Tracking island colonization history and phenotypic shifts in Indian Ocean bulbuls (*Hypsipetes*: Pycnonotidae)

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Molecular phylogenies of island organisms provide useful systems for testing hypotheses of convergent or parallel evolution, since selectively neutral molecular characters are likely to be independent of phenotype, and the existence of similar environments on multiple isolated islands provides numerous opportunities for populations to evolve independently under the same constraints. Here we construct a phylogenetic hypothesis for *Hypsipetes* bulbuls of the western Indian Ocean, and use this to test hypotheses of colonization pattern and phenotypic change among islands of the region. Mitochondrial sequence data were collected from all extant taxa of the region, combined with sequence data from relevant lineages in Asia. Data are consistent with a single *Hypsipetes* colonization of the western Indian Ocean from Asia within the last 2.6 Myr. The expansion of *Hypsipetes* appears to have occurred rapidly, with descendants found across the breadth of its western Indian Ocean range. The data suggest that a more recent expansion of *Hypsipetes madagascariensis* from Madagascar led to the colonization of Aldabra and a secondary colonization of the Comoros. Groupings of western Indian Ocean *Hypsipetes* according to phenotypic similarities do not correspond to mtDNA lineages, suggesting that these similarities have evolved by convergence or parallelism. The direction of phenotypic change cannot be inferred with confidence, since the primary expansion occurred rapidly relative to the rate of mtDNA substitution, and the colonization sequence remains uncertain. However, evidence from biogeography and comparison of independent colonization events are consistent with the persistence of a small grey continental bulbul in India and Madagascar, and multiple independent origins of large size and green plumage in insular island populations of the Comoros, Mascarenes and Seychelles. © 2005 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2005, **85**, 271–287.

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INTRODUCTION

Island-dwelling species have long been used as case studies in convergent evolution (Wallace, 1880; Carlquist, 1974; Givnish, 1998; Grant, 1998). Since island colonization is a replicated historical event in many groups of organisms, similar trends among independent evolutionary lineages can reveal much about general evolutionary forces (Grant, 1998). The geographical isolation of many oceanic island environments makes them inaccessible to a large proportion of continental community diversity; selection pressures experienced by the few continental species reaching an oceanic island may strongly contrast with those experienced in their continental source, yet have much in common with those experienced by related populations reaching other islands. Selection pressures on islands differ from those of continents as a

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result of mild island climate, greater availability of vacant ecological niches and the relative scarcity of predators and competing species (MacArthur & Wilson, 1967; Case, 1978; Roughgarden, 1995). Species which are successful in colonizing an island may further experience higher population densities and associated intraspecific competitive pressures than in their mainland source (MacArthur, Diamond & Karr, 1972).

Whether neighbouring islands or archipelagos are colonized by the same or independent evolutionary lineages, similarities in environmental pressures among independently evolving populations may lead to the evolution of similar phenotypes through parallel or convergent evolution, producing homoplasy. Parallel evolution occurs when two lineages share a common ancestral state and independently undergo the same phenotypic change, while convergent evolution occurs when the same phenotype arises in two lineages from phenotypically different ancestors (Harvey & Pagel, 1991). Implicit in these definitions is the idea that convergent evolution takes place by different developmental pathways in different lineages, while parallel evolution takes place by the same developmental pathways (Futuyma, 1998).

Studies aimed at reconstructing the evolution of individual characters in order to uncover patterns of homoplasy are therefore particularly useful for generating hypotheses concerning the mechanisms behind morphological and ecological diversification in island species. Rigorous examination of such patterns depends on the construction of a robust phylogenetic framework, independent of the traits of interest (Harvey & Pagel, 1991). Only within the last 15 years has the advent of molecular phylogenetic techniques enabled us to infer patterns of morphological evolution using phylogenetic hypotheses which are truly independent of phenotype. A number of such recent studies of island organisms have concluded that phenotypic similarities result from convergent evolution (see Emerson, 2002 for a review). However, evolutionary convergence and parallelism can act at a number of different levels, ranging from the insular woodiness found in many plant taxa (Carlquist, 1974; Böhle, Hilger & Martin, 1996) to the ecomorphs found within Caribbean *Anolis* lizards living in different microhabitats (Losos *et al*., 1998). Further research is needed to establish how widely these phenomena occur, why evolutionary homoplasy occurs in some lineages but not in others (Leal, Knox & Losos, 2002), and to understand the selective pressures and developmental pathways giving rise to convergent and parallel changes in phenotype.

Hypsipetes bulbuls of the western Indian Ocean region provide a good example of the difficulty in unravelling the evolutionary origins of phenotypic diversity in the absence of an independent phylogenetic hypothesis. *Hypsipetes* has a disjunct distribution; it is found throughout tropical Asia, while on the other side of the Indian Ocean it occupies Madagascar and the surrounding islands (Fig. 1). It is, however, absent from continental Africa, much closer to Madagascar than is India. *Hypsipetes madagascariensis madagascariensis* (Statius Müller) is common throughout Madagascar and the lowlands of the Comoros archipelago, as well as occurring on Gloriosa, and differs little in colour and size from *Hypsipetes* in India (*H. m. psaroides* Vigors, *H. m. ganeesa* Sykes and *H. m. nigrescens* Stuart Baker). *H. m. rostratus* (Ridgway) of Aldabra is also morphologically close to these forms. By contrast, all the other *Hypsipetes* taxa of the western Indian Ocean (*H. borbonicus* (J. R. Forster), *H. parvirostris parvirostris* Milne Edwards & Oustalet, *H. p. moheliensis* (Benson), *H. crassirostris* Newton and *H. olivaceus* Jardine & Selby) are morphologically highly diverged from the Indian forms. All are much greener in colour, while the latter three are appreciably larger in size (Table 1).

Existing hypotheses for the origins and relationships of western Indian Ocean *Hypsipetes* can be grouped into two. First, the green and grey bulbuls may represent two independent evolutionary lineages, corresponding to two independent colonizations of the western Indian Ocean. Since the extant *Hypsipetes* of India are similar in colour and general appearance to *H. m. madagascariensis*, it has been proposed that Indian Ocean *H. madagascariensis* (with subspecies *H. m. madagascariensis* and *H. m. rostratus*) represents a recent colonization by Indian stock, while the five green bulbuls represent an earlier one (Danis, 1940; Benson, 1984). The second hypothesis is that western Indian Ocean *Hypsipetes* are a monophyletic group derived from a single colonization of the region from India. In the latter case the phenotypic similarity between green bulbuls from disparate archipelagos must represent either an example of homoplasious evolution in insular environments (Louette, 1987), or, if they share phenotypic characteristics by descent, a very odd pattern of colonization between the Comoros, Seychelles and Mascarenes, avoiding the large intervening landmass of Madagascar.

In this study we use mitochondrial sequence data from all extant western Indian Ocean *Hypsipetes* taxa, along with a sampling of taxa in Asia, to address the origin and diversification of *Hypsipetes* of the western Indian Ocean. Islands of the region are exceptionally diverse in geological origin and age (0.015–88 Myr, Fig. 2), providing many local calibration points for the dating of evolutionary events. Therefore, using topological and branch length information, combined with estimates of divergence times and geological data, we seek to determine which pat-
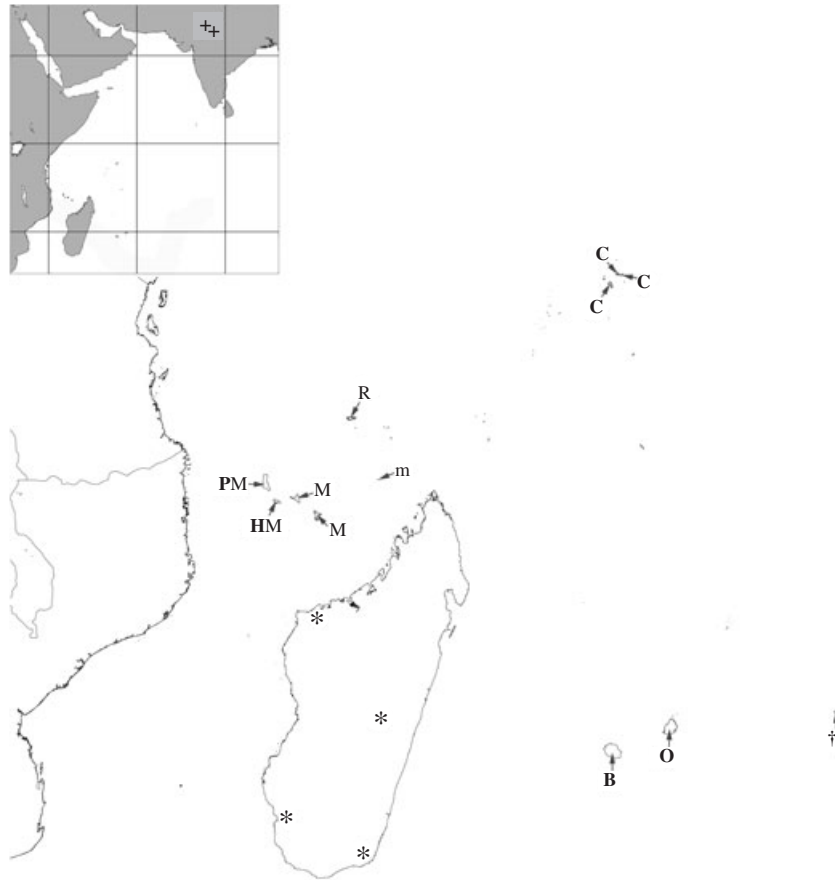
Figure 1. Distribution of *Hypsipetes* of the western Indian Ocean with inset map showing proximity to India. Roman lettering indicates 'grey' populations, bold 'green'. The use of capitals indicates sampled populations, lower case unsampled. *: sampling localities of *H. m. madagascariensis* ('grey'), common throughout Madagascar. +: sampling localities of *H. m. psaroides* ('grey') in India and Pakistan. We follow the taxonomy of Louette & Herremans (1985) and Horne (1987) for all western Indian Ocean forms, and that of Rand & Deignan (1960) for Asian forms. *Abbreviations:* M, *H. m. madagascariensis*; R, *H. m. rostratus*; C, *H. crassirostris*; P, *H. parvirostris parvirostris*; H, *H. p. moheliensis*; B, *H. borbonicus*; O, *H. olivaceus*; †, extinct *Hypsipetes* sp. of Rodrigues.

tern of colonization is most consistent with Indian Ocean *Hypsipetes* relationships, and why taxa in the disparate archipelagos of the Comoros, Seychelles and Mascarenes exhibit similar green phenotypes, while the population on the intervening large island of Madagascar is grey and resembles that of India.

MATERIAL AND METHODS

SAMPLING

We obtained mtDNA sequence data from 32 individuals representing all the extant western Indian Ocean *Hypsipetes* taxa. Two or more individuals of each taxon and of each island population of *H. m. madagascariensis* were sampled (Fig. 1, Table 1), with the exception of the population of Anjouan (for which we were only able to obtain one

sample) and of Gloriosa (which we did not have the opportunity to sample).

Blood samples were obtained during collecting expeditions to Madagascar, the Comoros, Mascarenes, granitic Seychelles and Aldabra between July 1999 and February 2002, and were preserved in Queen's lysis buffer (Seutin, White & Boag, 1991; Seutin *et al.*, 1993). All blood samples were taken non-destructively from mist-netted individuals. We were also generously loaned blood and tissue samples by the Zoological Museum University of Copenhagen (ZMUC), Jersey Zoo (JZ), The Field Museum of Natural History (FMNH) and the Louisiana State University Museum of Natural Science (LSUMNS). These included *H. madagascariensis* from Madagascar and Asia, *H. philippinus* (J. R. Forster) from the Philippines, and outgroup samples from the genera *Criniger*, *Andropadus*, *Bleda*, *Chlorocichla*, *Pycnonotus* and

Table 1. Taxonomy, distribution and phenotype of *Hypsipetes* of the western Indian Ocean and sampled continental populations. We follow the taxonomy of Louette & Herremans (1985) and Horne (1987) for all western Indian Ocean forms, and that of Rand & Deignan (1960) for continental forms

Species	Subspecies	Distribution	Sampling localities	Colour	Size
Western Indian Ocean forms					
madagascariensis	*madagascariensis*	Madagascar	Madagascar (South, West & Central)	Grey	Small
		Lowland Grande Comore	Lowland Grande Comore		
		Lowland Moheli	Lowland Moheli		
		Lowland Anjouan	Lowland Anjouan		
		Lowland Mayotte	Lowland Mayotte		
		Gloriosa	(not sampled)		
madagascariensis	*rostratus*	Aldabra	Aldabra	Grey	Small
parvirostris	*parvirostris*	Highland Grande Comore	Highland Grande Comore	Green	Small
parvirostris	*moheliensis*	Highland Moheli	Highland Moheli	Green	Large
crassirostris		Granitic Seychelles	Mahe, Praslin, La Digue	Green	Large
olivaceus		Mauritius	Mauritius	Green	Large
borbonicus		La Réunion	La Réunion	Green	Small
Continental forms sampled					
madagascariensis	*psaroides*	Himalayas	Pakistan & Western India	Grey	Small
philippinus	*philippinus*	Northern Philippines	Luzon (West & East)		

Figure 2. The geology of the western Indian Ocean. Continental landmasses are indicated in green, volcanic islands in red, coralline and sand islands in blue. Dates correspond to island age in Myr. *Abbreviations:* GC, Grande Comore; MH, Moheli; AJ, Anjouan; MY, Mayotte; AD, Aldabra; AP, Assumption; CM, Cosmoledo; AV, Astove; GS, Gloriosa; FQ, Farquhars; AM, Amirantes; RE, La Réunion; MU, Mauritius; RO, Rodrigues. Modified from Warren *et al.* (2003).

Phyllastrephus. The outgroup genera are all grouped in the Pycnonotidae with *Hypsipetes* (Sibley & Monroe, 1990), and their close relationship to *Hypsipetes* is supported by a recent molecular systematic study (Pasquet *et al.*, 2001). We further sampled *Sylvia* as a more distant outgroup (Sibley & Monroe, 1990). Since the only blood samples that we could obtain from Asian *H. madagascariensis* were from birds in captivity at Jersey Zoo of undetermined Asian origin, we also obtained samples from skins of *H. m. psaroides* of India and Pakistan from The Natural History Museum, Tring, UK (BMNH).

A total of 351 bp of sequence data was obtained for all individuals from the complete mitochondrial NADH dehydrogenase subunit 3 (ND3) gene. To improve the resolution of phylogenetic relationships we selected one sample from each of the principal lineages of interest in the ND3 dataset, and supplemented these data with full mitochondrial ATP synthase 6 (ATPase6), and 8 (ATPase8) sequences, providing a total of 1203 bp of sequence data.

DNA EXTRACTION, PCR AND SEQUENCING

Blood and tissue samples
DNA was extracted following the phenol-chloroform protocol of Seutin *et al.* (1993), except that the final suspension was purified by dialysis instead of ethanol precipitation. Polymerase chain reaction (PCR) amplification of two regions of the mitochondrial genome was performed for all individuals. The primer pair L10755 and H11151 (Chesser, 1999) was used to amplify and sequence a fragment which included the entire ND3 gene. For a subset of samples representing each of the principal evolutionary lineages, the primer pair CO2GQL and CO3HMH (http://nmg.si.edu/bermlab.htm) was used to amplify and sequence a 1018 base pair fragment which included the genes ATPase6, ATPase8, and adjacent regions. PCR conditions are listed in Table 2. The steps following PCR have been described elsewhere (Warren *et al.*, 2003).

Museum skin samples
Techniques appropriate for 'ancient DNA' were used for obtaining sequences from samples taken from museum skins. All steps prior to PCR were performed in a designated laboratory where no genetic or other biological work had been previously carried out, on a separate floor from the primary genetics laboratories. No PCR amplifications were carried out in the laboratory, nor were any PCR products or nonancient DNA extracts stored there. Work followed strict procedures to prevent contamination. Only new equipment was used, and gloves and lab coat were worn at all times. All benches and equipment, including pipettors, were cleaned with 10% bleach solution prior to initiating all

Table 2. Primers and experimental conditions used for *Hypsipetes* PCR amplification and DNA sequencing

PCR conditions						
Gene region	Primer names	Source	Denaturation	Annealing	Extension	Number of cycles
ATPase 6&8	CO2GQL & CO3HMH	http://nmg.si.edu/bermlab.htm	94 °C for 45 s	55 °C for 45 s	72 °C for 1 min	30
ND3	L10755 & H11151	Chesser, 1999	94 °C for 45 s	50 °C for 45 s	72 °C for 1 min	30

DNA extractions and PCRs. Filter tips were used in all steps prior to PCR.

Pieces of skin were taken from the feet of BMNH specimens following the procedure of Mundy, Unitt & Woodruff (1997). In the designated laboratory, each sample was first left to soak in 1 mL of 10 mM Tris pH 8 for 2 h to leach out tannins that otherwise interfere with DNA extraction. Samples were chopped finely, using a new sterile scalpel blade and gloves for each sample. Each chopped sample was added to 480 μL of digestion buffer (10 mM Tris pH 8, 6% Chelex resin), along with 15 μL of 20 mg/mL Proteinase K solution, and incubated at 55 °C for 3 h. In addition, a blank extraction control was included in each batch of extractions. Following digestion, each incubated tube was vortexed briefly, heated at 100 °C for 8 min (to inactivate any DNAse), and centrifuged for 3 min at 14 170 *g*. The supernatant was then washed and concentrated in Microcon YM-100 columns (Millipore UK Ltd) topped up with 10 mM Tris pH 8. Two concentration spins were performed at 480 *g* for 10 min. PCR reactions were prepared in the ancient DNA laboratory, while amplification and the steps following PCR were performed in the primary genetics laboratory following the procedures described above. Two to three rounds of PCR were used to amplify ND3 with the same primer sequences and PCR cycles used for the other *Hypsipetes* samples. Each round of PCR included a PCR blank, and the first round of PCR included a tube where the blank extraction control was used in place of extracted DNA. As with all tubes, the two control tubes were used to prime further tubes in the second and third rounds of PCR, while also adding another PCR blank for each new round of PCR.

PHYLOGENETIC ANALYSIS

ND3 for the full sample set

An unweighted parsimony analysis was performed on the combined dataset using the heuristic search algorithm, holding ten trees at each step and branch swapping on all trees, using the steepest descent option. In addition, the full data set excluding outgroups (non-*Hypsipetes* samples) was analysed in MODELTEST (Posada & Crandall, 1998) to determine the substitution model which best describes the data.

Bayesian analysis was performed using MrBayes v. 2.01 (http://morphbank.ebc.uu.se/mrbayes/) (Huelsenbeck *et al*., 2001). This program implements the Markov Chain Monte Carlo (MCMC) algorithm to approximate the posterior probability distribution of a large number of trees. Starting from random initial trees, it proposes a new tree by stochastically perturbing a previous tree. The new tree can either be accepted or rejected based on its probability; if accepted, it is subject to further perturbation. The pro-

portion of the time that any tree is visited in the chain is taken as an approximation of its posterior probability (PP). Furthermore, the PP of any clade is the sum of the PPs of all the trees that contain that clade (Huelsenbeck *et al*., 2001). Base frequencies were estimated from the data. Four Markov chains were run simultaneously for 2 million generations, and sampled every ten generations. Variation in the maximum likelihood (ML) scores in this sample was examined graphically. The trees generated prior to stationarity were discarded, and the consensus phylogeny and posterior probability of the nodes were determined from the last 150 000 trees in the chain. To check our results and guard against the possibility of multiple optima, we ran this analysis a second time for 5 million generations and used the last 450 000 trees to determine the consensus phylogeny and posterior probability of nodes.

ATPase6, ATPase8 and ND3 data for the restricted sample set

The congruence of the ATPase6, ATPase8 and ND3 data sets was checked using the partition homogeneity test (Farris *et al*., 1995) implemented in PAUP*, and data from these three genes were combined for further analysis. The combined data set was analysed in MODELTEST. The optimal model thus defined was used to determine the ML distances for both a neighbour-joining (NJ) analysis in PAUP* and a ML analysis in PAUP* with the heuristic search algorithm. In addition, an unweighted parsimony analysis was performed as above. Bayesian analysis followed the above procedures, with the first analysis run for 2 million generations. The consensus phylogeny and posterior probability of its nodes were determined from the last 150 000 trees. The final analysis was run for 5 million generations and the last 450 000 trees were used to obtain the consensus.

ESTIMATION OF DIVERGENCE TIMES AND DIRECTION OF ISLAND COLONIZATION

Rate heterogeneity was first tested using PAUP* to determine whether branch lengths were consistent with a molecular clock. Ages were assigned to nodes in the tree based on geological estimates of island ages. Where closely related taxa are found on neighbouring islands we consider that the age of the younger island represents an approximate estimate for the maximum age of the split between these lineages. Fleischer, McIntosh & Tarr (1998) and Warren *et al*. (2003) summarize eight assumptions made when dating nodes with this method.

Based on the Bayesian tree for the full ATPase6, ATPase8, and ND3 data, we employed the Topology Plus Branch Length method (Thorpe *et al*., 1994),

which makes use of tree topology and branch lengths to infer sequence and direction of inter-island colonization under the assumption of speciation following dispersal. This method has a number of underlying assumptions (Thorpe *et al.*, 1994), but basically argues that when an ancestral population (a) on island A colonizes island B, the resulting populations (a′) on island A and (b) on island B will not be equally divergent from (a) and that (b) will be more divergent than (a′) due to founder effects. Applying this principle to *Hypsipetes*, nodes gaining ≥ 95% Bayesian branch support were assigned the distribution occupied by the anagenically closest descendant.

CHARACTERIZATION OF PHENOTYPIC VARIATION

We chose to follow changes in body size and plumage colour through the tree, because these traits are easy to characterize from the data available and also appear to correlate with other biological characteristics of Indian Ocean *Hypsipetes*. For example, while grey forms are gregarious, green forms tend to be more furtive and are normally sighted alone or in pairs (Sinclair & Langrand, 1998; pers. observ.). Green forms also have reduced flight capabilities (Meirte, 1987). Although body size and colour are continuous variables, a clear dichotomy in both variables can be observed amongst *Hypsipetes* taxa within the western Indian Ocean region. We therefore categorize each taxon as either grey or green, following Louette (1987), and as either small or large (Table 1). Standard deviation in wing and bill length within Indian Ocean populations is high, while standard deviation in tarsus length is much lower (Louette & Herremans, 1985). These statistics accord well with studies of other avian groups, suggesting that tarsus length is the best estimator of body size (e.g. Merilä & Fry, 1998). *Hypsipetes* tarsus length data from the taxonomic literature indicate a natural break in length, with none of the taxa under study having mean tarsus length between 22.3 and 24.7 mm (Ali & Ripley, 1971; Louette & Herremans, 1985). We therefore define taxa with mean tarsus length less than 22.3 mm as 'small', and those with mean tarsus length greater than 24.7 mm as 'large'.

RESULTS

PHYLOGENETIC ANALYSIS
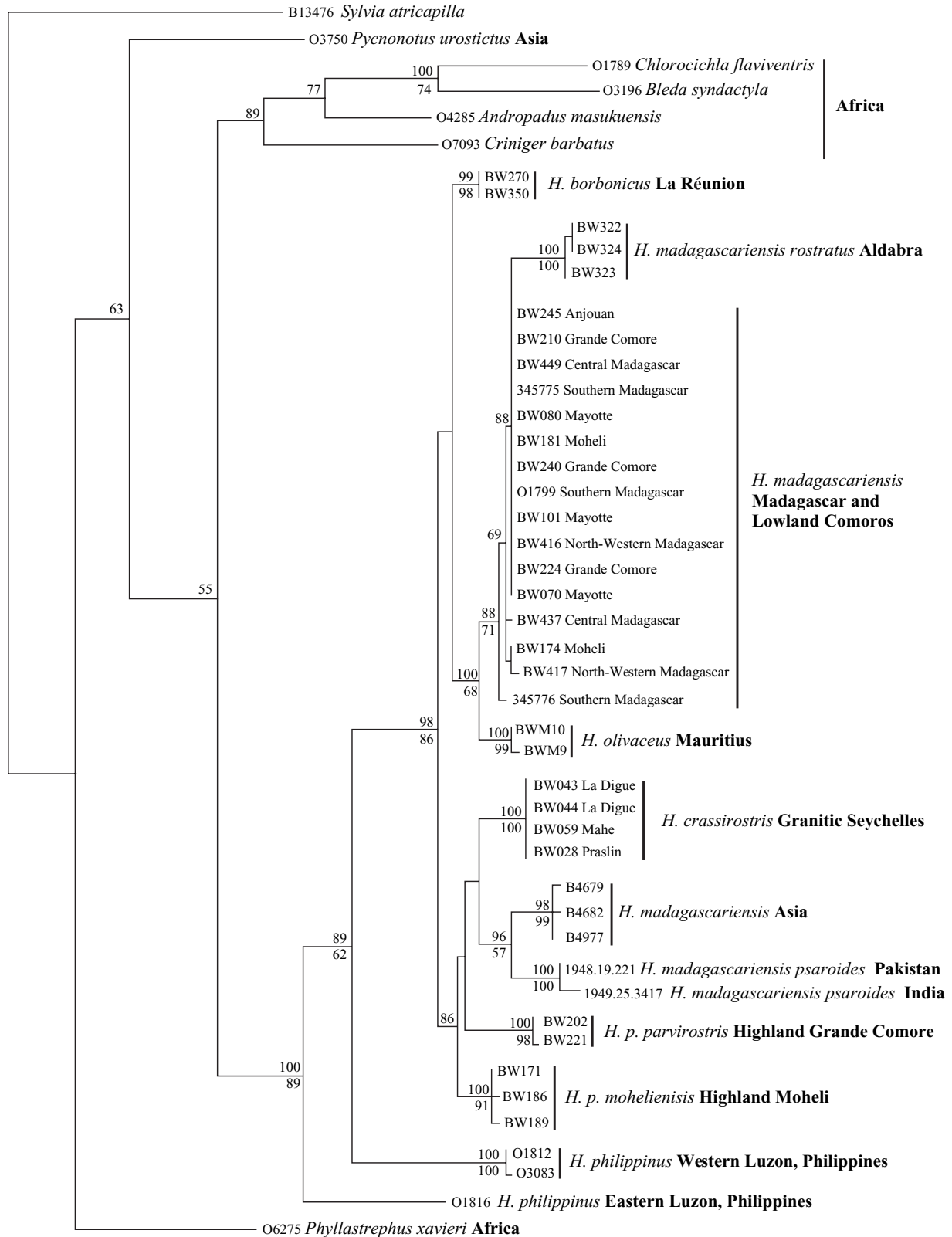
ND3 data for the full sample set

An unweighted parsimony analysis resulted in 52 most parsimonious trees with length 406. A strict consensus was calculated, and bootstrap values for this consensus tree were obtained (stepwise-addition, 1000 replicates). MODELTEST identified the TIM model of DNA substitution (Rodriguez *et al.*, 1990) with gamma shape parameter (TIM + G) as best describing the data under the Akaike information criterion. Estimates of substitution rates under this model are A-C, 1; A-G, 24.6115; A-T, 0.2143; C-G, 0.2143; C-T, 8.3114; G-T, 1. The gamma distribution shape parameter is estimated as 0.1917. For Bayesian analysis we used a GTR with gamma distribution shape parameter model of DNA substitution, given that it is the model available in MrBayes that best matches the TIM + G model.

Maximum parsimony (MP) and Bayesian analyses yielded identical tree topologies with respect to all nodes gaining ≥ 95% Bayesian branch support. The Bayesian tree (Fig. 3) demonstrates that *Hypsipetes* is a monophyletic genus (100% Bayesian branch support) and that all taxa from the western region form a monophyletic group which includes *H. madagascariensis* from Asia, but excludes *H. philippinus* from the Philippines (98% Bayesian branch support).

A basal polytomy in the clade containing Indian Ocean *Hypsipetes* separates five Indian Ocean lineages from a monophyletic lineage (96% Bayesian branch support) containing all our samples of *H. madagascariensis* from Asia. *H. madagascariensis* is therefore a polyphyletic group; the Asian and Malagasy lineages do not share an exclusive common ancestor. Within the Asian *H. madagascariensis* lineage, *H. m. psaroides* from India and Pakistan pairs as the sister to birds in Jersey Zoo obtained through the pet trade from Asia.

Of the five principal Indian Ocean lineages, four consist of single taxa: *H. borbonicus* of La Réunion, *H. crassirostris* of the granitic Seychelles, *H. parvirostris parvirostris* of Grande Comore and *H. p. moheliensis* of Moheli. In the fifth lineage, *H. olivaceus* of Mauritius pairs as the sister to *H. madagascariensis* of Madagascar, the Comoros and Aldabra with 100% Bayesian branch support. While these Indian Ocean *H. madagascariensis* haplotypes form a monophyletic group, this receives only 88% Bayesian branch support. Within the haplotypes, Aldabra birds (*H. m. rostratus*) form a distinct clade (100% Bayesian branch support) divergent from the Madagascar and Comoro haplotypes (minimum eight substitutions, 2.3% absolute distance). One Madagascar haplotype pairs as the sister to all other Indian Ocean *H. madagascariensis* haplotypes, while three haplotypes from Madagascar and one from the Comoros fall outside of a monophyletic group containing the samples from Aldabra and 12 other samples with identical haplotypes (eight from the Comoros and four from Madagascar). However, the latter two relationships gain only 88% Bayesian branch support. Regard-

B13476 *Sylvia atricapilla*

O3750 *Pycnonotus urostictus* **Asia**

100
77
74
O1789 *Chlorocichla flaviventris*
O3196 *Bleda syndactyla*

89
O4285 *Andropadus masukuensis*

O7093 *Criniger barbatus*

Africa

99
98
BW270
BW350
H. borbonicus **La Réunion**

100
100
BW322
BW324
BW323
H. madagascariensis rostratus **Aldabra**

63

55

88
BW245 Anjouan
BW210 Grande Comore
BW449 Central Madagascar
345775 Southern Madagascar
BW080 Mayotte
BW181 Moheli
BW240 Grande Comore
O1799 Southern Madagascar
BW101 Mayotte
69
BW416 North-Western Madagascar
BW224 Grande Comore
BW070 Mayotte
88
71
BW437 Central Madagascar
BW174 Moheli
100
68
BW417 North-Western Madagascar
345776 Southern Madagascar

H. madagascariensis
**Madagascar and
Lowland Comoros**

98
86
100
99
BWM10
BWM9
H. olivaceus **Mauritius**

100
100
BW043 La Digue
BW044 La Digue
BW059 Mahe
BW028 Praslin
H. crassirostris **Granitic Seychelles**

89
62
96
57
98
99
B4679
B4682
B4977
H. madagascariensis **Asia**

100
100
1948.19.221 *H. madagascariensis psaroides* **Pakistan**
1949.25.3417 *H. madagascariensis psaroides* **India**

86
100
98
BW202
BW221
H. p. parvirostris **Highland Grande Comore**

100
89
100
91
BW171
BW186
BW189
H. p. mohelienisis **Highland Moheli**

100
100
O1812
O3083
H. philippinus **Western Luzon, Philippines**

O1816 *H. philippinus* **Eastern Luzon, Philippines**

O6275 *Phyllastrephus xavieri* **Africa**

Figure 3. Bayesian analysis of the *Hypsipetes* mitochondrial ND3 dataset for all samples. Consensus of the last 450 000 trees after 5 million generations based on the GTR + G model. Bayesian branch support values are indicated above MP bootstrap values (stepwise-addition, 1000 replicates).

less of tree topology, haplotype diversity and divergence within the Madagascar samples (0–3 substitutions, 0.49% mean absolute distance) is higher than within the Comoro samples (0–2 substitutions, 0.13% mean absolute distance). Within the granitic Seychelles archipelago we find no divergence in ND3 between birds on the three main islands of Mahe, Praslin and La Digue.

ATPase6, ATPase8, and ND3 data for the restricted sample set

The partition homogeneity test (Farris *et al.*, 1995) on the combined mtDNA data (three partitions; 1203 bp) indicated that the ATPase6, ATPase8, and ND3 regions did not differ significantly ($P = 0.69$). We therefore combined these three data sets for further analysis. An unweighted parsimony analysis resulted in two most parsimonious trees with length 365 (CI = 0.721, RI = 0.764, RC = 0.550, HI = 0.279). MODELTEST again identified the TIM + G model of DNA substitution (Rodriguez *et al.*, 1990) as best describing the data under the Akaike information criterion. Estimates of substitution rates under this model are A-C, 1; A-G, 30.8293; A-T, 0.4432; C-G, 0.4432; C-T, 8.9684; G-T, 1. The gamma distribution shape parameter is estimated as 0.1481. We used these parameters and nucleotide frequencies in the NJ and ML analyses. For Bayesian analysis we again used a GTR with gamma distribution shape parameter model of DNA substitution, given that it is the model available in MrBayes that best matches the TIM + G model.

Since the ND3 data support a clear sister relationship of *H. philippinus* to our other *Hypsipetes* samples, we used *H. p. philippinus* as the outgroup in our combined (ATPase6, ATPase8 and ND3) analysis. ML, MP, NJ and two Bayesian analyses yield identical tree topologies with respect to all nodes gaining ≥ 95% Bayesian branch support, and in addition, tree topology for the combined data (Fig. 4) is fully concordant with the topology of the Bayesian tree for ND3 data alone, with respect to nodes gaining ≥ 95% Bayesian branch support in the ND3 tree.

The extra sequence data in the combined dataset increase the resolution of phylogenetic relationships in certain parts of the tree. All the Indian Ocean *Hypsipetes* appear to form a monophyletic group, excluding the Asian *H. madagascariensis*. Although this relationship receives only 87% Bayesian branch support, it is further supported by branch lengths (Fig. 4) which show that the internodal distance between nodes 1 and 2 is more than double that of any of the distances between nodes 2, 3, 4, 5 and 7. Five principal Indian Ocean *Hypsipetes* lineages (*H. p. moheliensis*, *H. crassirostris, H. p. parvirostris*, *H. borbonicus*, and *H. olivaceus* + *H. m. madagascariensis* + *H. m. rostra-*

tus) are separated by very short internodes that lack Bayesian branch support (all values less than 60%). Figure 5 presents a nucleotide saturation analysis of the combined mtDNA data, and plots the absolute number of observed substitutions against corrected patristic distances based on TIM + G. The slope of this relationship indicates that nucleotide substitutions are not saturated in these comparisons, and thus we infer that the basal diversification of Indian Ocean *Hypsipetes* was rapid relative to the rate of mtDNA substitution.

The ATPase6-ATPase8-ND3 data further adds to the ND3 data in confirming that Indian Ocean *H. madagascariensis* is a monophyletic group (100% Bayesian branch support) sister to *H. olivaceus*, and that haplotypes from Madagascar and the Comoros form a monophyletic group (99% Bayesian branch support) sister to *H. m. rostratus* of Aldabra.

ESTIMATION OF DIVERGENCE TIMES AND DIRECTION OF ISLAND COLONIZATION

For the purposes of dating the phylogeny we collapse nodes 3, 4 and 7 (Fig. 4), all of which gain very low (≤ 59%) Bayesian branch support. There are two nodes (2 and 6; Fig. 4) in the ML tree which can be used as calibration points, as the maximum age of divergence can be inferred. First, the youngest island occupied by an island endemic lineage branching from the polytomy at node 2 is Grande Comore. Therefore the maximum age for node 2 is taken as the earliest possible colonization of Grande Comore, and dated on the estimated origin of this island at 0.5 Mya. Second, *H. madagascariensis* is found on Madagascar, Aldabra, and the intervening islands of the Comoros, of which Aldabra is the youngest island. Therefore the maximum age for the node separating the Aldabra lineage from the Madagascar and Comoro haplotypes (node 6) is taken as the earliest possible colonization of the Aldabra group since the last inundation of Aldabra Atoll (0.125 Mya). A log likelihood test rejected the null hypothesis of rate constancy with all taxa included. Following the removal of multiple *H. madagascariensis* haplotypes from Madagascar and the Comoros with the retention of one sequence at random, the data failed to reject the null hypothesis (–lnL clock-enforced tree = 3255.61842, –lnL unconstrained tree 3250.48426, $\chi^2 = 10.27$, d.f. = 9, $P > 0.25$). We therefore used the ML tree constructed under the assumption of a molecular clock with the multiple *H. madagascariensis* haplotypes removed for estimating divergence times.

Clock calibrations based on the dating of nodes 2 and 6 yield closely compatible divergence times across the tree. However, divergence rates associated with these clock calibrations (13.9 and 15.3% per Myr,
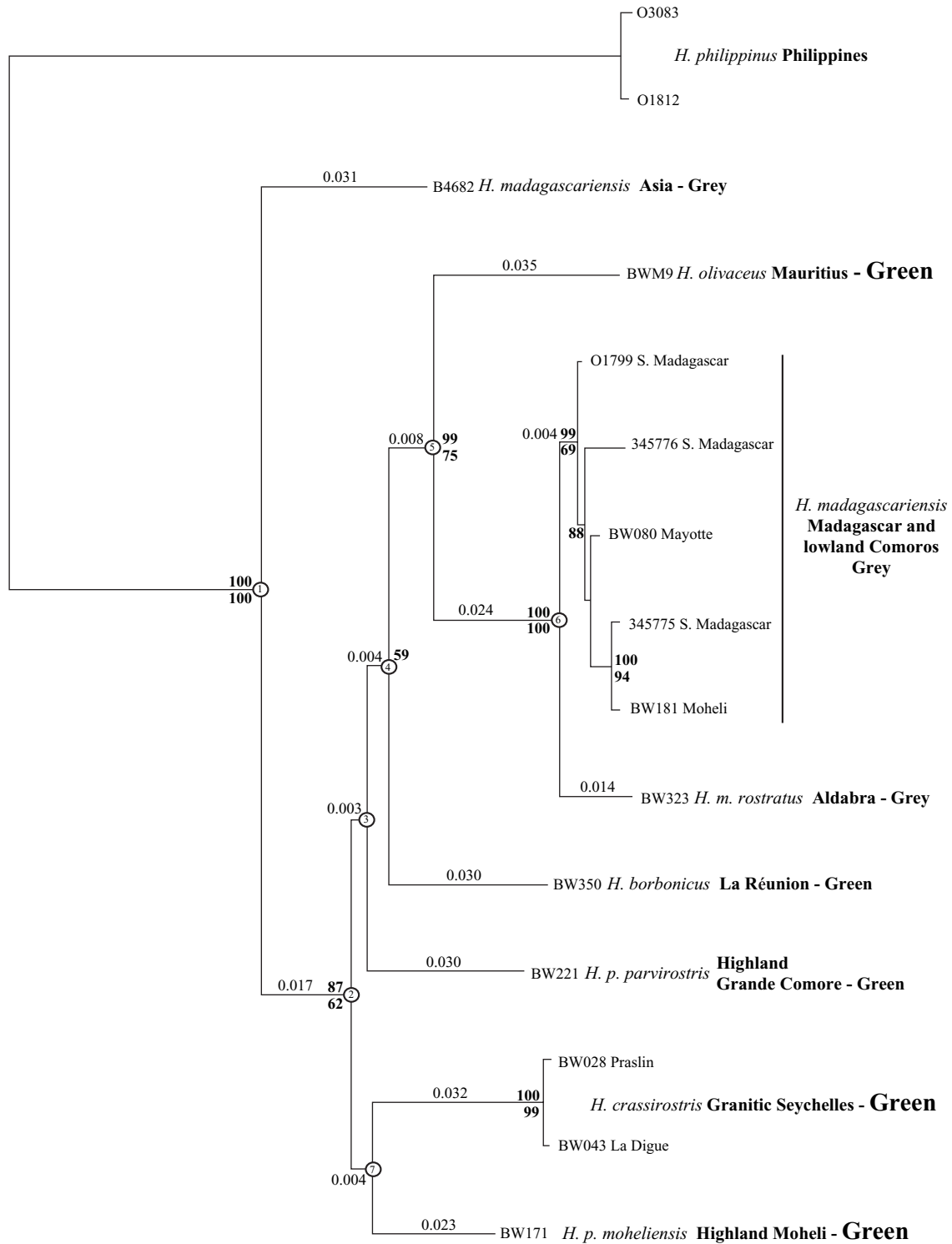
Figure 4. Bayesian analysis of the mitochondrial ATPase6, ATPase8 and ND3 dataset for the principal Indian Ocean *Hypsipetes* lineages. Consensus of the last 450 000 trees after 5 million generations based on the GTR + G model. Bayesian branch support values are indicated above ML bootstrap values (stepwise-addition, 1000 replicates). *Hypsipetes* taxa with the green plumage phenotype are labelled 'Green', while those with the grey phenotype are labelled 'Grey'. Large and small font sizes are used to distinguish, respectively, between taxa with large body size (*H. olivaceus*, *H. crassirostris* and *H. p. moheliensis*) and those with small body size (*H. madagascariensis*, *H. m. rostratus*, *H. borbonicus* and *H. p. parvirostris*).
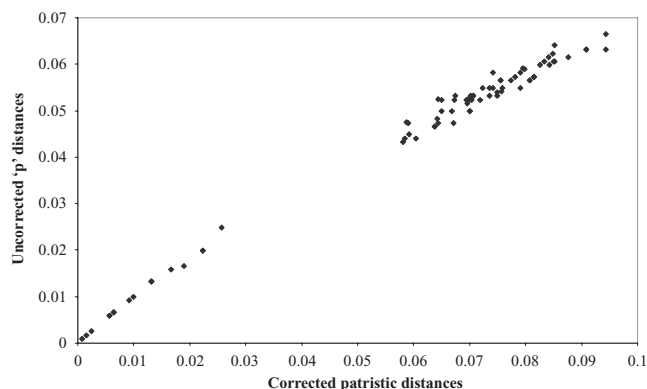
Figure 5. Plot of uncorrected p distances against corrected patristic distances (using the TIM + G model) for the combined *Hypsipetes* mtDNA dataset with outgroup excluded.

respectively) are much higher than the 2% tick rate of the commonly used passerine mtDNA cyt *b* clock (Fleischer *et al.*, 1998). For comparison we experimented by calibrating node 2 as 2.1 Mya, corresponding to the estimated origin of La Réunion, the second youngest island occupied by a lineage derived from this polytomy. The latter dating results in a tick rate of 3.3% per Myr and much older divergence times. Here we treat La Réunion and Grande Comore clock calibrations as maximum and minimum estimates for the timing of divergence events. On this basis, the divergence of western Indian Ocean *Hypsipetes* from continental stock (Fig. 4, node 1) is dated at 0.6–2.6 Mya, while the rapid early expansion of *Hypsipetes* in the western Indian Ocean originated at 0.5–2.1 Mya (Fig. 4, node 2). The divergence of *H. olivaceus* and *H. m. madagascariensis* (Fig. 4, node 5) is dated at 0.4–1.8 Mya.

The Topology Plus Branch Length method of Thorpe *et al.* (1994) applied to node 6 results in a colonization of Aldabra by the *H. madagascariensis* population of Madagascar and the Comoros. This result conforms to the geological history of the region. Since the early divergence of *H. madagascariensis* from *H. olivaceus* (within the interval of 0.4–1.8 Mya) predates the last complete submergence of the Aldabra Group (0.125 Mya), it follows that Aldabra was colonized from the population in Madagascar and the Comoros, and not vice versa. Whether the founding birds came from Madagascar or from the Comoros cannot be determined, as there is a lack of consistent mtDNA divergence between *H. madagascariensis* in these two areas. The Topology Plus Branch Length method cannot be applied to node 5 (between *H. olivaceus* and *H. m. madagascariensis*) because branch lengths within *H. m. madagascariensis* are variable in length, with some haplotypes being anagenically closer to

node 5 than *H. olivaceus*, while others are more distant.
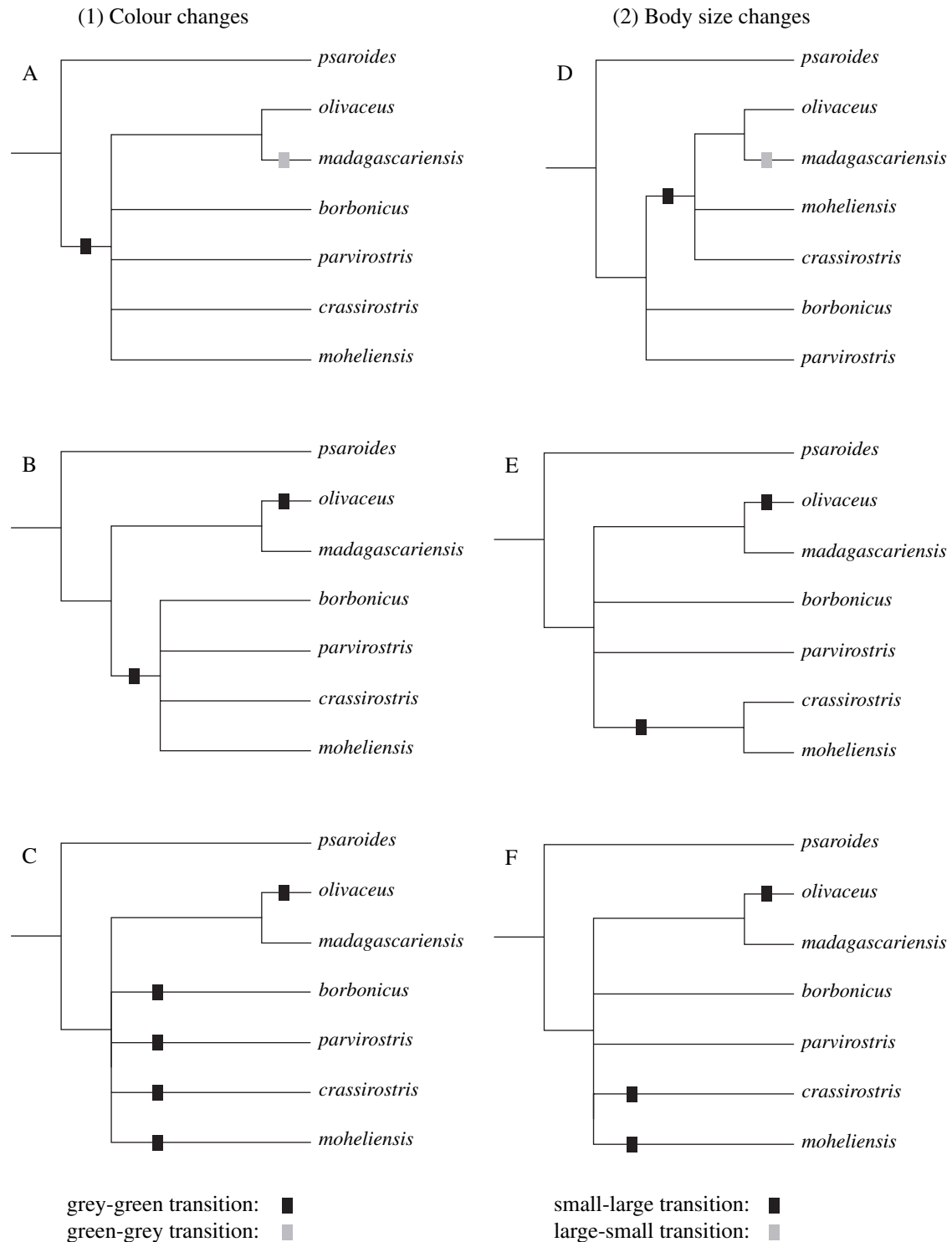
PHENOTYPIC CHANGES

Phenotypic traits were mapped onto the Bayesian phylogeny (Fig. 4). In interpreting patterns of phenotypic change we assume that the ancestral state was small and grey (a reasonable assumption given the characteristics of *H. madagascariensis* races on the Indian subcontinent), and weight gains and losses of phenotypic characters equally. Our results show that none of our four phenotypic bulbul groupings (grey, green, small and large) constitutes a monophyletic group, and that at least two changes in character state of both size and colour are required to yield the observed phylogenetic placement of forms. For clarity of presentation, Figure 6 illustrates trees in which node 2 (87% Bayesian branch support) is maintained. However, the same outcome is achieved when we collapse this node; following the evolutionary lineage giving rise to *H. madagascariensis* of Madagascar, either the lineage has evolved from grey to green to grey (Fig. 6A), or it has remained grey throughout, with between two and five independent origins of the green phenotype (Fig. 6B, C). Likewise, following body size changes through the same lineage results in either a small-large-small evolutionary sequence (Fig. 6, D), or a continuously small lineage giving rise to between two and three independent origins of large body size (Fig 6E, F).

DISCUSSION

PHYLOGENETIC RELATIONSHIPS AND HISTORICAL ORIGINS OF INDIAN OCEAN BULBULS

The polyphyletic relationships of the grey and green *Hypsipetes* of the western Indian Ocean provide clear evidence that these lineages do not result from independent colonizations of the region. Our data do not, therefore, support the hypotheses of Danis (1940) and Benson (1984). As we know from the ND3 data that our Asian *H. madagascariensis* samples form a monophyletic group with the Indian and Pakistani *H. m. psaroides* samples, we take the phylogenetic placement of Asian *H. madagascariensis* to be representative of the placement of *H. madagascariensis* of the Indian subcontinent. Our results (Fig. 4) demonstrate that *H. madagascariensis* consists of separate evolutionary lineages in Asia and the western Indian Ocean and does not constitute a monophyletic group.

That *Hypsipetes* colonized the western Indian Ocean from Asia and not vice versa is supported by tree topology, as *H. philippinus* of the Philippines is sister to the entire monophyletic clade containing Indian and western Indian Ocean *Hypsipetes* (Fig. 4), and none of the

Figure 6. Possible sequences of character state changes leading to the observed phenotypic patterns in western Indian Ocean *Hypsipetes*. The placement of continental Asian *H. madagascariensis* is represented by the subspecies *psaroides*, while all other names refer to western Indian Ocean forms. A, grey-green-grey plumage sequence with two changes of character state. B, multiple origins of green plumage with a minimum of two changes of character state. C, multiple origins of green plumage with a minimum of five changes in character state. D, small-large-small body size sequence with two changes of character state. E, multiple origins of large body size with a minimum of two changes of character state. F, multiple origins of large body size with a minimum of three changes in character state.

genera closely related to *Hypsipetes* are native to the western Indian Ocean (Fig. 3; Pasquet *et al.*, 2001). The monophyly of western Indian Ocean *Hypsipetes* (Fig. 4) is therefore consistent with a single colonization of the region from Asia. Although this monophyletic relationship receives only 87% Bayesian branch support, it is further supported by the congruent topology of Bayesian, ML, MP and NJ trees, and the small internodal branch lengths between Indian Ocean forms compared with those between these forms and their closest continental relative. Further, the rapid basal diversification of Indian Ocean *Hypsipetes* provides clear evidence that a lineage of Asian origin underwent a wave of range expansion across the region within a single short period of time. Therefore, a single Asian origin for these forms is likely.

That an Asian bulbul lineage colonized the Indian Ocean rather than an African one might seem surprising given the much greater distance of Madagascar from Asia (3800 km) than from Africa (420 km). However, data on sea level changes over the last 0.5 Myr show many sea level fluctuations with lowstands of up to 139 m below present sea level, some of which persisted for up to 50 000 years at a time (Rohling *et al.*, 1998). Geological records going back further suggest lowstands of 80–100 m below present sea level at around 1.6 Mya and 2.4 Mya (Haq, Hardenbol & Vail, 1987). Assuming relatively constant ocean floor topography, a drop in sea level of 80 m or more would result in much larger landmasses being exposed in the granitic Seychelles and at other sites along the Mascarene bank, with a chain of smaller islands appearing between these landmasses and India. This chain of islands could have served as a series of stepping stones for a *Hypsipetes* colonization of the Indian Ocean from India at 0.6–2.6 Mya, greatly reducing the distance of open ocean to be crossed.

We infer from the basal polytomy in Indian Ocean *Hypsipetes* that, following initial arrival in the region, a rapid expansion occurred across Madagascar, the granitic Seychelles, Comoros and Mascarenes. Bones of an extinct species of *Hypsipetes* have been collected on Rodrigues (Cowles, 1987), the most easterly Mascarene island, and we speculate that this species may also be derived from this early expansion. With the exception of the Mascarenes, the colonization pattern proposed by Louette (1987) cannot be rejected. Under this hypothesis, *Hypsipetes* first reached Madagascar from India, and then proceeded to colonize the surrounding archipelagos. However, it is equally possible that *Hypsipetes* first arrived in one of the island archipelagos (e.g. the Seychelles, which are closer to India than is Madagascar), thence proceeding to Madagascar and the other archipelagos.

Following the early expansion of Indian Ocean *Hypsipetes*, four of the five resulting lineages show a continuous history of evolutionary independence with a lack of gene flow between islands. Our analysis does not support the conspecific status of the two races of *H. parvirostris* of highland areas of neighbouring Grande Comore and Moheli; their phylogenetic placement suggests that the two island populations were established during the rapid early expansion phase, and have been evolutionarily independent for as long as all other full species in the western Indian Ocean.

Within Indian Ocean *H. madagascariensis* our results are consistent with Louette's (1987) hypothesis that the populations of Aldabra and the Comoros represent recent colonizations from Madagascar. Given the result of the Topology Plus Branch Length method, in combination with divergence time estimates, we infer that the first of these islands to be colonized was Aldabra, following the last major inundation of this atoll at 0.125 Mya. Based on both ND3 and combined (ATPase6, ATPase8 and ND3) data, bulbuls from the lowlands of all four Comoro islands show no consistent divergence from the Madagascar population. However, ND3 haplotype diversity and divergence within our sample of Madagascar grey bulbuls is higher than that within our sample of the Comoro grey bulbuls. This fact, in combination with the much greater size of Madagascar compared with all four Comoro islands, leads us to favour a colonization of the Comoro lowlands from Madagascar rather than the reverse. In addition, the absence of consistent sequence divergence between Madagascar and Comoro populations of *H. m. madagascariensis* implies that either the colonization of these islands has been very recent, or that there is ongoing gene flow between the Comoros and Madagascar.

The sister relationship between Indian Ocean *H. madagascariensis* and *H. olivaceus* of Mauritius is contrary to Louette's (1987) Mascarene *Hypsipetes* colonization hypothesis, in which *Hypsipetes* proceeds in a stepwise fashion from Madagascar to La Réunion to Mauritius. While *H. m. madagascariensis* is found on the Comoros, on geographical grounds it would appear highly unlikely that *Hypsipetes* colonization has proceeded between the Mascarenes and Comoros, since the two archipelagos are separated by the much larger landmass of Madagascar. A direct colonization of Mauritius from Madagascar therefore seems most likely, especially given the much larger size of the latter.

PATTERNS OF CONVERGENCE IN INDIAN OCEAN BULBUL PHENOTYPE

The polyphyletic origins of *Hypsipetes* groupings (grey, green, small and large) demonstrate that phenotypic similarities between different taxa are homoplasious and result from evolution in similar environments, rather than shared common ancestry. However, the

question remains as to the direction of evolutionary change.

Two alternative hypotheses may be proposed. First, the evolutionary sequence may have proceeded from small-grey to large-green and back to small-grey (Fig. 6A, D). This implies that the ancestral lineage colonizing the Indian Ocean from India became green and then large on first arriving in the region, but later reverted to a small grey phenotype upon reaching the large 'continental island' of Madagascar. Second, the evolutionary sequence may consist of multiple origins of green and large phenotypes (Fig. 6B, C, E, F). This implies that the ancestral lineage colonized Madagascar and remained relatively undiverged morphologically from the parental population in India, but underwent at least two independent origins of large size and green plumage following colonization of the surrounding archipelagos (Seychelles, Comoros and Mascarenes).

While we have no way to categorically reject either of these two possibilities, several lines of evidence appear to support multiple origins of the green and large phenotypes. First, the distribution of the 'large' phenotype is inconsistent with a common origin for this character state, since large birds occupy disparate islands (the granitic Seychelles, Moheli and Mauritius). Indeed, the large *Hypsipetes* species appear to be those occupying the most inaccessible islands from Madagascar (Louette, 1987). Mauritius is more distant from Madagascar than La Réunion, and while Grande Comore and Moheli are roughly the same distance from Madagascar, Moheli is much the smaller in size. The granitic Seychelles is the most isolated of all the archipelagos under study. The same observations apply to the origin of green plumage, since the distribution of the green birds (Mascarenes, Seychelles, Grande Comore and Moheli) is breached in the middle by Anjouan, Mayotte and the large island of Madagascar.

Second, within our colour categories 'grey' and 'green', variation in colour among green populations is much greater than among grey ones (Ali & Ripley, 1971; Louette & Herremans, 1985). This observation is more consistent with a grey ancestral state and multiple origins of green, than with a grey-green-grey transition.

Third, the apparent trend for independent insular increase in body size and change in plumage from grey to green is replicated to a lesser extent in *Hypsipetes* colonizations which are independent of the primary expansion in the western Indian Ocean. Within the secondary expansion of *H. m. madagascariensis* from Madagascar to Aldabra and the Comoros, it appears that while these are all 'small' birds with respect to the other western Indian Ocean species, the same trend of insular increase in body size proposed for the primary

expansion is beginning to occur. Louette & Herremans (1985) showed that *H. m. rostratus* of Aldabra is slightly larger than *H. m. madagascariensis* throughout its range on the basis of mean tarsus length, and that this difference is significant. Further, the four Comoro island populations of *H. m. madagascariensis* are slightly larger than the Madagascar population on the basis of mean tarsus and bill length, although this difference is not statistically significant (Louette & Herremans, 1985). While the secondary expansion of western Indian Ocean *Hypsipetes* does not yet exhibit an insular change in plumage from grey to green, replication of this insular trend is found outside the region; *H. nicobariensis* Moore of the Nicobar islands in the eastern Indian Ocean is not thought to be related to the western Indian Ocean *Hypsipetes*. Nevertheless, it exhibits a 'green' phenotype despite being the likely descendant of a 'grey' continental form (Ali & Ripley, 1971; Louette, 1987).

An insular increase in *Hypsipetes* body size is consistent with the findings of a recent analysis across numerous bird groups (Clegg & Owens, 2002). This found strong evidence that birds follow the same 'island rule' as mammals, with large-bodied species diminishing in size on islands and small-bodied species increasing in size. With regard to the latter phenomenon, Clegg *et al*. (2002) summarize a number of different explanations. These include size-biased dispersal, evolution along the genetic line of least resistance in the direction of greatest genetic variance, and random walk away from small size. By contrast, large body size may be the result of natural selection in an insular environment (Clegg *et al*., 2002). With a reduced number of competitor species on islands, niche width is broadened. Selection for large bill size may allow exploitation of a greater range of resources (Grant, 1965), with body size increases being either a consequence or prerequisite of bill size increase. Finally, large body size may have fitness advantages in insular environments, where high population densities result in high intraspecific competition (MacArthur *et al*., 1972), and fitness disadvantages resulting from increased predation pressure may be reduced (Grant, 1965).

The pattern of plumage change in *Hypsipetes* is consistent with other reconstructions of avian plumage evolution (e.g. Omland & Lanyon, 2000) showing that plumage characters evolve rapidly and exhibit high levels of homoplasy. The insular change from grey to green may reflect differences in selective pressures between island and continental environments, and be highly correlated with behaviour and ecology. Grey forms appear to be generalist species found in a wide range of habitats, including humid forest, savannah and human landscapes. By contrast, green forms tend to be more specialized, being largely restricted to

native forest on islands where such forest still persists, although they are also found in gardens and plantations in the Seychelles, where native forest is absent (Ali & Ripley, 1971; Cheke, 1987a, b; Sinclair & Langrand, 1998; pers. observ.).

We suggest that the homoplasious evolution of green plumage may be a response to selective pressures in small island environments where the range of ecological conditions is less varied than in continental environments (or would have been, prior to human arrival). In addition, grey forms are highly gregarious, while green forms tend to be more solitary and furtive, normally sighted alone or in pairs (Ali & Ripley, 1971; Sinclair & Langrand, 1998; pers. observ.). These behavioural changes may reflect differences in the balance between predation pressure and intraspecific competition in island vs. continental environments. The relative scarcity of predators and competitors on islands may result in higher intraspecific competition and reduced gregariousness.

CONCLUSIONS

The *Hypsipetes* phylogeny provides strong evidence for a recent (max. 2.6 Mya) colonization of the western Indian Ocean by Asian bulbuls, with subsequent rapid expansion across the islands of the region. The timing of continental break-up between India and Madagascar (*c*. 88 Mya) therefore enables us to rule out a vicariant origin for *Hypsipetes* in the Indian Ocean as proposed by Danis (1940). The estimated timing of *Hypsipetes* arrival in the western Indian Ocean highlights a probable role for the former chain of islands between India and the Seychelles as stepping stones for dispersal to the Malagasy region.

Our phylogeny conforms with other molecular phylogenetic studies that have revealed homoplasious evolution among island organisms (Losos *et al.*, 1998; Goodacre & Wade, 2001; Rees *et al.*, 2001). In *Hypsipetes*, the weight of evidence from biogeography and comparison between independent evolutionary events support the persistence of small grey continental forms in India and Madagascar, giving rise to multiple independent origins of large size and green plumage on islands around Madagascar.

Further investigation of *Hypsipetes* morphometrics and ecology is needed to refine our understanding of the steps involved in the insularization process. Such study may help to determine whether or not common insular changes occur by the same developmental pathways in different lineages, and therefore whether homoplasy results from convergence or parallelism. This subject has received remarkably little attention, and it is indeed likely that a combination of both convergence and parallelism occurs in many cases. In addition, comparison between continental and insular island populations regarding morphological and ecological correlates of fitness may shed light on the forces driving insular phenotypic change.

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