Can Microsatellites Be Used to Infer Phylogenies? Evidence from Population Affinities of the Western Canary Island Lizard (Gallotia galloti)

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INTRODUCTION

Microsatellites (1- to 5-bp tandem repeats) are codominant, widely distributed in the genome, and highly variable in length, which has resulted in them being widely used to assess population genetic structure and in molecular ecological studies (Jarne and Lagoda, 1996). It has also been suggested that microsatellites may be used to study the evolutionary relationships between groups that have evolved independently for up to several million years (Goldstein et al., 1995a). However, they may possess attributes that limit their usefulness in this area. For example, the range of allele size within microsatellite loci may be limited (e.g., Garza et al., 1995) and they have high mutation rates. As a consequence of the combination of these properties, the potential genetic divergence may be limited. Mutation may be a homogenizing factor due to size homoplasy (i.e., leading to the appearance of alleles already existing) that may overcome the diversifying effect of genetic drift, especially in large populations. Therefore, genetic divergence measurements that use microsatellite data may plateau quickly with time (Nauta and Weissing, 1996; Garza et al., 1995; Paetkau et al., 1997).

Even so, microsatellite data have been used to reconstruct population/species phylogenies in a range of insects and vertebrates (Bowcock et al., 1994; Estoup et al., 1995a; Angers and Bernatchez, 1998; Sültmann and Mayer, 1997; Forbes et al., 1995; Berube et al., 1998; Paetkau et al., 1997; MacHugh et al., 1997).

Further evaluation of microsatellite data for reconstructing population phylogenies is important because currently population phylogenies are predominantly based on mtDNA data, and nuclear markers would have great utility in supplying an independent test of whether the mtDNA gene tree represents the organizational phylogeny (Moore, 1995). Use of additional mtDNA genes may increase the probability of resolving the gene tree, but all mtDNA constitutes a single linkage group and therefore additional mtDNA genes do not provide an independent test. The use of introns as...
nuclear genome markers tends to be more useful at higher taxonomic levels (Giannasi et al., 2001a), and the use of amplified fragment polymorphisms (AFLPs) in phylogenetic reconstruction in vertebrates may be appropriate (Giannasi et al., 2001b), but is not well established.

If microsatellites are to be used in this way, it is not yet clear which genetic distance measures should be used for studying microsatellite data. Several genetic metrics have been developed specifically for microsatellites (e.g., $R_s$; Slatkin, 1995), which use the variance in repeat numbers (VRN) and are compatible with the stepwise mutation model. Classic genetic distances (e.g., $F_{st}$; transformed for linearity with time) are based on the variance in allele frequencies (VAF) and are compatible with the infinite allele mutation model, which may, or may not, be appropriate to use to describe the microsatellite mutation processes. However, the mutation processes are unlikely to follow just one model, so over-generalization concerning the suitability of a given metric due to compatibility with a given mutation model is probably inappropriate (Gaggiotti et al., 1996 and references therein). If one assumes that the range of allele sizes is unconstrained, VRN metrics should be well suited to phylogenetic reconstruction as they are expected to be linear with time (Kimmel et al., 1996; Shriver et al., 1995; Takezaki and Nei, 1996). However, Nauta and Weissing (1996) have shown that if this assumption does not hold then even these genetic distances will rapidly asymptote with time, particularly in large populations, and Gaggiotti et al.'s (1999) simulation study has shown that the large variance of VRN metrics may lead to a poorer performance than VAF metrics unless sample size and loci numbers are large. Considering migration and mutation further complicates the situation. The performance of VRN and VAF metrics in estimating $N_m$ may be evaluated against varying migration/mutation rates (Gaggiotti et al., 1999), but it is not apparent from this how these parameters influence genetic distance. Phylogenetic reconstruction assumes that genetic distances (whatever the type) are not perturbed by migration, i.e., that the effect of migration is trivial relative to mutation. This is likely to be the case when considering isolated populations, e.g., on islands, but less likely to be so for parapatric populations. Consequently, one would expect microsatellite genetic distances to be useful for phylogenetic reconstruction when mutation rates are much higher than migration rates, but not when they are high enough in relation to the time scale for homoplasy to become a problem. Population size is also pertinent. Hence, one would expect microsatellites to be most useful in reconstructing phylogenies between closely related, small, allopatric populations. These expectations must be tested, so it is important to evaluate the performances of these different statistics on data sets with well-supported evolutionary relationships (e.g., Forbes et al., 1995; Paetkau et al., 1997).

The western Canary Islands and the lizard Gallotia galloti constitute an extensively studied island/endemic species system. The geological history of the archipelago is well known. Whereas Tenerife probably arose from several precursor islands (Ancochea et al., 1990), each western island is of independent volcanic origin and was not previously attached to another. These islands arose in an east to west series, Tenerife being the oldest at 15.7 million years and El Hierro the youngest at 1.2 million years (Carracedo, 1979; Guillou et al., 1996).

G. galloti occupies these islands at very high densities with large, at least partly contiguous, populations within islands. There is a substantial amount of morphological and molecular (Thorpe, 1996; Thorpe et al., 1994, 1996; Thorpe and Richard, 2001) variation within and between islands. The molecular phylogeny shows two primary lineages: one consisting of La Gomera and El Hierro and the other of Tenerife populations and La Palma, with La Palma and northeast Tenerife as sister groups (Thorpe et al., 1994). The primary split shown by the mtDNA gene trees is independently corroborated by nuclear random amplified polymorphic DNAs (RAPDs). Consequently, this split probably represents the primary division in the organismal phylogeny. These molecular data reveal phylogeographic patterns of colonization (Thorpe et al., 1994) and help to test evolutionary hypotheses (Thorpe, 1996; Thorpe et al., 1996; Thorpe and Richard, 2001). Phylogeographic interpretation of these data (Thorpe et al., 1994) suggests an origin on Tenerife with two westward colonizations to the younger islands as they arose from the seabed (from southwest Tenerife to La Gomera and then La Gomera to El Hierro and independently from northeast Tenerife to La Palma).

As this system has a wide range of molecular divergences (and associated times) it is a useful model to use to investigate the utility of microsatellites for phylogeny construction and to test the reliability of the different types of genetic distances in the case of large populations. Five microsatellite markers (Richard and Thorpe, 2000) have been used and a sample of populations chosen to represent different divergence times and demographic histories.

**MATERIAL AND METHODS**

**Sampling**

Noninvasive biopsies (tail tips naturally autotomized) were taken from 372 individuals representing a total of 12 populations of G. galloti (2 on each of La Palma and El Hierro, 1 on La Gomera, and 7 on Tenerife; Fig. 1). The Tenerife populations represent both the mtDNA lineages found on that island (Thorpe et al.,...
1996) with localities 19 and 6 being adjacent to the contact between them. The number of individuals analyzed per population range from 26 to 39. DNA extraction was performed with a standard phenol protocol (Sambrook et al., 1989).

Microsatellite Loci

Microsatellite analyses were performed with five dinucleotide microsatellite markers isolated from a G. galloti genomic library (A348, (AC)19; A49, (CA)10; B81, (TC)19; B821, (AC)12; B967, (GT)AT(GT)10) (Richard and Thorpe, 2000). Radioactive polymerase chain reaction (PCR) amplifications were carried out in 10 μl of a mixture containing 15 to 30 ng of DNA, 200 nM each primer (100 nM one of them labeled with 32P), 30 μM each dNTP, 1.5 mM MgCl2, 1× reaction buffer (standard MgCl2-free BRL buffer), and 0.4 units of Taq polymerase (BRL). An initial denaturing step of 3 min at 94°C was followed by 35 cycles (94°C for 30 s, 53 or 55°C for 30 s, and 72°C for 15 s) and 2 min at 72°C. PCR products were run through 6% denaturing sequencing polyacrylamide gels and visualized by autoradiography. Allele lengths were determined by comparison to the original clone and individuals with known allele size.

Statistical Analysis

Most of the data analysis was performed with ARLEQUIN 1.1 (Schneider et al., 1996) and GENEPOP 1.2 (Raymond and Rousset, 1995). For each population-locus combination, allele frequency and observed (H_o) and expected (H_e) heterozygosities (gene diversity) were computed. The results were also pooled across loci by Fisher’s combined probability method. We also tested our data set for departure from Hardy–Weinberg equilibrium (exact tests using a Markov chain, length 10,000). A permutation test (10,000 permutations) that used the expectation maximization (EM) algorithm (Slatkin and Excoffier, 1996) was used to test for linkage disequilibrium between each pair of loci.

Some genetic metrics, for example (δμ)^2 (Goldstein et al., 1995b), are designed specifically as genetic distances, whereas others, e.g., Fst, may be used to estimate gene flow (Slatkin, 1985), but can be converted to a dissimilarity coefficient (distance) by the appropriate transformation. Different pairwise genetic metrics have been chosen for evaluation on this data set. Two use the VAF: Nei’s (1972) standard distance GI and weighted Fst over loci, as in Weir and Cockerham (1984). Two use the VRN: (δμ)^2 from Goldstein et al. (1995b) and Rst from Slatkin (1995). We also used the proportion of shared alleles, Dst. Transformed Gst(Gst/(1 – Gst)) and Dst(Dst/(1 – Dst)), together with (δμ)^2, were computed with the package MICROSAT 1.5 (Minch et al., 1996). The metrics Fst and Rst were computed with ARLEQUIN 1.1 and a transformation was applied to Fst and Rst to linearize the distance with population divergence time (Reynolds et al., 1983; Slatkin, 1995). For convenience, transformed metrics are referred to in their untransformed state (i.e., Fst, Gst, Rst, and Dst) and Dst is grouped with VAF metrics.

The genetic structure of the populations was investigated by an analysis of molecular variance (AMOVA; Excoffier et al., 1992; Michalakis and Excoffier, 1996), a hierarchical analysis of variance of allele frequencies. We have used two metrics, the number of different
alleles ($F_{st}$) and the average squared size differences ($R_{st}$). This procedure was used to elucidate the among-island versus the within-island structure of population differentiation. The pairwise genetic distances generated were used to reconstruct trees of genetic similarity with the neighbor-joining algorithm in the package PHYLIP 3.57c (Felsenstein, 1995).

Comparison with Existing Data

Mitochondrial and whole-genome DNA data were available for each island (Thorpe et al., 1993, 1994). They consisted of mtDNA restriction fragment length polymorphisms (RFLPs) (four and six base cutters), sequence of three mtDNA genes (cytochrome b, cytochrome oxidase, and 12s rRNA genes which together constitute over 1000 bp) and whole-genome RAPDs. The data were available for some of the localities sampled in the present study (Fig. 1). The pairwise genetic distances calculated for RFLPs from 19 restriction enzymes ($DRFLP$) were taken from McGregor (1992). Kimura two-parameter genetic distance (Kimura, 1980) was calculated, with PHYLIP, from sequences presented in Thorpe et al. (1994). Genetic distances from mtDNA and microsatellite data were compared with the correlation coefficient ($r$) as a measure of relative correspondence between matrices. The trees were compared visually to elucidate congruence in the major phylogenetic divisions.

### RESULTS

Within-Population Data

Allele frequency distributions are available from the corresponding author. The loci all have a rather similar range of allele sizes (17–21 repeats) and there are no consistent trends in which a set of populations has larger, or smaller, sizes across all loci (Table 1). The variation in Tenerife generally encompasses that on the other islands except for the larger alleles of loci A49 and B967 on the south western islands of La Gomera and El Hierro. The mean number of alleles (MNA), over a range of loci, is considered to be a reasonable indicator of genetic variation within a population, given the assumption of mutation-drift equilibrium and similar sample sizes among populations (Nei, 1987). On the more recently colonized islands (La Gomera, El Hierro, and La Palma) MNA is low compared to Tenerife. This is supported by observed heterozygosity ($H_o$) and gene diversity ($H_e$) estimates (Table 1) which are low in these more recently colonized islands, particular El Hierro. Only locus B821 has a strong heterozygote deficit and this only for the populations of La Gomera and El Hierro. This may be due to null alleles, selection, or population subdivision (Wahlund, 1928), although the latter is unlikely if the deficit is limited to one locus.

### TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>Northeast Tenerife</th>
<th>Southwest Tenerife</th>
<th>La Gomera</th>
<th>El Hierro</th>
<th>La Palma</th>
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<tr>
<td></td>
<td>T19</td>
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<td>T26</td>
<td>T6</td>
</tr>
<tr>
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<td>30</td>
<td>31</td>
<td>26</td>
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<td>30</td>
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<td>$A_{349}$</td>
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<td>$H_e$</td>
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<tr>
<td>MNA</td>
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<td>11.8</td>
<td>10.8</td>
<td>11.6</td>
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<td>$H_e$</td>
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<td>0.866</td>
<td>0.809</td>
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**TABLE 2**

<table>
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<tr>
<th>Source of variation</th>
<th>df</th>
<th>Percentage of variation</th>
<th>Significance tests (10,000 permutations)</th>
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<td>$F_w$ Among islands</td>
<td>4</td>
<td>11.29</td>
<td>$P &lt; 0.0001$</td>
</tr>
<tr>
<td>Among populations</td>
<td>7</td>
<td>1.01</td>
<td>$P &lt; 0.0001$</td>
</tr>
<tr>
<td>within islands</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within populations</td>
<td>732</td>
<td>87.70</td>
<td>$P &lt; 0.0001$</td>
</tr>
<tr>
<td>Total</td>
<td>743</td>
<td></td>
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<tr>
<td>$R_w$ Among islands</td>
<td>4</td>
<td>24.35</td>
<td>$P &lt; 0.0001$</td>
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<tr>
<td>Among populations</td>
<td>7</td>
<td>1.95</td>
<td>$P &lt; 0.0001$</td>
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<tr>
<td>within islands</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Within populations</td>
<td>732</td>
<td>73.70</td>
<td>$P &lt; 0.0001$</td>
</tr>
<tr>
<td>Total</td>
<td>743</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note. The populations were grouped according to island (Fig. 1), with the populations from Tenerife forming two “island” groups according to their mtDNA haplotype representing their hypothesized derivation from ancient precursor islands (Thorpe et al., 1996) (southwest = T6, T48 [TSW], T29; northeast = T19, T39, T24 [TNE], T26; with localities T6 and T19 on the border of the transition between the two Tenerife haplotypes).

The tests for departure from Hardy–Weinberg equilibrium showed that most populations were in equilibrium for most loci (except B821 and B967 for one El Hierro population and B821 for La Gomera). Tests for pairwise linkage disequilibrium between loci with the EM algorithm to estimate haplotype frequencies showed no significance and the loci are therefore considered to be at linkage equilibrium.

Among-Population Data

AMOVA tests indicate that there is significant structure in the data at all levels irrespective of whether $F_w$ or $R_w$ estimates are used (Table 2). The main source of genetic variation is at the within-population level with substantial variation between islands and little, but still highly significant, variation between populations within islands.

Pairwise genetic distances exhibit a wide range of values with the lowest values between populations within islands, e.g., southwestern Tenerife, and the highest values for comparisons between islands, e.g., La Palma and El Hierro. Scatter diagrams (Fig. 2) show the mtDNA distances to be bimodal with substantial overlap in both VRN and $F_w$ microsatellite distances from high- and low-divergence mtDNA distances. However, the $D_m$ and $G_m$ distances show no such overlap. Even so, the bimodal nature of the mtDNA distances allows no critical test of linearity with microsatellite distances. The correlations between VAF microsatellite distances on the one hand and RAPD, mtDNA, RFLP, and mtDNA sequence distances on the other hand are all consistently higher than comparable correlations with VRN distances (Fig. 2).

The neighbor-joining method was used to recon-
million years for mtDNA, then the divergence time between major lineages is thought to be 2.8 mybp (Thorpe et al., 1994). However, the rate may be lower for ectothermal vertebrates (Gübitz et al., 2000) and a rate of 1% base pair changes per million years would give a divergence time of 5.6 mybp for these two major lineages (ca. million generations). This range of times is compatible with the geological history of the islands (Fig. 1). Whereas, conventionally, *G. galloti* is considered a single species, under the evolutionary species concept (Frost et al., 1992), some populations would probably be considered independent species. The La Gomera–El Hierro lineage (*G. caesaris*) could be considered a separate species relative to the Tenerife–La Palma lineage (*G. galloti, s.s.*) defined on the basis of fixed differences: i.e., differences in mtDNA base pairs (Thorpe et al., 1994), whether the blue markings (perhaps sexual signals) are on the forelimbs (as in *G. caesaris*) or on the head/trunk (as in *G. galloti, s.s.*), a crenulated collar (present in *G. caesaris*; rare in *G. galloti, s.s.*), and mature male snout–vent length (generally less than 9.2 cm for *G. caesaris*; greater than 9.2 cm for *G. galloti, s.s.*) (data in Thorpe, 1996). In addition to being recognizable as separate species under the evolutionary species concept, the time scale involved encompasses species divergence in many other groups (Avise et al., 1998). Consequently, the level of divergence between the two main clades in this model is comparable to species divergence in many vertebrates.

**FIG. 3.** Neighbor-joining trees reconstructed from different genetic distances: $(\delta_\mu)^2$ and linearized $D_{ps}, G_{st},$ and $F_{st}$. The linearized $R_{st}$ distance tree is not illustrated as it very similar to the tree based on $(\delta_\mu)^2$. Bootstrap values over 50% are given and a solid circle symbolizes the midpoint root.

**FIG. 2.** Comparison of pairwise genetic distances from microsatellite ($D_{ps}, G_{st}, F_{st},$ and $(\delta_\mu)^2$) and mtDNA (Kimura two-parameter and RFLP pairwise genetic distances) data. The $R_{st}$ plots are excluded as they are very similar to the $(\delta_\mu)^2$ plots, and RAPD plots are excluded as RAPDs did not differentiate between similar populations. The correlations between RAPD and microsatellite distances are $(\delta_\mu)^2$, 0.798; $R_{st}$, 0.709; $F_{st}$, 0.830; $D_{ps}$, 0.852; and $G_{st}$, 0.862. The mtDNA genetic distances are used here as a “time scale” for the divergence times between populations, assuming a molecular clock (in relation with Fig. 1). MtDNA lineage name abbreviations: La Gomera, G; El Hierro, H; La Palma, L; northeast Tenerife, TNE; southwest Tenerife, TSW.
The expectation that all microsatellite genetic distances may lose linearity after several thousand generations of divergence, essentially due to range constraints in allele sizes (Nauta and Weissing, 1996; Feldman et al., 1997), is not borne out by the scatter diagrams. If the relationship between all microsatellite distances and mtDNA distances is asymptotic over the range presented then one would expect all of the VAF distances, including $D_{st}$ and $G_{st}$, and not just the VRN $F_{st}$ distances to show overlap in distances between mtDNA-similar and mtDNA-divergent populations. This is not the case; so, this study presents no evidence for all microsatellite distances reaching an asymptote over the time scale under consideration (3–6 mybp). However, the rather bimodal nature of mtDNA divergence in this example (Fig. 2) does not allow a critical test of linearity.

Microsatellite distances based on VAF are consistently more highly correlated to mtDNA and RAPD genetic distances than those based on VRN (Fig. 2). The scatter diagrams show that this is generally due to a wider range of VRN distances at high mtDNA distances, which, in turn, may be due to the greater variance of VRN metrics. This comparison of distance matrices argues in favor of the use of VAF distances to elucidate historical relationships in the situation represented by this data set (i.e., a limited number of loci and moderate sample sizes), a conclusion that is in accord with Gaggiotti et al.’s (1999) investigation of population structure and gene flow.

Phylogenetic analyses of the previous molecular information (both mtDNA and RAPDs) provide a strongly corroborated organismal phylogeny for G. gallopi, sensu lato, which has two distinct lineages. One lineage comprises the adjacent islands of La Gomera and El Hierro and the other lineage consists of La Palma and northeast and southwest Tenerife. All the trees reconstructed from microsatellite data show these two lineages, irrespective of which mutation model was the basis for the genetic distance metric and despite the organismal model failing to conform entirely to the assumptions and optimal conditions for the use of microsatellites. Whereas, in a few species, numerous microsatellite loci are available, the number of loci used in this study is more typical of the number that has been available for most species. Consequently, the fact that the essentials of a well corroborated tree can be reconstructed from such a relatively small number of microsatellites argues for their utility in this area. This is in line with Estoup et al.’s (1995a,b) phylogenetic study of bees based on seven microsatellite loci, Berube et al.’s (1998) study of fin whales based on six microsatellite loci, and Forbes et al.’s (1995) study of sheep based on six microsatellite loci. These phylogenetic studies, which used relatively few loci, produced results broadly compatible with mtDNA and other data. However, several other phylogenetic studies have not shown this. For example, Paetkau et al.’s (1997) study of bears, based on eight microsatellite loci, gave less phylogenetic resolution at higher levels, a study of Pacific trout (Nielsen et al., 1997), based on three microsatellite loci, showed incongruence to mtDNA, and a study of brook charr (Angers and Bernatchez, 1998), based on five microsatellite loci showed incongruence between mtDNA and microsatellites, as the latter gave information at a finer scale.

Although cross-species amplification occurs, we are probably near the upper limit of the phylogenetic usefulness of microsatellites in this group as not all the primers are conserved in G. stehlini, the available outgroup. Consequently we had to use midpoint rooting, with its limitation of the assumption of equal rate of divergence. An alternative would be to use a more closely related outgroup, such as G. atlantica or one of the rare and endangered species, e.g., G. simonyi.

Failure to meet the assumptions and optimal conditions for the use of microsatellites for phylogenetic reconstruction is evident when one considers the finer details of the trees. During the colonization of La Gomera from Tenerife and that of El Hierro from La Gomera, strong bottlenecks and founder effects are expected to have occurred (although migration between these islands is negligible). Microsatellite genetic distances are expected to be strongly affected by bottlenecks and fluctuations of population size (Nauta and Weissing, 1996; Takezaki and Nei, 1996). Whereas all the trees based on VAF distances revealed the El Hierro populations as a coherent lineage (consistent with geography and mtDNA), the VRN trees failed in this respect. The VRN (but not the VAF) El Hierro–Tenerife distances are lower than the La Gomera–Tenerife distances even though El Hierro was probably colonized from La Gomera. This suggests either that these VRN distances have been more disrupted by these relatively recent bottlenecks than VAF distances and are more sensitive to the recent demographic history of the populations or that the greater variance of the VRN distances renders the finer details unreliable with moderate sample sizes and few loci.

The finer relationships within the La Palma/Tenerife lineage represent a different demography as migration among Tenerife populations may be high compared to mutation rates. In this case neither the VAF nor the VRN trees represent the relationships seen in the mtDNA trees (Thorpe et al., 1994, 1996), i.e., La Palma as a sister group to the northeast Tenerife lineage (from whence it was colonized), with a southwest Tenerife lineage as sister group to this northeast Tenerife/La Palma lineage. Hence, it appears that migration in Tenerife, as expected, has prevented a phylogenetic reconstruction congruent with the mtDNA data for this part of the tree.

In summary, the deeper phylogenetic division is consistently represented by all microsatellite trees while
the finer details are not, although trees based on VAF metrics appear to do marginally better at this than those based on VRN metrics. Against expectations, bottlenecks do not necessarily perturb microsatellite distances (at least not VAF distances), although, as expected, migration does appear to prevent phylogenetic reconstruction with microsatellites. Nevertheless, even with few loci and severe bottlenecks, both VAF and VRN distances may recover phylogenetic relationships among island populations that are millions of years old and may be useful in studying the phylogeography of populations and closely related species.

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