Contrasting response to Pleistocene climate change by ground-living and arboreal *Mandarina* snails from the oceanic Hahajima archipelago

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While the genetic impact of Pleistocene climate change on temperate species has been well characterized, especially in Europe and North America, an effect on the diversification of species on oceanic islands has been less well studied. This is perhaps a surprising observation given the traditional and continuing contribution of island species (e.g. Darwin's finches, *Partula* snails, Lord Howe Island palms) to understand speciation. Here, we combine mitochondrial and microsatellite data from the ground-living and arboreal *Mandarina* snails of the oceanic, subtropical Hahajima archipelago (Ogasawara, colloquially 'Galapagos of the Orient') to enable a comparative approach to understand the impact of the Pleistocene glaciations on their phylogeography. Prior work suggested that several narrowly divergent, ground-living species pairs of *Mandarina* populations on the outlying islands, as well as the low-lying southern and central parts of Hahajima, probably underwent bottlenecks and subsequent expansions during the recent Pleistocene. Here, the most striking finding is that largely arboreal species have deeply divergent, geographically restricted mitochondrial lineages, in contrast to a census size that is at least an order of magnitude lower than ground-living snails. As populations of both types are highly polymorphic at microsatellite loci, the systematic difference at the mitochondrial locus probably indicates a contrasting effect of the Pleistocene climate cycles on the two groups. We speculate that this may have partly come about owing to a reduced efficacy of natural selection on the more greatly structured populations of arboreal snails. If so, then a prediction is that the genome of other snails, or other species with limited mobility, will show a similar response to the Pleistocene climate cycles.

Keywords: Bonin Islands; effective population size; genetic draft; Hill–Robertson effects; land snail; oceanic island

1. INTRODUCTION

Although many species have been investigated from a phylogeographic perspective (Emerson *et al.* 2001; Hewitt 2004; Hofreiter *et al.* 2004), research effort has tended to concentrate on species from temperate continental regions. So, while many studies have shown that temperate species were confined to one or a few refugia during much of the Pleistocene because the environment was inhospitable or the land was glaciated (Hewitt 2004), the response of tropical and island species to climate change is less well understood (Schneider & Moritz 1999; Hugall *et al.* 2002; Dutech *et al.* 2003; Lessa *et al.* 2003; Bell *et al.* 2004; Flanagan *et al.* 2004). Thus, making predictions on the genetic

impact of Pleistocene climate change in the tropics and subtropics is inherently harder. Increasingly, researchers are addressing this gap in our knowledge (Trewick & Wallis 2001; Hugall *et al.* 2002; Jordan *et al.* 2005; Ciofi *et al.* 2006; Holland & Cowie 2007) and attending to the study of island species, which have already contributed so much to our understanding of speciation (e.g. Darwin's finches on the Galapagos, *Partula* snails from the Society Islands), and provided some of the most compelling recent examples of sympatric speciation (Barluenga *et al.* 2006; Savolainen *et al.* 2006).

Generally, changes in sea level are considered to be one of the most important causes for islands appearing and disappearing, so one main impact of high-latitude glaciations was to globally increase the area of land above sea level. At the greatest extent, when the sea level was approximately 100–130 m below that of the present day, many islands were both larger (e.g. top inset to figure 1) and had a greater range of elevations. Some islands became connected to nearby mainland or archipelagos became a single island. Some species
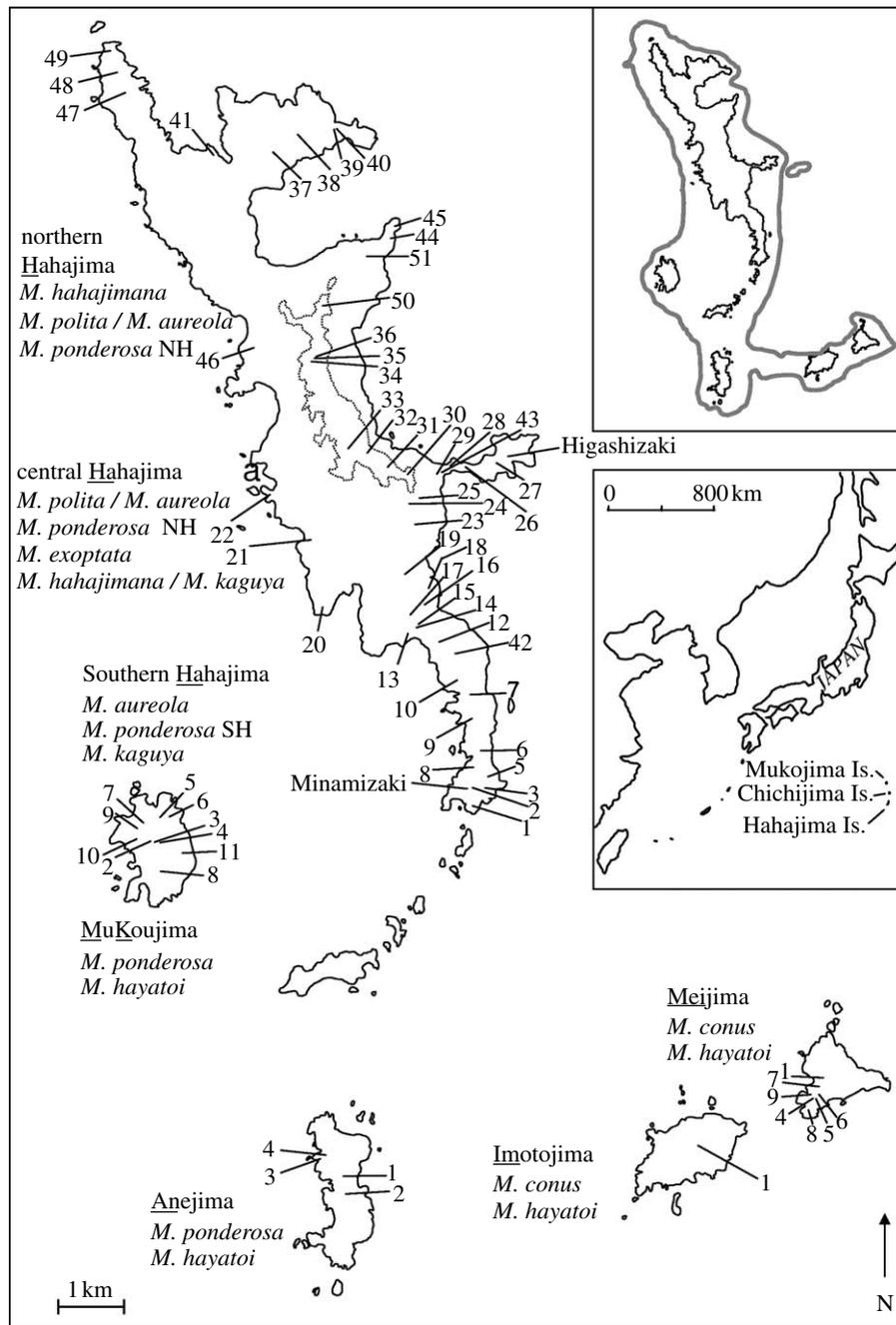
Figure 1. The Hahajima archipelago showing sample sites used in this study and in Davison & Chiba (2006b). Underlining indicates the site code name for each island. Minamizaki and Higashizaki are peninsulas of Hahajima that are referred to in the main text. Top inset: extent of land above sea when level was 100 m below present. The archipelago was a single island. Bottom inset: Ogasawara is approximately 1000 km south of Japan and is made up of three main archipelagos, the Mukojima, Chichijima and Hahajima islands.

may therefore have reached their greatest extent and maximum population size during the glaciations. A second main impact of the high-latitude glaciations was a global change in climate, which at high latitudes was expressed primarily as cooling and the formation of glaciers that impacted directly on species ranges. On oceanic islands, especially those in tropical regions, climatic effects are likely to have been much more subtle. One possible scenario is that decreased atmospheric CO_2 initiated arid conditions and decreased forest cover (Asahara 1999; Levis *et al.* 1999; Shen *et al.* 2005).

From an evolutionary perspective, island species are of interest because they frequently contain unique adaptations to specific habitats, a consequence of an adaptive radiation. If so, then a likely response to climate change was to track available habitat, so the geographical extent of refugia on islands would be dependent upon the range of available elevations and consequent habitat zones. An alternative is that species and species assemblages responded by adapting to changing climate/habitat, a process that could promote diversification. For example, in the Neotropics one explanation for a correspondence between pollen diversity and changes in global temperature is that fluctuating climate forced plants into refugial habitat islands, directly enabling the diversification of species in allopatry, and so creating the extraordinary diversity that is found there (Haffer 1969; Jaramillo *et al.* 2006).

Understanding the response of island species to Pleistocene climate change is therefore part of wider research on speciation.

In an ongoing project to understand speciation on islands, we are studying the genetics and ecology of *Mandarina* (Bradybaenidae), a land snail genus of oceanic Ogasawara (also known as the Bonin Islands, and colloquially, as the 'Japanese Galapagos'; Yamaoka 2007, figure 1). Twenty-four small islands (area greater than 0.6 km^2) make up three island groups of Ogasawara, Mukojima (aka Nakodojima), Chichijima and Hahajima, for which a large part of the flora (approx. 40%) and fauna (approx. 90%) is endemic, and largely derived from surrounding continental regions such as southeast Asia, Taiwan and the Japanese archipelago (Kobayashi 1978; Tomiyama & Kurozumi 1992; Ito 1998; Takayama *et al.* 2005). In the past, *Mandarina* has been studied from the perspective of understanding how their exceptional morphological and species diversity arose (Chiba 1999*a*, 2004), because the sister genus *Euhadra* in Japan inhabits an area that is orders of magnitude larger (see insets to figure 1), yet has only a few more species (approx. 22 compared with 15–20), and less morphological variation within and between species (Davison *et al.* 2005).

It has already been established using mitochondrial and nuclear markers that one or a few individuals of an ancestral *Mandarina* species colonized Ogasawara between 0.9 and 1.8 Ma, when the islands rose as a result of tectonic uplift (Chiba 1999*a*; Davison & Chiba 2006*a*). Speciation on different islands appears to have independently created similar ecotypes within different lineages and islands (Chiba 1999*a*). Also, the present-day species occur in several, genetically determined ecotypes, depending on their geographical location and local competitors (Davison & Chiba 2006*a*). An important feature of our research has been to investigate populations of *Mandarina* that are polymorphic for ecotype, in the hope that the data will shed light on the speciation process and the possible response to shifts in climate.

The specific aim of the study described here was to investigate in detail the phylogeographic structure and demographic history of *Mandarina* from one of the main Ogasawara island groups, the Hahajima archipelago (figure 1). We are particularly concerned with trying to obtain a general understanding of the impact of Pleistocene climate change, so we extended the initial study (Davison & Chiba 2006*b*) to include all the extant ground-living and arboreal species of the Hahajima archipelago, species for which a reasonable phylogeny is available (Davison & Chiba 2006*a*). For comparative purposes, it is unfortunate that few genetic studies have been carried out on other endemic species of Ogasawara (Ito *et al.* 1997; Ito 1998; Mukai *et al.* 2005; Takayama *et al.* 2005; Kaneko *et al.* 2007), although as mentioned, there are an increasing number of phylogeographic studies on other low-latitude islands, including several on snails (Goodacre 2002; Holland & Hadfield 2004; Rundell *et al.* 2004; Parent & Crespi 2006; Holland & Cowie 2007; Kameda *et al.* 2007).

2. MATERIAL AND METHODS

(a) Samples

Approximately 15 *Mandarina* (Bradybaenidae) species are recorded from Ogasawara. Each species is ultimately recognized by differences in the genitalia, but shell shape and banding pattern are useful in the first instance. In a previous study (Davison & Chiba 2006*b*), five species were sampled from 56 sites. In this study, the sampling was extended to a total of nine species from 73 sites, including a further 173 individuals for the mitochondrial locus and 124 individuals for each of 10 microsatellite loci.

On the main island of Hahajima, two main ecotypes of ground-living species usually coexist on a broad scale, though each is found in a different niche. One species tends to be associated with the litter of broad-leaved trees (*Mandarina polita* or *Mandarina aureola*) and the other with palm litter (*Mandarina ponderosa*; Chiba 1996, 1999*b*, 2004). *Mandarina ponderosa* on Hahajima take one of two forms, which are distinguished by shell and genital differences. As they are probably two separate species, they are referred to here as *M. ponderosa* southern Hahajima (SH) or northern Hahajima (NH). *Mandarina ponderosa* SH usually coexists with *M. aureola*, and *M. ponderosa* NH with *M. polita* (figure 1), but there is a central region where similar ecotypes may live together and hybridize (e.g. *M. polita*×*M. aureola* and *M. ponderosa* NH×SH). *Mandarina aureola* is also found in the extreme north of Hahajima (sites 47–49, figure 1).

On each of the small islands surrounding Hahajima, only one ground-living species is present (figure 1), generally either *M. ponderosa* or *Mandarina conus*. On Mukoujima, the genitalia of the ground-living species are similar to *M. ponderosa* SH from south Hahajima. On Anejima, the genitalia of the snails are approximately intermediate between both *M. ponderosa* NH and SH forms from Hahajima. On Imotojima and Meijima, the genitalia and shell morphology of the ground-living species are sufficiently distinct that they have been described as a separate species, *M. conus*.

In addition to the ground-living species described above and investigated in Davison & Chiba (2006*b*), we also sampled four other largely arboreal species: (i) *Mandarina hahajimana* ($n = 68$ for mitochondrial DNA), a morphologically variable species from the north and central parts of Hahajima, sometimes found in trees, but frequently also ground living, (ii) a largely arboreal species *Mandarina kaguya* ($n = 48$ for mitochondrial DNA, $n = 22$ for microsatellites) from central and southern parts of Hahajima, (iii) *Mandarina hayatoi* ($n = 43$ for mitochondrial DNA, $n = 41$ for microsatellites) of the outlying islands of Hahajima, including Mukoujima/Anejima and Imotojima/Meijima, and (iv) *Mandarina exoptata* ($n = 14$ for mitochondrial DNA), a geographically restricted (central highlands of Hahajima only) semi-arboreal species. In terms of distribution, *M. hahajimana* and *M. kaguya* are probably parapatric and possibly hybridize, whereas *M. kaguya* and *M. hayatoi* are restricted to separate islands. *Note.* The taxonomy of *Mandarina* has recently been revised (Chiba & Davison in press). Samples described here as *M. hayatoi* and *M. kaguya* were referred to as *M. hahajimana* in previous studies.

There is a sampling problem in that the census size of arboreal species, as judged by counts of live individuals, empty shells and fossils, is at least an order of magnitude lower than ground-living species (A. Davison & S. Chiba 2003, unpublished data). While it is therefore simple to collect approximately 20–100 individuals from a single

panmictic population of, for example, *M. ponderosa*, the same is generally not possible for *M. hahajimana*, *M. kaguya*, *M. hayatoi* and *M. exoptata*, except in some locations where they are largely ground living (e.g. *M. kaguya* on Higashizaki). For parts of the analysis, it was therefore necessary to make pooled, paired comparisons. For example, on Meijima, *M. hayatoi* were found at sites Mei1, 5, 7, 8 and 9, so for an analysis of microsatellite polymorphism, the same number of *M. conus* individuals was used from each site.

(b) DNA methods

For the present study, we were interested in the recent evolution of *Mandarina*, so efforts concentrated on a short (approx. 410 base pair (bp)), but highly variable region of the 16S ribosomal RNA (rRNA) gene, as well as polymorphism at 10 microsatellite loci (Davison *et al*. 2004). Briefly, genomic DNA was isolated using methods described by Teshima *et al*. (2003). Primers for PCR amplification of an approximately 900 bp of 16S rRNA gene fragment have been described by Chiba (1999*a*). All PCRs used Takara rTaq (Takara Biomedicals, Japan) and buffers, with annealing temperatures of 50°C. Cycle sequencing was carried out with the forward primer, using approximately 80–100 ng of PCR product in the reaction and the BigDye Terminator v. 3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems), and then only sequenced in the reverse direction if there were any ambiguities. DNA sequences were electrophoresed on an Applied Biosystems 310 Genetic Analyzer. Sequences were aligned using the ClustalX software, and then checked manually. The number of unique haplotypes was then counted. For most analyses, all insertion and deletion sites (indels) were removed, meaning that some pairs of haplotypes that differed by an indel only were pooled. This was necessary owing to potential alignment problems, and also because several of the analytical methods are not able to use gaps as characters or are not able to use them effectively.

Conditions for the amplification of microsatellite loci are described in Davison *et al*. (2004). To measure polymorphism at microsatellite loci, observed heterozygosity (H_o) and numbers of alleles (n_a) were counted using the Excel microsatellite toolkit (Park 2001). A Mann–Whitney *U*-test was used to determine whether there was a significant difference in the numbers of alleles per locus between pairs of populations. Pairwise population differentiation (F_{ST}) was estimated in Arlequin v. 3.10 (Schneider *et al*. 2000), in order to evaluate population of genetic structure.

(c) Phylogenetic and phylogeographic analyses

To assess the variation present within each population of five or more sampled individuals, nucleotide and haplotype diversities were calculated using Arlequin v. 3.10 (Schneider *et al*. 2000). Fu's F_s test of neutrality was used, also implemented in Arlequin, to test for the evidence of selection under an assumption of neutral evolution (Fu 1997). Previous phylogenetic analyses with longer sequences (approx. 1600 bp, Davison & Chiba 2006*a*), as well as analyses with the shorter sequences (A. Davison & S. Chiba 2006, unpublished data), showed that certain phylogeographic groups are consistently recovered, so these were treated separately in the population genetic analyses. The relationship between unique haplotypes was described using a median joining (MJ) network, with the sequences as nodes of a network instead of the terminal tips of a tree. This was carried out using the reduced median algorithm of Network v. 4.201 (www.fluxus-engineering.com; Bandelt *et al*. 1999).

Population bottlenecks followed by growth, or selective sweeps, may both lead to a Poisson distribution of substitutional differences between pairs of haplotypes (Slatkin & Hudson 1991; Rogers & Harpending 1992). The hypothesis of recent demographic expansion was therefore tested using mismatch analyses, carried out in Arlequin. Expected distributions were fitted to the observed mismatch distributions using a generalized least-squares method to estimate the demographic expansion parameters τ, θ_0 and θ_1. Parametric bootstrapping with 1000 pseudo-replicates was used to obtain confidence intervals around the parameter estimates and to test whether the observed mismatch distributions fitted the sudden expansion model.

3. RESULTS

The census size of arboreal species is normally at least an order of magnitude less than the ground-living species. In consequence, at some sites, it was only possible to sample a few individuals of the arboreal species, in comparison to the multiple samples of the ground-living species. For some analyses, therefore, samples from more than one site were pooled.

Overall, approximately 410 bp of the 16S rRNA gene fragment were sequenced from 173 individuals of *M. hahajimana*, *M. kaguya*, *M. hayatoi* and *M. exoptata*, the exact length varying according to the number of insertions–deletions (GenBank reference nos. EU622404–EU622478). Data were then combined with 606 sequences from five other species, *M. aureola*, *M. polita*, *M. ponderosa* SH, *M. ponderosa* NH and *M. conus*, previously described in Davison & Chiba (2006*b*), and compared. The most striking finding is that mitochondrial diversity is, in general, very high within species and populations of *M. hahajimana*, *M. kaguya* and *M. hayatoi* (electronic supplementary material, table 1), contrasting markedly with populations of *M. aureola*, *M. polita*, *M. ponderosa* SH, *M. ponderosa* NH and *M. conus* (table 1, Davison & Chiba 2006*b*), as well as *M. exoptata*, where nucleotide diversity is rarely greater than 1 per cent.

As phylogenies are not always ideal to describe the relationship between haplotypes within species, the MJ method was used to construct haplotype networks (figure 2). Units of analysis were paired species comparisons from populations living in the same geographical area: the islands of Meijima; Anejima; and Mukoujima, as well as the peninsula populations on the south and east of Hahajima (Minamizaki, sites Ha1, 2, 3, 5, 6, 7, 8, 9 and Higashizaki, site Ha27, respectively). Again, a striking pattern was evident—the haplotype network of populations of *M. aureola*, *M. polita* and *M. ponderosa* is characterized by a few narrowly divergent haplotypes that radiate from one or several common haplotypes, a signal that is characteristic of a population bottleneck and subsequent demographic or spatial expansion, or of a selective sweep. By contrast, the networks of *M. kaguya* and *M. hayatoi* are characterized by a series of deeply divergent haplotypes (grey shaded networks in figure 2).

We therefore tested the hypothesis that the patterns in the network may be a consequence of bottlenecks and expansions by carrying out a mismatch analysis. Consistent with the networks, we found that the mitochondrial genome of populations of strictly

Table 1. The mitochondrial diversity of *M. hahajimana*, *M. kaguya* and *M. hayatoi* is considerably greater than the other species.

| species		sample size	no. of haplotypes	nucleotide diversity	haplotype diversity
Hahajima archipelago					
M. hahajimana	Hahajima	68	26	0.063	0.93
M. kaguya	Hahajima	48	35	0.090	0.98
M. hayatoi	Mukoujima	10	9	0.037	0.98
	Anejima	17	9	0.011	0.85
	Meijima	12	9	0.092	0.94
M. exoptata	Hahajima	14	2	0.001	0.54
M. aureola	Hahajima	118	35	0.010	0.86
M. polita	Hahajima	113	48	0.016	0.96
M. ponderosa SH	Hahajima, Mukoujima	121	30	0.003	0.75
M. ponderosa NH	Hahajima, Mukoujima	118	24	0.006	0.79
M. conus	Meijima, Imotojima	58	18	0.009	0.90
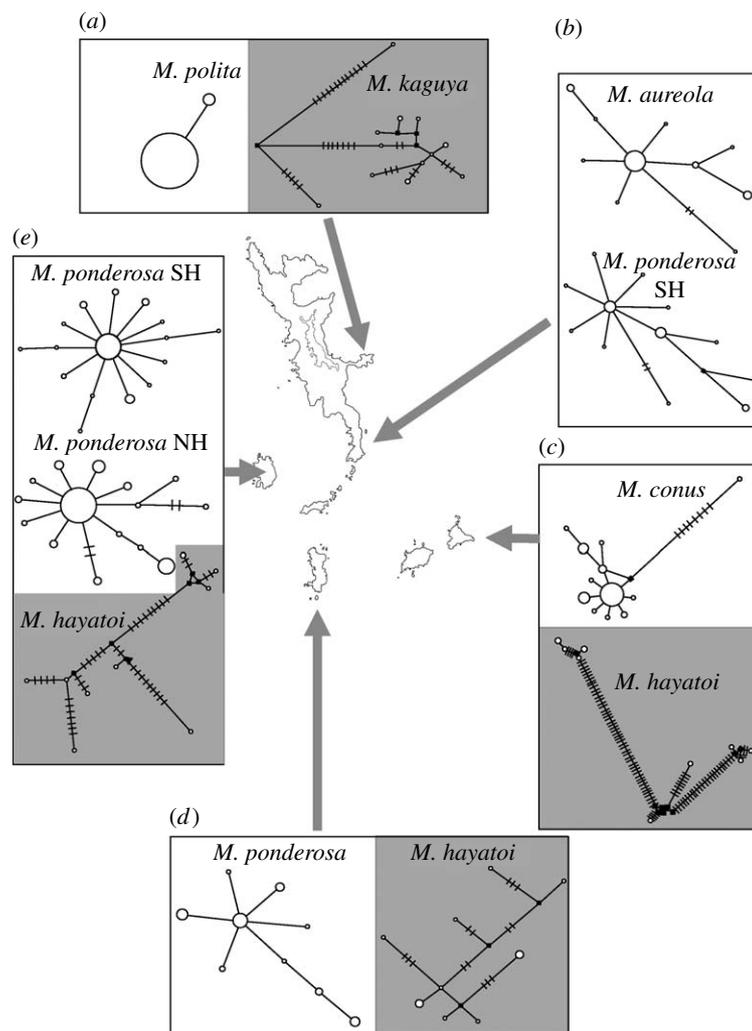
Figure 2. (*a*) Hahajima (Higashizaki), (*b*) Hahajima (Minamizaki), (*c*) Meijima, (*d*) Anejima and (*e*) Mukoujima. Paired comparisons of nucleotide diversity, illustrated using MJ networks, for population samples of ground-living and arboreal *Mandarina* species of the Hahajima archipelago. The size of the circle is proportional to the sample frequency; hatched lines indicate a number of hypothesized mutations between sampled haplotypes. Ground-living species (*M. aureola*, *M. polita*, both forms of *M. ponderosa*) are characterized by narrowly divergent haplotypes. By contrast, largely arboreal species (grey shading), such as *M. kaguya* and *M. hayatoi*, contain a mitochondrial diversity that is an order of magnitude greater.

ground-living snails from Meijima, Anejima, Mukoujima and the low-lying parts of Hahajima has undergone population bottlenecks. The main evidence for this is that the mismatch distributions are convincingly unimodal (figure 3), and there was also sometimes a strong deviation from neutrality (Fu's F_s; table 2). By contrast, the mismatch distributions of *M. kaguya* and *M. hayatoi* from the three outlying islands and two peninsulas are clearly multimodal (figure 3), although the conservative nature of the simulations run in ARLEQUIN v. 3.10 meant
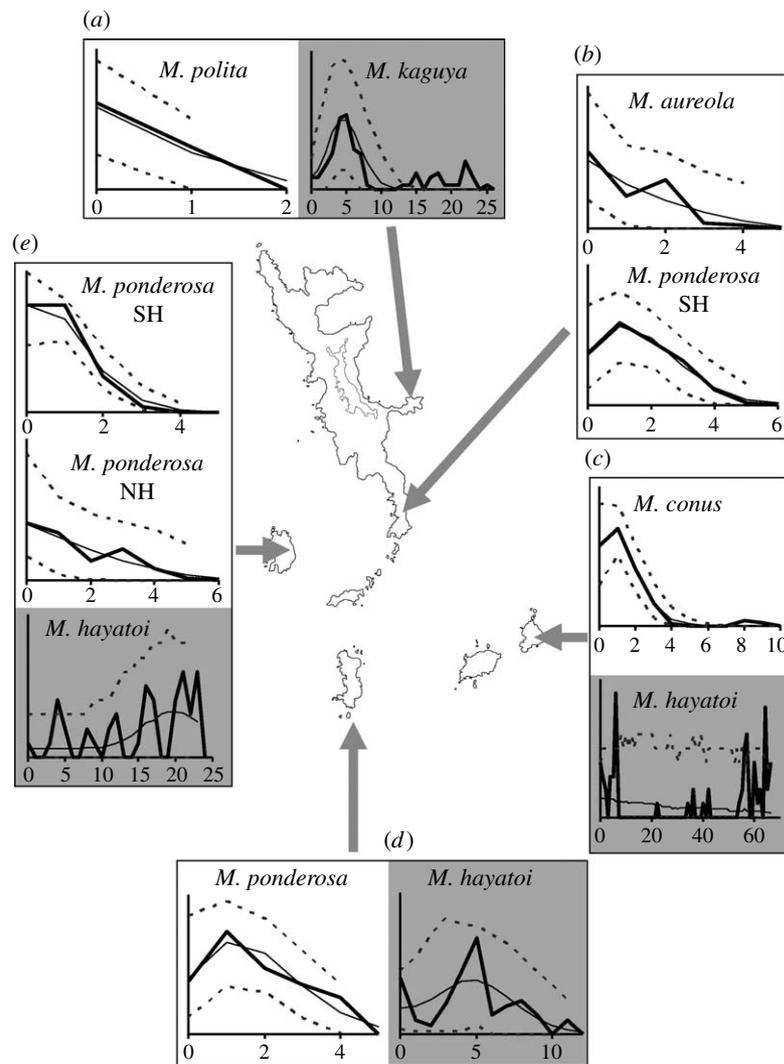
Figure 3. (*a*) Hahajima (Higashizaki), (*b*) Hahajima (Minamizaki), (*c*) Meijima, (*d*) Anejima and (*e*) Mukoujima. Paired comparisons of nucleotide diversity, illustrated using mismatch distributions (thick solid line), for population samples of ground-living and arboreal *Mandarina* species of the Hahajima archipelago. The simulated distributions (thin solid lines) and confidence intervals under the sudden demographic expansion model are also shown. While none of the observed distributions differed significantly from the simulated distribution, the patterns indicate different histories for the mitochondrial gene in ground-living versus arboreal species. By and large, the mismatch distributions of ground-living species have a unimodal distribution, indicative of a recent demographic or spatial expansion, whereas the arboreal species (grey shading) have multimodal distributions.

Table 2. Sample size of each of the haplotype groups, basic statistics and results from mismatch distribution analysis.

island or region	species	sample size	no. of haplotypes	nucleotide diversity	$p (F_s)$	p (SSD)	$p (H_{rag})$	τ	θ_0	θ_1
Meijima	*M. conus*	56	12	0.003	0.004**	0.75	0.64	1.16	0.04	99 999
	M. hayatoi	12	9	0.092	0.92	0.15	0.21	0.00	47	47
Anejima	*M. ponderosa*	37	9	0.004	0.13	0.43	0.13	1.81	0	30
	M. hayatoi	17	9	0.011	0.36	0.15	0.15	5.75	0	14
Mukoujima	*M. ponderosa* SH	81	16	0.002	0.000**	0.25	0.20	0.85	0	99 999
	M. ponderosa NH	78	15	0.003	0.001**	0.59	0.79	2.85	0	2
	M. hayatoi	10	9	0.037	0.29	0.11	0.40	21.96	0	70
Hahajima										
Minamizaki	*M. ponderosa* SH	39	12	0.004	0.003**	0.80	0.70	1.72	0	30
	M. aureola	69	10	0.002	0.07	0.36	0.47	2.10	0	1
Higashizaki	*M. polita*	11	2	0.001	0.36	0.37	0.43	0.44	0	99 999
	M. kaguya	15	11	0.020	0.08	0.27	0.57	5.05	0	41

** $p \leq 0.01$.

that the distribution of the differences in all cases was similar to that obtained from simulations that assume a recent demographic expansion (figure 3; table 2).

It has already been shown that population genetic structure, as inferred from mitochondrial DNA, is strong in *Mandarina* (Davison & Chiba 2006*b*), with

Table 3. Numbers of alleles and observed heterozygosities for 10 microsatellite loci. (Four paired comparisons were possible, using a ground-living (*M. ponderosa* or *M. conus* or *M. polita*) and arboreal (*M. kaguya*, *M. hayatoi*) species for each. In contrast to the mitochondrial data, there is no trend: the ground-living species are as polymorphic as the arboreal species.)

| | Meijima ($n = 20$) | | | | Anejima ($n = 40$) | | | | Mukoujima ($n = 22$) | | | | Higashizaki ($n = 42$) | | | |
| | *M. conus* | | *M. hayatoi* | | *M. ponderosa* | | *M. hayatoi* | | *M. ponderosa* | | *M. hayatoi* | | *M. polita* | | *M. kaguya* | |
	H_o	n_a	H_o	n_a	H_o	n_a	H_o	n_a	H_o	n_a	H_o	n_a	H_o	n_a	H_o	n_a
Mpo1	0.90	9	1.00	10	0.90	14	0.75	16	0.73	13	0.82	9	0.62	12	0.81	16
Mpo2	0.80	5	1.00	10	0.60	8	0.55	7	0.82	9	0.55	7	0.71	6	0.76	12
Mpo3	0.90	12	0.90	13	0.89	11	0.95	10	0.91	12	0.82	12	0.75	13	0.78	9
Mpo4	0.80	10	0.90	10	0.65	11	0.70	13	1.00	13	0.73	11	0.75	9	0.85	16
Mpo6	1.00	14	1.00	17	0.85	16	1.00	16	1.00	12	0.91	13	0.79	13	1.00	14
Mpo7	0.90	12	0.80	11	0.95	18	0.95	17	1.00	12	0.91	11	0.85	15	0.86	20
Mpo8	1.00	13	0.80	12	1.00	22	1.00	18	0.73	14	1.00	13	0.86	15	0.90	18
Mpo9	0.90	9	1.00	16	0.95	16	0.70	11	0.91	15	1.00	14	1.00	19	0.95	23
Mpo11	0.70	6	0.90	8	0.80	8	0.70	7	0.82	8	0.70	6	0.86	7	0.70	8
Equ 1	0	1	0.80	2	0.50	4	0.55	4	0.09	2	0.55	3	0	1	0.05	2
all loci	0.79	9.1	0.91	10.9	0.81	12.8	0.79	11.9	0.80	11	0.80	9.9	0.72	11	0.77	13.8

F_{ST} usually between 0.4 and 0.6, but sometimes equal to 1. Of relevance here is the finding that structure is especially marked between populations of arboreal species, especially *M. kaguya* and *M. hayatoi*. For example, both mitochondrial lineages and some haplotypes of *M. ponderosa* on Mukoujima are also found in *M. ponderosa* SH from southern Hahajima and *M. ponderosa* NH from northern Hahajima, indicative of recent short- and long-distance gene flow. In comparison, the lineages and haplotypes of *M. hayatoi* on Mukoujima are monophyletic, indicating a long-term independent history. Similarly, the lineages and haplotypes of *M. polita* on the Higashizaki peninsula are in common with geographically close populations in the highlands of central Hahajima, whereas the lineages and haplotypes of *M. kaguya* on Higashizaki are also monophyletic, so found nowhere else.

To further understand the nature of the potential bottlenecks, 124 snails were genotyped at 10 microsatellite loci. Then four pairwise comparisons were made, using the same number of snails per site per species (insufficient *M. kaguya* were collected from Minamizaki to enable a fifth comparison). The results are as striking as when viewed in the light of the mitochondrial data (table 3): all the samples are diverse in terms of numbers of alleles and heterozygosity, with no obvious evidence for a bottleneck (e.g. no significant differences in n_a between populations). Similarly, pairwise differentiation (F_{ST}) was an order of magnitude lower using microsatellites compared with mitochondrial DNA (approx. 0.4–1). Specifically, the average F_{ST} between ground-living snails on Meijima, Anejima and Mukoujima was 0.046; the F_{ST} for arboreal species was almost identical (0.045). Unfortunately, as it was necessary to pool samples, more sophisticated analyses such as tests for heterozygosity excess were not possible (Piry *et al.* 1999).

4. DISCUSSION

Two of the most widespread, largely arboreal species of *Mandarina* on the Hahajima archipelago, *M. kaguya* and *M. hayatoi*, are characterized by conspicuous mitochondrial diversity, and reciprocal monophyly between populations, in marked contrast to the ground-living species (Davison & Chiba 2006*b*). The difference is perhaps most marked on Higashizaki (figure 1), a peninsula that has a maximum habitable area of only approximately 0.2 km^2. There, the ground-living *M. polita* has only two mitochondrial haplotypes, differing at a single base, whereas *M. kaguya* has a much higher diversity: 38 polymorphic sites (out of approx. 410), with up to 6 per cent uncorrected sequence divergence, and none of the lineages found elsewhere. There is therefore no doubt that the mitochondrial genome has undergone a population bottleneck in at least four independent populations of ground-living snails on the outlying islands and peninsulas, suggestive of a single cause, most likely demographic events occurring during the Pleistocene climate cycles (Davison & Chiba 2006*b*). Over the same period of time, divergent lineages and marked mitochondrial genetic diversity were maintained in several arboreal species, especially *M. kaguya* and *M. hayatoi*, implying relative stability through the Pleistocene. In contrast to the mitochondrial data, the patterns of polymorphism at microsatellite loci are interesting owing to their similarity: pairs of species on the same peninsulas and islands are equally polymorphic (table 3). The events that led to a mitochondrial bottleneck, or a selective sweep, did not significantly affect the nuclear genome.

The situation in the central and highland parts of Hahajima is more complicated to interpret, but the difference between species in these regions is arguably less, when compared with the outlying regions and islands. For example, both *M. aureola* and *M. polita* from central Hahajima are characterized by a high degree of population structuring (F_{ST} approx. 0.6), and comparatively low genetic diversity, yet the haplotype networks are far from simple (Davison & Chiba 2006*b*). There is, in consequence, no evidence for a dominant effect of bottlenecks on the population structure of these species in central Hahajima. In addition, one widespread arboreal snail of north

and central Hahajima, *M. hahajimana*, is genetically diverse and has a strong population structure, like the other arboreal species, but close inspection shows that this is largely due to it containing two lineages with widely divergent mitochondrial haplotypes, each one of which contains little variation (in the electronic supplementary material, table 1, the frequency of these divergent haplotypes in populations is evident by the 'hh' or 'nh' designation). A final arboreal species, *M. exoptata*, is geographically restricted to the central highlands of Hahajima—mitochondrial variation is almost completely lacking.

Care must be taken not to over-interpret results from few genetic markers, especially since the one-quarter effective population size of mitochondrial DNA means that it is particularly susceptible to bottlenecks/selective sweeps and genetic drift. Nevertheless, the results of the paired comparisons are especially striking (figures 2 and 3). There is, after all, no *a priori* explanation for the mitochondrial DNA of one species or population to respond in a consistently different manner to demographic events compared with mitochondrial DNA of another species or population, unless there is an underlying difference in the biology (a role for sex-biased dispersal is ruled out because *Mandarina* are hermaphrodite). If the climate was less amenable during certain Pleistocene events, then why is the signature only evident in mitochondrial DNA, and why were only some species affected? The problem will be even more acute if population size is also considered—arboreal species have a census size that is at least an order of magnitude lower than ground-living species, so the conventional view is that equilibrium neutral diversity should be less, not more.

One possible explanation for the discrepancy is that if neutral genetic diversity is only dependent upon N_e and the mutation rate, then it might be that the mitochondrial mutation rate of *M. kaguya* and *M. hayatoi* is higher, especially given the lower census size. If so, then these two species contain high genetic diversity and deep mitochondrial lineages because the genetic diversity going into the bottleneck was high, so correspondingly more genetic diversity survived the bottleneck. Unfortunately, the depth of sampling in our study is insufficient to test this hypothesis, but we nonetheless consider this explanation unsatisfactory in isolation, because it leaves open the question as to why the rate of evolution is higher in one lineage over another?

A recent meta-analysis of mitochondrial DNA variation has reported no correlation between N_e and mitochondrial DNA polymorphism (Bazin *et al.* 2006). Briefly, an absence of recombination in mitochondrial DNA and Y chromosomes can in theory lead to inefficient natural selection, because novel advantageous mutations are generally not able to escape a poor background (Charlesworth & Charlesworth 2000; Marais 2007). Gillespie (2000) has argued that in large populations, natural selection is more efficient, and so there is therefore more opportunity for beneficial mutations to be fixed by a selective sweep, correspondingly reducing diversity.

We therefore speculate that a linked explanation for the patterns of diversity in *Mandarina* is that species with a high degree of mitochondrial diversity exist in extremely structured subpopulations (Thomaz *et al.* 1996). If there is limited gene flow between each of these populations, then mitochondrial diversity could be maintained through bottlenecks, because the relative independence of each deme (or refugium) would mean that selection is inefficient across the whole population, acting instead on the local population. In comparison, if some snails have a greater mobility, and so reduced population structure, then selection would act on the whole population, so that the mitochondrial genome would undergo selective sweeps. Differences in diversity between species would be a symptom of the response to Pleistocene climate cycling. This 'just-so' explanation is not only consistent with the observational data in *Mandarina* (and another slow-moving taxon, Galapagos tortoise, Caccone *et al.* 2004), but also consistent with the generally high rate of mitochondrial polymorphism in pulmonate snails (Thomaz *et al.* 1996; Davison 2006). For *Mandarina*, one important consideration may be that the movement of arboreal snails is in a vertical plane (i.e. up and down a tree), whereas ground-living species exist on a horizontal plane and also have a much greater census size.

In summary, as four or more independent populations of ground-living snails have a mitochondrial genome with a similar genetic signature, then the simplest explanation for differences in diversity signature is a Pleistocene-induced population bottleneck. If some arboreal species do not show this signature, then the explanation may be that they have a much greater population structuring. The reasons that microsatellite loci do not show the effects of this bottleneck in either group of species are unclear, but may simply be because autosomal loci are inherently less susceptible to bottlenecks (N_e is four times greater), but also because recombination enables selection to operate more efficiently, or finally, because the high mutation rate of microsatellites means that measures of diversity and population structure are underestimated (Hedrick 1999).

How did the Pleistocene climate cycles generally impact on the demographic history and speciation of *Mandarina*? To date, research on *Mandarina* has largely focused on ecological speciation, a result of divergent selection in sympatry (e.g. between the wholly overlapping dry litter of broad-leaved trees and wet litter of palms). The phylogeographic data presented here and in our previous work (Davison & Chiba 2006*a*,*b*) suggest that genetic drift and founder effects during bottlenecks may also have had a particular role in promoting divergence of geographical replacement species pairs with similar ecologies, such as *M. aureola*/*M. polita* and *M. kaguya*/*M. hayatoi*. The present-day species diversity of *Mandarina* is therefore a composite of these complementary processes occurring in sympatry and allopatry. More specifically, the genetic data probably indicate that separate populations of structured arboreal species maintained a degree of isolation for the duration of the Pleistocene, and were relatively stable in population size, whereas ground-living species were subject to bottlenecks and founder events. As new data accumulate on both the genetics and our understanding of the specific climate changes that took place over the course of the Pleistocene in subtropical regions

and on islands, it will be interesting to see whether other species of Ogasawara, or other model systems, were affected in the same way as arboreal or ground-living *Mandarina*. One prediction arising from this work is that the impact of demographic events on the genome will depend upon the population structure of the species in question.

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