

Explosive ice age diversification of kiwi

Jason T. Weir^{a,b,1}, Oliver Haddrath^{a,c}, Hugh A. Robertson^d, Rogan M. Colbourne^d, and Allan J. Baker^{a,c}

^aDepartment of Ecology and Evolutionary Biology, University of Toronto, Toronto, ON, Canada M5S 3B2; ^bDepartment of Biological Sciences, University of Toronto Scarborough, Toronto, ON, Canada M1C 1A4; ^cDepartment of Natural History, Royal Ontario Museum, Toronto, ON, Canada M5S 2C6; and ^dScience & Policy Group, Department of Conservation, Wellington, New Zealand

Edited by Scott V. Edwards, Harvard University, Cambridge, MA, and approved July 19, 2016 (received for review March 7, 2016)

Molecular dating largely overturned the paradigm that global cooling during recent Pleistocene glacial cycles resulted in a burst of species diversification although some evidence exists that speciation was commonly promoted in habitats near the expanding and retracting ice sheets. Here, we used a genome-wide dataset of more than half a million base pairs of DNA to test for a glacially induced burst of diversification in kiwi, an avian family distributed within several hundred kilometers of the expanding and retracting glaciers of the Southern Alps of New Zealand. By sampling across the geographic range of the five kiwi species, we discovered many cryptic lineages, bringing the total number of kiwi taxa that currently exist to 11 and the number that existed just before human arrival to 16 or 17. We found that 80% of kiwi diversification events date to the major glacial advances of the Middle and Late Pleistocene. During this period, New Zealand was repeatedly fragmented by glaciers into a series of refugia, with the tiny geographic ranges of many kiwi lineages currently distributed in areas adjacent to these refugia. Estimates of effective population size through time show a dramatic bottleneck during the last glacial cycle in all but one kiwi lineage, as expected if kiwi were isolated in glacially induced refugia. Our results support a fivefold increase in diversification rates during key glacial periods, comparable with levels observed in classic adaptive radiations, and confirm that at least some lineages distributed near glaciated regions underwent rapid ice age diversification.

kiwi | *Apteryx* | New Zealand | glaciation | diversification

Pleistocene climate cycles resulted in glaciation and the buildup of extensive ice sheets at temperate latitudes during periods of global cooling, followed by recession of ice sheets during brief interglacial periods. These cycles were originally proposed to have fragmented populations, resulting in speciation and the formation of much of today's species richness (1–5). Speciation was believed to have occurred primarily through habitat movements and fragmentation tied to global temperature fluctuations that accompanied the glacial advances. Glacially induced bursts of speciation were thought to have occurred in both temperate (2, 4) and tropical latitudes (3, 5). This paradigm was largely overturned with the advent of molecular dating, which placed the origin of the majority of species before the last several glacial cycles or even before the Pleistocene Epoch altogether, both in lowland forests of the Neotropics (6–9), as well as in southern temperate latitudes of the Northern Hemisphere (10–13). Although a small proportion of species did date to the Middle and Late Pleistocene in both, there seemed to be no associated increase in the rate of species origination (6, 12). The implication was that habitat movements and fragmentation caused by glacial cycles were inconsequential in driving diversification above background levels. However, these conclusions were formulated primarily for biomes not in direct contact with the actual glaciers and, in most cases, were based largely on phylogenetic dating using gene trees from just a single genetic marker—mitochondrial DNA (mtDNA hereafter).

In contrast, studies of diversification in biomes immediately adjacent to the expanding and retracting glaciers have uncovered more substantial evidence for glacially induced diversification. These studies leave little doubt that glaciation resulted in fine-scale differentiation at the population level within species (14, 15). More importantly, the small number of studies that have

focused on species diversification in biomes directly adjacent to the expanding and retreating glaciers have also found evidence for glacially induced speciation. This finding is true for high latitudes of North America, where speciation was commonly initiated in boreal birds (16), and to a lesser extent in mammals and other groups whose geographical ranges were directly fragmented by expanding ice sheets (17). It may have also been true for high altitudes of the Neotropics, where glaciation in the Andes resulted in direct fragmentation, leading to speciation along its eastern and western slopes (6, 18). In contrast, less glacially induced speciation seems to have been associated with biomes adjacent to ice sheets in Eurasia (14, 15) or glaciers in the Himalayas (19).

New Zealand provides an ideal opportunity to study the potential effect of glaciation on speciation. Most of New Zealand lies within a few hundred kilometers of glaciated regions although glaciation was most extensive on the South Island and only localized on the North Island (20). The Southern Alps, which bisect the Southern Island along a northeast to southwest axis, were extensively glaciated during ice age cycles, effectively separating the east coast of the South Island from the rest of New Zealand, which, during glacial periods, was interconnected between the west coast of the South Island and the North Island by a land bridge (21, 22). Along the southern half of the west coast, glacial ice extended to the paleo-coastline, with a series of small ice-free refugia forming during the last glacial maximum (22), which presumably would have occurred during earlier maxima as well. Direct fragmentation by glacial ice could have provided opportunities for speciation in these areas. In contrast, localized Pleistocene glaciation on the North Island was not extensive enough to directly fragment habitats by glacial ice although habitats were shifted and potentially fragmented due to

Significance

The role of Pleistocene ice ages in driving a recent burst of diversification is controversial. We used thousands of loci to test the timing and rates of diversification in kiwi—a flightless avian group endemic to New Zealand. Not only did we discover many kiwi taxa—we found 16 or 17 genetically distinct lineages within the currently recognized five species—but we found that most diversification dates to the seven major glacial advances that characterized the latter half of the Pleistocene ice ages and that directly fragmented New Zealand into a series of glacial refugia. Rates at which new kiwi taxa originated increased fivefold during these major cycles, thus linking rapid kiwi diversification to glacial periods.

Author contributions: J.T.W. and A.J.B. designed research; J.T.W. and O.H. performed research; J.W. and O.H. analyzed data; J.T.W. wrote the paper; and H.A.R. and R.M.C. collected samples.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database and the National Center for Biotechnology Information Sequence Read Archive. For a list of accession numbers, see [Dataset S1](#) and [SI Appendix, Table S5](#).

¹To whom correspondence should be addressed. Email: jason.weir@utoronto.ca.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1603795113/-DCSupplemental.

global cooling, potentially initiating the speciation process there. Additionally, the repeated emergence and submergence of the land bridge between the North and South Islands was tied to glacially induced sea-level changes. During glacial periods, the land bridge would have provided a dispersal corridor between islands whereas submergence of the land bridge during interglacials would have isolated populations in each island, leading to the initiation of speciation.

Events other than those tied to glacial cycles may have also driven speciation in New Zealand. For example, the uplift of the Southern Alps occurred primarily from 8.5 Ma to the present and has been previously linked to preglacial diversification (22). Other possible events include extensive volcanism on the North Island, with thick ash fall isolating lineages. Volcanism has occurred in New Zealand for millions of years (22), but the eruption of Taupo Volcano at 26 ka was notable because it was the second largest volcanic explosion in the past 250 ka and covered much of the North Island in as much as 2 m of ash fallout (23).

To address the role of glaciation and other events in driving diversification in New Zealand, we focused on a group of endemic, flightless ratite birds—the kiwi (order Apterygiformes), which, before the arrival of humans, occurred primarily in rainforest habitat (and less frequently in a variety of other habitats) through much of the North and South Island. An accurate assessment of the number of distinct lineages is necessary to determine whether diversification dates to key glacial periods. However, our current understanding of kiwi diversity is poor, largely because kiwi have not developed substantial differences in plumage or morphology that would aid taxonomists in recognizing species differences. Until recently, kiwi were classified as only three species, the great spotted (*Apteryx haastii*), little spotted (*Apteryx owenii*), and brown (*Apteryx australis*) kiwi, each of which is distinctive in size or plumage. mtDNA analyses discovered three deeply diverged lineages within the brown kiwi that are now recognized as distinct species, despite being morphologically cryptic (24–26): the North Island brown kiwi (*Apteryx mantelli*), the rowi or Okarito brown kiwi (*Apteryx rowi*), and the tokoeka or southern brown kiwi (*A. australis*). mtDNA also suggested that geographically isolated populations within some of these species might represent distinct lineages (25, 27, 28), but previous studies suffered from low sample sizes and the use of a single genetic marker (mtDNA). Multilocus data and extensive geographic sampling are required to properly assess kiwi diversity.

Here, we harnessed the precision of thousands of genetic markers in a coalescent population genetic and phylogenetic framework to characterize the number of distinct lineages of kiwi, to determine whether dates of lineage splitting coincided with glacial cycles, and whether kiwi experienced population bottlenecks during glacial periods, as expected if they were fragmented into glacial refugia. If glacial cycles drove diversification, we expected that the majority of kiwi diversity would date to the major glacial advances of the Middle and Late Pleistocene (0.78 Ma to 0.012 Ma) when the buildup of ice was most extensive, and that kiwi lineages affected by glaciation would show bottlenecks over the course of the last glacial cycle. Alternatively, kiwi diversification may largely predate Pleistocene glacial periods or may date to glacial periods but without evidence of glacially induced bottlenecks, suggesting that factors other than glaciation drove their diversification.

Results

Phylogeny and Lineage Delimitation. A Bayesian phylogeny using 1,710 bp of mtDNA for 203 individuals (including sequences from previously published Holocene aged fossil kiwi) (26, 27) showed all five currently recognized species to be reciprocally monophyletic with strong nodal support (Fig. 1). The phylogeny also suggested extensive differentiation between extant populations within *A. australis* and *A. mantelli* and fossil populations

in *A. australis*, *A. owenii*, and *A. rowi* (Fig. 1A). To determine whether these populations also represented genetically distinctive lineages in the nuclear genome, we used a combination of principal coordinate analysis (PCoA), analyses of population structure (29), and Bayesian species delimitation (30).

Four allopatric populations occur within *A. mantelli* (Northland, Coromandel, Taranaki, and Eastern) that corresponded to four largely monophyletic clades uncovered in the mtDNA phylogeny (Fig. 1). The mtDNA phylogeny possessed a single individual that did not group with other members of its population, but, otherwise, individuals from each population were supported as reciprocally monophyletic. These four populations were also distinct in PCoA and in structure analyses of the SNP data although the structure analysis identified some individuals in the Taranaki and Coromandel populations to be of mixed ancestry (Fig. 1C). These four lineages exhibited moderate genetic differentiation with pairwise genome-wide F_{st} between lineages ranging from 0.12 to 0.20 (and significantly greater than 0) (*SI Appendix, Table S1*) and pairwise genetic distances from 0.00114 to 0.00130 (versus 0.00097 to 0.0012 within lineages). Bayesian species delimitation of the nuclear data supported these four populations as distinct lineages (posterior probability = 1.0). All of these analyses point to four genetically distinctive, although shallowly diverged, lineages within *A. mantelli* that we suggest should be recognized as distinct subspecies.

A. australis has three extant allopatric populations in Haast, Fiordland, and Stewart Island. These populations were divided into four strongly supported reciprocally monophyletic clades in the mtDNA phylogeny, with the Fiordland region differentiated into distinctive northern and southern clades (Fig. 1). PCoA plots of nuclear data showed four distinct clusters corresponding to the four clades, albeit with the two Fiordland clades grouping closely together. Structure analyses best supported only three lineages in the nuclear data (with North and South Fiordland clades combined) using the traditional Evanno method for choosing the best number of populations (31). When we set the number of populations to four (as done in Fig. 1), then the analysis of structure uncovered the North and South Fiordland clades as distinct but indicated some admixture between these. These four lineages exhibited moderate to substantial genetic differentiation, with pairwise genomewide F_{st} between lineages ranging from 0.21 to 0.63 (and significantly greater than 0) (*SI Appendix, Table S1*) and pairwise genetic distances from 0.00127 to 0.00173 (versus 0.00067 to 0.0011 within lineages). Bayesian species delineation likewise supported four distinct lineages (posterior probability = 1.0). These analyses together strongly support four distinct lineages within *A. australis* that we suggest should be recognized at least at the subspecies level although some could represent full species and further study is needed.

We included previously published ancient mtDNA samples from Holocene fossil kiwi (28) in our analysis. Our mtDNA phylogeny recovered five or six extinct clades that were genetically differentiated from each of the 11 extant kiwi clades (Fig. 1A). These extinct clades included two strongly supported extinct clades of *A. australis*, two of *A. rowi*, and one or two less clearly supported extinct clades of *A. owenii*. We treat all of these as distinct lineages here (for a total of six extinct lineages) but caution that two of these are represented by only a single individual and that more fossil sampling would be desirable for confirmation of lineage distinction in these cases.

We constructed a species tree using the SNP data for each of the 11 extant lineages. With the exception of a single node, the species tree was fully resolved (Fig. 1C) and largely in agreement with the topology of the mtDNA phylogeny. Within *A. australis*, the species tree consistently placed Northern and Southern Fiordland as sisters (posterior probability = 1.0) in contrast to the mtDNA phylogeny, but consistent with the close grouping of these lineages on the SNP-based PCoA plot (Fig. 1D). The

at 16–19 Ma (32), fossil kiwis are known only from the Holocene, and none provide an internal calibration point. We therefore used the best supported external avian calibrations (*SI Appendix, Table S2*) and 16 nuclear gene regions to date the basal divergence in kiwi to 5.96 Ma [95% credible interval (CI) 2.9–10.8] (*SI Appendix, Fig. S3*), a date similar to other studies using smaller datasets (33, 34). We used this date to time-calibrate a phylogeny generated in BEAST2 (35) using our mtDNA dataset (including extinct lineages), in which node ages represent gene coalescent dates. These node ages largely predate the major glacial advances of the Middle and Late Pleistocene (see blue lineage through time plot in Fig. 2) (*SI Appendix, Fig. S4*). However, coalescent dates are expected to predate the actual dates of lineage splitting and are unreliable indicators of divergence times for events near the present (36).

To obtain high quality estimates of lineage divergence times, we used a population genetic modeling approach (37) that estimated dates along our best supported species tree topology (*SI Appendix, Fig. S5*). With the exception of the basal split within kiwi (which varied across models by about 1 million years), dates were highly consistent across models (with and without migration), and across genomic datasets (with and without mtDNA and extinct lineages included) (*SI Appendix, Table S3*). We focused on results of the model that included both extinct mtDNA lineages and migration (Fig. 2). The basal split occurred at 3.85 Ma (range 1.87–7.0 using the upper and lower 95% CI calibration dates). The split between *A. australis* and *A. rowi/A. mantelli* occurred at 1.56 Ma (range 0.76–2.83), between *A. rowi* and *A. mantelli* at 1.12 Ma (0.54–2.02), and between *A. haastii* and *A. owenii* at 0.55 Ma (0.27–0.99). Intraspecific splits between genetically differentiated lineages of kiwi ranged between 0.11 Ma (0.05–0.20) and 0.72 Ma (0.27–1.02). These dates of splitting postdate the node age estimates from the ultrametric phylogeny generated in BEAST2 from the mitochondrial data (Fig. 2), suggesting that the gene tree coalescent dates estimated by BEAST2 overestimate lineage-splitting events in kiwi.

Diversification Rate Analyses. The lineage through time (LTT) plot had a steep increase in slope toward the present, as expected if origination rates increased through time. Likewise, diversification rate models in which lineage origination rates increased toward the present had much better support (combined Akaike weight of 0.83) than null models in which origination rates did not change (Table 1). The best fit diversification rate model (VR λ) supported an exponential increase in origination rate to the present, with no background extinction. Origination rates increased 44-fold from the base of the kiwi phylogeny to the present. Net diversification rates likewise showed a significant and substantial increase during the Middle and Late Pleistocene (*SI Appendix, Table S4*).

Effective Population Size Through Time. Effective population size (N_e) was estimated over the last glacial cycle to test for population bottlenecks associated with glaciations. All lineages except *A. owenii* showed a sharp population decline during glacial cycles, followed by a rebound toward the end of the glacial period. N_e ranged from about 5,000 to 100,000 for these lineages during the last interglacial at 130 ka and declined to an estimated low of less than 300 birds per lineage (although confidence intervals were often large) (*SI Appendix, Fig. S8*). For most lineages, the timing of this low occurred between 28 ka and 50 ka, but, in the two southernmost lineages from Stewart Island and South Fiordland, the low occurred at 17 ka. N_e then rebounded to Holocene levels that were generally the same or lower compared with levels in the previous interglacial. *A. owenii* showed a very different pattern, with very low initial N_e followed by a gradual increase to about 10,000 in the Holocene. This latter result is consistent with low haplotype variation in fossil mtDNA across the historical geographic range of *A. owenii*, suggesting that the species may have more recently expanded its range to cover most of the South Island (28).

Table 1. Support for different models of diversification fit to divergence times for 17 lineages of kiwi

Model	<i>n</i>	Delta AIC	Akaike weights	Increase/decrease in λ ?
Without extinction				
CR λ	1	6.42	0.017	
λ -2rates	3	4.28	0.051	Increase
λ -3rates	5	2.51	0.123	Increase
VR λ	2	0.00	0.433	Increase
With extinction				
CR λ -CR μ	2	2.65	0.115	
VR λ -CR μ	3	2.00	0.159	Increase
CR λ -VR μ	3	4.65	0.042	
VR λ -VR μ	4	4.00	0.059	Increase

Models allow origination (λ) and/or extinction (μ) rates to remain constant through time (CR), to vary as a linear function of time (VR), or for λ to change discretely after two or three breakpoints in time (λ -2rates, λ -3rates). An increase or decrease in best fit values of λ toward the present is indicated for all models in which λ is allowed to vary.

Discussion

Critical to our understanding of the role of glaciation as a driver of kiwi diversification is a proper characterization of the number of genetically distinct taxa. However, the number of distinct taxa has been difficult to establish, given that kiwi are largely nocturnal and possess only minor if any plumage differences between populations within currently named species, or even between some species. Here, we applied Bayesian species delimitation and analyses of population structure on a genomewide dataset of thousands of loci, obtained from all currently recognized kiwi species throughout their geographic ranges, and found that the number of distinct kiwi lineages is greatly underestimated by current taxonomic practice. Together with previously published ancient DNA samples from fossils, our analysis brings the total count of genetically diagnosable kiwi taxa that existed within the Holocene to no fewer than 16 or 17, and the number that survive to the present to 11 (Fig. 1), which contrasts sharply with the three species that were recognized in the early 2000s on the basis of morphological differences, and the five species that are currently recognized (24). Although it is unlikely that all of these kiwi lineages represent distinct biological species (indeed, plots of genetic structure show some genetically admixed individuals) (Fig. 1C), they do at least represent incipient species (e.g., subspecies) that, if given enough time, may evolve into full species. Because reproductive isolation in birds generally requires at least a million years to evolve (38), it is unlikely that lineages initiated by the major glacial advances of the Late Pleistocene will have had enough time to achieve strong levels of reproductive isolation (39, 40). For this reason, genetically distinct lineages, regardless of their levels of reproductive isolation, define the appropriate unit to address the role of Pleistocene glaciation in driving diversification.

Pleistocene glaciation was most extensive during the major glacial cycles of the past 0.78 million years, and, if these cycles were influential in diversification, we would expect many diversification events to date to this period. Node ages estimated from our mtDNA dataset date primarily to the early Pleistocene, with only three diversification events postdating the onset of severe glacial cycles at 0.78 Ma (Fig. 2, blue curve). However, the node ages on our time-calibrated tree represent dates of gene coalescence that are expected to predate lineage-splitting events. Indeed, our estimated dates of population splitting using a population genetic modeling approach were younger than the mtDNA gene coalescence dates and placed the bulk of kiwi diversification during the major glacial cycles of the past 0.78 million years. The first splitting event within kiwi dates to the Pliocene, during the

period of major uplift of the Southern Alps and before the onset of glaciation. All subsequent kiwi diversification events date to the last 1.5 million years, with two splitting events during the end of the early Pleistocene, and 13 events dating to the severe glacial cycles of the Middle and Late Pleistocene when the Southern Alps and surrounding lowland regions were heavily glaciated (Fig. 3). The importance of the Middle and Late Pleistocene in diversification remains true even when considering the wide confidence intervals associated with our use of external calibration points to date kiwi (Fig. 2, red dashed lines).

The sharp upturn of the lineage through time plot suggests an acceleration in the per capita rate of lineage origination during intense glacial periods of the Pleistocene (Fig. 2) although high background extinction rates can also result in such upturns (6). A model with increasing origination rates fit the data better than a model in which extinction alone drove the pattern (Table 1). The best fit diversification model ($VR\lambda$) supported a 44-fold increase in origination rate from the basal divergence event within kiwi to the present. Likewise, net diversification rates were low during the Pliocene (0.25 lineages per lineage per million years, 95% CI 0–0.75) and Early Pleistocene (0.32; 95% CI 0.01–0.63) but increased substantially and significantly during the Middle and Late Pleistocene (rate = 1.75; CI 1.48–2.01) (*SI Appendix, Table S4*) to rates that exceed most classic adaptive radiations (e.g., Galapagos Finches, 1.25; *Dendroica* warblers, 1.25; Tanganyika cichlids, 1.4; Malawi Cichlids, 5; Hawaiian *Drosophila*, 1.25) (see table 12.1 in ref. 41). This more than fivefold increase in net

diversification rate during the Middle and Late Pleistocene over earlier rates suggests that glaciation may have played a key role in accelerating rates of kiwi diversification.

These results contrast sharply with diversification rate analyses of the Nearctic avifauna, which showed no evidence of an increase in origination rates during key glacial periods of the Pleistocene (12, 13, 42). However, these studies relied on gene coalescent dates that are expected to predate the actual time of population splitting. The discrepancy between mtDNA coalescent dates and estimated population divergence dates for kiwi suggest the possibility that the failure of many previous studies to find a connection between Pleistocene glaciation and diversification may have arisen because coalescent dates often substantially predate population splitting dates, thus artificially making most origination events within the intense glacial periods seem to date to earlier time periods. Analyses using population genetic models that harness the power of the multigene coalescent to infer species splitting times would shed new light on the potentially important role of the Middle and Late Pleistocene glacial cycles in driving diversification in other geographic regions.

The association of rapid diversification with key glacial periods suggests, but does not demonstrate, that kiwi diversification was driven by glacial cycles. Here, we provide two additional lines of evidence that glaciation and its associated effects on habitat fragmentation were responsible. First, the current ranges and the location of Holocene fossils demonstrate that the 13 lineages within the brown kiwi clade were allopatric or parapatric during

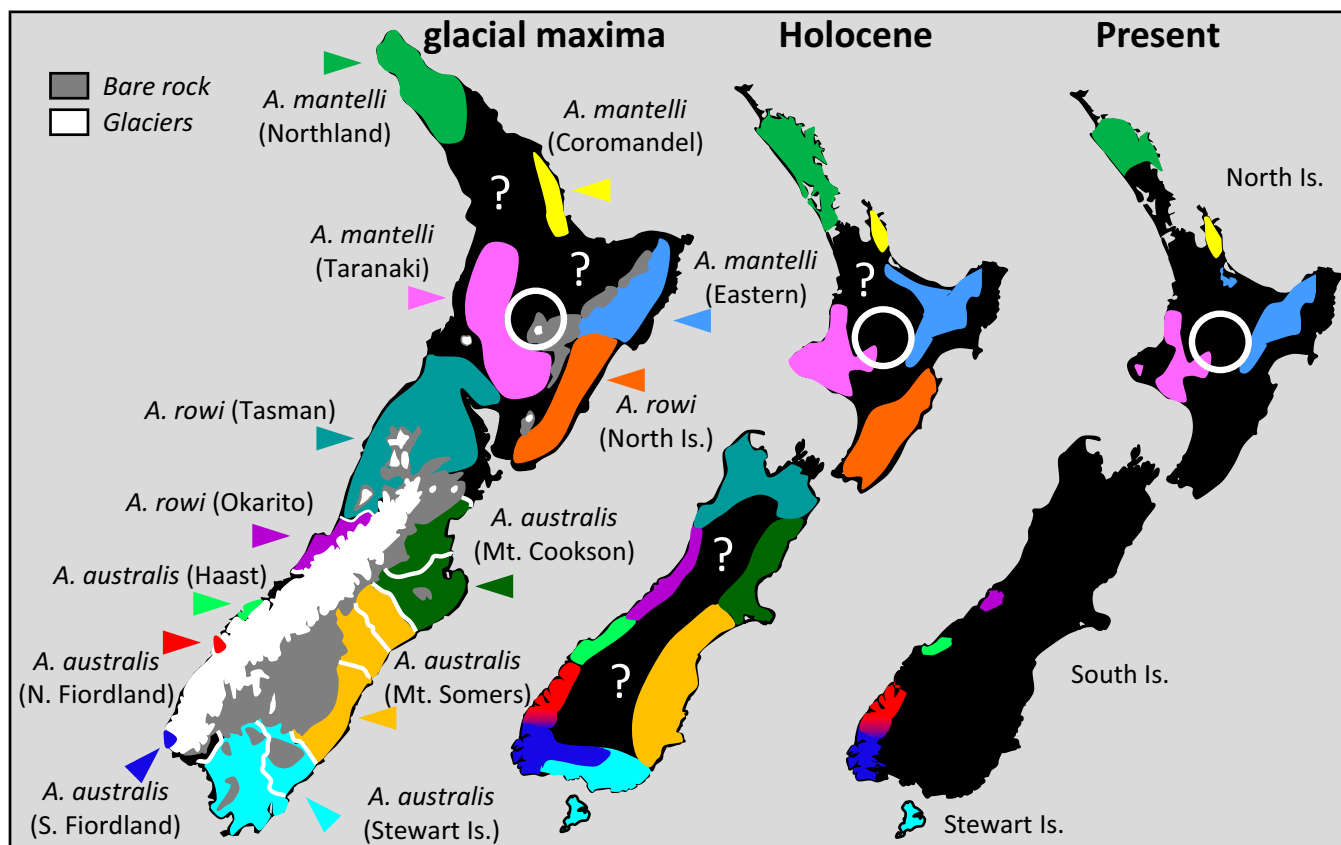


Fig. 3. Distribution of glacial ice, bare rock, coastlines, and a likely scenario for the geographic distributions of 13 genetically differentiated lineages of the brown kiwi clade during the last glacial maximum (~20,000 yBP), the early Holocene before human arrival, and at the present. Land bridges connected the North Island, South Island, and Stewart Island during glacial maxima. The massive Taupo Volcano of the North Island is indicated by the white circle. Eruptions of this volcano occurred repeatedly over the past 300,000 years, possibly isolating the four lineages of *A. mantelli* by extensive ash fields unsuitable for kiwi foraging on fossorial arthropods. White lines indicate major glacial rivers, some of which may have functioned as geographic barriers between kiwi lineages. Not all colored clades correspond between Fig. 1 and Fig. 3.

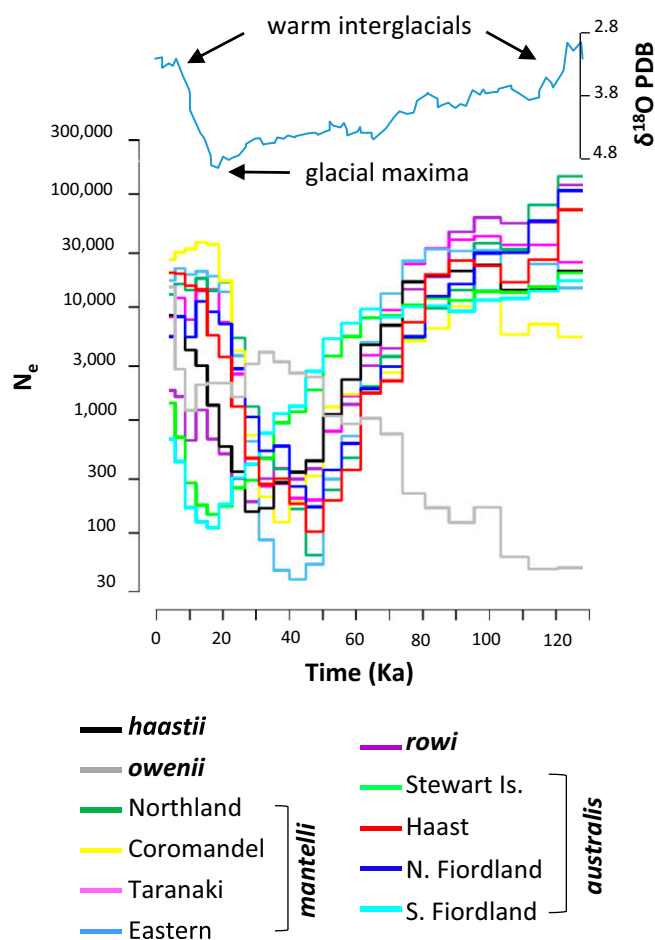


Fig. 4. Effective population size (N_e) through time (fit between 3 and 130 ka) over the last glacial cycle for the 11 extant lineages of kiwi. N_e is plotted on a log₁₀ scale. The global averaged paleo-temperature record as recorded by the high-resolution deep-sea oxygen record ($\delta^{18}\text{O}$) taken from Zachos et al. (53). High values of $\delta^{18}\text{O}$ indicate cool periods (notice reversed axis in contrast to Fig. 2).

the Holocene to present. It is thus likely that the clade largely retains the geographic signature of where speciation was initiated. The eight lineages in this clade from the South Island were distributed in a ring-like fashion around the Southern Alps (Fig. 3). During the Middle and Late Pleistocene, the Southern Alps were heavily glaciated (20), with glaciers descending to the ocean along much of the southern half of the west coast (Fig. 3). Here, a series of small, now inundated refugia are reconstructed for the last glacial maximum along the paleo-coastline, each separated from adjacent refugia by glacial fingers extending into the ocean (Fig. 3) (22). Similar refugia would presumably have occurred during previous glacial cycles. The Haast and two Fiordland lineages of *A. australis* almost certainly evolved in such refugia because their current geographical ranges are isolated in small geographical regions along the coast adjacent to the now inundated refugia (Fig. 3). Our dating suggests that Stewart Island, Haast, the extinct Mount Somers lineage, and an ancestral Fiordland lineage diverged two or three glacial cycles ago, followed by subsequent divergence of the Fiordland region into North and South Fiordland populations, probably during the last one or two glacial advances. The Haast and the two Fiordland lineages may have been directly fragmented from each other and adjacent lineages (e.g., Stewart Island and Mount Somers populations of *A. australis* and probably the Okarito population of *A. rowi*) by glacial fingers

(Fig. 3). Given the current geographic ranges of these lineages in areas that were completely covered by glaciers, it is hard to imagine how they could have formed without direct fragmentation by ice into refugia. In contrast, two extinct lineages of *A. australis* occurred along the east coast and one extinct lineage of *A. rowi* along the north-west coast, where glaciers are not reconstructed to have extended all of the way to the coastline but did produce outwash plains. Regions of unsuitable habitat or large glacial-fed rivers and their outwash plains that bisected lowlands in these regions may have instigated the formation of these lineages during glacial advances (Fig. 3).

Evidence of population bottlenecks forms our second line of evidence that glaciation was responsible for kiwi diversification. If the geographic ranges of kiwi were fragmented and isolated in small refugia of suitable habitat, then we should expect to find population bottlenecks during glacial cycles. Here, we estimated effective population sizes over the last such cycle and found that all but one of the 11 extant kiwi lineages exhibited severe population bottlenecks toward the middle of this cycle, as expected (Fig. 4). This finding was true of both the South and North Islands, suggesting that glaciation and its associated effects on habitat fragmentation may have affected both similarly, despite the lack of direct fragmentation by ice in the North Island. Alternatively, the repeated eruption of volcanoes on the North Island, in particular the massive Taupo Volcano, may have been instrumental in promoting lineage diversification and/or population bottlenecks there. Major eruptions of Taupo commenced 0.34 Ma, with many explosive eruptions over the past 65 ka (43). Given its central location on the North Island (Fig. 3), fallout of ash from this volcano was as much as two meters thick in central regions of the North Island but had little impact on the South Island (23). This ash fallout may have impeded kiwi feeding. Kiwi insert their long bills deep into soil to catch worms and other arthropods. Soil with the wrong texture and humus content is known to impede both feeding and the digging of nesting burrows (44). Thus, thick ash fallout may have repeatedly isolated *A. mantelli* into a series of refugia along the periphery of the North Island, with intervening ash-covered areas providing poor habitat for kiwi and reducing gene flow between the resulting four allopatric populations of *A. mantelli*. Although we cannot discount the possible effect of ash fallout in driving bottlenecks on the North Island, we note that the patterns of N_e through time are remarkably similar between the North and South Island despite the fact that ash fallout, which was carried eastward by trade winds, had little if any impact on the South Island (23). Given this consideration, we feel the bottlenecks in North Island lineages are likely the result of glacially induced population declines in fragmented habitat, rather than, or in addition to volcanism.

The South Island seems to have played a key role in the history of kiwi and other ancient New Zealand terrestrial lineages. From 30 to 21 Ma, the North Island is believed to have been largely submerged during what is referred to as the “Oligocene Drowning” (45), with many ancient terrestrial New Zealand lineages surviving only on the South Island (22). The absence of pre-Holocene kiwi fossils on the North Island but the presence of fossils back to ~18 Ma on the South Island (32) are consistent with this hypothesis and suggest that most of the history of kiwi occurred on the South Island. A land bridge connection between the South and North Island was established only at ~1.5–2 Ma, after the closure of the deep water Manawatu Strait (22). Glacially induced sea level fluctuations then repeatedly resulted in land bridge connections with the North Island during glacial periods of low sea level, followed by submergence of the land bridge during interglacial high sea level periods. Our ancestor state reconstructions for kiwi support an origin of the crown group of kiwi on the South Island with the oldest colonization of the North Island at either 1.1 or 1.6 Ma (Fig. 5), after the initiation of the land bridge. These reconstructions are remarkably similar to those reported for another flightless ratite group—the moas—that are also proposed to have originated in the

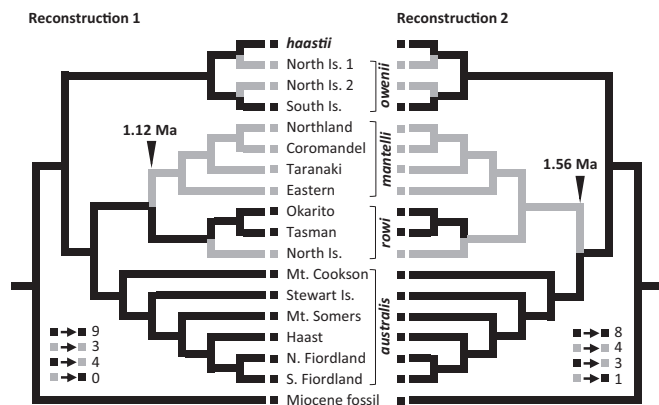


Fig. 5. The two most parsimonious reconstructions for geographic distribution in the North Island (gray) and the South Island (black) of New Zealand. The age of the node (as estimated using our coalescent model from genomic data) subtending the oldest colonization of the North Island is shown for each reconstruction. The reconstruction includes all known Holocene fossil lineages, as well as the only known pre-Holocene kiwi—the Miocene-aged fossil *Proapteryx* from the South Island. The number of intra- and interisland diversification events is shown for each reconstruction without including the node connecting *Proapteryx* to other kiwi. Stewart Island is here treated as part of the South Island complex, but treating it as its own character state has no effect on reconstructions between the North and South Islands.

South Island and colonized the North Island at the same time as kiwi (22). Reconstructions for kiwi support four distinct colonizations across the land bridge (most in a northward direction), each resulting in the formation of sister lineages in the North and South Island (Fig. 5). Our reconstructions also support eight or nine in situ diversification events for the South Island and three or four for the North Island (Fig. 5), thus highlighting the important role of both interisland and within-island diversification in the rapid Middle and Late Pleistocene buildup of kiwi diversity.

In conclusion, both genomewide nuclear DNA and mtDNA support the recognition of a number of genetically distinctive, but morphologically cryptic, taxa within currently named kiwi species. Future research should focus on assessing levels of reproductive isolation between these lineages, some of which our nuclear data suggest may hybridize. Because kiwi use vocalizations extensively for both courtship and territorial defense, vocal differentiation likely plays a key role in species discrimination and reproductive isolation. Analyses of vocal differences between morphologically cryptic taxa are greatly needed. Likewise, kiwi have an acute sense of smell, but it remains unknown whether smell differentiates kiwi taxa. Our dating of lineage divergence times supports a burst of diversification associated with the severe glacial cycles in the Middle and Late Pleistocene and suggests that the past several glacial cycles were especially instrumental in generating the cryptic lineages we characterize here. All but one kiwi lineage experienced severe population size bottlenecks during the last glacial cycle, consistent with glacially induced fragmentation and isolation in glacial refugia. These results support the important role of expanding and retracting glacial ice in driving range fragmentation and initiating allopatric speciation in biomes close to glaciated regions and are consistent with a growing number of studies linking recent bursts of diversification to glaciation in New Zealand (e.g., refs. 22 and 46–49).

Methods

Sampling Design. Whole DNA was obtained from 155 individuals (62 *A. mantelli*, 18 *A. rowi*, 14 *A. haastii*, 6 *A. owenii*, and 55 *A. australis*) from throughout the geographic ranges of each kiwi species (Dataset S1). Approval was obtained from the Royal Ontario Museum's Animal Care Committee (Animal Utilization Protocols 2008-03, 2009-07) to work with kiwi.

DNA Sequencing. mtDNA was sequenced for 153 of 155 individuals (1,039 bp of *cytochrome b* and a 671 bp of the *control region*) (Dataset S1) using standard methods (33). We also included previously published (28) mtDNA sequences from the same genes, for an additional 50 individuals primarily representing extinct populations obtained from Holocene fossils and museum skins (8 *A. rowi*, 10 *A. haastii*, 19 *A. owenii*, and 13 *A. australis*) (Dataset S1). We used genome-reduction genotype-by-sequencing (50) to obtain genomewide datasets for 96 individuals. We followed other authors (37) by retaining loci that were spaced at least every 50 kbp and were at least 10 kbp from coding regions along the kiwi reference genome (51). After data quality filtering for coverage, we retained 96 kiwi individuals (Dataset S1). See further details in *SI Appendix, Section 1*.

Lineage Delimitation. Seventeen distinct lineages were uncovered on our mtDNA phylogeny. The genomewide distinctness of extant lineages was characterized using principal coordinate analyses (PCoA), structure plots (29), and Bayesian species delimitation (30) of the SNP data, and genomewide pairwise F_{st} and JC69 genetic distances were calculated between lineages (*SI Appendix, Table S1*). For details, see *SI Appendix, Section 2*.

Phylogenetic Analyses. Three key Bayesian phylogenetic analyses were performed on the kiwi dataset as follows: (i) An mtDNA gene tree was generated for 204 individuals; (ii) a species tree was generated for three individuals per supported kiwi lineage from a sample of 1,000 SNPs; and (iii) an ultrametric phylogeny was generated in BEAST2 (35), with a single individual per lineage using 1,710 bp of mtDNA. For details, see *SI Appendix, Section 3 and Section 5*.

Phylogenetic Dating. We used 17 well-supported external fossil and biogeographic calibration points on a phylogeny of birds generated using 16 nuclear genes (17,205 bp) for 45 species (*SI Appendix, Table S5 and Fig. S3*) to date the kiwi crown group. This phylogeny dated the basal split within kiwi to 5.96 Ma (for further details, see *SI Appendix, Section 4*). We treated this date as an averaged date of gene coalescence in subsequent calibrations. We used this date to directly calibrate the BEAST2 phylogeny, where node ages likewise represent dates of gene coalescence.

Gene coalescent dates are expected to predate actual population-splitting dates by an amount equivalent to $2N_eG$ years, where N_e is the effective population size and G is the generation time. Thus, to obtain dates of population splitting along our best supported species tree topology, we used the multigene coalescent in a population genetic modeling framework using the program G-PHOCs (37) (*SI Appendix, Table S3*). We ran separate analyses with and without gene flow between geographically adjacent lineages within species for two datasets. The first dataset had 380,790 base pairs from 4,231 nuclear loci and included three individuals (six haploid sequences) per species. The second dataset was the same as the first but also included the two mitochondrial genes (treated as a single nonrecombining locus) and the six extinct lineages for which only mtDNA was available. For the latter, we used the best supported species tree topology for extant lineages and the topological placement of extinct lineages from phylogenies that included mtDNA. For details of all dating methods, see *SI Appendix, Section 5*.

Diversification Rates. We used the R package *Laser* (52) to fit eight models of diversification that constrain origination rates to be constant through time or allow origination rates to change as a function of time. *Laser* allows origination rates only to decline through time so we adjusted the code to allow for both increases and decreases in rate through time. The likelihood fit of models was compared using the Akaike information criterion (AIC) and Akaike weights. All fits were performed on the G-PHOCs analysis of divergence times, with geneflow and with mtDNA included.

Ancestor State Reconstructions. We performed parsimony-based ancestor state reconstruction of distribution in the North and South Islands to determine where kiwi originated, the number of in situ diversification events within each island, and the number of interisland diversification events. For details, see *SI Appendix, Section 6*.

Effective Population Size. Effective population size for each extant lineage was estimated in 24 discrete time windows from 3 ka to 130 ka, spanning the entire last glacial cycle. For details, see *SI Appendix, Section 7*.

ACKNOWLEDGMENTS. We gratefully acknowledge the Ngati Hine, Ngati Hei, Tuhoë, Ngati Tuwharetoa, Ngati Raukawa, and Ngai Tahu peoples, who permitted genetic analyses of kiwi blood samples obtained from their lands. Simon Boitard provided advice on estimating effective population sizes, and two anonymous reviewers greatly helped to improve the manuscript.

Computations were performed on the General Purpose and Sandybridge supercomputers at the SciNet High Performance Computing Consortium, which is funded by the Canadian Foundation for Innovation, the Government of Ontario, and the University of Toronto. This research was funded by

Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grants RGPIN-2016-06538 (to J.T.W.) and 038397 (to A.B.), NSERC Discovery Accelerator Grant 492890 (to J.T.W.), and the University of Toronto Scarborough VPR-Research Competitiveness Fund (J.T.W.).

- Rand AL (1948) Glaciation, an isolating factor in speciation. *Evolution* 2(4):314–321.
- Mengel RM (1964) The probable history of species formation in some northern wood warblers (Parulidae). *Living Bird* 3(4):9–43.
- Haffer J (1969) Speciation in amazonian forest birds. *Science* 165(3889):131–137.
- Hubbard JP (1973) Avian evolution in the aridlands of North America. *Living Bird* 12: 155–196.
- Diamond AW, Hamilton AC (1980) The distribution of forest passerine birds and Quaternary climatic change in tropical Africa. *J Zool* 191(3):379–402.
- Weir JT (2006) Divergent timing and patterns of species accumulation in lowland and highland neotropical birds. *Evolution* 60(4):842–855.
- Rull V (2008) Speciation timing and neotropical biodiversity: The Tertiary-Quaternary debate in the light of molecular phylogenetic evidence. *Mol Ecol* 17(11):2722–2729.
- Rull V (2011) Neotropical biodiversity: Timing and potential drivers. *Trends Ecol Evol* 26(10):508–513.
- Hoon C, et al. (2010) Amazonia through time: Andean uplift, climate change, landscape evolution, and biodiversity. *Science* 330(6006):927–931.
- Klicka J, Zink RM (1997) The importance of recent ice ages in speciation: A failed paradigm. *Science* 277(5332):1666–1669.
- Klicka J, Zink RM (1999) Pleistocene effects on North American songbird evolution. *Proc R Soc Lond B Biol Sci* 266(1420):695–700.
- Zink RM, Klicka J, Barber BR (2004) The tempo of avian diversification during the Quaternary. *Philos Trans R Soc Lond B Biol Sci* 359(1442):215–219, discussion 219–220.
- Lovette IJ (2005) Glacial cycles and the tempo of avian speciation. *Trends Ecol Evol* 20(2):57–59.
- Hewitt GM (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. *Biol J Linn Soc Lond* 58(3):247–276.
- Hewitt GM (1999) Post-glacial re-colonization of European biota. *Biol J Linn Soc Lond* 68(1–2):87–112.
- Weir JT, Schluter D (2004) Ice sheets promote speciation in boreal birds. *Proc Biol Sci* 271(1551):1881–1887.
- Arbogast BS, Kenagy GJ (2008) Comparative phylogeography as an integrative approach to historical biogeography: Guest editorial. *J Biogeogr* 28(7):819–825.
- Weir JT (2009) Implications of genetic differentiation in neotropical montane forest birds. *Ann Mo Bot Gard* 96(3):410–433.
- Johansson US, et al. (2007) Build-up of the Himalayan avifauna through immigration: A biogeographical analysis of the *Phylloscopus* and *Seicercus* warblers. *Evolution* 61(2):324–333.
- Suggate RP (1990) Late pliocene and quaternary glaciations of New Zealand. *Quat Sci Rev* 9(2):175–197.
- Newnham RM, Lowe DJ, Williams PW (1999) Quaternary environmental change in New Zealand: A review. *Prog Phys Geogr* 23(4):567–610.
- Bunce M, et al. (2009) The evolutionary history of the extinct ratite moa and New Zealand Neogene paleogeography. *Proc Natl Acad Sci USA* 106(49):20646–20651.
- Wilson CJ (2001) The 26.5ka Oruanui eruption, New Zealand: An introduction and overview. *J Volcanol Geotherm Res* 112(1–4):133–174.
- Baker AJ, Daugherty CH, Colbourne R, McLennan JL (1995) Flightless brown kiwis of New Zealand possess extremely subdivided population structure and cryptic species like small mammals. *Proc Natl Acad Sci USA* 92(18):8254–8258.
- Burbidge ML, Colbourne RM, Robertson HA, Baker AJ (2003) Molecular and other biological evidence supports the recognition of at least three species of brown kiwi. *Conserv Genet* 4(2):167–177.
- Ornithological Society of New Zealand (2010) *Checklist of the Birds of New Zealand, Norfolk and Macquarie islands, and the Ross Dependency, Antarctica* (Te Papa Press, Sefton, New Zealand).
- Shepherd LD, Lambert DM (2008) Ancient DNA and conservation: Lessons from the endangered kiwi of New Zealand. *Mol Ecol* 17(9):2174–2184.
- Shepherd LD, et al. (2012) Ancient DNA analyses reveal contrasting phylogeographic patterns amongst kiwi (*Apteryx* spp.) and a recently extinct lineage of spotted kiwi. *PLoS One* 7(8):e42384.
- Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* 155(2):945–959.
- Yang Z, Rannala B (2010) Bayesian species delimitation using multilocus sequence data. *Proc Natl Acad Sci USA* 107(20):9264–9269.
- Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol Ecol* 14(8):2611–2620.
- Worthy TH, et al. (2013) Miocene fossils show that kiwi (*Apteryx*, *Apterygidae*) are probably not phyletic dwarves. *Paleornithological Research 2013—Proceedings of the Eighth International Meeting of the Society of Avian Paleontology and Evolution*, eds Göhlich UB, Kroh A (Natural History Museum, Vienna), pp 63–80.
- Haddrath O, Baker AJ (2012) Multiple nuclear genes and retroposons support vicariance and dispersal of the palaeognaths, and an Early Cretaceous origin of modern birds. *Proc R Soc Lond B Biol Sci* 279(1747):4617–4625.
- Mitchell KJ, et al. (2014) Ancient DNA reveals elephant birds and kiwi are sister taxa and clarifies ratite bird evolution. *Science* 344(6186):898–900.
- Bouckaert R, et al. (2014) BEAST 2: A software platform for Bayesian evolutionary analysis. *PLOS Comput Biol* 10(4):e1003537.
- Edwards SV, Beerli P (2000) Perspective: Gene divergence, population divergence, and the variance in coalescence time in phylogeographic studies. *Evolution* 54(6): 1839–1854.
- Gronau I, Hubisz MJ, Gulko B, Danko CG, Siepel A (2011) Bayesian inference of ancient human demography from individual genome sequences. *Nat Genet* 43(10): 1031–1034.
- Weir JT, Price TD (2011) Limits to speciation inferred from times to secondary sympatry and ages of hybridizing species along a latitudinal gradient. *Am Nat* 177(4): 462–469.
- Avise JC, Walker D (1998) Pleistocene phylogeographic effects on avian populations and the speciation process. *Proc Biol Sci* 265(1395):457–463.
- Avise JC, Walker D, Johns GC (1998) Speciation durations and Pleistocene effects on vertebrate phylogeography. *Proc Biol Sci* 265(1407):1707–1712.
- Coyne JA, Orr HA (2004) *Speciation* (Sinauer Associates, Sunderland, MA).
- Zink RM, Slowinski JB (1995) Evidence from molecular systematics for decreased avian diversification in the pleistocene Epoch. *Proc Natl Acad Sci USA* 92(13):5832–5835.
- Wilson CJN, et al. (1995) Volcanic and structural evolution of Taupo Volcanic Zone, New Zealand: A review. *J Volcanol Geotherm Res* 68(1):1–28.
- Elliott A, Imboden C (1992) *Ostrich to Ducks*, Handbook of the Birds of the World, eds del Hoyo J, Sargatal J (Lynx Edicions, Barcelona), Vol 1.
- Cooper A, Cooper RA (1995) The Oligocene bottleneck and New Zealand biota: Genetic record of a past environmental crisis. *Proc Biol Sci* 261(1362):293–302.
- Trewick SA, Wallis GP (2001) Bridging the “beech-gap”: New Zealand invertebrate phylogeography implicates Pleistocene glaciation and Pliocene isolation. *Evolution* 55(11):2170–2180.
- Trewick SA (2001) Scree weta phylogeography: Surviving glaciation and implications for Pleistocene biogeography in New Zealand. *NZ J Zool* 28(3):291–298.
- McCulloch GA, Wallis GP, Waters JM (2010) Onset of glaciation drove simultaneous vicariant isolation of Alpine insects in New Zealand. *Evolution* 64(7):2033–2043.
- Weston KA, Robertson BC (2015) Population structure within an alpine archipelago: Strong signature of past climate change in the New Zealand rock wren (*Xenicus gilviventris*). *Mol Ecol* 24(18):4778–4794.
- Elshire RJ, et al. (2011) A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PLoS One* 6(5):e19379.
- Le Duc D, et al. (2015) Kiwi genome provides insights into evolution of a nocturnal lifestyle. *Genome Biol* 16(1):147.
- Rabosky DL (2006) Likelihood methods for detecting temporal shifts in diversification rates. *Evolution* 60(6):1152–1164.
- Zachos J, Pagani M, Sloan L, Thomas E, Billups K (2001) Trends, rhythms, and aberrations in global climate 65 Ma to present. *Science* 292(5517):686–693.