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Complex patterns of genetic and phenotypic divergence in an island bird and the consequences for delimiting conservation units

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Abstract

Substantial phenotypic and genetic variation is often found below the species level and this may be useful in quantifying biodiversity and predicting future diversification. However, relatively few studies have tested whether different aspects of intraspecific variation show congruent patterns across populations. Here, we quantify several aspects of divergence between 13 insular populations of an island endemic bird, the Vanuatu white-eye (*Zosterops flavifrons*). The components of divergence studied are mitochondrial DNA (mtDNA), nuclear DNA microsatellites and morphology. These different aspects of divergence present subtly different scenarios. For instance, an mtDNA phylogenetic tree reveals a potential cryptic species on the most southerly island in Vanuatu and considerable divergence between at least two other major phylogroups. Microsatellite loci suggest that population genetic divergence between insular populations, both between and within phylogroups, is substantial, a result that is consistent with a low level of interisland gene flow. Finally, most populations were found to be strongly morphologically divergent, but no single population was morphologically diagnosable from all others. Taken together, our results show that, although many measures of divergence are concordant in this system, the number of divergent units identified varies widely depending on the characters considered and approach used. A continuum of divergence and a degree of discordance between different characters are both to be expected under simple models of evolution, but they present problems in terms of delimiting conservation units.

Keywords: birds, conservation, islands, phylogeny, subspecies

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Islands have been described as natural laboratories by evolutionary biologists and ecologists alike and have played a pivotal role in the development of these fields (e.g. Darwin 1859; Wallace 1880; Mayr 1942; MacArthur & Wilson 1967; Lack 1971; Diamond 1975; Grant 1986). Perhaps more than any other vertebrate group, birds are excellent colonizers of, and speciators on, islands and archipelagos (Newton 2003). Indeed, Gaston & Blackburn (1995) classified almost 20% of all bird species as island endemics. Unfortunately, insular bird species are also among the most threatened in their Class (Collar *et al*. 1994), which places a premium on consistently delimiting appropriate units of insular diversity for conservation.

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After more than a century of debate (see Darwin 1859 for an early discussion of this issue), the criteria for delimiting species is still contentious (e.g. Hey 2001; Coyne & Orr 2004; de Queiroz 2005) and this includes insular taxa. For instance, under the biological species concept (BSC) reproductive isolation is required for species status to be conferred on a population. This means that a degree of subjectivity is necessary in deciding whether allopatric populations have diverged enough to prevent interbreeding (Wilson & Brown 1953; Helbig *et al*. 2002; Coyne & Orr 2004). An alternative to the BSC, the phylogenetic species concept (PSC) offers greater objectivity with respect to allopatric taxa; species are identified as monophyletic clusters that share a derived character (Cracraft 1983). However, a drawback of this method is that it offers little information regarding the potential for future interbreeding (Helbig *et al*. 2002).

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Application of the PSC generally leads to greater splitting (Agapow *et al*. 2004), a trend that tends to be more pronounced on islands (Peterson & Navarro-Sigüenza 1999).

It is of little surprise that in a BSC framework, the taxonomic rank below the species, the subspecies, has also attracted enduring controversy (e.g. Wilson & Brown 1953; Barrowclough 1980; Mayr 1982; O'Brien & Mayr 1991; Zink 2004). Subspecies are defined as populations that occupy different ranges or habitats, possess phylogenetically concordant traits, and differ from each other in natural history (O'Brien & Mayr 1991). Typically, studies that address relationships among subspecies using molecular phylogenies have found many to be paraphyletic (Barrowclough 1980; Zink 2004). Interestingly, the incidence of paraphyly is lower on islands (Phillimore & Owens 2006), which may be attributable to faster rates of drift and fixation of alleles in small populations that experience little gene flow. Deciding on appropriate units for conservation below the species level is a major area of current discussion, with different sources of information favoured (e.g. Moritz 1994; Crandall *et al*. 2000; Zink 2004; Haig *et al*. 2006). One of the problems is that intraspecific geographical variation in different characters is often discordant (Wilson & Brown 1953). Studies that compare the extent of neutral genetic divergence and phenotypic divergence between populations have reported a weakly positive correlation or no correlation between the two and that phenotypic divergence tends to exceed neutral genetic divergence (e.g. Merilä 1997; McKay & Latta 2002; Nicholls *et al*. 2006; Saether *et al*. 2007; Leinonen *et al*. 2008). Most tests addressing phenotypic evolution among insular taxa have found that phenotypic divergence departs from a neutral null expectation, implying an important role for selection (e.g. Clegg *et al*. 2002b; Harmon & Gibson 2006). A lack of congruence between different classes of information relates both to the continuum of divergence that exists as different loci evolve at different rates, and to the different evolutionary processes that are involved in the divergence of different characters (Crandall *et al*. 2000).

Our aim in this study is to quantify three distinct aspects of interisland divergence across 13 insular populations of an endemic passerine bird, the Vanuatu white-eye (*Zosterops flavifrons*). This species represents an excellent case study as it has diversified into seven recognized subspecies in a single archipelago (Mayr 1945; Mees 1969). First, we use a phylogenetic approach to identify clusters of populations that have been on independent evolutionary trajectories long enough for mitochondrial genes to coalesce. It is, however, possible for two populations to be polyphyletic on a gene tree, but for them to have been exchanging few or no individuals for many generations (Hudson & Coyne 2002; Funk & Omland 2003). We therefore use microsatellites to test for population genetic divergence at neutral nuclear loci and calculate the proportion of total neutral genetic variation that is distributed among island populations. We complement this with an individual-based clustering approach, which we use to test for genetic structure in the absence of prior geographical information. Last, we conduct quantitative analyses of morphological divergence among islands using (i) a multivariate variance-partitioning approach, which is a variant of the Q_{ST} statistic commonly used by evolutionary biologists (Spitze 1993), and (ii) univariate diagnosability tests, which are advocated by some museum taxonomists (Patten & Unitt 2002; Cicero & Johnson 2006). We interpret the results of these tests and the degree of congruence or conflict between them in the light of evolutionary theory and consider the implications for the conservation of insular endemics.

Methods

Study system

The Vanuatu white-eye is endemic to the Vanuatu archipelago in Melanesia, where it occurs on every major island (Mees 1969). Seven subspecies have been described (Mayr 1945; Mees 1969), a number that is only exceeded among birds in Vanuatu by the island thrush (*Turdus poliocephalus*, with eight subspecies in the archipelago) and is in the top decile for avian subspecies richness globally (Phillimore *et al*. 2007). These subspecies vary only slightly in plumage colour and pattern, size and tail length and are divided into two groups on the basis of plumage: those that are 'yellow' (*efatensis*, *flavifrons* and *gauensis*); and those that are 'darker' (*brevicauda*, *macgillivrayi*, *majuscula* and *perplexa*) (Mayr 1945; Mees 1969; see Fig. 1). Throughout this study, we use the term 'population' to refer to single-island populations of the Vanuatu white-eye, which will only correspond to subspecies status where a subspecies is a single-island endemic.

Data collection

Birds were caught using mist nets and traps on 13 islands in the Vanuatu archipelago during February–May 2004 (A.B.P.) and February–April 2006 (S.M.C.). A total of 432 *Zosterops flavifrons* were caught (Fig. 1). The following standard morphological measurements were taken for each bird: culmen length to posterior of nostril, culmen depth and width (both taken at anterior of nostril), length of the tarso-metatarsus, flattened wing chord length, maximum tail length and body mass (Clegg *et al*. 2002b). Blood samples were taken via brachial venipuncture from each bird and stored either in eppendorfs containing 95% ethanol (A.B.P.) or on EDTA treated filter paper (S.M.C.) (Petren 1998). As use of numbered or coloured bands was not authorized for these populations, the tip of the right-sided outer tail feather of each captured bird was clipped to avoid resampling.

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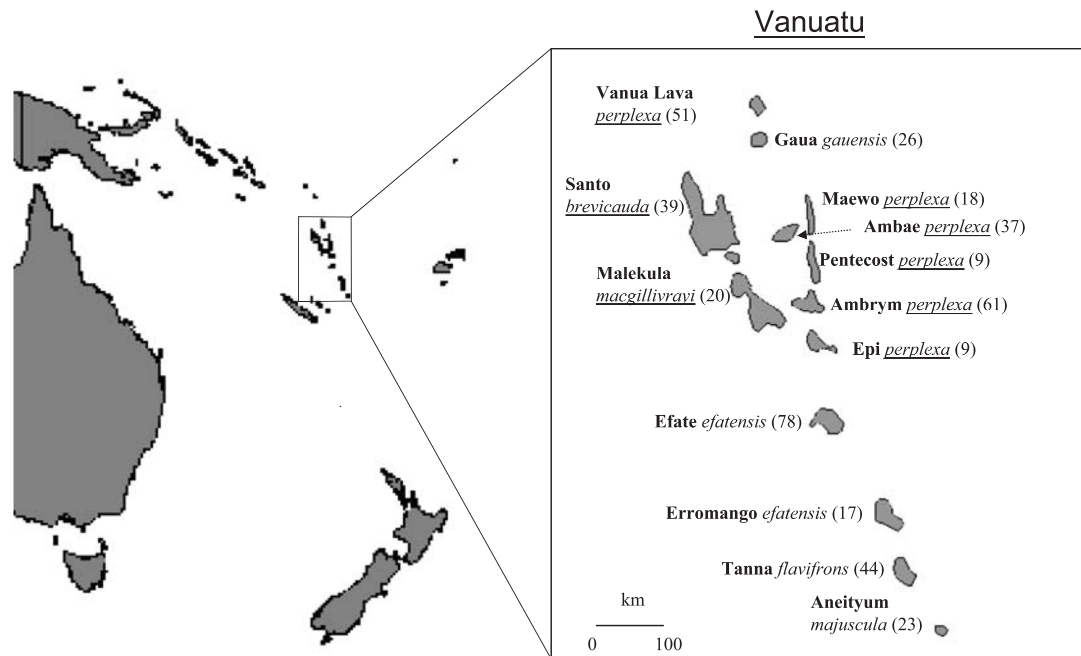

Fig. 1 Map of Vanuatu and the distribution of subspecies and sample sizes (yellow subspecies are in plain text and darker subspecies are underlined).

Molecular methods

DNA was extracted from all individuals, following an ammonium acetate protocol (Nicholls *et al*. 2000). A further 12 blood samples from Santo were kindly provided by J. Kirchman (New York State Museums). We selected four to five individuals for phylogenetic analysis from each of the islands. In addition, we included a sample from *Zosterops rennellianus* (kindly provided by C. Filardi) and *Zosterops lateralis* (collected on Tanna) as potential outgroups. We amplified two mitochondrial protein coding regions: the L10755 and H11151 primers (Chesser 1999) amplified a region including the ND3 gene; and the primers CB1 and CB2 amplified a portion of cytochrome *b* (cyt *b*) (Palumbi 1996). These genes were chosen as they have already been used in a phylogeographical study of *Zosterops* species (Warren *et al*. 2006). Amplifications were performed in 20 μL reactions, including 30–40 ng template DNA, 0.5 μM of each forward and reverse primer, 0.25 μM dNTPs, 1× reaction buffer (Bioline), 0.1 U *Taq* polymerase (Bioline), 2 mM $MgCl_2$ (Bioline). Thermal cycling consisted of an initial denaturation at 94 °C for 3 min, followed by 35 cycles (40 cycles for cyt *b*) of: 94 °C for 30 s, 54 °C (55 °C for cyt *b*) for 30 s (45 s for cyt *b*) and 72 °C for 35 s (40 s for cyt *b*). A final 10-min incubation period at 72 °C completed the reaction. Amplified fragments were electrophoresed on a 1% agarose gel, stained with ethidium bromide and excised for purification with a Qiaquick gel extraction kit (QIAGEN). The purified DNA fragments were quantified against a 100 base pair (bp) ladder (Bioline) on an ethidium bromide-stained agarose gel. The purified fragments were used as template for Big Dye terminator cycle sequencing reactions (Applied Biosystems [ABI]). Cycle sequence products were precipitated in ethanol and electrophoresed on an ABI 3730 sequencer. SEQSCAPE (ABI) was used to score bases and align sequences. The sequences for ND3 and cyt *b* are deposited in EMBL under Accession nos AM946092–AM946148 and AM946034–AM946091, respectively.

A total of 438 individuals were screened at seven microsatellite loci using primers that had been developed for *Z. lateralis* [*ZL12*, *ZL18*, *ZL38* (Degnan *et al*. 1999) and *ZL45*, *ZL46*, *ZL50* and *ZL54* (Frentiu *et al*. 2003, 2008)] and a further three loci characterized in *Acrocephalus sechellensis* [*Ase19*, *Ase28*, *Ase64* (Richardson *et al*. 2000)], that had been successfully used across a wide range of species (see the passerine microsatellite cross-utility database maintained on the BIRDMARKER webpage at http://www.shef.ac.uk/misc/groups/molecol/deborah-dawson-bird-markers.html). Polymerase chain reaction (PCR) was conducted using standard protocols or QIAGEN Multiplex PCR kits. Standard PCR consisted of 10 μL reactions containing 20 ng DNA, 0.5 μM of each primer (one fluorescently labelled), 0.25 mM dNTPs, 1× reaction buffer (Bioline), 0.5 U *Taq* polymerase (Bioline), and primer-dependent concentrations of $MgCl_2$. Cycling conditions were: 94 °C for 3 min, followed by 34 cycles of 94 °C 30 s, T_A for 30 s (see Table S1, Supplementary material for further details), 72 °C for 30 s. Primer sets were multiplexed post-PCR prior to running on an Applied Biosystems 3730 DNA analyser. For QIAGEN Multiplex PCR, we followed the manufacturer's

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recommendations, modified to a 4 μL reaction volume and used the recommended touchdown PCR cycle for multiplex PCR. Additionally, each individual was genetically sexed using primers P2 and P8 (Griffiths *et al*. 1998). Allele size scoring was conducted using the software GENEMAPPER version 3.0 (Applied Biosystems).

Phylogenetic analyses

We included published *Zosterops* sequence data from *Z. atricapillus* (GenBank Accession nos DQ328498.1, DQ328398.1), *Z. nigrorum* (DQ328455.1, DQ328355.1), *Z. palpebrosus* (DQ328449.1, DQ328349.1) and *Z. senegalensis* (DQ328443.1, DQ328343.1) as additional potential outgroups (Warren *et al*. 2006). We used the partition rate homogeneity test in PAUP* 4.0 (Swofford 2003) to test whether there was a significant difference in the phylogenetic signal of the two genes. We then used an Akaike information criterion-based approach in MODELTEST version 3.7 (Posada & Crandall 1998) to identify an appropriate nucleotide substitution model. Phylogenetic reconstruction was implemented in BEAST version 1.4.6 (Drummond & Rambaut 2007), using a relaxed clock approach (Drummond *et al*. 2006) with a mean substitution rate set at 1% per lineage per million years (Weir 2006). Rates of substitution were assumed to be uncorrelated between adjacent branches and were drawn from a lognormal distribution. A neighbour-joining tree was used as a prior distribution for the tree, and a Yule prior was used for branching rates. We conducted two runs of 10 million generations, each with sampling conducted every 1000 generations. TRACER (Rambaut & Drummond 2003) was used to assess convergence, whether the two chains were mixing, and whether the estimated sample size (ESS) for each parameter was of sufficient size to obtain good parameter estimates (i.e. ESS > 200). One million generations were discarded as burn-in from each run, leaving us with a posterior distribution of 18 000 ultrametric trees. We obtained a rooted ultrametric consensus tree where branch lengths represent divergence measured in units of time.

Reciprocal monophyly of clades in phylogenetic trees has variously been advocated as a means of delimiting species (e.g. Cracraft 1983) and intraspecific units (e.g. Ball & Avise 1992; Moritz 1994; Zink 2004). We therefore tested which of our island populations or groups of populations form well-supported monophyletic clades on the basis of Bayesian posterior support. We also implemented a coalescent model in a likelihood framework to identify the number and membership of species clusters (described in Pons *et al*. 2006; Fontaneto *et al*. 2007). We preferred this method to several alternative tree-based methods (see Sites & Marshall 2004), because clusters are identified using an entirely objective criterion and the number of clusters identified is not a function of sample size. We compared the maximum-likelihood solution to a null model, under which all lineages followed the same coalescent and branching process through time (i.e. corresponding to all individuals belonging to a single cluster), using a likelihood-ratio test with three degrees of freedom. We identified any other clustering solutions that were found within two log-likelihoods of the maximum-likelihood solution. This analysis was conducted in R (R Development Core Team 2007) using script provided by T. Barraclough (Imperial College London).

Population genetic analyses of microsatellites

Deviation from Hardy–Weinberg equilibrium was assessed for each locus-population combination using GENEPOP version 3.4 (Raymond & Rousset 1995). An exact test for deviation from Hardy–Weinberg equilibrium was implemented using a Markov chain approach (Guo & Thompson 1992; Raymond & Rousset 1995), to find the probability of obtaining an allele frequency distribution that was equally or less likely than that which was observed. This probability value, combined with Weir & Cockerham's (1984) F_{IS} value, identified cases in which there was a significant deficit (negative F_{IS}) or excess (positive F_{IS}) of homozygotes. We tested linkage disequilibrium between loci for all pairwise population combinations and for each locus in turn using a Markov chain contingency table approach in GENEPOP (Raymond & Rousset 1995). A global P value across populations was estimated using Fisher's exact test (Fisher 1932). Bonferroni corrections for multiple comparisons were applied to these tests (Holm 1979; Rice 1989; but see Moran 2003; Nakagawa 2004).

The proportion of the total genotypic variation that is distributed among, rather than within, populations, denoted F_{ST}, is commonly used to quantify neutral genetic divergence among populations (Wright 1965). Although this measure assumes an infinite alleles model of mutation, it was preferred as it has been shown to perform better than other estimates of population structure when the number of loci and the number of samples per population are small (Gaggioti *et al*. 1999). Here we estimate F_{ST} using θ (Weir & Cockerham 1984). However, F_{ST}-based approaches are influenced by the genetic variation within populations; two populations that share no alleles but have high heterozygosity will have lower F_{ST} values than two equally divergent populations each with low heterozygosity (Hedrick 2005). To address this, and make θ estimates comparable, we adopted the straightforward standardization procedure of Meirmans (2006). Pairwise θ was divided by the maximum possible value, which we estimated by coding alleles using the RECODEDATA package, such that the within-population variation was maintained and among-population variation maximized, i.e. no allele sharing between populations (Meirmans 2006).

We used an individual-based Bayesian clustering method implemented in STRUCTURE 2.0 to assess genetic structure in the absence of prior geographical information (Pritchard

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et al. 2000). The analysis was conducted separately for populations of the darker form vs. the yellow forms due to the substantial phylogenetic divergence between the two groups (see mtDNA results). The population from Aneityum was not included in this analysis, as our mtDNA gene tree suggests that it is not monophyletic with other *Z. flavifrons*. For both sets of analysis, we used a burn-in length of 10^5 and run-length of 10^6 iterations, with the default program settings and no prior population information. We evaluated the number of genetic clusters (k) from 1 to 8 for the darker group, and from 1 to 5 for the yellow group. The posterior probabilities (calculated from model likelihoods) for each value of k were compared to identify the most likely number of genetic clusters in each case. The proportion of membership of each population in each of the identified clusters was taken from the posterior distribution from the best run for the most likely value of k.

Multivariate morphological analyses

Repeatability between the two measurers (A.B.P. and S.M.C.) was assessed on 25 live blue tits (*Cyanistes caeruleus*) measured in the field (tarsus, wing and tail) and 22 *Z. flavifrons* skins measured at Natural History Museum at Tring, UK (culmen length to posterior nostril, culmen width and depth at anterior of nostril). The repeatability obtained in the field for tarsus and wing was greater than 0.9, while that of the tail was < 0.6; we thus excluded tail measurements from subsequent analyses. The *Z. flavifrons* specimens derived from six islands, consequently measurer identity was nested within island in a linear mixed-effects model (Pinheiro & Bates 2000). A correction factor of 0.064 was added to the ln-transformed culmen length measurements made by A.B.P., giving a between-measurer repeatability for culmen length of 0.74. Repeatabilities for culmen width and depth were 0.90 and 0.68, respectively.

We used multivariate analysis of variance (MANOVA) of the ln-transformed measurements to test whether there were significant differences between adult birds from the different island populations. Sex was included as a fixed effect in this model. The orthogonal axes that maximized morphological divergence between island populations were calculated from the output of the MANOVA, following the approach of McGuigan *et al*. (2005). Divergence of morphology between islands in multidimensional trait space (D) was calculated from the sum of squares cross-product (SSCP) matrix of islands (H) and error (E), respectively, following equation 6.1 in Rencher (1998). Canonical variates, which maximize interpopulation variance, were extracted from the D matrix. Unless otherwise indicated, all analyses were conducted in the R statistical programming environment (R Development Core Team 2007).

To examine phenotypic divergence between pairs of islands, we conducted pairwise MANOVA with island and sex as fixed effects. The SSCP matrices for the island and error term in each test were used to estimate the variance–covariance matrix for each term. By summing the eigenvalues from the resulting variance–covariance matrices, an estimate of the total variation in morphospace distributed between (v_b) and within islands (v_w) was obtained (Hadfield & Owens 2006). We then used P_{ST} (equation 1, Leinonen *et al*. 2006), which is the phenotypic analogue of Q_{ST} (Spitze 1993), to quantify morphological divergence between populations. P_{ST} differs from Q_{ST} in that both v_b and v_w may also include environmental effects, whereas for Q_{ST}, a quantitative genetic design is used so that only the additive genetic variance components of v_b and v_w are estimated (Leinonen *et al*. 2006). We estimated P_{ST} under the assumption that all phenotypic differences between populations are solely genetic-based and that the heritability, h^2, of morphological traits is 0.5. This value was chosen as heritability estimates of adult morphological traits in an island population of *Z. lateralis chlorocephalus* are in the range 0–0.74 (S.M.Clegg, unpublished data). We also examined the sensitivity of P_{ST} estimates to (i) different environmental effects, where v_b is replaced with $0.5v_b$, and (ii) different heritability values, where $h^2 = 0.25$ and 0.75. We tested the significance of a correlation between the pairwise P_{ST} and the degree of neutral genetic divergence, θ, using a Mantel test with 10 000 permutations.

$$P_{ST} = \left(\frac{v_b}{v_b + 2v_w h^2}\right) \quad \text{(eqn 1)}$$

Next, we tested whether pairs of populations were statistically diagnosable along univariate morphological axes (Amadon 1949; Patten & Unitt 2002). Two populations are considered diagnosable (D_{ij} and D_{ji} values > 0) if ≥ 99% of the distribution of traits in population i (the population with larger trait size) are larger than 75% of the distribution of traits in population j and ≥ 75% of the distribution of traits in population i are larger than 99% of the distribution of traits in population j (Patten & Unitt 2002). We applied this test for each population pairwise comparison for wing length and relative culmen length (measured as culmen length/tarsus length), as these variables were found to correspond to important axes of morphological divergence. We favoured simple morphometric measurements over multivariate approaches in this instance, because diagnosability methods were developed to provide a straightforward means of distinguishing populations. As the sexes of this species show significant differences in morphology (Phillimore 2006), tests were conducted separately for males and females.

Results

Phylogenetic analyses

We obtained a 351-bp region of ND3 and 308-bp region of cyt *b* from 55 *Z. flavifrons* plus six individuals of potential

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outgroup species. For ND3, 270 (77%) and 48 (14%) sites were invariable and parsimony-informative, respectively. For cyt *b*, the corresponding figures were 246 (80%) and 38 (12%). Using the partition rate homogeneity test, we uncovered no significant conflict in the phylogenetic signal between the two protein-coding genes ($P = 0.968$); consequently, we partitioned the concatenated sequence for phylogenetic analysis allowing parameters to vary between the two genes. Of the nucleotide substitution models implemented in BEAST (Drummond & Rambaut 2007), HKY + *I* + *G* was preferred.

Our reconstructed mitochondrial gene tree casts some doubt on the monophyly of *Z. flavifrons*, with a group including *Z. f. majuscula* (from Aneityum in the far south) and the outgroup taxa having a Bayesian posterior support of 0.68 (Fig. 2). There was robust evidence of a deep split between two *Z. flavifrons* clades that correspond to previously described yellow (*gauensis*, *efatensis* and *flavifrons*) and darker (*brevicauda, macgillivrayi* and *perplexa*) forms (Mayr 1945), and there was some evidence that this clade may not be monophyletic. Of the yellow forms, the Tanna (the nominate race) and Erromango populations were monophyletic. The *efatensis* population on Erromango appears to be sister to the Tanna population (*flavifrons*) rather than the *efatensis* population on Efate, rendering *efatensis* paraphyletic. The gene tree revealed a deep split within the darker clade, between the monophyletic Santo and Vanua Lava populations and the remaining darker island populations. The affinities of individuals from the remaining darker populations were not well-resolved, with only Epi and Pentecost possessing monophyletic clades. On this basis, phylogenetic monophyly of the *perplexa* subspecies can be rejected.

Individuals from outgroup species were excluded from our analysis of the number and identity of independently evolving lineages. Four distinct *Z. flavifrons* lineages, some made up of multiple populations, were identified; these were: (i) Ambae, Ambrym, Epi, Maewo, Malekula and Pentecost; (ii) Santo and Vanua Lava; (iii) Efate, Erromango, Gaua and Tanna; and (iv) Aneityum (Fig. 2). The four-lineage solution was not significantly more likely than the null hypothesis that all lineages belonged to a single cluster (likelihood ratio = 1.08, d.f. = 3, $P = 0.78$). Furthermore, the confidence interval for the maximum-likelihood solution included many other different numbers of clusters, suggesting either the absence of independently evolving units or low statistical power.

Population genetic analyses

A total of 438 individuals were genotyped at 10 polymorphic microsatellite loci (for sample sizes and heterozygosities see Table S2, Supplementary material). *ZL38* and *Ase64* showed a highly significant deficit of heterozygotes in multiple populations (Table S3, Supplementary material); therefore, these loci were excluded from subsequent analyses due to a risk of null alleles (Brookfield 1996) and the possibility that the locus is under selection. Seventeen of the remaining 104 island-locus combinations showed significant departures from Hardy–Weinberg equilibrium. The Vanua Lava population had an excess of homozygotes at 7/8 loci, which may indicate inbreeding.

Combined across populations, all but two cases of pairwise locus tests for linkage disequilibrium were nonsignificant ($P > 0.15$). In one case, *ZL12* × *ZL46*, $P = 0.007$; however, this was not significant following Bonferroni correction for multiple comparisons (adjusted $\alpha = 0.001$) and was due to significant values in only two of the 13 populations, Aneityum ($P = 0.01$) and Efate ($P = 0.031$). Therefore, both loci were retained. Linkage disequilibrium between *ZL54* and *Ase28* was suggested from the combined *P* value ($P < 0.0001$). On closer inspection, this result was due to a highly significant value in a single population, Malekula, and all other *P* values for this locus pair comparison were > 0.12; therefore, these loci can be considered independent and were also retained in the analysis.

Pairwise θ and standardized θ values reflected the deeper phylogenetic groupings very well (Table 1). The pairwise θ values between populations that are in different, highly divergent phylogenetic clades (i.e. the yellow clade, the two darker clades, and the Aneityum population) were generally > 0.1 (0.2 using standardized θ) and in some cases much higher than this; for example, comparisons involving Gaua were typically between 0.35 and 0.45 (0.56 and 0.80 using standardized θ). The absolute values of the large θ estimates between highly divergent clades should, of course, be interpreted cautiously due to the risk of back-mutation leading to underestimation (Hedrick 1999) and the likely departure of populations from migration/drift balance.

Within each clade (i.e. between populations within the yellow clade and two darker clades), pairwise θ-values were generally smaller than those between clades, agreeing with the mtDNA phylogenetic inferences. Nevertheless, substantial population genetic structuring was evident from both island-based and individual-based approaches within these clades. Pairwise θ values within the yellow group reached 0.404 (standardized $\theta = 0.627$), between Tanna and Gaua, and within the darker group it reached 0.217 (standardized $\theta = 0.458$), between Vanua Lava and Pentecost. Within both the yellow and darker groups, some populations with close mtDNA phylogenetic affinities were found to be highly divergent at microsatellite loci, e.g. Efate and Gaua (standardized $\theta = 0.40$) and Malekula and Ambae (standardized $\theta = 0.23$). While varying degrees of differentiation were exhibited between populations, even the smallest θ values were significantly greater than zero.

The STRUCTURE analysis (Pritchard *et al.* 2000) supported the presence of several genotypic clusters within both the yellow and darker forms. The most likely number of genetic

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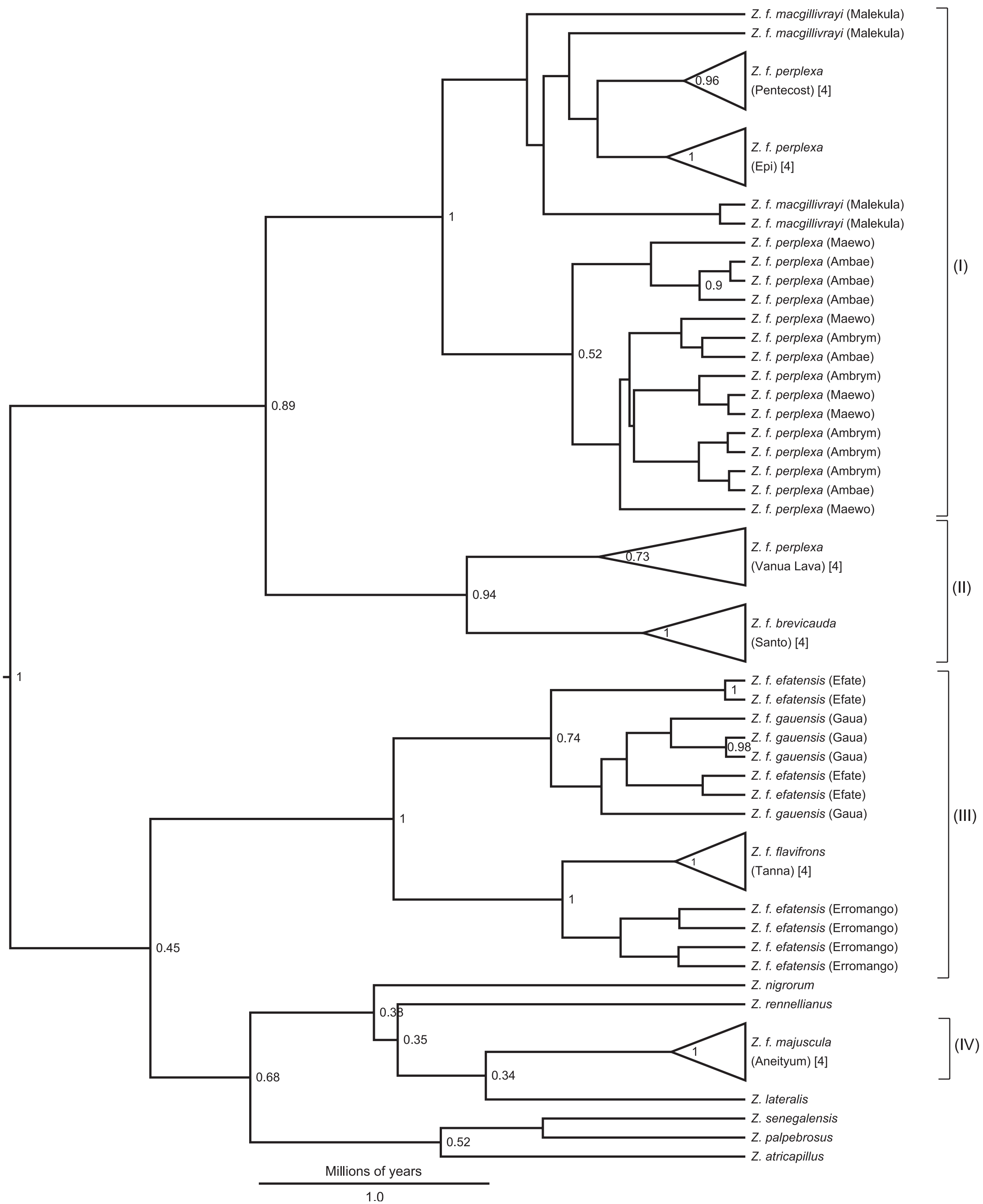

Fig. 2 Maximum clade credibility tree for *Zosterops flavifrons* subspecies based on 659 bp of ND3 and cyt *b*. Numbers below nodes represent Bayesian support (in the most recent million years only values > 0.5 are reported). Numbers in square parentheses indicate the number of specimens. Branching within reasonably supported (Bayesian posterior support > 0.5) monophyletic island clades was collapsed. Roman numerals denote the maximum likelihood selection of independently evolving clades (Fontaneto *et al*. 2007). Yellow *flavifrons* subspecies are in plain text and darker subspecies are underlined.

clusters within the yellow group was $k = 4$ (for two out of three independent runs, the posterior probability that $k = 4$ was > 0.99, and for the third run > 0.73). Clusters 1 and 2 were distributed on Efate, cluster 3 on Gaua and cluster 4 on Erromango and Tanna (Table 2a). The most likely number of genetic clusters in the darker group was $k = 5$ (two of the three independent runs returned a posterior probability > 0.99 and the third run a posterior probability > 0.88 for $k = 5$). Clusters 1 and 3 were associated with the eastern islands of Ambae, Maewo, Pentecost and Ambrym, clusters 2 and 5 with Santo, Malekula and Epi (although some affinities of this last population with the other eastern

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Table 1 Pairwise θ and standardized θ values calculated based on microsatellite data

	Ambae	Ambrym	Epi	Maewo	Malekula	Pentecost	Santo	Vanua Lava	Efate	Gaua	Erromango	Tanna	Aneityum
Ambae	—	**0.129**	**0.339**	**0.150**	**0.227**	**0.191**	**0.264**	**0.354**	0.590	0.689	0.443	0.674	0.585
Ambrym	**0.065**	—	**0.201**	**0.076**	**0.190**	**0.108**	**0.226**	**0.328**	0.551	0.651	0.311	0.543	0.509
Epi	**0.163**	**0.092**	—	**0.268**	**0.233**	**0.227**	**0.228**	**0.418**	0.575	0.584	0.246	0.423	0.529
Maewo	**0.079**	**0.038**	**0.126**	—	**0.215**	**0.184**	**0.263**	**0.274**	0.637	0.729	0.394	0.595	0.574
Malekula	**0.111**	**0.089**	**0.098**	**0.103**	—	**0.223**	**0.143**	**0.252**	0.520	0.610	0.215	0.478	0.365
Pentecost	**0.103**	**0.055**	**0.109**	**0.099**	**0.109**	—	**0.371**	**0.458**	0.464	0.598	0.357	0.589	0.526
Santo	**0.131**	**0.108**	**0.102**	**0.129**	**0.066**	**0.186**	—	**0.213**	0.521	0.676	0.195	0.447	0.377
Vanua Lava	**0.169**	**0.151**	**0.175**	**0.128**	**0.110**	**0.217**	**0.096**	—	0.697	0.802	0.403	0.579	0.560
Efate	0.400	0.351	0.396	0.447	0.349	0.338	0.335	0.435	—	**0.400**	**0.378**	**0.495**	0.487
Gaua	0.414	0.367	0.346	0.452	0.351	0.391	0.380	0.433	**0.299**	—	**0.558**	**0.627**	0.682
Erromango	0.236	0.156	0.118	0.210	0.105	0.196	0.097	0.190	**0.268**	**0.351**	—	**0.110**	0.318
Tanna	0.382	0.292	0.228	0.341	0.257	0.349	0.239	0.298	**0.349**	**0.404**	**0.064**	—	0.479
Aneityum	0.344	0.281	0.301	0.345	0.203	0.332	0.207	0.295	0.360	0.467	0.194	0.302	—

The lower triangle contains pairwise F_{ST} values estimated using Weir and Cockerham's (1984) θ. The upper triangle contains pairwise values that have been standardized following Meirmans (2006). Values in bold represent pairwise values between island populations within the yellow and darker clusters (excluding Aneityum).

Table 2 Proportion of membership of each predefined population in each inferred cluster for (a) yellow plumage group, and (b) darker plumage group

(a)

	Cluster			
Population (sample size)	1	2	3	4
Gaua (24)	0.029	0.048	**0.904**	0.019
Efate (79)	**0.418**	**0.487**	0.066	0.030
Erromango (17)	0.096	0.065	0.023	**0.816**
Tanna (40)	0.041	0.023	0.013	**0.923**

(b)

	Cluster				
Population (sample size)	1	2	3	4	5
Vanua Lava (51)	0.029	0.07	0.027	**0.818**	0.056
Maewo (18)	**0.412**	0.058	**0.439**	0.029	0.062
Ambae (37)	**0.436**	0.053	**0.412**	0.040	0.059
Pentecost (9)	**0.433**	0.030	**0.484**	0.008	0.045
Ambrym (61)	**0.414**	0.062	**0.429**	0.020	0.075
Epi (9)	0.108	0.247	0.142	0.016	**0.487**
Santo (49)	0.032	**0.505**	0.030	0.045	**0.388**
Malekula (21)	0.078	**0.343**	0.078	0.027	**0.475**

Proportions > 0.33 are highlighted in bold. Clustering was conducted on microsatellites using STRUCTURE (Pritchard *et al*. 2000). Note that no geographical population information was used to identify cluster membership.

Table 3 Trait loadings and eigenvalues of the canonical variates for morphology

Trait	CV1	CV2	CV3	CV4	CV5
Culmen length	0.194	–0.817	0.161	0.314	–0.079
Culmen depth	0.032	0.375	0.640	0.046	0.346
Culmen width	0.117	0.390	–0.192	0.493	–0.345
Tarsus	0.366	0.195	0.292	–0.806	–0.617
Wing	0.902	0.030	–0.665	–0.074	0.612
Eigenvalue	2.566	0.465	0.130	0.089	0.055
% explained	77.6	14.1	4.0	2.7	1.7

islands was also evident), and cluster 4 was strongly associated with the Vanua Lava population in the north (Table 2b). In many cases, the genotypic data and phylogenetic data agreed at this finer geographical scale. The close phylogenetic relationship between Tanna and Erromango was also seen in the genotypic data, with the Bayesian method identifying them as a single cluster. However, departures from associations identified from the phylogenetic analysis were also noted. In particular, both Bayesian clustering and θ values suggest that Santo and Malekula are closely associated, whereas the mtDNA evidence points to a closer relationship between Santo and Vanua Lava. Additionally, the mtDNA distinction between Pentecost and other eastern islands (Ambrym, Maewo and Ambae) is not shown by the genotype clustering.

Multivariate morphological analyses

A summary table of the means and standard deviations for the measurements is provided in Table S4, Supplementary material. MANOVA revealed that the populations show highly significant differences in morphospace ($P < 0.0001$). The divergence between populations in five-dimensional morphospace was dominated by the first canonical variate (CV) (Table 3), which accounts for 77.6% of the total variance.

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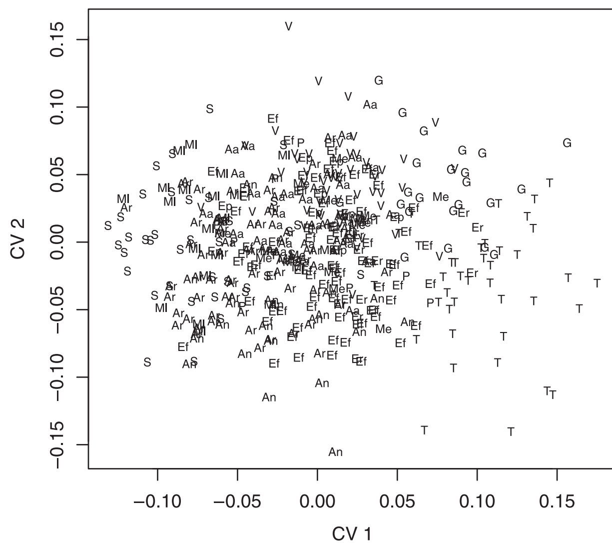

Fig. 3 The positions of individual birds on different islands along the first two morphological canonical variates. Canonical variates (CVs) were calculated following McGuigan *et al*. (2005); note that we did not correct for sex when calculating CVs on the basis of the trait loadings in Table 3 and individual trait values. Individuals from island populations are represented as follows: Aa, Ambae; Ar, Ambrym; An, Aneityum; Ef, Efate; Ep, Epi; Er, Erromango; G, Gaua; Me, Maewo; Ml, Malekula; P, Pentecost; S, Santo; T, Tanna; and V, Vanua Lava.

This CV reflected variation in body size, particularly wing and tarsus lengths. Individuals from different islands occupy overlapping but clearly different areas of trait space along the first CV, with individuals from Gaua and Tanna being particularly large and those from Malekula and Santo being particularly small (Fig. 3). The second CV corresponded to relative culmen length and accounted for a further 14.1% of the variance. Population divergence along CV2 was less pronounced, although individuals from Aneityum and Vanua Lava are notable for long (small CV2) and short (large CV2) relative culmen length values, respectively. The remaining three CVs comprised just over 8% of the variance, and consequently, we did not attempt biological interpretation of these axes.

Pairwise MANOVA revealed significant differences between island populations in five-dimensional morphospace in 73 out of 78 cases (Table S5, upper triangle, Supplementary material). The five nonsignificant results were returned for the Efate × Maewo, Efate × Pentecost, Maewo × Pentecost, Malekula × Santo and Epi × Vanua Lava comparisons. Four of these nonsignificant results involved populations that belong to the *perplexa* subspecies.

P_{ST} values (Table S5, lower triangle) demonstrated that considerable morphological divergence exists between many populations, but that there was substantial variation in the extent of divergence. At the upper extreme was the Tanna population, which shared a $P_{ST} > 0.88$ with all other populations. At the other end of the scale were the neighbouring islands of Maewo and Pentecost, which shared a negative P_{ST} with each other, implying that the populations occupy the same trait space. Some of the phenotypic divergence agreed with the phylogenetic and population genetic analyses; for example, the Aneityum, Tanna, and Gaua populations were all highly divergent (Fig. 4d). Other results, however, were in conflict. For instance, Efate and Pentecost, although highly divergent at neutral loci were only very weakly divergent in morphospace, $P_{ST} = 0.056$. P_{ST} values exceeded standardized θ in 70/78 cases. This general finding was relatively robust to varying the test assumptions. For example, when the additive genetic component of between population differences was reduced to 0.5, the proportion of cases where $P_{ST} > \theta$ declined to 65/78 and 57/78 when h^2 was fixed at 0.5 and 0.75, respectively. When heritability was fixed at 0.75 and 0.25 the corresponding ratios were 69/78 and 76/78, respectively. There was, however, no significant correlation between the pairwise values of P_{ST} and θ ($r = -0.22$, $P = 0.166$, after 10 000 Mantel test permutations).

A striking result from the pairwise diagnosability tests was that no island population was completely diagnosable from all others for either wing or relative culmen length (Table S6, Supplementary material). Four pairs of island populations had wing length (which was inferred to be one of the most important axes of island divergence from the CV loadings — Table 2) D_{ij} and D_{ji} scores > 0, and all of these involved comparisons of females on Tanna to smaller-winged populations. None of the 78 possible population comparison returned a positive D_{ij} and D_{ji} score for relative culmen length (also inferred to be an important axis of divergence from the CVs) for either males or females.

Comparison of divergence patterns across multiple data sets

The number of distinct units identified using different characters and methods showed considerable variation and some conflict (Fig. 4). Using a population genetic approach, divergence tended to be greater between rather than within mtDNA clades. Moreover, some of the populations that were not monophyletic on a phylogenetic tree (e.g. Efate and Gaua, Fig. 4b) were found to be strongly divergent at microsatellite loci (Fig. 4c). In one case, the reverse was true, as individuals from the Santo population were found to have microsatellites that clustered with individuals from Epi and Malekula, which are two populations from which it is phylogenetically distinct on an mtDNA tree. Pairwise P_{ST} values revealed that the majority of island populations were phenotypically more divergent from one another than they were at neutral genetic loci (Fig. 4d). Using the same phenotypic data in a diagnosability framework, no populations were sufficiently divergent from all others, although the colour differences

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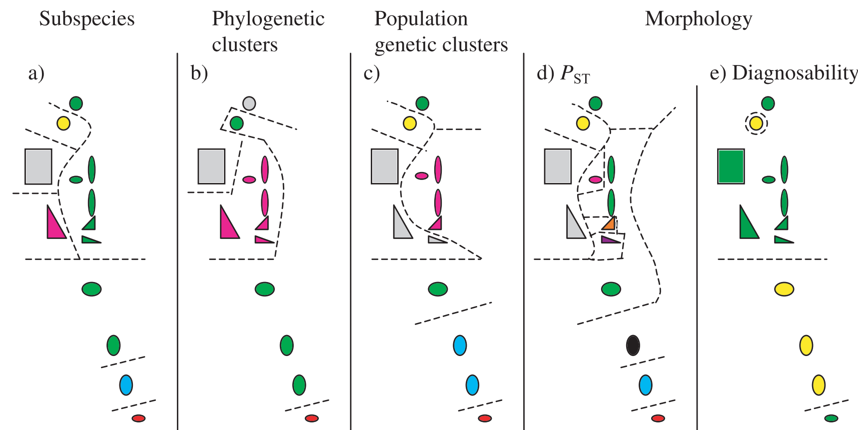

Fig. 4 Schematic maps of the clusters of related individuals identified using different data and/or methods. Membership of a cluster is indicated by colour. (a) Subspecies correspond to those recognized by Mayr (1945). (b) Phylogenetic clusters were identified using the method of Fontaneto *et al*. (2007). (c) Membership of population genetic clusters was assessed using Bayesian clustering methods; see Table 2. (d) Membership of P_{ST} clusters used an arbitrary cut-off of 0.25 for membership. This arbitrary cut-off value if for illustrative purposes and could be made more inclusive or exclusive by lowering or raising the value. (e) Diagnosability clusters are those in which every member of one cluster is diagnosable from every member of the other. Here, two diagnosable clusters on the basis of differences in plumage colour and pattern are presented. Alternatively, if morphometric information alone were used, all populations would belong to a single cluster.

between the yellow and darker phylogroups (Mayr 1945) mean that two units are probably diagnosable using this approach (Fig. 4e). Thus, an approximate spectrum across characters and methods from highest to lowest extent of divergence is: morphology (P_{ST}) > population-based microsatellite analysis > individual based microsatellite analysis > mtDNA phylogeny > morphology (diagnosability).

Some of the units identified using phylogenetic, population genetic and phenotypic methods agreed well with subspecies designation, such as Aneityum (*majuscula*), and to some degree Gaua (*gauensis*) and Tanna (*flavifrons*) (Fig. 4a). Others had mixed support depending on the data set considered, e.g. Santo (*brevicauda*) was phylogenetically (mtDNA) distinct, but had similar microsatellite genotypes to *macgillivrayi* on nearby Malekula and these two populations were extremely similar morphologically. The most geographically widespread subspecies (*perplexa*) was found to have a complex pattern of divergence, comprising at least three phylogenetic clusters and two to three population genetic clusters.

Discussion

We identified considerable divergence among insular populations of a single recognized species of island-dwelling bird. The Vanuatu white-eye is paraphyletic on a mitochondrial gene tree and we argue that it is likely to represent the product of at least two independent invasions of the archipelago. The multiple monophyletic island groups within the Vanuatu white-eye lead us to infer that these clades are likely to have experienced long periods of isolation with very little, if any, gene flow between most islands. Insular populations of the Vanuatu white-eye, including those that were polyphyletic on the mitochondrial gene tree, were all significantly divergent at nuclear microsatellite loci, although a close association between some sets of neighbouring populations was revealed by the genotype clustering method. Moreover, the majority of population comparisons showed levels of phenotypic divergence that were statistically significant and exceeded levels of neutral divergence. Given that this species contains considerable evolutionary and phenotypic diversity, it was surprising then that none of the island populations was diagnosable from all others using the 75% diagnosability rule (Patten & Unitt 2002).

Our results show that these island populations contained a large amount of independent evolutionary history, in agreement with several other studies on insular birds (Slikas *et al*. 2000, e.g. Phillimore & Owens 2006; Warren *et al*. 2006; Smith & Filardi 2007; Smith *et al*. 2007). As the Aneityum (*majuscula*) population of Vanuatu white-eye was more closely related to *Zosterops lateralis* than any other *Zosterops flavifrons* population on an mtDNA gene tree and was strongly divergent from all other populations at nuclear loci, we suggest that this may be a cryptic species, *Zosterops majuscula* (Murphy & Mathews 1929). A study on the white-eyes of Micronesia also identified very deep phylogenetic splits among insular populations (Slikas *et al*. 2000), perhaps reflecting the difficulties of delimiting species in this relatively phenotypically uniform genus (Lack 1971). *Zosterops minutus* of Lifou, New Caledonia, should be included in future phylogenetic analyses assessing the sister relationship of *Z. f. majuscula* on Aneityum, due

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to its proximate geography and similar colouration. The deep phylogenetic divergence among the remaining Vanuatu white-eye populations is concordant with a long-recognized divergence in colouration (Mayr 1945; Mees 1969). Within the yellow clade, the affinity of the Gaua population (*gauensis*) with the Efate population of *efatensis,* in particular, was raised earlier by Mees (1969) in his taxonomic study of Zosteropidae. A striking aspect of this affinity, corroborated by mitochondrial and to a lesser extent nuclear DNA microsatellites (of all pairwise comparisons involving Gaua, the standardized θ with Efate is the lowest), is that the Gaua population is more closely related to a population that lies several hundred kilometres to the south than it is to populations on any of the intervening islands or on Vanua Lava (located approximately 30 km to the north of Gaua). It seems quite likely that members of the yellow and darker clades will have dispersed onto islands inhabited by members of the alternate phylogroup at some point in time. Therefore, given the lack of evidence for recent introgression, we infer that that some form of pre- or postzygotic isolation may exist between populations belonging to these two clades.

Across all Vanuatu white-eye populations, the major axis of morphological divergence was body size. Indeed, body size has been identified as a major axis of variation and divergence among other white-eye species and subspecies (Mees 1957, 1961, 1969; Lack 1971; Clegg *et al*. 2002a; Frentiu *et al*. 2007). Phenotypic divergence between populations generally exceeded the neutral genetic divergence, an outcome that is often taken as evidence for directional selection (Spitze 1993; Merilä & Crnokrak 2001). However, great care must be taken in drawing such conclusions from these data, as we did not conduct a formal test or use a quantitative genetic design. Moreover, concerns have been raised about both the validity of the assumptions required (e.g. Hendry 2002; Saether *et al*. 2007) and the statistical approaches used (O'Hara & Merilä 2005) in Q_{ST} vs. F_{ST} tests. Unfortunately, despite identifying significant phenotypic divergence between most pairs of populations, we can say little about the degree of ecological exchangeability, as we do not know whether the phenotypic divergence that we observe has an adaptive basis (Crandall *et al*. 2000; Rader *et al*. 2005).

While morphological differentiation between the majority of island populations was pronounced, very few populations were found to be diagnosable using a standard univariate approach (Amadon 1949; Patten & Unitt 2002). In fact, no pairwise population comparison with a $P_{ST} < 0.9$ (indicating that < 90% of the total morphological variation lies between populations) was found to be diagnosable using this method. Considering the failure of this method to diagnose even strongly phenotypically divergent populations, the arbitrary 75% criterion appears to be very stringent and shows little relation to the evolutionary processes at play. It is straightforward to show using simulations that for two populations to be diagnosable at the 75% level, the P_{ST} for the trait in question (assuming a heritability of 0.5, that between-population differences have a solely genetic basis and a sample size for each population of 100) should exceed approximately 0.91. Whereas, if $P_{ST} = 0.5$, this translates to a diagnosability criterion in the region of just 9%. Therefore, P_{ST} (or ideally Q_{ST}, if a quantitative genetics design to control for environmental variance is possible) may be preferable to the 75% criterion for identifying subspecies on the basis of continuous traits. Moreover, the behaviour of the Q_{ST} statistic has been demonstrated under a variety of evolutionary scenarios (e.g. Spitze 1993; Goudet & Buchi 2006), which means that its use would serve to bridge a gap between evolutionary biology and intraspecific systematics. A drawback of using P_{ST} or Q_{ST} to identify conservation units is that, like the diagnosability approach, these methods would require an arbitrary threshold.

We found that subspecies limits showed some correspondence with the units identified using both phenotypic and molecular data (Fig. 4). Of the seven subspecies considered here, three (*brevicauda*, *flavifrons* and *majuscula*) were monophyletic. The genetic and phenotypic distinctness of the latter population was supported by microsatellite and morphological data. However, both *brevicauda* from Santo and *flavifrons* from Tanna were part of larger genetic clusters when nuclear microsatellites were used. While *flavifrons* was highly divergent in terms of morphometrics, *brevicauda* was found to be morphologically very similar to *macgillivrayi* on Malekula. Of the others: (i) *efatensis* on Efate and Erromango was paraphyletic and should perhaps be split into two subspecies; (ii) *gauensis* on Gaua was not monophyletic, although it was highly divergent from other populations at microsatellite loci and in terms of morphometrics; (iii) *macgillivrayi* on Malekula was paraphyletic with respect to several *perplexa* populations and, based on microsatellites, grouped in a genotypic cluster with populations on neighbouring islands (Santo and Epi); and (iv) *perplexa* was paraphyletic and comprised up to three genotypic clusters. A phylogenetic study of three species that were each monotypic in Vanuatu identified no reciprocally monophyletic units (Kirchman & Franklin 2007). In comparison, a phylogeographical analysis of *Zosterops lateralis* in Vanuatu, which has two to three subspecies described in the archipelago (Mayr 1945; Mees 1969), identified two putative evolutionary units, one monophyletic single-island clade and one widespread paraphyletic group of populations (Phillimore 2006). Moreover, a phylogeographical study conducted on 23 species in the Solomon Islands found that species that are represented by multiple subspecies tended towards greater intraspecific phylogenetic divergence (Smith & Filardi 2007). Overall, there appears to be some correspondence between insular subspecies richness and intraspecific phylogenetic diversity. Thus, in the absence of detailed phylogenetic and population genetic information,

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subspecies richness may be a useful proxy for the number of conservation units in insular systems.

Conservation in island environments

An objective means of quantifying biodiversity within and between forms would help in making informed conservation decisions (Moritz 2002), but different traits and/or approaches can lead to very disparate outcomes (Wilson & Brown 1953). In this study, we found that divergence between populations at mitochondria, nuclear loci and morphology were broadly concordant, although there were a number of discrepancies. Some of the conflicts between the approaches suggested that there may be differences in the mode of evolution of the different characters; for instance, Ambae and Ambrym populations were paraphyletic at mitochondrial loci and weakly divergent at microsatellite loci, but divergent in terms of morphometrics, which is consistent with morphology evolving in a non-neutral manner. Other differences in the number and identity of divergent populations reflect variation in stringency between different methods. Depending on the approach employed, the 13 populations we examined may constitute anything between two and 13 conservation units. From an evolutionary perspective, these different degrees of population divergence reflect the continuum from populations to species (Mayr 1942; Crandall *et al*. 2000).

In order to conserve biodiversity, it is desirable to maintain the processes that are essential for future diversification (Crandall *et al*. 2000; Moritz 2002). Islands are important promoters of intraspecific diversification (Lack 1947; Mayr & Diamond 2001; Phillimore *et al*. 2007) and, as such, their importance for conservation is strongly advocated (Whittaker & Fernandez-Palacios 2007). Interestingly, few cases of island species colonizing continents have been observed (but see Nicholson *et al*. 2004; Filardi & Moyle 2005), which would appear to place a limit on the potential for insular taxa to generate continental diversity. Given that each of the five most species-rich avian families is primarily continental (Sibley & Monroe 1990), it could be argued that islands are not among the most important engines of global diversity. It seems that elevated intraspecific diversification rates on islands do not beget elevated interspecific diversification rates (Phillimore *et al*. 2006, 2007). A potential explanation for this paradox is that extinction rates may also be elevated in insular settings (see Ricklefs & Bermingham 1999; Steadman 2006). Taken together, the available evidence suggests that islands are important promoters of divergent but potentially short-lived populations.

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Supplementary material

The following supplementary material is available for this article:

Table S1 Microsatellite primer sets and PCR conditions for *Zosterops flavifrons*

Table S2 Summary of sample size, number of alleles, observed heterozygosity and expected heterozygosity for microsatellite loci on each island

Table S3 Tests of departure from Hardy-Weinberg equilibrium across populations and loci

Table S4 Summary statistics of morphometrics obtained from different populations

Table S5 Pairwise P_{ST} values and the significance derived from pairwise MANOVA tests

Table S6 Pairwise diagnosability scores for (a) wing length and (b) culmen length to tarsus length ratio

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