Short Communication

Phylogeography of the spotted skink (Oligosoma lineoocellatum) and green skink (O. chloronoton) species complex (Lacertilia: Scincidae) in New Zealand reveals pre-Pleistocene divergence

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1. Introduction

The New Zealand skink fauna is diverse, comprising at least 28 species in two endemic genera, Oligosoma and Cyclodina (Daugherty et al., 1994; Gill and Whitaker, 2001). The taxonomy of New Zealand skinks has been problematic, complicated by cryptic species, overlapping morphological characters among species, and wide variation within many species across their ranges (Hardy, 1977). There is no comprehensive molecular phylogeny for New Zealand skinks, so many species remain undescribed, and the origin and evolutionary history of the fauna remain largely unknown. For example, the age of the entire New Zealand skink fauna has been variously dated to the Pliocene, the Miocene and the Oligocene (reviewed in Smith et al., 2007).

Oligosoma lineoocellatum (spotted skink) and Oligosoma chloronoton (green skink) form the most taxonomically difficult species complex of all New Zealand lizards (Whitaker et al., 2002). During its taxonomic history various populations have been split off from and then returned to the complex (McCann, 1955; Hardy, 1977). Hardy’s (1977) taxonomic revision defined the modern species boundaries. In particular, Hardy (1977) formally recognised a second species long suspected to exist within the complex, naming it Leiolopisma chloronoton (Prior to 1995, Oligosoma skinks were part of the non-endemic genus Leiolopisma; Patterson and Daugherty, 1995). Genetic studies have been equivocal as to the status of O. chloronoton, with some supporting its distinctiveness (Hardy, 1977; Hay, 1998) but others finding no genetic differentiation from O. lineoocellatum (Towns et al., 1985). The complex is thought to contain cryptic species (Whitaker and Gaze, 1999).

Oligosoma lineoocellatum and O. chloronoton show substantial intraspecific geographic variation in body size and morphology (Hardy, 1977). Oligosoma lineoocellatum reaches body sizes of up to 111 mm snout–vent length (SVL) (Hardy, 1977; Gill and Whitaker, 2001). Its distribution within the North Island is patchy and restricted (Fig. 1b); it occurs in just a few locations on the North Island, near Napier and in the Wellington region (Gill and Whitaker, 2001; Towns et al., 2002). O. lineoocellatum is also found on islands in Cook Strait, and in the Marlborough Sounds (Towns et al., 2002), and it is widespread on the South Island (Gill and Whitaker, 2001) (Fig. 1b). Oligosoma chloronoton reaches a maximum body size of 125 mm SVL (Whitaker et al., 2002), and is restricted to the southern South Island and surrounding islands (Towns et al., 2002; Whitaker et al., 2002) (Fig. 1b). Both O. lineoocellatum and O. chloronoton occur in coastal areas and through mid-altitudes up to the sub-alpine zone, to a maximum of 1700 m (Towns et al., 2002; Whitaker et al., 2002). They inhabit a wide range of habitats but prefer open shrubland, grassland or tussock habitat with stones, logs or vegetation for shelter (Towns et al., 2002; Whitaker et al., 2002). O. lineoocellatum may have been more widely distributed in the past, with Quaternary sub-fossils showing that it was once present in Otago, south of its current distribution limit (Worthy, 1997).
New Zealand’s recent geological and climatic history has been complex (Cooper and Millener, 1993; Daugherty et al., 1993). Most of its mountains date from the Pliocene and are the result of a single, continuing cycle of tectonism which began in the Miocene (Gage, 1980; Suggate, 1982). From the late Pliocene, New Zealand experienced several glacial periods, marked by the formation of a continuous complex of extensive valley glaciers and ice fields along the Southern Alps (Suggate, 1990). Tectonic land movements and sea level changes during glacial cycles caused large-scale alterations to coastlines, including the formation of intermittent land bridges joining the two main islands of New Zealand (North Island and South Island) (Lewis et al., 1994). The relative impacts of these processes on biogeographic patterns in New Zealand taxa has long been a subject of debate. For example, ongoing debates include: (i) whether Pliocene tectonism was a more important factor than glacial climate change in shaping current distribution and diversity patterns (McGlone, 1985; Wardle, 1988; McGlone et al., 2001; Trewick and Wallis, 2001) and (ii) whether land bridges that formed during glacial periods allowed the interchange of terrestrial taxa between the North and South Islands (Lewis et al., 1994; Worthy and Holdaway, 2002).

It has been suggested that the current distributions of *Oligosoma lineoocellatum* and *O. chloronoton* and patterns of variation within the complex were shaped by Pleistocene glacial cycles, specifically migration across land bridges and range shifts into and out of glacial refugia (Hardy, 1977). Here we re-examine Hardy’s (1977) hypothesis that Pleistocene processes suffice to explain distribution and genetic patterns within *O. lineoocellatum* and *O. chloronoton*, by using mtDNA sequence data (*ND2*, *ND4* and *cytb*) and a phylogeographic framework. We also examine taxonomic issues within the species complex, including the genetic distinctiveness of *O. lineoocellatum* and *O. chloronoton*, and the possibility of cryptic species.

2. Materials and methods

2.1. Sampling

*Oligosoma lineoocellatum* and *O. chloronoton* are classified as threatened species, and some populations are thought to be extinct, so we used existing specimen collections for our study. We obtained samples from the National Frozen Tissue Collection (NFTC; Victoria University of Wellington, New Zealand) and from ethanol-preserved museum specimens from Te Papa (National
Museum of New Zealand, Wellington) for sites covering the entire known range of *O. lineoocellatum* and *O. chloronoton*. We also obtained some samples from private collections (Fig. 1b and Table 1). Two outgroup species, *O. otagense* (Otago skink) and *C. ornata* (ornate skink), were chosen based on a wider phylogenetic study of all endemic New Zealand skinks (Chapple, Daugherty and Ritchie, unpublished data).

2.2. DNA extraction, amplification and sequencing

Total genomic DNA was extracted using a standard phenol and chloroform protocol (Sambrook et al., 1989) followed by ethanol precipitation. The oligonucleotide primers listed in Table 2 were used with PCR to amplify fragments from three mitochondrial loci for each sample: *ND2*, *ND4* (plus *tRNA-His* and part of *tRNA-Ser*), and *cytochrome b*. Previous work using these fragments of the mitochondrial genome showed that they are sufficiently variable for intraspecific studies in squamate reptiles (Chapple and Keogh, 2004; Chapple et al., 2004, 2005; Keogh et al., 2005).

PCR was performed in 25 µL volumes containing 1 µL of DNA template, 1× reaction buffer (67 mM Tris–HCl (pH 8.8), 16 mM (NH₄)₂SO₄, 1.5 mM MgCl₂), 0.4 mg/mL BSA, 200 µM each dNTPs, 0.4 µM of each primer and 1 U of BioTherm DNA Polymerase (GeneCraft), or in 25 µL volumes containing 1 µL of DNA template, 1× reaction buffer (20 mM Tris–HCl (pH 8.4), 50 mM KCl), 1.5 mM MgCl₂, 0.4 mg/mL BSA, 200 µM each dNTPs, 0.4 µM of each primer and 1 U of Platinum *Taq* Polymerase (Invitrogen). All reactions were performed on an Eppendorf Mastercycler thermocycler. A typical PCR profile consisted of initial denaturation for 3 min at 94 °C, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 20 s at 55 °C and extension for 50 s at 72 °C, and a final extension step for 5 min at 72 °C. When amplification failed using this protocol, we used a gradient of annealing temperatures between 40 and 60 °C to obtain PCR product.
PCR products were purified using High Pure PCR Product Purification columns (Roche Diagnostics). The purified product was sequenced directly using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and then analysed on an ABI 3730 capillary sequencer.

2.3. Phylogenetic analyses

Sequence data were edited manually using ContigExpress in Vector NTI Advance 9.1.0 (Invitrogen). DNA sequences were aligned using ClustalX (Thompson et al., 1997) executed in MEGA 3.1 (Kumar et al., 2004). We translated all sequences to confirm that protein coding regions did not contain premature stop codons.

A partition-homogeneity test executed in PAUP* 4.0b10 (Swofford, 1998) confirmed that the phylogenetic signal from all three loci (ND2, ND4 and cytb) was concordant (100 replicates; $P = 0.11$), and the sequences for each individual were therefore concatenated to create a single dataset which was used for subsequent phylogenetic analyses. A dataset for ND2 only was also constructed. It contained ND2 sequences from all of the individuals in the combined dataset, plus six additional sequences for individuals not included in that analysis and for which it was problematic to obtain PCR product, presumably because the museum specimens are several decades old (Table 1).

To determine the most appropriate model of evolution for the datasets, log-likelihood scores were generated using PAUP*, and used to conduct a hierarchical likelihood ratio test (hLRT) in ModelTest 3.7 (Posada and Crandall, 1998). The ModelTest analysis also provided estimates of base frequencies, substitution rates, the proportion of invariable sites, and the among-site substitution rate variation for each dataset.

The model and parameters obtained using ModelTest were used as settings in PAUP* to generate a maximum likelihood (ML) phylogenetic tree for the combined dataset and the ND2-only dataset. Heuristic searching was used, with the tree bisection-reconnection algorithm and random addition of sequences from the datasets. MrBayes 3.1.2

Fig. 1 (continued)
(Ronquist and Huelsenbeck, 2003) was used for Bayesian analysis of each dataset. To increase confidence that the analyses obtained a sampling of the full tree space rather than becoming trapped in local optima, a full analysis of each dataset was run twice for each dataset. The analysis was run for 3,000,000 generations and was sampled every 100 generations. The program Tracer 1.3 (Rambaut and Drummond, 2003) was used to check for chain convergence. The first 25% of sampled trees was discarded as the burn-in phase, with the last 22,500 trees used to estimate the Bayesian posterior probabilities.

Bootstrap values and Bayesian posterior probabilities were used to assess branch support. The datasets were too large to do ML bootstraps, so neighbour-joining bootstrap analyses were performed for each dataset, using PAUP*. We used the distance corrections recommended by the ModelTest analyses, and ran 1000 bootstrap replicates for each dataset. Branches supported by bootstrap values of 70% or greater (Hillis and Bull, 1993) and/or posterior probability values greater than or equal to 0.95 (Wilcox et al., 2002) were considered to be supported by the data (Fig. 2a and b).

To estimate the time since the divergence of lineages within the O. lineoocellatum and O. chloronoton species complex, we calibrated the evolutionary rate of ND2 by re-analysing data from Macey et al. (1998) for agamid

### Table 1

Museum registration numbers and sampling localities for samples used in this study

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<th>Sample</th>
<th>Species</th>
<th>Museum tissue code</th>
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Museum collections: FT and CD, National Frozen Tissue Collection (NFTC), housed at Victoria University of Wellington, New Zealand; RE, Museum of New Zealand Te Papa Tongarewa.
genus *Laudakia*. Specifically, we re-calculated the evolutionary rate for *Laudakia* using only the 550 bp fragment of ND2 used in the present study (e.g., Smith et al., 2007). We calculated average between-group nucleotide differences across each of the calibrated nodes from Macey et al. (1998) (1.5, 2.5 and 3.5 Mya), plotted them against time and used the slope of the linear regression to calculate a rate of evolution for our 550 bp fragment of ND2. This resulted in an evolutionary rate of 1.4% per My (0.7% per lineage, per My) and is slightly faster than the rate of 1.3% per My found by Macey et al. (1998).

### 3. Results

The final combined dataset contained sequences from 35 individuals from 23 locations, as well as two outgroup sequences. For each individual, we obtained sequences from the mitochondrial loci ND2 (550 bp), ND4 plus tRNA-His and part of tRNA-Ser (773 bp), and cyt b (610 bp). After concatenation, the aligned data set contained 1933 characters, of which 583 (30%) were variable, 438 (23%) were parsimony-informative. For the ingroup only, 151 (27%) sites were variable, and 133 (24%) were parsimony-informative. Base frequencies were unequal (A = 31.3%, T = 22.6%, C = 32.1%, G = 14.0%). A $\chi^2$ test executed in PAUP* confirmed the homogeneity of base frequencies across all taxa in the dataset (df = 108, $P = 1.00$).

The ND2-only dataset contained sequences from 41 individuals from 28 locations, as well as two outgroup sequences. The aligned dataset contained 550 characters, of which 187 (34%) were variable and 144 (26%) parsimony-informative. For the ingroup only, 151 (27%) sites were variable, and 133 (24%) were parsimony-informative. Base frequencies were unequal (A = 31.3%, T = 22.6%, C = 32.1%, G = 14.0%). A $\chi^2$ test executed in PAUP* confirmed the homogeneity of base frequencies across all taxa in the dataset (df = 126, $P = 1.00$).

The hLRT implemented using Modeltest selected the GTR + I + G substitution model as the most appropriate for the combined dataset ($-\ln L = 8271.0635$) and the TrN + G model as the most appropriate for the ND2-only dataset ($-\ln L = 2699.0759$). For both datasets, there was a strong bias towards transition substitutions (combined dataset rate matrix: $A \leftrightarrow C = 5.40$, $A \leftrightarrow G = 119.78$, $A \leftrightarrow T = 3.35$, $C \leftrightarrow G = 4.42$, $C \leftrightarrow T = 49.12$, $G \leftrightarrow T = 1.00$). For the combined dataset, Modeltest estimated the gamma shape parameter to be 2.4145 and the proportion of invariable sites to be 0.0615. For the ND2-only dataset, the gamma shape parameter was estimated to be 0.2343.

All phylogenetic analyses recovered three strongly-supported clades (Fig. 2a and b). Clades 1 and 2 represent *O. lineoocellatum*, while Clade 3 represents *O. chloronoton*. The average within-clade, uncorrected genetic distances for Clades 1, 2 and 3 were 2.1%, 3.9% and 5.5%, respectively. Average genetic distances between clades ranged from 8.3% (Clades 1 and 2) to 9.3% (Clades 1 and 3). The relationships among the three clades were not resolved. We identified nine regional subclades within the phylogeny: five subclades representing *O. lineoocellatum* and four subclades within *O. chloronoton* (Fig. 2a and b). Uncorrected genetic distances between representative members of different subclades ranged between 4.1 and 10.5% (Table 3). Average within-subclade genetic distances were between 0.06% and 1.4% (Table 4). The nine subclades were supported by high bootstrap values (100% in all cases) and posterior probabilities (1.00 in all cases) (Fig. 2a).

### 4. Discussion

#### 4.1. Taxonomic implications

Morphological differences between northern populations classified as *O. lineoocellatum*, and southern populations classified as *O. chloronoton* have long been recognised. However, the taxonomic status of southern populations as a separate species has been debated. McCann (1955) included them within *L. lineoocellatum*, as *L. lineoocellatum* ‘form parvicephallum’. However, using wider sampling, especially of *L. lineoocellatum* in the North
Island, Hardy (1977) recognised southern populations as a new species, which he named \textit{L. chloronoton}. Genetic studies using electrophoresis of haem compounds (Hardy, 1977) and mitochondrial loci (16S rRNA; Hay, 1998) have also distinguished these species from one another, but because they used very limited sampling, especially within \textit{O. chloronoton}, they are considered equivocal (Whitaker et al., 2002). However, our genetic data show \textit{O. chloronoton} to be a strongly-supported monophyletic grouping, separated from the two major lineages of \textit{O. lineoocellatum} by genetic distances over 9.0%. Our data therefore provide strong support for Hardy’s (1977) hypothesis that \textit{O. lineoocellatum} and \textit{O. chloronoton} are separate species.

Morphological differences within \textit{O. lineoocellatum}, between northern and southern populations were recognised by McCann (1955). He renamed Cook Strait populations as \textit{Leiolopisma festivum}, based on larger body size and higher scale counts when compared with southern populations. Hardy (1977) examined additional specimens (McCann examined only one specimen from the North Island) and did not find sufficient differences among populations to justify this subdivision. He therefore returned \textit{L. festivum} to synonymy with \textit{L. lineoocellatum}. However,
our phylogeny identifies a two major lineages within *O. lineoocellatum*, separated genetically by a distance of 8.3%, and geographically by the northern end of the Southern Alps. These lineages are as genetically distinct from one another as from *O. chloronoton*. Our data therefore provide support for McCann’s (1955) hypothesis that *O. lineoocellatum* contains at least two species. However, our data support subdivision across the Southern Alps, rather than between Cook Strait populations and all other populations of *O. lineoocellatum*, as suggested by McCann (1955).

Thus, our data suggest that further morphological work on this complex is warranted, with the description of new species where appropriate.

### 4.2. Phylogeographic implications

Hardy (1977) proposed a pivotal role for Pleistocene glacial cycles in shaping distributions and intraspecific patterns of variation within New Zealand endemic skink species. However, the genetic pattern revealed in *O. lineoocellatum*...
Shallower genetic divergences are observed across waterways that have intervened in the complex’s distribution during New Zealand’s recent history. During the Pliocene, the lower North Island and the South Island were joined by dry land, while a sea way separated them from the upper North Island (Lewis et al., 1994). The genetic distance between lineages from Napier and Wellington, areas which would have been separated by the Pliocene sea way, is only 1.3%, corresponding to divergence occurring 0.9 Mya, during the Pleistocene. The Pliocene sea way between Wellington and Taranaki is therefore probably not the cause of divergence between these lineages. It may be instead that lineages from the southern North Island colonised northward during the Pleistocene, when the sea way had receded.

The North and South Islands are now separated by Cook Strait, which first formed in the mid-Pleistocene (C. 0.45 Mya; Lewis et al., 1994). Dry land intermittently bridged Cook Strait during glacial periods, extending from the South Island to the Taranaki region, which is some 200 km north of Wellington (Lewis et al., 1994). The genetic distance between Nelson and Wellington populations, located on either side of Cook Strait, is 4.6%, corresponding to a divergence time of approximately 3.3 Mya. The depth of genetic divergence between populations on either side of Cook Strait and the current distribution of O. lineoocellatum in Wellington (the northern end of the Pliocene land bridge) rather than in Taranaki (the northern end of Pleistocene land bridges) suggests that migration occurred across the Pliocene land bridge between the modern-day North and South Islands, but not across late Pleistocene land bridges.

Molecular studies on the brown kiwi (Apteryx australis; Baker et al., 1995), cicadas (Maoricicada campbelli; Buckley et al., 2001) and bats (Mystacina tuberculata; Lloyd, 2003) have dated divergences between North and South Island clades to C. 0.9 Mya, while a study of carnivorous land snail species (Waimuia umula), found genetic distances across Cook Strait that suggested separation for at least 4 My (Efford et al., 2002). Our results, taken together with these studies, support the idea that if glacial land bridges existed across Cook Strait during the late Pleistocene, they were not
sufficiently long lived to provide suitable routes for the migration of terrestrial species.

Genetic structure across a waterway is also evident within *O. chloronoton*. Foveaux Strait, which intervenes in the distribution, forms a shallow sea strait between the South Island and Stewart Island and was bridged during glacial periods (Newnham et al., 1999). The latest opening of this sea way occurred only C. 11,500 years ago (McGlone and Wilson, 1996). However, there is 5.1% uncorrected genetic distance between the most closely-related haplotypes on either side of this waterway suggesting that the lineages had already diverged before the onset of glacial cycles. Tiwai Point on the South Island’s south coast is notable for being the only location at which we found sympatric haplotypes. One of these mainland haplotypes (OCH8) was more closely related to Codfish Island haplotypes than to other mainland haplotypes. Sympatry of haplotypes in the grand skink, *O. grande*, was interpreted as evidence of admixture following expansion from glacial refugia (Berry and Gleeson, 2005). Since haplotypes within *O. lineocellatum* and *O. chloronoton* are not sympatric elsewhere, we suggest that the most likely explanation for sympatry of these haplotypes is secondary contact, due to the migration of individuals across an historic Foveaux Strait land bridge.

Genetic distances between lineages within the complex reach over 10%, suggesting that the complex may be over 7 million years old. Given the depth of divergence within this species complex, it seems unlikely that the origin of the entire New Zealand skink fauna lies in the Pliocene as some studies have suggested (Robb, 1973; Towns, 1974; Bull and Whitaker, 1975; Hardy, 1977), or even within the last 7 My (Smith et al., 2007).

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**References**


