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RESEARCH ARTICLE

Phylogeography of *Leiopelma hochstetteri* reveals strong genetic structure and suggests new conservation priorities

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Abstract *Leiopelma hochstetteri* belongs to a singular anuran lineage endemic to New Zealand that diverged from other frogs about 200 million years ago. The species now is reduced to a series of isolated populations in the northern half of the North Island and Great Barrier Island. We have used mitochondrial and nuclear sequence data to examine the genetic affinities of extant populations of *L. hochstetteri*. Phylogenetic reconstructions reveal that populations are highly structured. Each of the geographically isolated populations harbours independent mtDNA lineages as well as different degrees of nuDNA differentiation. Moreover, molecular dating reveals that this structure originated in the early Pleistocene. This pattern of genetic structure likely results from unfavourable climatic conditions during the Pleistocene combined with the low dispersal ability of the species. Isolated populations in forested refugia existed even in the southern part of the distribution of the species during glacial cycles. Previously published variation in chromosome numbers and isozyme data are consistent with the new evidence. We identified 13 evolutionary significant units (ESUs) that should serve as the focus for future management and conservation of this species.

Keywords Phylogeography · *Leiopelma* · New Zealand · Conservation · Pleistocene

Introduction

New Zealand's endemic frog genus *Leiopelma* is comprised of three species of which Hochstetter's frog, *Leiopelma hochstetteri*, is sister to all others in the group

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(Holyoake et al. 2001). The closest relative of *Leiopelma* is the North American genus *Ascaphus* and recent molecular dating suggests that they diverged about 200 million years ago (Ma) (Roelants et al. 2007). The Leiopelmatidae thus represent a unique evolutionary legacy of special importance for conservation biology. Living *Leiopelma* frogs appear very similar in their morphology to Jurassic-era frog fossils, suggesting that they have not changed much from their ancestral form (Estes and Reig 1973).

Subfossil remains indicate that *Leiopelma* species were once more widely distributed throughout New Zealand (Worthy 1987). However, the arrival of humans and, with them, mammalian predators, such as rats, has resulted in the extinction of several endemic frog species (Worthy 1987; Bell 1985). The remaining species continue to be threatened by habitat destruction, exotic predators, population fragmentation, and the consequences of small population size (Daugherty et al. 1994; Waldman and Tocher 1998). Today their conservation status ranges from low risk to critically endangered (Daugherty et al. 1994; Newman 1996; Stuart et al. 2008).

Hochstetter's frog was the first of the New Zealand native frog species to be recognised taxonomically (Fitzinger 1861) and has been protected since 1922 (Bell 1985, 1994). In general, *L. hochstetteri* inhabits rocky streambeds in pristine native forest, which today is highly fragmented (McGlone et al. 1993; Didham et al. 2005). Although the most abundant of the remaining endemic frogs, *L. hochstetteri* is categorised as "vulnerable" by the IUCN (International Union for the Conservation of Nature, Bell et al. 2004), and has a "sparse" ranking under the New Zealand Department of Conservation threatened species ranking system (Molloy et al. 2002; Hitchmough 2002).

Leiopelma hochstetteri currently occupies a fragmented range and lives in scattered populations across the North Island and on Great Barrier Island with the highest population density on the Coromandel Peninsula (Newman 1996). Subfossil remains identified as *L. hochstetteri* appear throughout the North Island and even in the northern half of the South Island, suggesting its range was once much wider (Worthy 1987). Recently, a new population of *L. hochstetteri* was discovered at Maungatautari in the Waikato region (Baber et al. 2006). Our lack of knowledge of the distribution of extant populations highlights the need for more research on this important species.

Amphibians often show strong genetic differentiation across small geographic scales as a consequence of their low dispersal ability (Driscoll 1998a, b; James and Moritz 2000; Shaffer et al. 2000; Zeisset and Beebee 2008). The dispersal tendencies of *L. hochstetteri* are unknown but the frogs are habitat specialists preferring narrow zones along rocky streambeds (Green and Tessier 1990) and their daily movements are limited (Tessier et al. 1991). As a consequence of restricted gene flow and genetic drift, populations thus are likely to be highly genetically differentiated from one another.

Cytogenetic work previously revealed considerable variation among *L. hochstetteri* populations in the average number of supernumerary chromosomes and in the presence and morphology of a unique, univalent sex chromosome (Green et al. 1993; Green 1994). Isozyme data also partially support this framework with some populations being clearly differentiated but others appear indistinguishable (Green 1994). These genetic subdivisions raise the possibility that cryptic species exist. Regardless, if the cytogenetic and isozyme data are correct, then every population may be an important component of the species' evolutionary potential. Therefore, effective conservation management practise should focus on protecting these historically isolated, genetically distinct assemblages (Ryder 1986; Waples 1991; Moritz 1994).

This view is further supported by palaeo-vegetation studies that revealed major modification of vegetation patterns during the Quaternary (e.g., McGlone 1988; McGlone et al. 1993) with likely impact on the phylogeography of *L. hochstetteri* and other species. During the Last Glacial Maximum (LGM), continuous closed forest was limited to areas north of about 38°S latitude, where most *L. hochstetteri* populations now occur. Additionally, paleoclimate modelling suggests that mean annual temperature was about 2.5–4°C cooler with less precipitation than today (Drost et al. 2007) which implies that during this period most of New Zealand would not have been suitable for *L. hochstetteri*. Assuming niche conservatism, *L. hochstetteri* probably would have been restricted to northern refuge areas whereas southernmost populations are likely either: (1) to be recently derived from northern refugia and therefore display little genetic structure, or (2) to have remained in situ in small patches of forest during glacial cycles and thus display strong genetic structure.

To distinguish between these two hypotheses and to gain some insight into the relationships among populations and the timing of historical phylogeographic events, we examined DNA sequence data both from the mitochondrial (mtDNA) genes (Cytochrome *b* and 16S) and the nuclear (nuDNA) gene Tyrosinase. We determined the relationships among all known Hochstetter's frog populations using phylogenetic tree reconstructions and haplotypic network analyses and investigated the timing of the diversification within the species. These results, together with insights from karyological and isozyme data, have enabled us to identify populations with unique genetic characteristics that may require special consideration for conservation management of this species.

Materials and methods

Samples

We extracted total genomic DNA from 104 *L. hochstetteri* samples, preserved in 70% ethanol, obtained from 21 populations spanning the entire range of the species (Supplementary Table 1) using a modification of the Chelex protocol of Walsh et al. (1991). Approximately 2 mm^2 of tissue was suspended in 300 µl of digestion buffer containing 5% Chelex. Proteinase K and RNase were added to final concentrations of 100 µg/ml and the samples were incubated overnight at 37°C. The samples were centrifuged at 13,000 rpm to precipitate debris. The supernatant was transferred to a fresh tube and an equal volume of 5% Chelex in TE added. The sample was centrifuged once more at 13,000 rpm, and the supernatant removed and stored at −20°C.

In addition, single DNA samples of *L. archeyi* and *L. hamiltoni*, from a previous study (Holyoake et al. 2001) were obtained for use as outgroups (Supplementary Table 1).

PCR amplification and sequencing

We amplified mitochondrial and nuclear genes partial sequences by polymerase chain reaction (PCR). For Cyt *b*, we used primers designed using comparative sequence alignments (Bowsher 2000), JB1F 5′-ATGAAACTTCGG CTCTCTTMRGG-3′, JB36R 5′-TCTTCTACTGGTTGAC CTCCAATTCA-3′ and two additional primers designed for this study CBLeioF 5′-TAATCCGAAATATCCATG CC-3′, CBLeioR 5′-AGGTGATTATRATGTAGAAGCC-3′. For 16S, we used primers from Salducci et al. (2005) and two additional primers designed for this study 16F3Leio 5′-TTACCAAAAACACCGCCTC-3′, 16RLeio 5′-TATCC CCAGGGTAACTTAG-3′. For Tyrosinase we used primers Tyr H and Tyr I from Bossuyt and Milinkovitch (2000) and two additional primers designed for this study TyrLeioF2 5′-TACAACAGGACATGCAAGTGTC-3′, TyrLeioR 5′-AGTAAGGAATGGTGAAGTTCTC-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, 5 µl of 10× reaction buffer (500 mM KCl, 100 mM Tris–HCl, pH 9.0), 1.5 mM $MgCl_2$ and 1 unit of *Taq* polymerase. All reactions were denatured for 2 min at 95°C prior to initiation of the PCR. For all reactions, the cycling parameters were 35 cycles of 95°C/30 s, 52–55°C/30 s, and 72°C/45 s, followed by a final extension step of 72°C/4 min.

Following amplification, we examined the integrity and size of PCR products by agarose gel electrophoresis and purified the products using Pall Acroprep filter plates Omega 30 K to remove residual primers and dNTPs. Purified PCR products were sequenced using ABI Big Dye V3.1 in both directions, purified using Whatman hydrophilic GF/C filter plates, and then resolved on an automated sequencer at the University of Canterbury sequencing facility. Sequences were edited and aligned with Sequencher 4.1 (Gene Code Corp). Newly determined sequences were deposited in GenBank (Supplementary Table 1).

Phylogenetic analyses

Individual sequences were aligned using Clustal X (Thompson et al. 1997) using the default gap penalties. Cyt *b* and 16S fragments were then concatenated. We used Modeltest 3.7 (Posada and Crandall 1998) to choose the substitution models that best fit each of the three codon positions of the Cyt *b* fragment and the 16S rRNA fragment as a whole using the Akaike Information Criterion (Akaike 1974). These four models (Supplementary Table 3) were subsequently used for partitioned Bayesian analysis performed with MrBayes 3.1 (Huelsenbeck and Ronquist 2001) on the University of Canterbury Supercomputer (http://www.ucsc.canterbury.ac.nz). Bayesian analysis consisted of two independent runs of 2.0×10^7 generations with random starting trees and 10 Markov chains (one cold) sampled every 1,000 generations. Adequate burn-in was determined by examining a likelihood-by-generation plot. We also employed maximum parsimony (MP) with PAUP 4.0b10 (Swofford 2000). Support for proposed clades was assessed via 1,000 nonparametric bootstrap pseudoreplicates (Efron 1979; Felsenstein 1985) with the heuristic search option, tree bisection-reconnection (TBR) branch swapping and 10 random taxa addition replicates. Finally we chose the substitution models that best fit the Cyt *b* + 16S fragment as a whole with Modeltest 3.7 using the Akaike Information Criterion. This model (Supplementary Table 3) was subsequently used in Neighbour-Joining (NJ) with PAUP 4.0b10. Support for proposed clades was assessed via 1,000 nonparametric bootstrap pseudoreplicates. We considered relationships with posterior probabilities ≥0.95 and/or bootstrap percentages ≥75% to be strongly supported. We treated *L. archeyi* + *L. hamiltoni* as a monophyletic outgroup for all the tree reconstructions, a topology supported by previous phylogenetic analyses (e.g., Holyoake et al. 2001).

Haplotypic network analyses

For an alternative, non tree-based, graphical representation of the number of nucleotide differences among haplotypes and their interrelationships, we used TCS 1.21 (Clement

et al. 2000) to create a statistical parsimony network of haplotypes (Templeton et al. 1992) separately on the mitochondrial genes and on the nuclear gene. Because some haplogroups were not connected to each other with the 95% limit of probability of parsimony, we attempted to connect them by decreasing the connection threshold to 90%. We subsequently used Nested Clade Analysis (NCA) according to the criteria of Templeton (1998) to examine the relationships among mtDNA haplotypes.

Heterozygous positions on the nuDNA sequences were coded using IUPAC ambiguity code. Eighty-two out of 86 individuals harboured one ($n = 11$) or no ambiguous sites ($n = 71$). However, four individuals were polymorphic for more than one segregating site. To determine the most likely alleles for these individuals we ran PHASE (Stephens et al. 2001; Stephens and Donnelly, 2003) implemented in DNAsp 4.5 (Rozas et al. 2003). We used default conditions, including 1,000 iterations (of which 500 were used as burn-in), which were sufficient to reach stationarity, and a thinning interval of 1. To improve reliability, we ran the algorithm multiple times with different seeds for the random number generator. We chose the run with the highest average value for the goodness of fit.

IBD analysis

A Mantel test was performed using the program IBDWS 3.15 (Jensen et al. 2005) to determine whether significant isolation by distance exists among populations. Genetic distances were computed with PAUP 4.0b10 using the best model estimated with Modeltest for the total alignment. Raw and log-transformed geographical distances were used with 30,000 randomisations. Log-transformation is recommended in a two-dimensional stepping-stone model (Rousset 1997), which seems suitable for *L. hochstetteri* given the strong association with rocky streams. Reduced major axis (RMA) regression was used to estimate the slope and intercept of the isolation by distance association.

Molecular dating

To provide a time frame for the evolutionary history of *L. hochstetteri* populations we obtained coalescence-based estimates using IMa (Hey and Nielsen 2007) as provided by CBSU (Computational Biology Service Unit) Web Computing Resources (http://cbsuapps.tc.cornell.edu accessed July 2008). We estimated the time of basal diversification of the species by splitting populations into two groups according to the NCA and combining data from the three loci Cyt *b*, 16S and Tyrosinase. The program treats autosomal and mtDNA data with different inheritance scalars. Using this method we were able to simultaneously estimate the effective population size of the two groups, the ancestral population size before population splitting, and the time since population divergence.

Coalescence-based analyses were run several times to estimate appropriate parameter values to bound a "well-behaved" posterior distribution. Convergence was determined by evaluating the consistency of model values for each parameter. We set the migration parameter to 0 because we were attempting to estimate the time of divergence between allopatric groups defined on mtDNA divergence (Fig. 1) and to avoid any potential bias in the analysis due to nuDNA shared ancestral polymorphism. The rate of evolution of the nuDNA locus, which is an exon, is much lower than the mtDNA loci (11–6 times lower, Table S4) and the coalescence time is higher due to the diploid nature of the loci, consequently the low level of nuDNA polymorphism observed in our data is mostly ancestral and allele sharing among populations is more likely inherited than acquired by migration. All the runs comprised 2×10^6 generations sampled every 100 preceded by a burn-in of 1×10^5 generations and using 40 chains with terms of geometric increment model set as Term1 = 0.95 and Term2 = 0.7. A generation time of 6 years was used (B. Bell, personal communication).

A realistic molecular rate of evolution (μ) is needed to ensure correct dating. Given the remote phylogenetic relationship of *Leiopelma* species to any other living frogs (Roelants et al. 2007), estimating the rate of molecular change is particularly challenging. Roelants et al. (2007) studied diversification in the class Amphibia through time and estimated the time of divergence between *L. hochstetteri* and *L. archeyi* at 50.2 Ma (with 95% CI 72.6–31.9 Ma). Given the genetic pairwise distance calculated between *L. archeyi* + *L. hamiltoni* and *L. hochstetteri* using the best fitting model evaluated with Modeltest 3.7, we estimated μ for each of the three loci. Having derived μ we calculated the population divergence time (T) using the formula $t = T \times u$ where t is generated by the program and u is geometric mean of the substitution rates of each loci. We calculated u as $\mu \times k$, where μ is the mutation rate per site per year and k is the length of the sequence. We also used combined mtDNA pairwise distances and the same molecular rates of evolution to give an alternative estimate of basal time of divergence.

Results

Sequence data were obtained from 104 Hochstetter's frogs representing all 21 known populations. 830 bp were obtained for Cytochrome *b* and 424 bp for 16S for all the individuals. A 434 bps fragment of the nuDNA marker

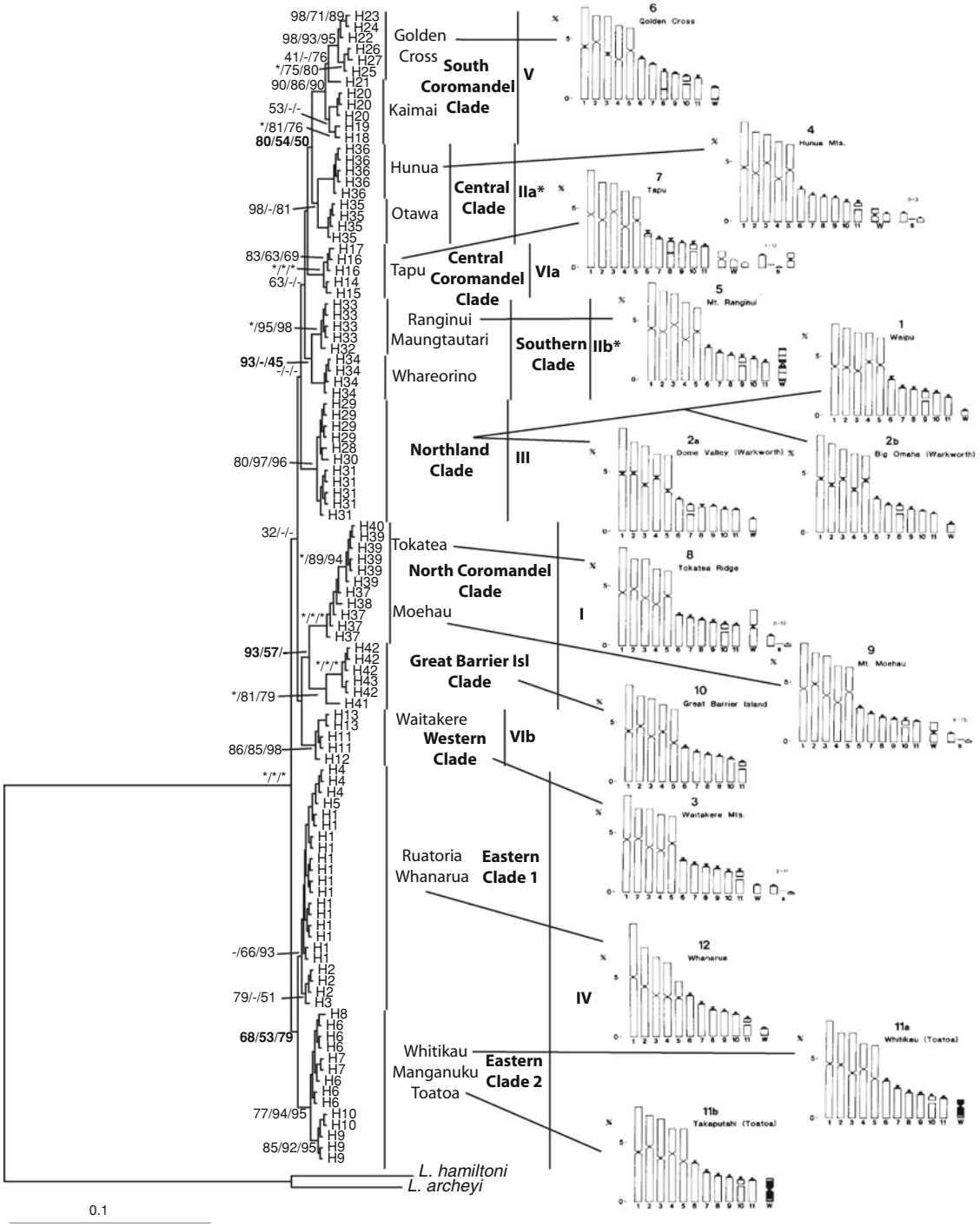

Fig. 1 Phylogram hypothesised from 1,254 bp of mtDNA (830 bp for Cyt *b* and 424 bp for 16S) with Bayesian analysis using 104 *L. hochstetteri* individuals and rooted on *L. hamiltoni* and *L. archeyi*. Node supports are indicated with first posterior probability, secondly MP bootstrap support and then NJ bootstrap support. For Bayesian analysis we used a partitioned model of evolution combining one model for each codon position on the Cyt *b* fragment and one for the 16S fragment that were determined with Modeltest 3.7. pp = 1 and 99 and bootstrap values = 100 and 99 are indicated with *asterisks*. Clades are indicated according to the NCA. Incongruences between tree topology and NCA grouping are indicated for higher clades with *asterisks*. Karyotypes adapted from Green et al. (1993) are indicated for each corresponding populations to compare the two kinds of data

Tyrosinase also was obtained for 86 individuals representing all focal populations except Mount Maungatautari a population already known to have close affinity with that at Mt Ranginui (Supplementary Table 1, Baber et al. 2006). Sequences for each gene region also were obtained for the two outgroups, with the exception of the 16S fragment of

L. hamiltoni for which we used GenBank sequence X86275.

Phylogenetic trees

Forty-three distinct haplotypes were identified (Fig. 1) and haplotypes were not shared among populations except between a few geographically proximate populations (H1 and H4 shared between Whanarua and Ruatoria—Eastern clade 1; H6 shared between Manganuku and Toatoa—Eastern clade 2; H29 shared between Brynderwyn and Waipu—Northland clade; H31 shared between Warkworth and Waipu—Northland clade; and H33 shared between Ranginui and Maungatautari—Southern clade). All populations or groups of populations sharing haplotypes formed independent, highly divergent clades that were well supported in phylogenetic reconstructions.

However, relationships among these clades were largely unresolved. Few displayed unambiguous interrelationships: Tokatea and Moehau populations formed one higher clade in North Coromandel (*/*/*), Hunua and Otawa clades formed one higher clade (Central clade—II, 98/-/81), and Golden Cross and Kaimai clades formed one higher clade (V, 90/86/90). Some additional nodes were not highly supported but were recovered by at least two of the three methods of phylogenetic reconstruction (in bold in Fig. 1): Ranginui + Maungatautari and Whareorino populations formed one higher clade in the South (Southern clade—II, 93/-/45), North Coromandel clade formed one higher clade with the Great Barrier Island clade (I, 93/57/-), Eastern clades 1 and 2 formed one higher clade in the eastern part of the distribution (IV, 68/53/79) and clade V and the central clade formed one higher clade which is not strongly supported (80/54/50). However, other relationships that were not significantly supported also were recovered in the network reconstruction.

Statistical parsimony networks

The mtDNA network more clearly resolved relationships among haplogroups within the species (Fig. 2). However, comparison of the topology of the phylogenetic tree and the haplotype network showed no incongruence except for the relationship between clade V and the central clade.

The central haplotype was sampled in Ruatoria and Whanarua and is included in Clade IV. This central clade in the network was recovered as the basal group in Bayesian phylogenetic reconstruction but with very low posterior probability (Fig. 1). NCA at the highest step recovered a dichotomy between clades A, which grouped Great Barrier Island, north Coromandel, Northland, Southern and Central clades, and B, grouping eastern, western, central Coromandel, Kaimai and Golden Cross clades. We used this dichotomy as a basal split in the species in the absence of a resolved relationship on the tree reconstruction. The strong genetic structure was apparent when mapped (Fig. 3) with the geographical position of the populations and lower level clades forming clusters. However, higher level clades (Clades A, B, VI) did not form obvious geographical clusters.

With nuclear data twenty-five different alleles were recovered from the PHASE runs (Fig. 4, Supplementary Table 1). Allele sharing was observed among populations and corresponding mtDNA clades. However, partial differentiation is evidenced by some groups (defined by mtDNA results) displaying little or no shared alleles. No allele sharing was observed in the Tapu population (Central Coromandel Clade) and very little allele sharing was observed in the Hunua (4/5 private alleles) and Northland (4/5 private alleles) populations. Finally, ongoing differentiation was visible in the Otawa clade, Kaimai clade + Golden Cross clade (clade V) and North Coromandel clade, which collectively share very few alleles with the other populations.

Genetic distances

The highest uncorrected genetic distances between populations were 2.7% between Great Barrier Island and Golden Cross for Cyt *b* (Supplementary Table 2) and 1.8% between Great Barrier Island and Hunua for 16S. The mean corrected distance between populations clustering in A versus B is 0.0142. Thus, using the time of divergence estimated by Roelants et al. (2007) and the corrected genetic distance (Supplementary Table 4) for Cyt *b* and 16S between *L. hochstetteri* and the outgroup (*L. hamiltoni* + *L. archeyi*), we estimated μCyt *b* to be 0.0057 substitutions/site/Ma (95% CI 0.0039; 0.0089) and μ16S to be 0.0033 sub./site/Ma (95% CI 0.0023; 0.0051). Using these rates of molecular evolution we estimated mtDNA divergence among clades A versus B of 1.32 Ma (95% CI 0.84–1.90). The use of corrected genetic distances and raw geographic distance did not detect significant IBD ($Z = 482.2925$, $r = 0.0819$, $P = 0.2111$). However, when geographical distance was log transformed, IBD was detected ($Z = 6.1190$, $r = 0.2093$, $P = 0.0059$).

Molecular dating with coalescence method

Using the geometric mean of the rates of evolution for the three loci and the estimated t with IMa the time of basal divergence within *L. hochstetteri* obtained was 1.79 Ma (95% CI 0.91; 3.67). The posterior distributions of parameters followed published expectations and chains mixed well (ESS > 50 except for *t* and TMRCA of Tyrosinase which were <15).

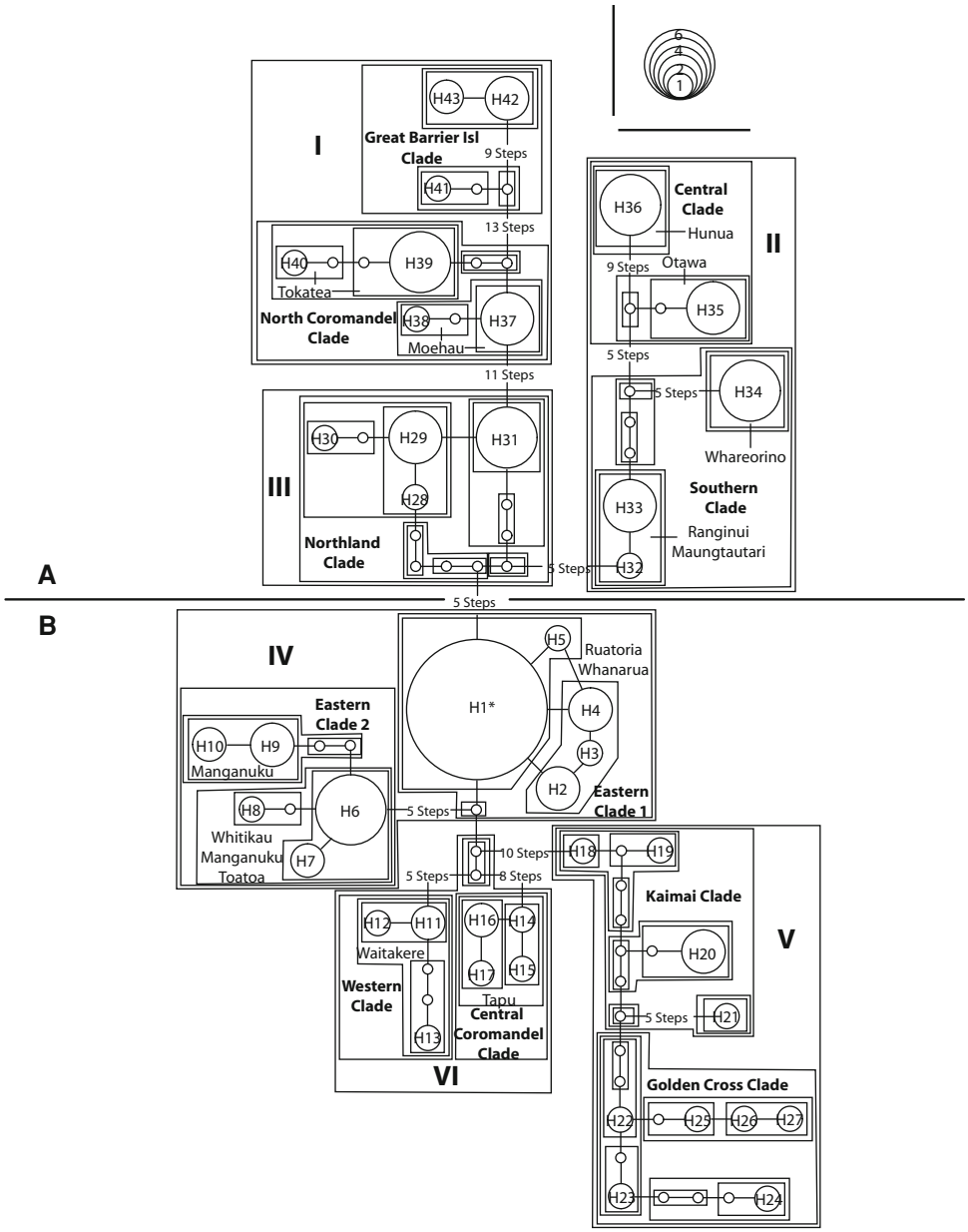

Fig. 2 Haplotype network hypothesised from 1,254 bp of mtDNA (830 bp for Cyt *b* and 424 bp for 16S) using 104 *L. hochstetteri* individuals. The size of the circle representing haplotypes is proportional to the number of individuals with the haplotype number indicated in the *circle*

Discussion

Genetic structure

Our analyses have revealed a high degree of haplotypic differentiation among populations. Almost every sampled locality possessed a distinct mtDNA haplogroup (Figs. 1, 2), leading to a highly structured relationship between genetic diversity and geography (Fig. 3). Some of these lineages cluster together in eight well-delimited geographically definable clades. The precise relationships among these clades are not well resolved and better resolution will require additional data. However, the phylogenetic trees and network (Figs. 1, 2) obtained from our mitochondrial sequence data suggest very little historic interconnectivity between populations in geographic proximity. Consequently, most populations are effectively isolated from each other and are evolving independently. For example, the Coromandel Peninsula harbours three major mtDNA lineages despite their geographical proximity and habitat continuity.

The extensive nuDNA allele sharing among populations and the corresponding, predominantly unique, mtDNA haplogroups that define these (Fig. 4) either result from gene flow or ancestral polymorphism with an absence of substitution since the populations diverged. We think this pattern mainly results from a combination of slower rates of evolution and the longer time necessary for lineage

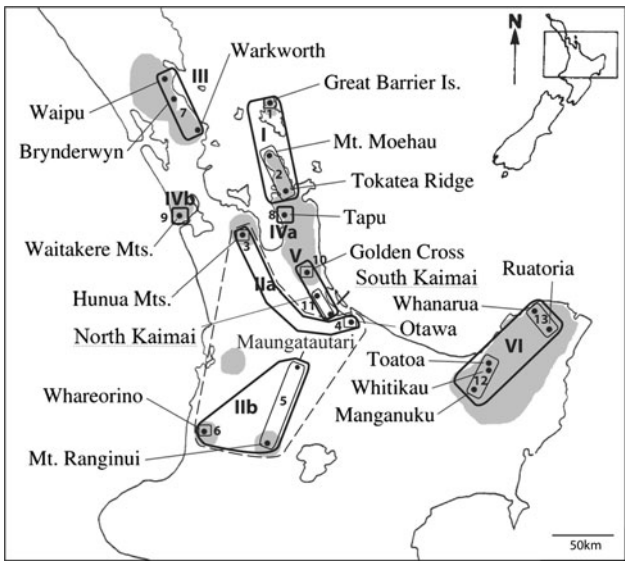

Fig. 3 Distribution of *Leiopelma hochstetteri* showing the locality of sampled populations and NCA results. *Shaded areas* represent the extant range of these frogs (Newman 1996). Since this map was drawn the Pirongia forest population probably became extinct and other populations (Otawa, Kaimai, Mt Maungatatauri) were discovered. *Fine black* limits group genetically similar or very close populations which can represent ESUs ($n = 13$). *Large black* limits group significantly supported higher clades ($n = 8$). *Black dashed* limits represent clade II which is not supported by tree reconstruction

sorting for the Tyrosinase gene polymorphisms compared to those observed for the mtDNA genes (Maddison 1997; Knowles and Carstens 2007) rather than any recent gene flow. Geographically distant populations that display highly divergent mtDNA lineages like eastern, western and southern populations, share most of their nuDNA alleles. However, in some instances the nuDNA data reinforces the notion of ongoing differentiation among populations (e.g., Tapu, Hunua, Northland), supporting further the general observation of strong population structure derived from mtDNA, as well as earlier work that used isozymes (Green 1994) and karyotypic differences (Green et al. 1993; Green 1994).

The genetic distances (2.7% max for Cyt *b*; 1.8% max for 16S) among lineages are not unexpected for amphibians (Vences and Wake 2007) and lie well below the values suggested to warrant consideration as "candidate" species (Vences et al. 2005; Fouquet et al. 2007). However, if additional data from independent nuclear loci were to confirm the lack of post fragmentation gene flow among populations, the species status of some of the entities described here might deserve further consideration and revision. The use of more rapidly evolving DNA markers such as microsatellites may help resolve relationships further and in conjunction with additional ecological morphological and behavioural data may help us understand the differential adaptive responses to local environmental conditions among populations.

Quaternary evolutionary history

Molecular dating suggests that the genetic differentiation observed among populations originated during the early Pleistocene (mtDNA pairwise distances estimate 1.32 Ma (95% CI 0.84; 1.90)) and coalescence analysis indicates that populations initially split from 1.79 Ma (95% CI 0.91; 3.67). Mueller's (2006) estimates for salamanders (μCyt $b = 0.0062$ substitutions/site/Ma (± 0.16) and $\mu 16S = 0.0007$ substitutions/site/Ma (± 0.03)) are close to our values for Cyt *b* but are five times lower for $\mu 16S$ (μCyt *b* 0.0057 substitutions/site/Ma (95% CI 0.0039; 0.0089) and $\mu 16S$ 0.0033 substitutions/site/Ma (95% CI 0.0023; 0.0051)). This difference for 16S probably is due to the fact that we only used a fragment of the gene while Mueller (2006) used the entire gene that includes both loops and hairpin structures that have different rates of evolution.

The current distribution of the species in the northern part of the North Island, mostly along coastal areas seems associated with warmer temperatures in those locales. The lack of a northward extension into tropical latitudes for New Zealand has maximised the influence of Quaternary climatic oscillations on the frost-sensitive components of its biota (Gardner et al. 2004). Consequently, drastically unfavourable cold climatic conditions associated with Pleistocene glaciations probably severely reduced the range of *L. hochstetteri*. Within New Zealand, the middle Pleistocene was characterised by the onset of both catastrophic volcanic eruptions in the central North Island (0.75 Ma; Healy 1992; Stevens et al. 1995) and extreme climatic oscillations (0.85 Ma; Stevens et al. 1995). These phenomena caused large fluctuations in the extent of forest and presumably the range of *L. hochstetteri* throughout the late Pleistocene up to the time of human settlement. Given the basal polytomy among mtDNA lineages (despite the use of a relatively large amount of data: 1,254 bp of mtDNA sequence), the absence of significant IBD when geographical distances are not log transformed, and the strong geographical structure observed, fragmentation of the historic range of *L. hochstetteri* probably happened rapidly, leaving little trace of inter-populational relationships. Moreover, low dispersal tendencies and ecological requirements probably prevented contacts with secondary long distance dispersal.

Late Pleistocene climate oscillations caused massive fluctuations in forest cover throughout New Zealand (McGlone et al. 1993, 2001). During warmer interglacial periods and interstadia, forest covered 85–90% of New Zealand, whereas at the glacial maxima continuous forest

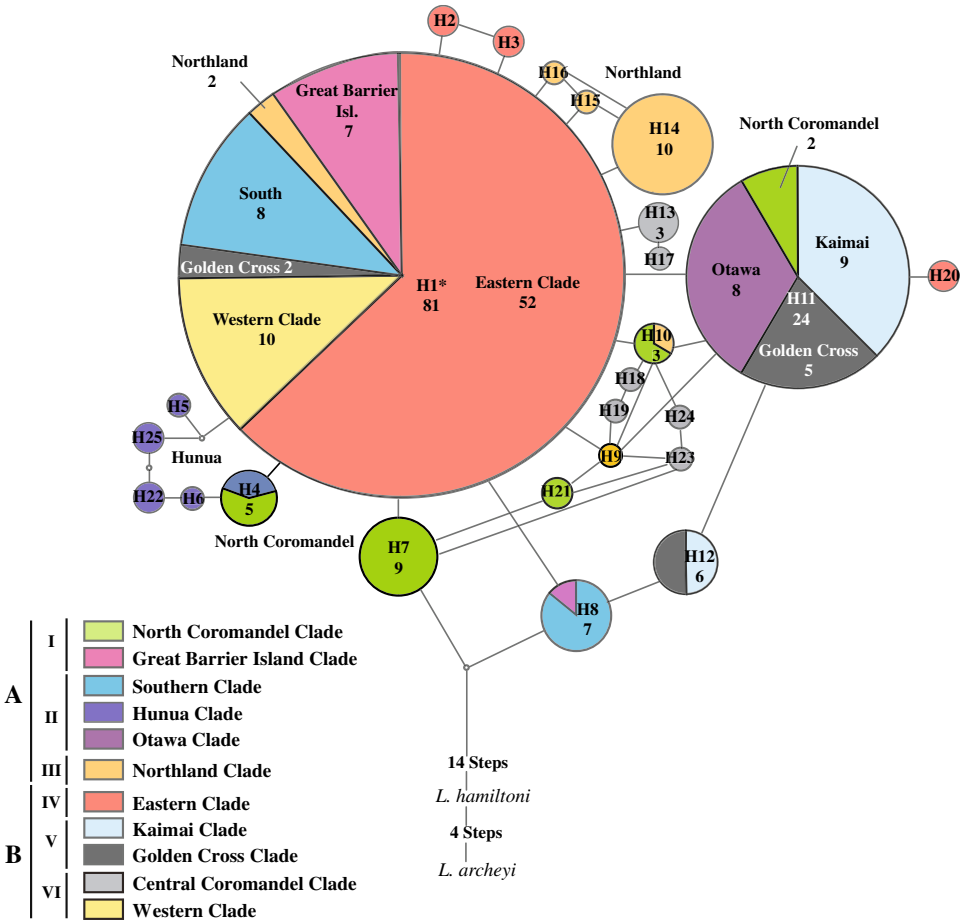

Fig. 4 Haplotype network hypothesised from 432 bp of Tyrosinase exon 1. Haplotypes are represented by *circles* whose sizes are proportional to their frequency, numbered from 1 to 25, with frequency when >3. *Colours* correspond to mtDNA clades indicated in the caption as well as higher mtDNA clades

cover was limited to Northland and the northwest North Island, with only scattered fragments of forest in favourable microclimates persisting elsewhere. The multiple *L. hochstetteri* lineages of Pleistocene origin support this scenario and suggest the existence of the multiple scattered isolated populations that survived glacial cycles. Even the southern and eastern populations display several independent mitochondrial lineages of Pleistocene origin.

We interpret this pattern as indicating that populations have remained in situ during glacial cycles, probably in scattered patches of forest. The only observed relationships that may have a more recent origin lie within the central clade, which clusters the Hunua and Otawa populations, and within the southern clade which brings together the Ranginui, Maungatautari and Whareorino populations (Figs. 1, 2). These relationships may be a consequence of climatic conditions being less favourable southward, which constrained these populations to a common region. The Hunua population displays unique nuDNA alleles while the Otawa population shares one nuDNA allele with its two closest populations of Kaimai and Golden Cross (Figs. 3, 4). Secondary contact after a range expansion could potentially have led to gene flow among these populations that created such a pattern. The populations in the Southern clade (Ranginui, Maungatautari and Whareorino), in the most southern part of the distribution, are presumably subject to the most variable climatic fluctuations. These populations also may have been affected by the volcanism common around Lake Taupo since the Pleistocene (Sparks et al. 1995).

More recently, human induced degradation of the New Zealand environment has increased the fragmentation (e.g., Pirongia forest park hosted a population that is believed to have gone extinct in the 1980s, A. Haigh personnel communication) and probably has driven other populations and lineages to extinction, which perhaps has accentuated the phylogeographic structure of *L. hochstetteri*. In this study we have identified 13 independent mtDNA lineages isolated across a small area in the northern part of the North Island of New Zealand—a phylogeographic pattern never before reported for the New Zealand biota. Comparable phylogeographic breaks have been recovered in many organisms like the Auckland tree weta *Hemideina thoracica* (Morgan-Richards 1997), the stream crayfish *Paranephrops planifrons* (Apte et al. 2007), the skinks of the *Cyclodina* genus (Chapple et al. 2008) and the lesser short-

tailed bat *Mystacina tuberculata* (Lloyd 2003a, b) but never with as many lineages and none have a comparable geographic pattern. The depth of the divergence among these lineages is not surprising for amphibians (Vences and Wake 2007) but the number of isolated lineages in a reduced area and the karyological variability are unique features of this species.

Comparison with previous data

The relationships among populations of *L. hochstetteri* that we found are, in general, concordant with the pattern of diversity of sex chromosomes and B-chromosomes previously documented in this species (Green et al. 1993; Green 1994). Each mtDNA lineage corresponds to very different karyotypes (Fig. 1). For instance, populations with high numbers of B-chromosomes (Waitakere, Hunua, Mt Moehau, Tokatea, and Tapu) are limited to Groups I, IIa and VI a and b (Figs. 2, 3; Table 1) and the cytogenetic distinctions between northern, central and southern Coromandel populations are evident in Groups I, VI and V, respectively. Nevertheless, the phylogeographic relationships that we found indicate that much of the cytogenetic diversity in Hochstetter's frog is relatively recent in origin. In particular, although the populations of Mt. Moehau (northern Coromandel Peninsula) and Great Barrier Island cluster together with Tokatea Ridge in Group I of our molecular analysis, none are more different in chromosomal terms. Frogs from Mt. Moehau have the greatest number of B-chromosomes of any examined population and females possess the univalent W chromosome characteristic of all North Island populations. Great Barrier Island frogs, though, have no B-chromosomes and no discernible sex chromosome or sex chromosome pair. As these populations appear to have been isolated since the Pleistocene, these findings support the hypothesis that B- and univalent sex chromosomes have rapidly evolved (Green et al. 1993).

The isozyme data are also concordant in some respects with the clear segregation of the Hunua population which is also recovered independently with mtDNA and nuDNA as well as Ranginui and Golden Cross populations (Table 1). However, the isozyme data analysed by Green (1994) showed the remaining populations to be similar while the mtDNA and nuDNA data presented here clearly segregate them. This discordance could be due to recent secondary contact with gene flow among North Island populations (except Hunua, Ranginui and Golden Cross) or, as suggested by Green (1994), that populations have diverged recently. However, the mtDNA, Tyrosinase and karyological evidence suggest this not the case. DNA sequence data provide finer scale relationships, and their analysis by more sophisticated models of evolution allows more precision in reconstructing populational relationships and the timing of splits than could ever be achieved with isozyme data.

Conservation implications

Our findings have important implications for the development of management plans to ensure the conservation of these frogs. The importance of maintaining the evolutionary potential of species has been embodied in the concept of 'evolutionarily significant units' (ESUs) to identify historically isolated, genetically distinct assemblages of a biological species for protection (Ryder 1986; Waples 1991; Moritz 1994). Waples (1991) considers that a population or group of populations that (1) is substantially reproductively isolated from other conspecific population units; and (2) represents an important component of the

Table 1 Summary of the different types of data concordant with mtDNA lineages supporting the 13 ESUs

	mtDNA	Tyrosinase	Isozyme	Karyology	ESU	Major clades
Great Barrier Island clade	X			X	1	I
North Coromandel clade	X	X		X	2	I
Hunua clade	X	X	X	X	3	IIa
Otawa clade	X		NA	NA	4	IIa
Maungatautari Ranguniui clade	X		X	X	5	IIb
Whareorino clade	X	X	NA	NA	6	IIb
Northland clade	X	X		X	7	III
Tapu clade	X	X		X	8	IVa
Waitakere clade	X			X	9	IVb
Golden cross clade	X		X	X	10	V
Kaimai clade	X		NA	NA	11	V
Eastern clade 2	X			X	12	VI
Eastern clade 1	X			X	13	VI

Major lineages according NCPA are also indicated

evolutionary legacy of the species should deserve ESU status. Additionally, ESUs should (1) be reciprocal monophyletic for mtDNA alleles; and (2) demonstrate significant divergence at nuclear loci (Avise 1994; Moritz 1994). For the purposes of conservation management, we delimit 13 ESUs that warrant conservation as independent units for management purposes (Fig. 3; Table 1) by all of these criteria. Moreover, the reciprocal monophyly of populations based on mtDNA data are corroborated by nuDNA sequence data, allozyme data and karyological data. This demonstrates the historical isolation that has produced unique and irreplaceable combinations of genotypes in each group of populations.

Conclusion

This study provides a comprehensive picture of the genetic structure and basic elements of the evolutionary history of an emblematic New Zealand frog, which is also one of the most unique anurans in the world. Moreover, our study is the first (see Apte et al. 2007; Gardner et al. 2004; Lloyd 2003a, b) to reveal such a heterogeneous pattern of intraspecific genetic diversity in a terrestrial organism in the North Island of New Zealand. This should encourage further research on the effect of Pleistocene climatic fluctuations on New Zealand fauna in this under studied region.

More complete documentation of the relationships among the remaining populations of *L. hochstetteri* may require the use of polymorphic nuclear genetic markers which could provide additional resolution of the phylogenetic relationships among these populations. Highly variable nuclear DNA markers such as DNA microsatellites (Buschiazzo and Gemmell 2006), which are biparentally inherited unlike the predominantly maternally inherited mtDNA (White et al. 2008), and increasingly easy to isolate de novo (Abdelkrim et al. 2009) would also be useful tools for examining population variability (He), effective population size (Ne), male versus female dispersal and gene flow and, potentially, individual reproductive success data. Such information will be increasingly important if we are not only to set priorities and goals for native frog conservation but also to monitor outcomes.

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