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Determining the species status of one of the world's rarest frogs: a conservation dilemma

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Abstract

New Zealand's native frogs (genus *Leiopelma*) are considered to be archaic amphibians of exceptional scientific interest that appear to have remained virtually unchanged for 160–200 million years. They are among the rarest extant amphibians and are highly restricted in distribution, confined to isolated, highly disjunct, populations on the North Island and a few small offshore islands in Cook Strait. Previous investigations have suggested, based on patterns of allozyme variation, that the Stephens Island frog *(Leiopelma hamiltoni*) and Archey's frog (*L. archeyi*) are sister taxa to the exclusion of the Maud Island frog, a species in close geographical proximity to the Stephens Island frog and previously viewed as a population of this species. As a consequence of these data, a new species, *L. pakeka*, the Maud Island Frog, has been described. This new species definition has dramatically enhanced the conservation status of *L. hamiltoni*, of which there are probably fewer than 150 individuals. In this study we re-examine the systematics of the Leiopelmatidae using mtDNA sequence analyses. Partial 12 S ribosomal RNA and cytochrome *b* (Cyt *b*) gene sequences were obtained for 57 frogs from six populations representing all four extant *Leiopelma* species. Contrary to previous reports we find *L. pakeka* and *L. hamiltoni* to be monophyletic. The amount of variation evident between these present species (<1% for Cyt *b*) is comparable to that seen between populations of *L. archeyi*. Based on these data, classification of *L. pakeka* and *L. hamiltoni* as separate species appears to be unwarranted, but they may be sufficiently distinct to warrant classification as evolutionarily significant units.

INTRODUCTION

Endemic New Zealand frogs, *Leiopelma* spp., are among the most primitive living in the world today. Morphologically they appear to be very similar to Jurassic-era frogs, suggesting that they have not changed much over the past 200 million years (Estes & Reig, 1973). Subfossil remains indicate that *Leiopelma* spp. were once widely distributed throughout New Zealand (Worthy, 1987). Since the arrival of humans and with them mammalian predators such as rats (Worthy, 1987; Holdaway, 1996), several species of native frogs have become extinct. The remaining species continue to be threatened by habitat destruction, exotic predators, population fragmentation and the consequences of small population size (Daugherty, Patterson & Hitchmough, 1994; Waldman & Tocher, 1998). Today their conservation status ranges from low risk to critically endangered (Daugherty *et al*., 1994; Baillie & Groombridge, 1996; Newman, 1996).

Leiopelma hochstetteri is widely distributed, but most populations are small and isolated, restricted to streambeds. Substantial cytogenetic variation among sites suggests that each population should be considered a distinct conservation unit (Green, 1994). Although more geographically restricted, *L. archeyi* (Archey's frog) populations have been considered stable (Bell, 1994) but recently some have undergone precipitous declines (Bell, 1999).

Of particular concern are the *Leiopelma* frogs endemic to two small offshore islands (Maud and Stephens) in the Cook Strait between the North and South Islands. *Leiopelma hamiltoni*, one of the rarest frogs in the world, occupies a 600 m^2 boulder tumble on Stephens Island that probably can support no more than 200 individuals (Newman, 1990, 1996; Brown, 1994). Frogs on Stephens Island were presumably able to survive even as larger populations on the South Island went extinct because the island historically has been free of mammalian predators (except for a brief period in the late 1890s when a

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feral cat population existed: Worthy, 1987; Bell, 1994; see Fig. 1). Another population of frogs, consisting of 19 000 individuals (Bell & Bell, 1994), exists in a 15 ha remnant of coastal forest on Maud Island, which is also free of mammalian predators. Maud Island frogs were regarded as a population of *L. hamiltoni* (Stephenson, 1961) but recently they have been described as a new species, *L. pakeka* (Bell, Daugherty & Hay, 1998).

Bell *et al*. (1998) examined allozyme variation in five populations of native frogs that comprise the three previously recognized species and the Maud Island frog. Among the 12 allozyme loci for which they obtained electrophoretic data, they found two loci that showed fixed differences (malate dehydrogenase and peptidase) and one locus that differed significantly in allele frequency (lactate dehydrogenase) between Maud and Stephens Island populations. Phylogenetic analyses of these data suggested that *L. hamiltoni* and *L. archeyi* are sister taxa to the exclusion of the Maud Island frog (Fig. 2). Furthermore, subtle morphometric differences between frogs on the two islands lent support for the split of *L. hamiltoni* from the newly described *L. pakeka* (Bell *et al*., 1998).

The description of Maud and Stephens Island frogs as distinct species has dramatically increased the conservation significance of both taxa, as each has been restricted to a single population (Bell *et al*., 1998). The conservation implications are dramatic: *L. hamiltoni* now may be the rarest recognized frog species in the world whereas previously it was only listed as vulnerable (Baillie & Groombridge, 1996: 1996 IUCN Red List of threatened animals). The New Zealand Department of Conservation ranks *L. hamiltoni* as an '*A*' category species, the highest priority species for conservation action, comparable to the critically endangered category of the IUCN (Molloy & Davis, 1994; Baillie & Groombridge, 1996).

Considering the extreme conservation importance of *L. hamiltoni* we set out to re-evaluate the phylogenetic relationships of the extant *Leiopelma* species using mtDNA sequence analysis, with particular emphasis on the relationship between *L. pakeka* and *L. hamiltoni*. In this paper we show that, based on mtDNA sequence analysis, *L. hamiltoni* and *L. pakeka* may be sister species or, more likely, allopatric populations of the same species, to the exclusion of *L. archeyi*. Within this context, we discuss whether management efforts should be directed toward maintaining genetically distinct populations or genetically diverse populations.

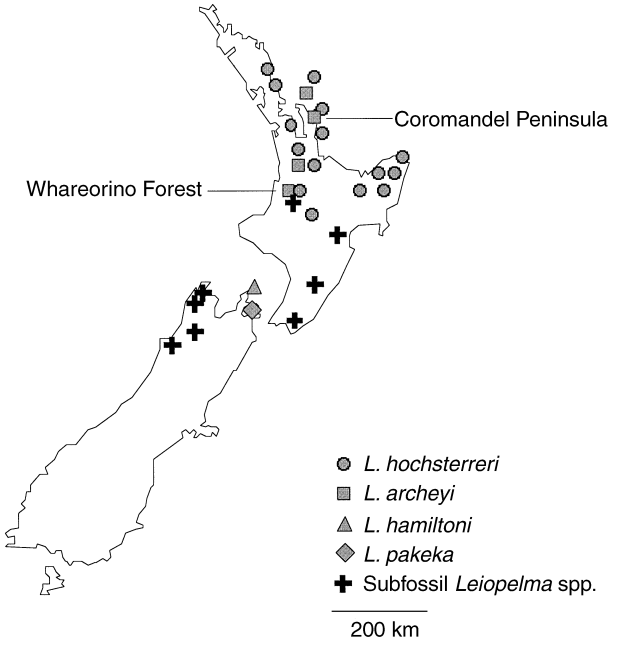

Fig. 1. Distribution map of extant and extinct species of *Leiopelma* in New Zealand (after Worthy, 1987; Bell, 1994 and Bell *et al*. 1998).

MATERIALS AND METHODS

Collection of tissue

Toe clips were obtained from all four *Leiopelma* species. Two populations of *L. archeyi* were sampled on the North Island, near Tapu, Coromandel (37°00′S, 175°35′E; $n = 5$) and Whareorino Forest, King Country (38°23′S, 174°47′E; $n = 6$). Samples of *L. hochstetteri* were obtained near Tapu, Coromandel (37°00′S, 175°35′E; $n = 5$). *Leiopelma hamiltoni* was sampled from Stephens Island, Cook Strait (40°40′S, 174°00′E; $n = 18$), and *L. pakeka* from Maud Island, Cook Strait (41°01′S, 173°53′E; $n = 13$). Individuals were toe-

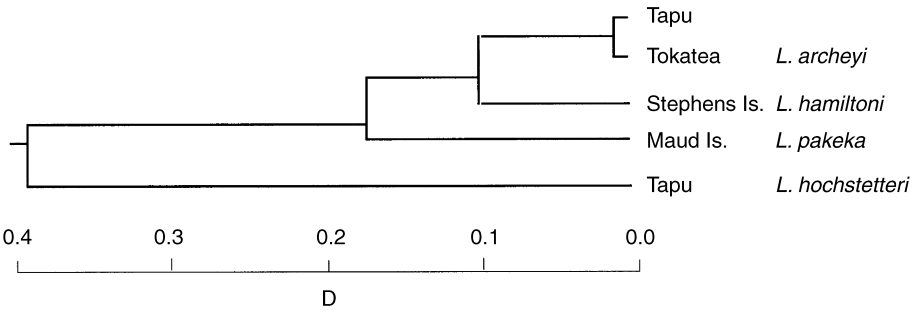

Fig. 2. Unweighted pair group method with arithmetic mean (UPGMA) analysis, based on Nei's standard distance (D) from 12 allozyme loci (after Bell *et al*., 1998), showing the genetic relationships of *Leiopelma* species.

clipped to facilitate ongoing population studies. Tissues thus obtained were saved for genetic analyses and stored in 95% ethanol at –20°C.

DNA extraction

DNA was extracted from toe clips using a modified chelex protocol (Walsh, Metzger & Higuchi, 1991). Each sample was incubated in 200 µl of 5% chelex (Bio-Rad), 0.1% Tween-20, 100 µg/ml Proteinase K in TNE (50mM Tris.Cl, 100mM NaCl, 10mM EDTA, pH 8.0) for 3–5 h at 50°C with intermittent mixing. Following digestion the sample was boiled for 10 min to inactivate the Proteinase K. The sample was then centrifuged briefly at 12 000 rpm in a microfuge, the supernatant retained, and the sample re-extracted with a further 200 µl 5% chelex in TNE, pH 8.0. The sample was re-centrifuged, the supernatant removed, and the sample stored at –20°C.

Polymerase chain reaction amplification and sequencing

Amplification of mitochondrial 12 S rRNA and cytochrome *b* (Cyt *b*) gene partial sequences was achieved by the polymerase chain reaction (PCR) using 'universal' primers modified from Kocher *et al*. (1989): 12 S rRNA, L1091 5′-CAAACTGGGATTAGATACCCCACTAT-3′, H1478 5′-AGGGTGACGGGCGGTGTGT-3′; Cyt *b*, L14841 5′-CCATCCAACATCTCAGCATGATGAAA-3', H15149 5′-CCCCTCAGAATGATATTTGTCCTCA-3′. PCRs were carried out in 25 µl reaction mixtures containing 50 ng of template DNA, 10 pmol of each primer, 5 nmol of each dNTP, 2.5 µl of 10× reaction buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0), 1.8 mM $MgCl_2$ and 0.5 units of *Taq* polymerase (Boehringer Mannheim). All reactions were denatured for 2 min at 80°C prior to initiation of the PCR. For all 12 S rRNA reactions, and for *L. hamiltoni*, *L. pakeka* and *L. archeyi* (Tapu) Cyt *b* reactions, the cycling parameters were 30 cycles of 94 °C/30 s, 48 °C/30 s, and 72 °C/1 min followed by a final extension step of 72 °C/4 min. For *L. archeyi* (Whareorino) and *L. hochstetteri* (Tapu) Cyt *b* amplifications, the annealing temperature was reduced to 44 °C.

Following amplification the integrity and size of PCR products were examined using agarose gel electrophoresis and the products were purified by ethanol precipitation to remove residual primers. PCR products were then sequenced using a thermosequenase cycle sequencing kit (Amersham Pharmacia Biotech). One primer was end-labelled with [γ^{32}]dATP using Polynucleotide Kinase (Boehringer Mannheim) and cycle sequencing reactions were performed according to the manufacturer's instructions using the following parameters: 20 cycles of 95 °C/30 s, 50 °C/30 s, 70 °C/1 min, followed by 10 cycles of 95 °C/30 s, 70 °C/1 min. Radiolabelled sequencing products were then electrophoresed on a 6% (w/v) denaturing polyacrylamide gel using a Gibco/BRL Labs S2 sequencing apparatus. Gels were dried and exposed to X-ray film for 12–72 h. For each individual, several PCR products were sequenced in both directions to ensure sequence fidelity.

Phylogenetic analysis

Individual sequences were aligned using ClustalW (Thompson, Higgins & Gibson, 1994) then identical sequences were filtered and collapsed in MacClade v3.06 (Maddison & Maddison, 1996). Maximum parsimony analyses were performed using the PAUP 3.11 package (Swofford, 1993). Bootstrap analyses (Felsenstein, 1985), based on 1000 replications, were performed within PAUP to provide an estimate of the statistical significance of the maximum-parsimony tree topology. Outgroup sequences were obtained from the published literature: *Colostethus pratti* (Ruvinsky & Maxon, 1996; U39968) and *Rana lessonae* (Ploetner, 1998; AJ222652) were used as outgroups for the 12 S analysis; *Colostethus talamancae* (Summers *et al*., 1996; U70140) and *Rana lessonae* (T. J. C. Beebee, AF084047, unpubl. Genbank submission) sequences were used as outgroups for the Cyt *b* analysis. In the 12 S phylogeny a Genbank derived sequence from *Ascaphus truei* (Hay *et al*., 1995; X86225) was added as an ingroup because Hay *et al*. (1995) previously showed it to be monophyletic with the Leiopelmatidae. All other sequences used are from this study.

RESULTS

Sequences were obtained for 12 S rRNA and Cyt *b* partial genes (299 bp and 300 bp, respectively) from 47 individual frogs representing the four described species (Genbank accession numbers AF231455–AF231462 and AF231463–AF231465 for Cyt *b* and 12 S rRNA partial sequences, respectively). Our *L. hamiltoni* and *L. pakeka* 12 S rRNA sequences differ by a one nucleotide T indel from the previously published 12 S rRNA sequence of Maud Island *L. hamiltoni* (currently *L. pakeka*) (Hay *et al*., 1995). None of the new sequences appear to be pseudogene derived. The majority of Cyt *b* sequence differences occur at the third codon position and, indeed, all inter-sequence comparisons show transition/transversion ratios consistent with those previously reported for mtDNA (Lopez *et al*., 1997).

An exhaustive maximum-parsimony analysis identified a single most-parsimonious tree (minimal-length tree) for each of the two genes sequenced. These phylogenies (Fig. 3) differ significantly from that postulated by Bell *et al*. (1998; Fig. 2). In particular we find no support for the contention that *L. archeyi* and *L. hamiltoni* are sister taxa.

Rather, our data strongly support the hypothesis that *L. hamiltoni* and *L. pakeka* are sister taxa. These two species possess identical 12 S rRNA and nearly identical Cyt *b* partial gene sequences and form a distinctive clade (Fig. 3). This clade excludes *L. archeyi*, a finding contrary to that of Bell *et al*. (1998). The bootstrap sup-

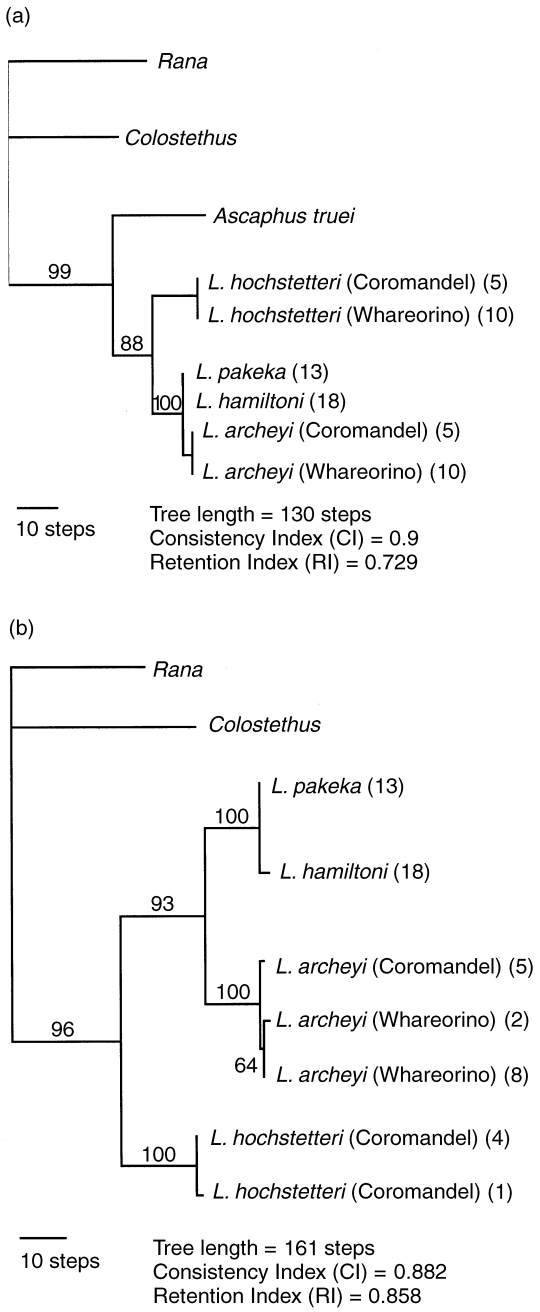

Fig. 3. Maximum parsimony phenogram based on (a) 12 S rRNA sequence data, (b) Cyt *b* sequence data. Trees were generated from 1000 bootstrap replications and branch lengths shown are proportional to the minimum number of changes associated with the respective branches. Bootstrap confidence values above 50% are shown. Numbers in parentheses are the numbers of individuals sequenced from each taxon.

port for a clade of *L. pakeka* and *L. hamiltoni* that is distinct from *L. archeyi* is 100% along both dividing branches within the Cyt *b* dendrogram (Fig. 3(b)). The general lack of sequence divergence within the 12 S rRNA gene between species (1% between *L. archeyi* and an *L. hamiltoni–L. pakeka* clade) can account for the lack of branch placement confidence between these obviously phylogenetically close species. In addition, Cyt *b* analysis suggests that *L. pakeka* is slightly more similar to *L. archeyi* than to *L. hamiltoni*, a result that contradicts that of Bell *et al.* (1998). This is not seen in our 12 S rRNA analysis, as *L. hamiltoni* and *L. pakeka* have identical partial sequences.

Constraining our Cyt *b* data to fit the predictions of Bell *et al.* (1998) we find that the minimum-length tree increases from 161 to 171 steps. This difference in tree length is highly significant ($P = 0.0015$) when examined using the Kishino-Hasegawa test (Kishino & Hasegawa, 1989). Re-examining the data obtained from our unconstrained search of tree space, we found 87 trees that were equally or more parsimonious than the tree produced using constraints.

Between the Whareorino and Tapu populations of *L. archeyi*, 12 S rRNA partial sequences do not vary but a fixed 0.66% difference is evident between Cyt *b* partial sequences. This is the same level of variation seen between *L. hamiltoni* and *L. pakeka* Cyt *b* partial sequences. This suggests that, based on sequence divergence alone, not only do *L. hamiltoni* and *L. pakeka* form a phylogenetically close clade but there may be only population-level variation between them. Neither *L. pakeka* nor *L. hamiltoni* show any within-population variability as each population is fixed for its particular Cyt *b* haplotype.

Mitochondrial DNA analysis (Fig. 3) confirms that *L. hochstetteri* has diverged considerably from the other Leiopelmatidae. This result is consistent with previous studies of allozymes (Bell, 1994; Green, 1994), morphology and ecology (Bell, 1994). Only minimal intra- or inter-population variation is apparent in any of the leiopelmid species. As predicted by Hay *et al.* (1995), in the 12 S rRNA dendrogram, *Aschaphus truei* is shown as a divergent sister taxa to all of the extant Leiopelmatidae.

DISCUSSION

Phylogenetic relationships of the Leiopelmatidae

Previous studies using allozyme and morphological variation hypothesized that *L. archeyi* and *L. hamiltoni* were sister taxa to the exclusion of *L. pakeka* (Bell *et al.*, 1998). This contention is not supported by the present geographical locations of these species (Fig. 1) nor by mtDNA sequence data from this study. In the 12 S rRNA amplicon there is no sequence difference between *L. hamiltoni* and *L. pakeka*. In comparison, three fixed differences (1%) are observed between the *L. hamiltoni–L. pakeka* clade and the two populations of *L. archeyi*. In the Cyt *b* amplicon, whilst there are two fixed differences between *L. pakeka* and *L. hamiltoni*, the hypothesis that these taxa are phylogenetically close and distinct from *L. archeyi* is strongly supported (Fig. 3(b) bootstrap values). In addition, we find that the genetic divergence between *L. pakeka* and *L. hamiltoni* is of the same magnitude as that between allopatric populations of *L. archeyi*. These data suggest that *L. hamiltoni* and *L. pakeka* are sister taxa or possibly geographically isolated populations of the same species.

The species status of *L. pakeka* and the implications for native frog conservation

When *L. pakeka* was elevated to species status in 1998, *L. hamiltoni* became arguably the most endangered frog species on earth. Our mtDNA data contradict the allozyme and morphological data presented by Bell *et al*. (1998) that served as the basis for the recognition of this new species. While both data sets identify differences between *L. pakeka* and *L. hamiltoni*, our data suggest that these are no greater than that observed between isolated populations of the same species.

Our findings have important implications for the development of management plans to ensure the conservation of these frogs. *Leiopelma hamiltoni* is currently a Category A endangered species (Molloy & Davis, 1994) represented by a single population of <150 frogs in a 600 m^2 rock pile (colloquially known as the 'frog bank'). Applying the 1996 IUCN guidelines, *L. hamiltoni* would rank as one of the world's most critically endangered frogs (Ballie & Groombridge, 1996). In contrast, the *L. pakeka* population may be as large as 19 000 frogs and is represented by two physically distinct populations following a successful translocation of frogs to Motuara Island, another small island in the Cook Strait (Tocher & Newman, 1997). In accordance with the IUCN guidelines the species is considered vulnerable to extinction (Ballie & Groombridge, 1996). If *L. pakeka* is once again recognized as a population of *L. hamiltoni*, the conservation status of *L. hamiltoni* will decrease, albeit to vulnerable, since conservation agencies can more easily marshal support for, and implement plans to protect, a species than an evolutionarily distinct population. Moreover, plans may be drawn up to translocate individuals between populations in an attempt to increase effective population size or to increase genetic diversity by interbreeding in captive rearing programmes.

In recent years the importance of maintaining the evolutionary potential of species has been formally recognized with the adoption of 'evolutionarily significant units' (ESUs) to protect historically isolated, genetically distinct assemblages of a biological species (Ryder, 1986; Waples, 1991; Moritz, 1994). An ESU is a population that is isolated from other conspecific population units, and it embodies an important component of the evolutionary legacy of the species. Moritz (1994) suggests that 'ESUs should be reciprocally monophyletic for mtDNA alleles and show significant divergence of allele frequencies at nuclear loci'. While the genetic criteria for recognizing ESUs may be overly restrictive, they can be applied with consistency and offer the advantage of being theoretically sound (Moritz, 1994). Most importantly, ESUs avoid the issue of 'how much divergence is enough?' by considering the pattern rather than the amount of genetic divergence.

Our sequence data suggest that *L. pakeka* and *L. hamiltoni* are sister taxa or populations of the same species. The level of mtDNA divergence between these populations is very low (0.66% at Cyt *b*). However, there is reciprocal monophyly between these two populations at mtDNA loci (our data), as well as fixed and allele frequency differences at nuclear allozyme loci (Bell *et al*., 1998). This pattern of genetic differentiation, together with their allopatry, suggests that for conservation management purposes these two populations should be considered as separate ESUs.

Of course, conservation management decisions need to be based on broader considerations, and a major dilemma facing conservation biologists is whether we should conserve populations because they are genetically distinct, or promote the maintenance of genetically more diverse species. Compounding this dilemma are demographic problems resulting from small population size. Preserving genetically distinctive species may prove of little value if the population size has dropped below a critical level (Lande, 1993; Lynch, Conery & Bürger, 1995). In resolving the dilemma, we cannot afford to lose sight of the reality that resources available to managers are limited. Ensuring the viability of ESUs, even if possible, might come at the cost of sacrificing other potentially more important projects such as those designed to detect and conserve cryptic but genetically distinct species.

Leiopelma hamiltoni and *L. pakeka* fit the criteria of being distinct ESUs and therefore warrant conservation as independent units for management purposes. However, compared to other native New Zealand frog species, only small levels of genetic variation have been detected within either of these populations using allozyme markers and mtDNA (see Green, 1994; Bell *et al*., 1998; and this study). For example, Bell *et al*. (1998) used 12 allozyme loci to assay genetic variation in 11 *L. hamiltoni* and 59 *L. pakeka* and observed no within-population genetic variation for *L. hamiltoni* and only one polymorphic locus in *L. pakeka* (H_{ave} = 0.03). Augmenting these data, we identified no within-population variation in Cyt *b* partial sequences from the 18 *L. hamiltoni* and 13 *L. pakeka* we analysed. In contrast, we identified three population-level variants in *L. archeyi* (two populations, n = 5 and 6, respectively) and two population variants in *L. hochstetteri* (one population, n = 5), which were equal to the difference observed between *L. pakeka* and *L. hamiltoni* (Fig. 3.).

Therefore, the maintenance of diversity and continued population viability may be better served by joint management to preserve the species. The alternative strategy – separate management – places what remaining diversity exists in significant jeopardy because for these frogs the demographic concerns may considerably outweigh those of genetics. For example, the latest census data from Stephens Island suggest that considerably less than 150 *L. hamiltoni* remain. The prognosis for this species is bleak and it may be impractical to attempt to preserve what amounts to shallow genealogical distinctiveness in a population that is exceptionally vulnerable to environmental and demographic stochasticity as well as strong inbreeding depression (Waldman & McKinnon, 1993).

CONCLUSION

In our opinion *L. pakeka* should not be considered to be a separate species, but it is certainly worthy of consideration as an evolutionarily significant unit (ESU) based upon its genetic and geographical differentiation from *L. hamiltoni*. Additional investigations, employing nuclear genetic markers such as microsatellites, are needed if we are to gain further insight into the evolutionary processes that have altered the genetic composition of these two groups since their isolation by allopatry approximately 10 000 years ago. Studies of behaviour and in particular the mating systems of these groups will also be necessary if we are to clarify the evolutionary consequences of the observed genetic differentiation. Therefore, at this time, we recommend a cautious approach to the future conservation management of these frogs. If management resources are sufficient, each population should be maintained as a discrete entity. Additional studies need to be undertaken, particularly in developing approaches for translocation and captive rearing that will ensure the survival of these unique frogs should more active management strategies be deemed necessary in the future. However, our results raise the clear possibility that other heretofore unrecognized ESUs of equal or higher conservation value may exist among the extant Leiopelmatidae.

Acknowledgements

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