigaus* grasshoppers were analyzed (Table 1). One hundred and forty-four individuals of *Sigaus australis* complex grasshoppers were surveyed using SSCP analysis of a ≈380 bp 12S fragment, comprising 13 *S. obelisci*, 11 *S. childi* and 120 *S. australis*. In addition one *S. childi*, one *S.* "undescribed", one *Sigaus* Species A, three *S. homerensis* and 10 *S. australis* (six locations) were sequenced without SSCP data (see below). The rare and localized species, *Sigaus takahe*, could not be obtained, SSCP revealed 23 haplotypes (hereafter referred to as putative haplotypes) labeled a–w (Table 2). Two putative haplotypes (a and i) were found in two species (*S. childi* and *S. australis*). Four other putative haplotypes (b, c, e, k) were present in samples from more than one site. In total, three COI and four 12S–16S haplotypes were sequenced from Alexandra *S. childi* individuals.

Sequence data

COI and 12S–16S DNA sequences from 40 and 29 individuals, respectively, were used in analyses (Table 2). These include four 12S–16S sequences representing *Sigaus* outgroup species (*S. campestris*, *S. minutus*, *S. villosus*). These data consisted of aligned DNA sequences of 540 bp and 603 bp for COI and 12S–16S, respectively. Sequencing of multiple representatives of SSCP putative haplotypes for the COI and 12S–16S fragments revealed some minor additional diversity, i.e., some sequenced haplotypes from different locations (with the same putative haplotype) were similar but not identical. However, the 12S portion of 12S–16S sequence fragments from individuals with the same putative haplotype were in fact identical, confirming the discriminating power of SSCP. COI nucleotide sequences were translated to amino acid sequences to check for stop codons and shifts in reading frame that might indicate the presence of nuclear mitochondrial copies, but none were detected. Sequences are deposited on GenBank with the accession numbers EF544487–EF544562.

Phylogenetics and genetic distance

Initial analyses using MP, NJ and ML of 12S–16S mtDNA sequence data supported an ingroup comprising sequences from *Sigaus australis*, *S. obelisci*, *Sigaus* species A and *S. childi*, as distinct from an outgroup

Table 2
Summary of DNA data obtained from *Sigaus* grasshoppers. Putative haplotype grouping inferred using SSCP are given along with haplotype groupings based DNA sequencing and phylogenetic analysis of DNA COI and 12S−16S sequences

		12S-SSCP		Sequence		
Species	Location	Haplotype	n	COI	12S−16S	Haplogroup
S. australis	Mt Sutton	m	3	2	–	–
S. australis	Mt Dobson	e	3	1	1	Sa.I
S. australis	Sealy Tarrra	e	7	2	1	Sa.I
S. australis	Mt Dobson	h	5	1	1	Sa.I
S. australis	Craigburn	k	1	–	–	Sa.I
S. australis	Fog Peak/Torlessa	k	3	–	1	Sa.I
S. australis	Mt Dobson	t	2	–	1	Sa.I
S. australis	Mt Dobson	u	2	–	1	Sa.I
S. australis	Mt John		(1)	–	1	Sa.I
S. australis	Alexandra–Conroy Dam	a	1	–	1	Sa.II
S. childi	Alexandra–Earnsleugh	a	2	1	–	Sa.II
S. childi	Alexandra–Harpin Little Valley Road	a	4	1	1	Sa.II
S. childi	Alexandra–Earnsleugh	i	1	1	1	Sa.II
S. childi	Alexandra–Graveyard Gully	i	1	–	–	
S. australis	Alexandra–Little Valley Road	i	2	2	1	Sa.II
S. australis	Alexandra–Conroy Dam	L	1	1	1	Sa.II
S. australis	Mt St Bathans	n	1	–	1	Sa.II
S. australis	Mt St Bathans	o	3	1	1	Sa.II
S. australis	Mt Sutton	q	1	1	1	Sa.II
S. australis	Dunstan		(1)	1	–	Sa.II
S. australis	Lindis Pass		(2)	2	–	Sa.II
S. childi	Alexandra–Graveyard Gully	s	3	–	1	Sa.II
S. childi	Alexandra–Little Valley Road		(1)	–	1	Sa.II
S. species A	Alexandra–Earnsleugh		(1)	1	–	Sa.II
S. australis	Rob Roy		(2)	2	–	Sa.III
S. homerensis	Earl Mountains		(3)	3		Sa.III
S. australis	Harris Saddle	c	9	2	1	Sa.III
S. australis	Alexandra–Little Valley Road	c	3	–	1	Sa.III
S. australis	Mt Scott	c	5	–	–	Sa.III
S. australis	Remarkables	d	8	1	1	Sa.III
S. australis	Old Woman Ra.	g	8	1	1	Sa.III
S. obelisci	Old Man Ra.	p	13	1	1	Sa.III
S. australis	Danseys Pass	b	15	1	1	Sa.IV
S. australis	Rock and Pillar Ra.	b	13	1	1	Sa.IV
S. australis	Flagstaff Hill	f	8	1	1	Sa.IV
S. australis	Mt St Bathans	j	5	1	1	Sa.IV
S. australis	Kakanui Mnts.	v	1	1	–	Sa.IV
S. australis	Rocky Too	w	8	1	1	Sa.IV
S. australis	Crawford Hilly	r	2	1	–	Sa.IV
S. australis	Danseys Pass		(2)	2	1	Sa.IV
S. australis	Rock and Pillar Ra.		(2)	2	–	Sa.IV
S. "undescribed"	Alexandra–Little Valley Road		(1)	1	1	Sa.IV
Ingroup	Total individuals SSCP screened		144			
	Total individuals including non-SSCP		160	40	29	
Outgroup				1	4	
Total sequences				41	33	

comprising *S. villosus*, *S. campestris* and *S. minutus*. Bootstrap resampling indicated strong support for a phylogenetic pattern that places *S. childi* within the *S. australis* complex rather than the *S. campestris* complex where it had formerly been assigned (Morris, 2002) (Fig. 2 inset). All further analyses of the ingroup thus revealed, using a single taxon (*S. villosus*) to represent the outgroup, returned four haplogroups (I–IV) comprising the same combination of haplotypes (Fig. 2, Table 2). All analyses grouped DNA sequences (true haplotypes) derived from individuals with the same SSCP putative-haplotype. Analysis of COI sequences returned trees (Fig. 3) with the same haplotype groupings as 12S−16S sequence data (Fig. 2). For both sets of data (for which the sampling was not entirely complimentary) bootstrap resampling indicated consistent support for these four haplogroups, although haplogroup IV sequences tended to form an internal polytomy in consensus trees. An additional (fifth) COI lineage (putative haplotype m) represented by three individuals

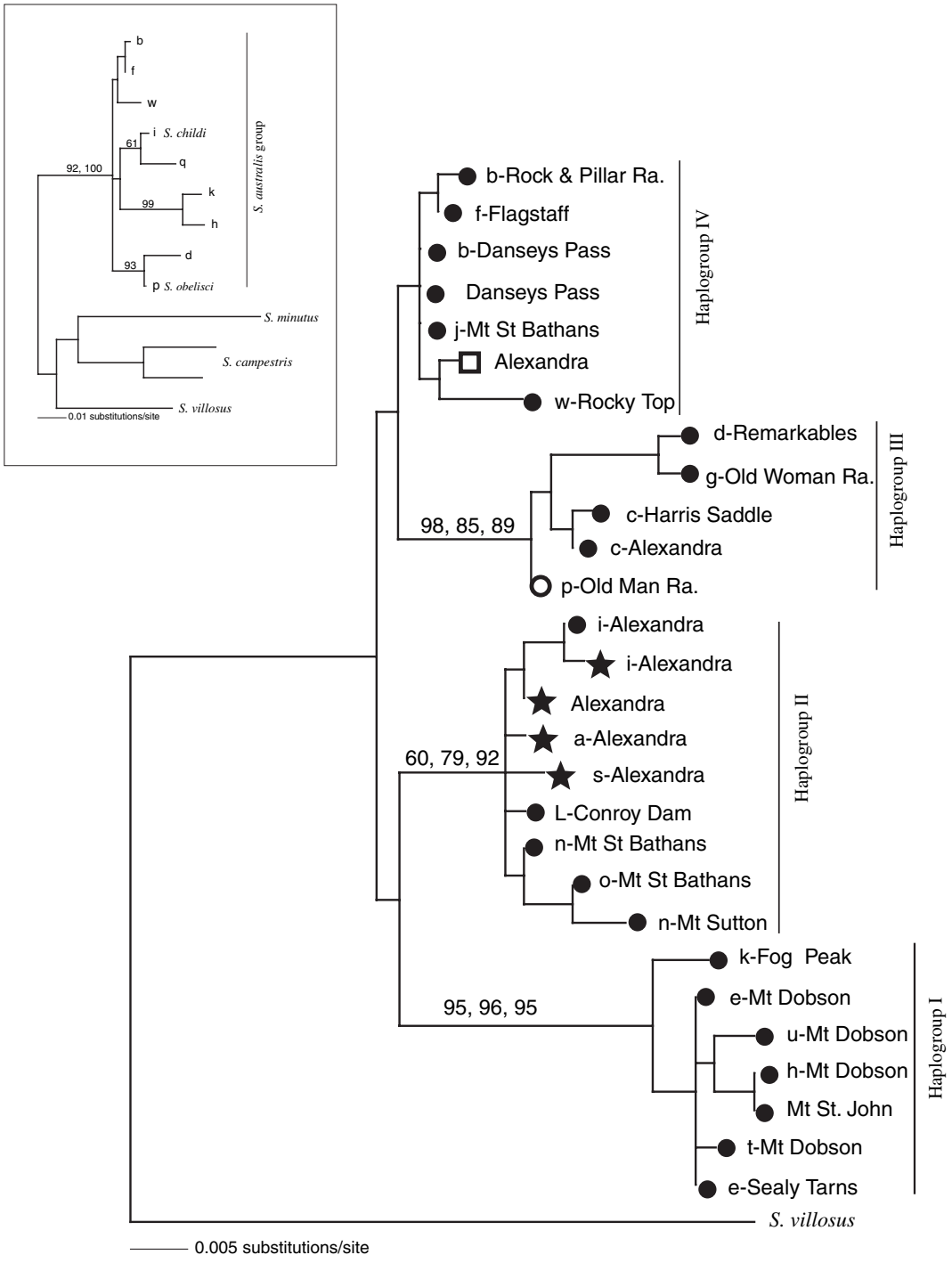

Fig. 2. ML tree of 12S–16S data from *S. australis* complex with a single outrgroup taxon (*Sigaus villosus*). Analysis used TrN + I + G model selected by Modeltest v3.06 using hieracrhical likelihood ratio tests. Inset: ML analysis of 12S–16S data with reduced number of ingroup taxa and three outgroup taxa. Analysis used GTR + I + G model selected by Modeltest v3.06 using Akaike information criteria. The results of 1000 bootstrap resampling replications using ML, MP and NJ optimality criteria, respectively, are given on relevant edges. Terminal labels indicate morphological species (filled circle *S. australis*, open circle *S. obelisci*, opensquare *S.* ''undescribed'', filled star *S. childi*, open star *Sigaus* species A), sampling location, and SSCP putative haplotype (a, b, c, etc.) where scored.

(three SSCP; two sequenced) from Mt Sutton failed to group with other sequences indicating the presence of additional significant diversity. All analyses are consistent in revealing the polyphyly of *S. australis* haplotypes with respect to those from *S. obelisci*, *S. homorensis*, *Sigaus* species A and *S. childi* (Fig. 3, 4).

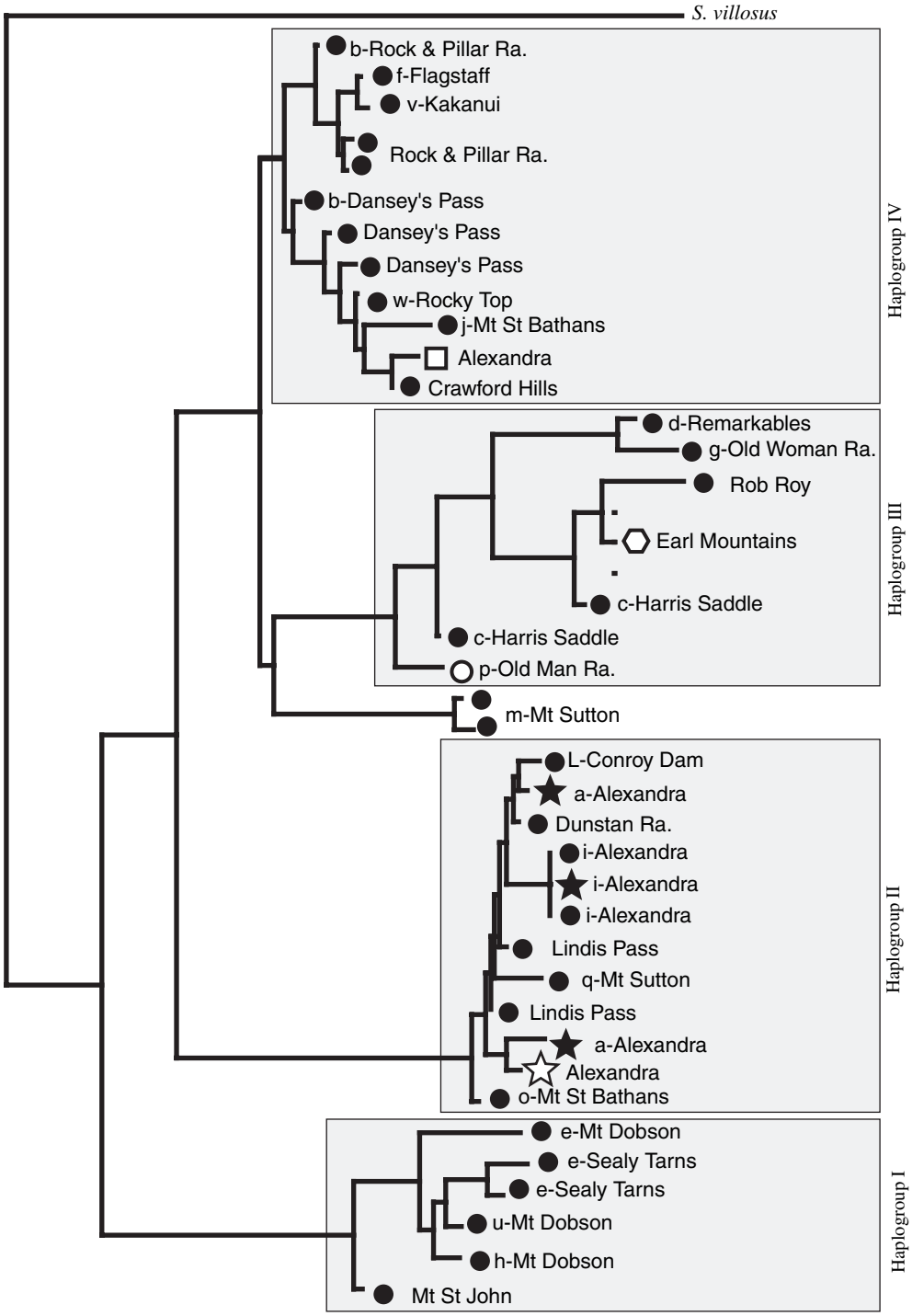

Fig. 3. NJ tree of COI mtDNA sequence data from *S. australis* compex with a single outgroup taxon (*Sigaus villosus*). Terminal labels indicate morphological species (filled circle *S. australis*, open circle *S. obelisci*, open square *S.* "undescribed", filled star *S. childi*, open star *Sigaus* species A, open hexagon *S. homerensis*), sampling location, and SSCP putative haplotype (a, b, c, etc.) where scored.

The maximum COI distance between the ingroup and outgroup (*S. villosus*) sequences was 24% (ML—TrN + G). Genetic distances inferred from the 12S–16S data were, as expected, lower than those inferred from COI. For example, the ML distance using 12S–16S between the ingroup and outgroup (*S. villosus*) sequences (12.8%) was about half that inferred from COI. For COI data, genetic distances among the ingroup sequences had

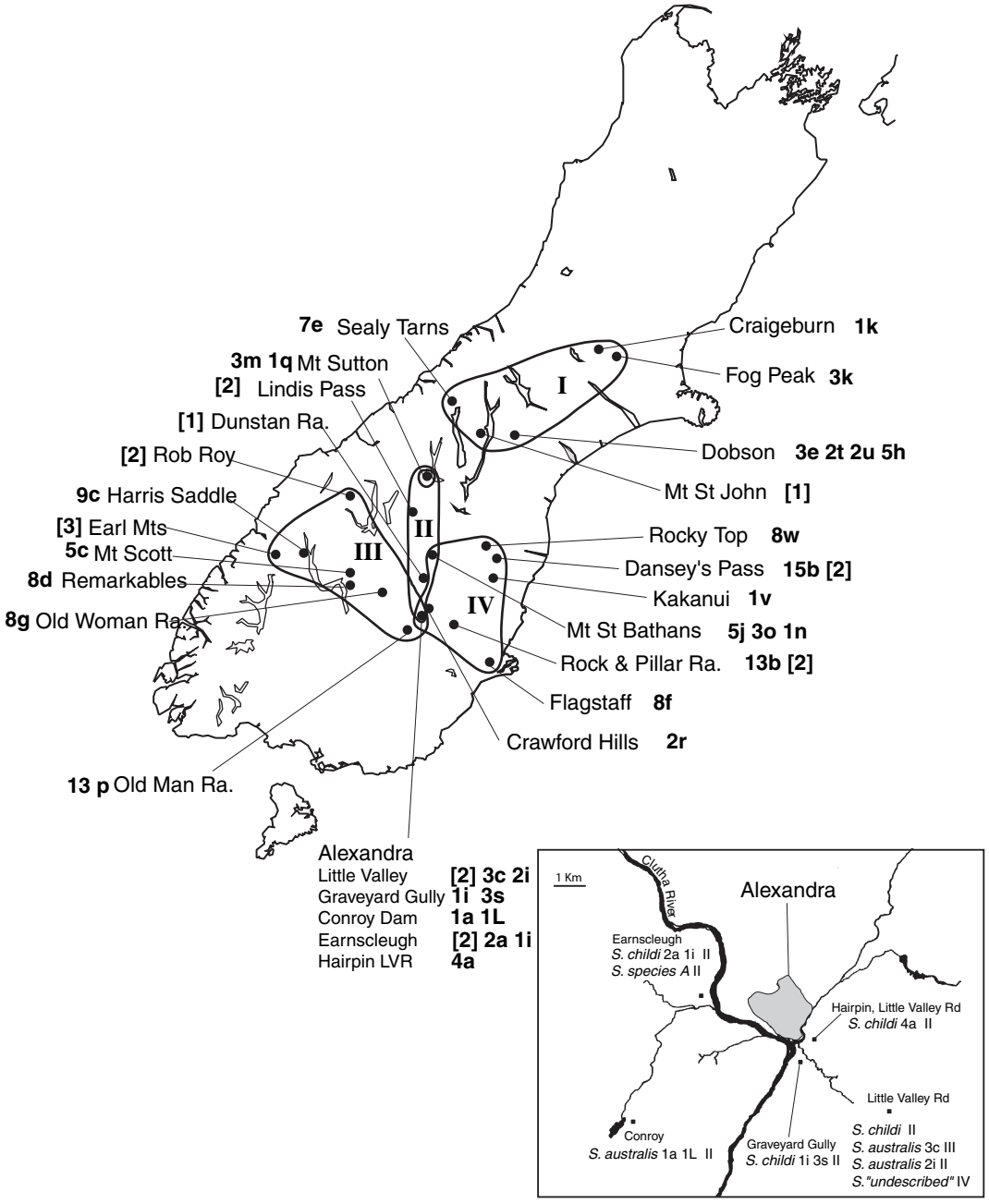

Fig. 4. Distribution of haplotypes from *S. australis* complex taxa, with contour plots representing mtDNA COI haplogroups. Inset of Alexandra area showing sample locations and distribution of species and haplotypes.

a maximum of 8.3% (P distance) or 15.9% (ML), with a mean of 7.4% (ML). Average genetics distances within haplogroups were (K2P/ML): haplogroup I 1.5%/1.7%, haplogroup II 1.6%/1.6%, haplogroup III 2.6%/3.4% and haplogroup IV 1.5%/1.6%. Most (92%) of pair-wise intraclade K2P genetic distances were smaller or equal to 3%, whereas most (98%) of interclade pair-wise distances were greater than 3% (Fig. 5). As indicated by phylogenetic analysis, genetic distances among pairs of *S. australis* (typical morphotype) grasshoppers ranged across the spectrum with no evidence of a barcode "gap" necessary to distinguish between species (Hebert et al., 2004b) (Fig. 5).

Phylogeography versus taxonomy

Sigaus australis populations tend to have unique mtDNA haplotypes. The general pattern of low diversity

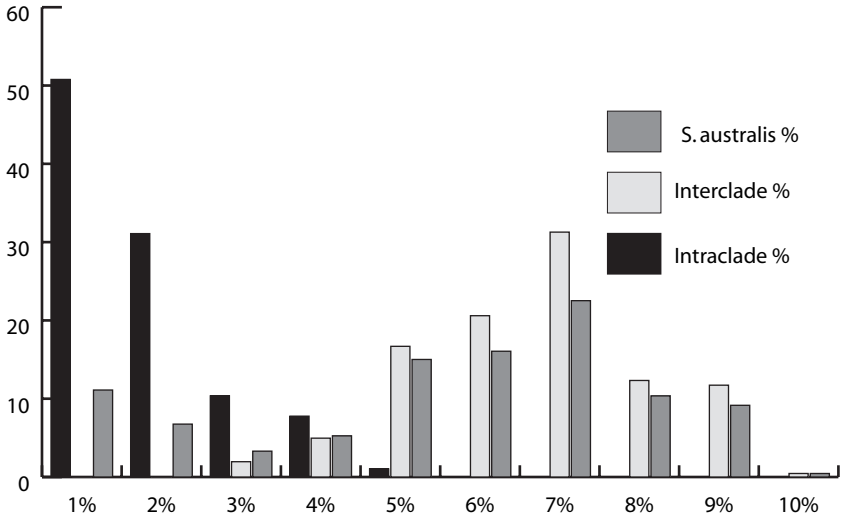

Fig. 5. Frequency distribution of pair-wise K2P genetic distances (expressed as percentage difference) from COI mtDNA sequences from grasshoppers of the *Sigaus australis* complex (i.e., including data from all species indicated as part of the complex by phylogenetic analysis). Frequency values for each distribution sum to 100%. Black bars—pooled distances between samples within each of the four main haplogroups (I–IV); pale gray bars—distances between samples in separate haplogroups (I–IV); dark gray bars—distances among all *S. australis* morpho-species grasshoppers.

at sites inferred from SSCP haplotyping is confirmed by sequence data. Most locations had a single and usually unique haplotype, although three closely related haplotypes were evident in the Mt Dobson sample. In contrast, three haplotypes (n, o, j) at Mt St Bathans correspond to two haplogroups (II and IV), and five SSCP haplotypes (a, c, i, s, L) at Alexandra correspond to three haplogroups (II, III and IV) (Fig. 3).

Individuals that yielded haplogroup I DNA sequences came from the northern-most extent of the *S. australis* range in the central waist of South Island (Fig. 4). Central South Island is an area noted for low species endemicity associated with the New Zealand beech-gap (a region of distributional discontinuity for *Nothofagus* beech and many other taxa; Trewick and Wallis, 2001). Genetic distances (COI) between haplogroup I and other ingroup sequences were relatively high (mean ML 10.7%).

Each of the three southern haplogroups comprised sequences from individuals collected in geographically distinct (but parapatric) ranges that meet at Alexandra. Haplogroup III comprises putative haplotypes (in brackets) from *S. australis* (c, d, g) and *S. obelisci* (p) distributed from Alexandra westwards. Putative haplotype p was unique to, and shared by all 13 *S. obelisci* individuals collected on the Old Man Range. In contrast, c was present in grasshoppers from three locations including Alexandra. Haplogroup IV comprised putative haplotypes from individuals of *S. australis* (b, f, j, v, w, r) and a single individual of *S.* ''undescribed'' collected north-east of Alexandra. Haplogroup II included *S.* species A, all 12 *S. childi* (a, i, s) surveyed plus some *S. australis* (a, i, L, n, o, q). Therefore some individuals of these latter two species shared the same putative haplotypes (two *S. australis* and two *S. childi* had putative haplotype i, one *S. australis* and six *S. childi* had a). Furthermore two *S. australis* from Little Valley Road had the same COI sequence haplotype as an *S. childi* from Alexandra (Earnscleugh), and an *S. australis* from Dunstan had the same COI sequence haplotype as an *S. childi* from Alexandra (Hairpin, Little Valley Road) (Fig. 4 inset).

Discussion

Phylogenetic analysis of mtDNA sequence data fails to reveal groupings of haplotypes consistent with the recognized taxonomy for the *Sigaus* grasshoppers surveyed. Instead, a complex is indicated comprising one widespread taxon (*S. australis*) plus a number of described (and undescribed), localized species. In the absence of the morphologically distinctive species such as *S. childi*, diversity of *S. australis* could be interpreted in either of two ways; *S. australis* could be treated as a single geographically structured species (phylogeographic approach) or as a number of allopatric (or parapatric) but morphologically cryptic species (barcoding approach). However, the presence of morphologically distinct species nested within the phylogeny and genetic diversity of *S. australis* (i.e., mismatch of genealogy and taxonomy) demands a more complex, and in evolutionary terms, interesting explanation.

Spatial structure of Sigaus australis

Molecular studies of alpine taxa often reveal a strong association between sequence similarity and spatial proximity. This reflects the isolation of habitats that are limited by altitude; sub/alpine taxa occupy geographically near-stationary habitats through time by tracking microclimate altitudinally as global climate changes. Such habitats are akin to oceanic islands in terms of the restriction on gene flow exerted by their ecological isolation from one another and in North America have been referred to as sky islands (Heald, 1951; Knowles, 2000). The recent environmental history of New Zealand is dominated by two contiguous geophysical phases: the Pliocene ($\approx$ 5 Mya) emergence of the axial mountains of South Island such that it now comprises $\approx$60% mountain land (Stevens, 1981; Whitehouse and Pearce, 1992; Batt et al., 2000) (Fig. 1) and the Pleistocene ($\approx$ 2 Mya) glaciation (Fleming, 1979; Daugherty et al., 1993). Today New Zealand, and in particular South Island, has a diverse and extensive alpine biota (e.g., Fleming, 1963; Wardle, 1963; Mark and Adams, 1995) that includes adaptive radiations of several plant groups (Raven, 1973; Wagstaff and Garnock-Jones, 1999; Lockhart et al., 2001; Winkworth et al., 2002) and specialized insects (Dumbleton, 1969; Arensburger et al., 2004) some of which tolerate freezing (Sinclair et al., 1999). It is primarily on these montane ranges that *Sigaus* grasshoppers are found.

The northern *S. australis* lineage (haplogroup I) is located in a region corresponding to the New Zealand ''beech-gap'' geographic zone. This area is characterized by comparatively low endemicity and is bounded (north and south) by areas of higher endemicity and disjunct species distributions (Wardle, 1963; Burrows, 1965; Dumbleton, 1969; McGlone et al., 2001; Trewick, 2001a). The presence here of a distinct *S. australis* lineage suggests a protracted period of isolation throughout many episodes of Pleistocene climate change, rather than colonization of the area at the end of the Pleistocene. On the basis of estimates of genetic distance between haplogroup I and other *S. australis* COI haplotypes and employing a standard rate calibration of 2–2.3% per million years as an approximate guide (Brower, 1994; Juan et al., 1995; Fleischer et al., 1998), this split may originate in the Pliocene (ML distance 0.107–5 Mya). This spatial pattern and estimated time of divergence is similar to that identified for the alpine scree weta (Trewick et al., 2000; Trewick, 2001a), and other organisms (Buckley et al., 2001; Trewick and Wallis, 2001; Trewick, 2001b; Heenan and Mitchell, 2003). In the case of the alpine scree weta, evidence from morphology and allozymes did not support treatment as more than one species (Morgan-Richards and Gibbs, 1996).

The southern part of the *S. australis* range contains comparatively more genetic, and also more morphological diversity (as judged by current taxonomy) than the northern populations. Elevated diversity in the southern South Island is common across the biota and probably relates to its relatively larger area, habitat diversity and more stable geophysical history compared with the central South Island (McGlone, 1985; McGlone et al., 2001; Wallis and Trewick, 2001). Genetic diversity of southern *S. australis* complex is nevertheless spatially structured: haplogroup II in southern central, haplogroup III in south-west, haplogroup IV in south-east. Each haplogroup is apparently allopatric or parapatric, a pattern typical of subdivision in the Pleistocene (e.g., Ribera and Vogler, 2004). Intriguingly, these three haplogroups meet in the semiarid environs of the Alexandra township (Fig. 4), as do other insect species pairs (*Phaulacridium* grasshoppers—Westman and Ritchie, 1984; *Prodontria* beetles—Emerson and Wallis, 1994; Wallis, 2001). In the absence of morphological or ecological correlates for this phylogeographic structure it would be presumptive to infer that haplogroups correspond to distinct species. However, for the purposes of conservation management and recognition of biodiversity, populations grouped by haplotype data could be treated as separate evolutionary significant units (ESUs) pending further study (Moritz, 1994).

Taxonomic diversity and the DNA barcode in the Sigaus australis complex

Applying a barcode approach to these data using information from phylogeny and genetic distance would result in the recognition of at least four groups of populations as putative species even though little if any corresponding ecological or morphological difference exists between them (e.g., two sympatric barcode ''species'' on Mt St Bathans, Fig. 2). But, applying a strict threshold approach fails to identify numerous individuals and populations (allopatric and sympatric) that are morphologically and behaviorally distinct.

A major potential problem for barcoding arises when mitochondrial DNA sequences for a given real species fail to coalesce to the exclusion of DNA sequences from other recognized species. In such cases a DNA barcoder would be misled into either underestimating true taxonomic diversity or assigning an individual to the wrong species. Shared barcodes might be the result of hybridization or retention of ancestral polymorphism, both are more likely (but not exclusively) among recent species (Mallet and Willmott, 2003). Near the town of Alexandra where *Sigaus childi* is found, either mitochondria have been exchanged recently via introgression (hybridization) or they have been retained by incomplete lineage sorting through a recent speciation event (Funk and Omland, 2003). In the present instance either

or both scenarios could be inferred. If hybridization has been involved in the evolution of *Sigaus childi* it was not restricted to a single ancestral event but rather has been extensive and recent with multiple similar haplotypes being shared between species. The fine-scale sharing of haplotypes among individuals of *S. australis*, *S. childi* and *S.* species A in haplogroup II contrasts with the high average ML genetic distances (mean 0.95) between haplogroup II COI and other haplogroups (which indicate coalescence in the Pliocene).

Although *S. childi* and *S. australis* have overlapping body size ranges they differ in shape, genital morphology, coloration and patterning (Jamieson, 1999a; Morris, 2002). Furthermore, *S. childi* is exceptional among *Sigaus* grasshoppers for being restricted to a localized, lowland, semiarid environment, displaying pronounced morphological crypsis that implicates selection by visual predators. This would suggest that the production of hybrid offspring would be expected to exert very high fitness costs. If *S. childi* was isolated during the Holocene in an arid lowland environment, by expansion of lowland woodland and elevation of the alpine zone (occupied by *S. australis*), then it is conceivable that the present level of haplotype sharing is the result of very recent hybridization following human clearance of woodland ($\approx$ 600 years BP) (McGlone, 2001; Wardle, 2001).

Alternatively, the observed distribution of mtDNA haplotypes might reflect incomplete lineage sorting across a recent isolating barrier. Such a barrier might be spatial (e.g., allopatric separation across a valley or mountain system), ecological (e.g., fine-scale structuring of vegetation type as in *Phaulacridium* in the same landscape; Westman and Ritchie, 1984) or related to sexual selection (mate recognition). Individuals of the two species could only share identical haplotypes (in the absence of hybridization) if these species formed very recently indeed. *Sigaus childi* could have evolved during the Holocene while isolated from other grasshopper populations by elevation of the alpine zone and expansion of woodland in neighboring low altitude areas (McGlone et al., 1995, 2003; Clark et al., 1996).

Other *Sigaus* species (*S. homerensis*, *S. obelisci*, *S.* "undescribed") included in this analysis have paraphyletic relationships (on mtDNA) with *S. australis*. *Sigaus obelisci* and *S. homerensis* are morphologically distinct from other *S. australis*, including those populations represented in haplogroup III. These localized morphospecies with narrow geographic ranges might be the products of stochastic fixation of morphological variation in small populations. But are these paraphyletic morphospecies more or less significant than taxa defined by haplogroups?

Although mtDNA (COI in particular) has proved useful in revealing the phylogeographic structure of many New Zealand alpine insects (e.g., Trewick et al., 2000; Trewick and Wallis, 2001; Buckley et al., 2001; Arensburger et al., 2004; Chin and Gemmell, 2004), including grasshoppers (Trewick, 2001b), the application of such sequences as DNA barcodes proves unsatisfactory as a taxonomic endeavor. *Sigaus childi* appears to be adapted to a specific, limited, non-alpine habitat and yet it cannot be distinguished from *S. australis* (as currently defined) using mtDNA alone. Similarly, the allopatric species *S. obelisci* and *S. homerensis* cannot be convincingly distinguished on the grounds of mtDNA phylogeny or genetic distance. An analogous situation of spatial structuring but poor fit of genealogy with taxonomy exists in another grasshopper genus in the same landscape (*Brachaspsis*—Trewick, 2001b).

Conclusions

Studies that demonstrate the efficacy and reliability of barcoding have been criticized for poor sampling. Known or putative taxa are usually represented by one or two individuals, even though phylogeographic studies have shown that in many instances interpopulation variation is high. Undersampling will tend to increase the likelihood of phylogenetic analysis yielding "monophyletic" clusters of sequences for each taxon sampled. To say that shared barcodes are "uncommon and their impacts are parochial" (Hebert and Gregory, 2005) is dismissive to say the least. There are insufficient examples exploring intraspecific diversity among sister species to know this (Moritz and Cicero, 2004) and what data are available show that paraphyly is rather common (Funk and Omland, 2003). DNA barcoding studies of invertebrates indicate that the method is particularly misleading when applied to incompletely sampled and taxonomically poorly characterized groups (Meyer and Paulay, 2005; Meier et al., 2006). The results from the present study of *Sigaus australis* complex corroborate these concerns; haplotype sharing and paraphyly essentially invalidate the DNA barcoding approach, and it is clear that without a robust taxonomy undersampling could yield misleading interpretations of biodiversity.

Will et al. (2005) paraphrase the apocryphal Samuel Johnson quote—"your work, Sir, is both new and good, but what's new is not good and what's good is not new"—as comment on DNA barcoding. This fairly well describes the situation that has arisen through the repackaging of existing methods. But, at the same time there remains confusion about what DNA barcoding is about. For the purposes of discriminating among haplotype diversity (with appropriate interspecific/geographic sampling) the COI locus has useful qualities because of the extreme non-linearity in its accumulation of substitutions, but this and other traits makes it a poor candidate for phylogenetics and therefore molecular

systematics (Rubinoff et al., 2006). Unfortunately, barcoding, which is a quasi-taxonomic enterprise, and molecular systematics, which looks at broader relationships, have been confused (e.g., DeSalle et al., 2005).

Neither DNA barcoding nor traditional taxonomy can satisfactorily describe the evolutionary history (and future) of the *Sigaus* grasshopper complex, but a combination of natural history and population genetics could. It is at this evolutionary coal face that taxonomy has always been most challenging. In most cases the problem is not merely one of the time needed to handle and process information (Hebert et al., 2003); underlying the taxonomic impediment is the fact that life is complex (Ebach and Holdrege, 2005). DNA barcoding is just one of the tools available and necessary for systematics. No one said that studying evolution would be easy, and the ''grandeur in this view of life'' (Darwin, 1859) will be lost if we take the easy way out.

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