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Molecular phylogeny of the Indian Ocean *Terpsiphone* paradise flycatchers: Undetected evolutionary diversity revealed amongst island populations

Rachel M. Bristol [a,*], Pierre-Henri Fabre [b], Martin Irestedt [c], Knud A. Jønsson [b], Nirmal J. Shah [d], Vikash Tatayah [e], Ben H. Warren [f], Jim J. Groombridge [a]

[a] Durrell Institute of Conservation and Ecology, School of Anthropology and Conservation, University of Kent, Canterbury CT2 7NR, United Kingdom
[b] Center for Macroecology Evolution and Climate at the Natural History Museum of Denmark, University of Copenhagen, Universitetsparken, 15, DK-2100 Copenhagen Ø, Denmark
[c] Molecular Systematics Laboratory, Swedish Museum of Natural History, P.O. Box 50007, SE-104 05 Stockholm, Sweden
[d] Nature Seychelles, P.O. Box 1310, The Centre for Environment and Education, Roche Caiman, Mahe, Seychelles
[e] Mauritian Wildlife Foundation, Grannum Road, Vacoas, Mauritius
[f] UMR PVBMT, Université de La Réunion-CIRAD, 7 chemin de l'IRAT, Ligne Paradis, 97410 Saint Pierre, Réunion, France

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ABSTRACT

We construct a molecular phylogeny of *Terpsiphone* flycatchers of the Indian Ocean and use this to investigate their evolutionary relationships. A total of 4.4 kb of mitochondrial (cyt-*b*, *ND3*, *ND2*, control region) and nuclear (*G3PDH*, *MC1R*) sequence data were obtained from all species, sub-species and island populations of the region.

Colonisation of the western Indian Ocean has been within the last two million years and greatly postdates the formation of the older islands of the region. A minimum of two independent continent-island colonisation events must have taken place in order to explain the current distribution and phylogenetic placement of *Terpsiphone* in this region. While five well-diverged Indian Ocean clades are detected, the relationship between them is unclear. Short intermodal branches are indicative of rapid range expansion across the region, masking exact routes and chronology of colonisation.

The Indian Ocean *Terpsiphone* taxa fall into five well supported clades, two of which (the Seychelles paradise flycatcher and the Mascarene paradise flycatcher) correspond with currently recognised species, whilst a further three (within the Madagascar paradise flycatcher) are not entirely predicted by taxonomy, and are neither consistent with distance-based nor island age-based models of colonisation. We identify the four non-Mascarene clades as Evolutionarily Significant Units (ESUs), while the Mascarene paradise flycatcher contains two ESUs corresponding to the Mauritius and Réunion subspecies. All six ESUs are sufficiently diverged to be worthy of management as if they were separate species.

This phylogenetic reconstruction highlights the importance of sub-specific molecular phylogenetic reconstructions in complex island archipelago settings in clarifying phylogenetic history and ESUs that may otherwise be overlooked and inadvertently lost. Our phylogenetic reconstruction has identified hidden pockets of evolutionary distinctiveness, which provide a valuable platform upon which to re-evaluate investment of conservation resources within the *Terpsiphone* flycatchers of the Indian Ocean.

1. Introduction

As the global biodiversity crisis intensifies with rising human activity, there is an increasing need to prioritise the allocation of finite conservation resources. The number of species worldwide threatened with extinction far exceeds the conservation resources available and predictions suggest this trend is worsening (Butchart et al., 2004; Hazevoet, 1996; Isaac et al., 2007; Myers et al., 2000; Pimm et al., 1995), forcing conservation planners to increasingly prioritise which populations to protect or restore. Priority setting approaches have frequently focused on measures of endemism and restricted range (e.g. Myers et al., 2000; Olson et al., 2001; Stattersfield et al., 1998), however numerous studies advocate that evolutionary distinctiveness should also be an important consideration (e.g. Crozier, 1997; Faith, 1992; Isaac et al., 2007; Witting and Loeschcke, 1995). In practice however, evolutionary distinctiveness is often overlooked, largely due to a lack of complete species and subspecies-level phylogenies (Isaac et al., 2007).

The concept of Evolutionarily Significant Units (ESUs) was developed to provide an objective approach for prioritising taxa

* Corresponding author.
E-mail addresses: rmb33@kent.ac.uk (R.M. Bristol), phfabri@snm.ku.dk (P.-H. Fabre), Martin.Irestedt@nrm.se (M. Irestedt), kajonsson@snm.ku.dk (K.A. Jønsson), nirmalshah@natureseychelles.org (N.J. Shah), vtatayah@mauritian-wildlife.org (V. Tatayah), ben.warren@cirad.fr (B.H. Warren), J.Groombridge@kent.ac.uk (J.J. Groombridge).

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for conservation management and to ensure important phylogenetic diversity is not overlooked (Ryder, 1986) as taxonomy does not necessarily reflect underlying phylogenetic diversity (Avise, 1989; Zink, 2004). A recent advance in the objective identification of ESUs is Pons et al.'s (2006) general mixed Yule coalescent (GMYC) model. The method makes use of coalescence theory to identify a point of transition between species-level and population-level evolutionary processes. The success in the application of this method across a wide range of taxa (e.g. Poulakakis et al., 2012; Vuataz et al., 2011) suggests that it is likely to become a key tool in the objective allocation of finite conservation resources across regions and communities. Nowhere is this approach likely to be more important than in island systems, as a result of their high frequency of cryptic evolutionary distinctiveness.

The islands of the western Indian Ocean are known for their high levels of biodiversity, endemism and investment of conservation efforts, and as a result have become a natural focus for evolutionary research (e.g. Fuchs et al., 2008; Groombridge et al., 2002; Kundu et al., 2012; Raxworthy et al., 2002; Rocha et al., 2009; Warren et al., 2003, 2005, 2006, 2010). This region's endemic biodiversity has suffered high levels of extinction including well-documented cases such as the Dodo (*Raphus cucullatus*), and solitaire (*Pezophaps solitaria*), as well as several species of parrot, owl, rail and giant tortoise. Réunion Island alone has lost 61% of its native landbird fauna since human arrival, of which at least 12 species were endemic to the island (Cheke and Hume, 2008; Probst and Brial, 2002). Fortunately, these islands still contain remnant populations of a plethora of other endemic species however many have suffered drastic declines and are now reduced to tiny relict populations relying on intensive and sustained conservation efforts to prevent further extinctions. Consequently, phylogenetic studies focused on radiations of island populations can play a particularly important role; identifying evolutionary significant units can help streamline biodiversity conservation within these island settings.

Against this backdrop of historical extinctions, the endemic taxa of the Indian Ocean islands are frequently comprised of different island forms that collectively show the full range of extinction threat status between them, from very common to extremely endangered, making this oceanic region an important focus for examining evolutionary processes within a conservation context. Previous molecular phylogenies of subspecies and island forms within the region have exposed pockets of phylogenetic diversity

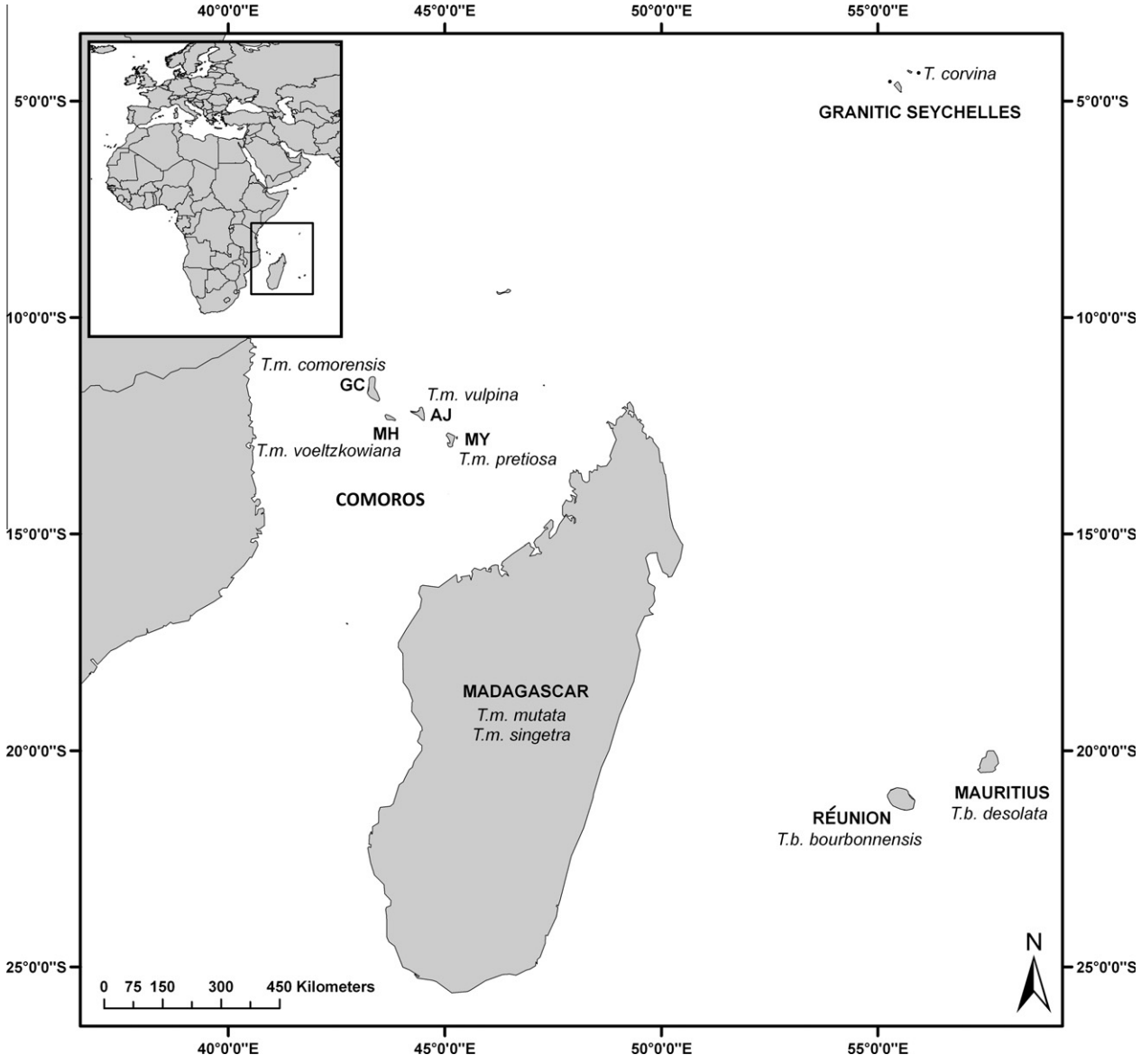

Fig. 1. Distribution of *Terpsiphone* taxa of the western Indian Ocean. GC, Grande Comore; MH, Moheli; AJ, Anjouan; MY, Mayotte.

that do not align to current taxonomy, e.g. Hypsipetes bulbuls (Warren et al., 2005), Phelsuma geckos (Austin et al., 2003) and Coracopsis parrots (Kundu et al., 2012), highlighting a widespread need to clarify phylogenetic history and refocus conservation priorities.

The *Terpsiphone* paradise flycatchers of the western Indian Ocean are a visually stunning and intriguing group that illustrate the need for fine-scale molecular phylogenetic information to determine evolutionary distinctiveness amongst their different island forms in order to prioritise conservation efforts. *Terpsiphone* paradise flycatchers are a globally widespread and highly speciose genus of Monarchidae passerines, occurring over most of sub-Saharan Africa, southern and eastern Asia, the Philippines and the western Indian Ocean islands. There are 12 or 13 recognised species depending on authors (BirdLife International, 2011; Coates et al., 2006; IUCN, 2011; Sibley and Monroe, 1990). Levels of threat and conservation status vary enormously. For example, the Seychelles paradise flycatcher (*Terpsiphone corvina*) is listed as Critically Endangered, three other species in the genus (*T. bedfordi*, *T. atrocaudata*, *T. cyanescens*) are listed as Near Threatened and the remainder are widespread and listed as Least Concern (IUCN, 2011). Three *Terpsiphone* species are endemic to the western Indian Ocean, (*T. corvina* in the Seychelles, *T. mutata* in Madagascar and the Comoros and *T. bourbonnensis* in Mauritius and Réunion). These three species are split into eight subspecies found amongst the different islands (see Fig. 1 for locations of different species and subspecies). The critically endangered Seychelles paradise flycatcher is restricted to just 10 km^2 with a total population of c. 300 individuals (Currie et al., 2003a,b). Several studies have examined habitat requirements, threats and conservation action strategies for this species, but its evolutionary distinctiveness remains unconfirmed. Elsewhere, the Mascarene paradise flycatcher (*T. bourbonnensis*) is common on Réunion (*T. b. bourbonnensis*), but the sub-species on Mauritius (*T. b. desolata*) is extremely rare with a population of a 100–223 pairs (Safford, 1997) while accurate population estimates for the *T. mutata* subspecies and island forms are lacking.

The high levels of biodiversity and endemism that have evolved in this part of the World have stemmed from two key characteristics, namely the position of the western Indian Ocean in relation to the neighbouring continental land masses, and the region's unusually complex geological history (Duncan and Hargraves, 1990; Raxworthy et al., 2002.) Situated between Africa to the west and Asia to the north-east, the western Indian Ocean islands' biota displays affinities with both Africa and Asia, with the origins of different taxonomic groups appearing to come from either one or the other of these continents. For example the western Indian Ocean islands were colonised by kestrels and sunbirds from Africa (Groombridge et al., 2002; Warren et al., 2003) and Scops owls and bulbuls from Asia (Fuchs et al., 2008; Warren et al., 2005). Additionally the western Indian Ocean islands show an unusually diverse range of geological ages and origins. They can be grouped into three broad categories based on geology and age (Warren et al., 2003; see Table 1 for details of island ages, geology and sources of information). A geological anomaly within the Indian Ocean is the presence of very shallow regions, such as the Seychelles Bank, a shallow submarine platform of some 43,000 km^2 that seldom exceeds 65 m depth (Camoin et al., 2004) and other shallow, currently submarine areas of substantial size. Sea level low-stands are known to have exceeded 80 m below present levels at least six times during the past 500,000 years (Bintanja et al., 2005; Camoin et al., 2004; Rohling et al., 1998) with at least 12 further episodes where sea level exceeded 65 m below present levels within the last 3.5 million years (Bintanja et al., 2005; Miller et al., 2005; Rohling et al., 1998; Siddall et al., 2003). During these sea-level low stands, some of which persisted for thousands of years, the Seychelles' land mass has been up to 180 times its present size and additional large islands would have been present in the western Indian Ocean providing stepping-stones between landmasses facilitating dispersal of individuals across the region during the Pliocene and Pleistocene (Cheke and Hume, 2008; Warren et al., 2010). Consequently, the evolutionary history of *Terpsiphone* flycatchers is likely to be complex and not easily discerned from current geography alone.

Here, we present a comprehensive molecular phylogenetic reconstruction for the western Indian Ocean *Terpsiphone* flycatchers based on a 4429 base pair (bp) DNA sequence dataset comprising six genes (two nuclear and four mitochondrial loci). We use this molecular phylogeny to: (i) identify the evolutionary origins and routes of radiation of the western Indian Ocean paradise flycatchers, and (ii) clarify the evolutionary distinctiveness of the different island forms, in particular the critically endangered Seychelles paradise flycatcher, and re-evaluate priorities for their conservation.

2. Materials and methods

2.1. Taxon sampling

We obtained samples for genetic analysis from 34 individuals covering all three species (*Terpsiphone mutata*, *Terpsiphone corvina* and *Terpsiphone bourbonnensis*) and nine populations (see Fig. 1) from the western Indian Ocean islands. Our sample contained representatives of between two and six individuals from each population (see Table 2 for details of all samples used in this analysis). In addition we included representatives of both African and Asian *Terpsiphone* flycatchers to determine closest continental affinities of the western Indian Ocean island *Terpsiphone* flycatchers. We also included the São Tomé paradise flycatcher *Terpsiphone atrochalybeia* as its plumage colouration is very similar to *T. corvina*. The Black-naped Monarch *Hypothymus azurea* was chosen as an outgroup to root the phylogenetic reconstructions because *Hypothy-*

Table 1
List of western Indian Ocean island ages, geology and sources of information.

Island	Geology	Age	Source
Granitic Seychelles Islands	Granite	Separated from Africa c.130 Mya/India c.64 Mya	Coffin and Rabinowitz (1987); Kingdom (1990), Rabinowitz et al (1983)
Madagascar	Granite	Separated from Africa c.130 Mya/India c.88 Mya	Coffin and Rabinowitz (1987); Kingdom (1990), Rabinowitz et al (1983)
Mayotte	Volcanic	7.7–15 Mya	Emerick and Duncan (1982), Nougier et al. (1986)
Moheli	Volcanic	c.5 Mya	Emerick and Duncan (1982), Nougier et al. (1986)
Anjouan	Volcanic	c. 3.9–11.5 Mya	Emerick and Duncan (1982), Nougier et al. (1986)
Grande Comore	Volcanic	c.0.13–0.5 Mya	Emerick and Duncan (1982), Nougier et al. (1986); R. Duncan pers. comm. in Warren et al., 2003
Mauritius	Volcanic	c.7.8 Mya	Duncan and Hargraves (1990), McDougall and Chamalaun (1969)
Reunion	Volcanic	c.2.1 Mya	Chevallier and Vatin-Perignon (1982), Duncan and Hargraves (1990)
South-east Seychelles Islands	Coral/ sand	$\leqslant$0.015–0.125 Mya	Radtkey (1996), Thompson and Walton (1972)

Table 2
List of all samples used in this analysis.

Species	Sample ID number	Location	Sample type[a]
Terpsiphone bourbonnensis bourbonnensis	38	Réunion	Fresh blood
Terpsiphone bourbonnensis bourbonnensis	90	Réunion	Fresh blood
Terpsiphone bourbonnensis bourbonnensis	222	Réunion	Fresh blood
Terpsiphone bourbonnensis bourbonnensis	255	Réunion	Fresh blood
Terpsiphone bourbonnensis bourbonnensis	302	Réunion	Fresh blood
Terpsiphone bourbonnensis bourbonnensis	353	Réunion	Fresh blood
Terpsiphone mutata pretiosa	71	Mayotte	Fresh blood
Terpsiphone mutata pretiosa	98	Mayotte	Fresh blood
Terpsiphone mutata pretiosa	106	Mayotte	Fresh blood
Terpsiphone mutata vulpina	120	Moheli	Fresh blood
Terpsiphone mutata vulpina	183	Moheli	Fresh blood
Terpsiphone mutata vulpina	111	Moheli	Fresh blood
Terpsiphone mutata voeltzkowiana	500	Anjouan	Fresh blood
Terpsiphone mutata voeltzkowiana	501	Anjouan	Fresh blood
Terpsiphone mutata voeltzkowiana	502	Anjouan	Fresh blood
Terpsiphone mutata voeltzkowiana	503	Anjouan	Fresh blood
Terpsiphone mutata	419	Madagascar	Fresh blood
Terpsiphone corvina	29	La Digue	Fresh blood
Terpsiphone corvina	39	La Digue	Fresh blood
Terpsiphone corvina	42	La Digue	Fresh blood
Terpsiphone corvina	46	La Digue	Fresh blood
Terpsiphone corvina	52	La Digue	Fresh blood
Terpsiphone corvina	68	La Digue	Fresh blood
Terpsiphone bourbonnensis desolata	Tb001	Mauritius	Fresh blood
Terpsiphone mutata comorensis	PH94	Grande Comore	Fresh blood
Terpsiphone cinnamomea	116848	Philippines	Fresh blood
Terpsiphone viridis	134397	Tanzania	Fresh blood
Terpsiphone mutata vulpina	ZMB 2000/17393	Anjouan	Museum tissue
Terpsiphone mutata pretiosa	ZMB 2000/17408	Mayotte	Museum tissue
Terpsiphone mutata mutata	ZMUC 116849	Berenty, Madagascar	Museum tissue
Terpsiphone mutata comorensis	MNHN 36 A03	Grande Comore	Museum tissue
Terpsiphone mutata mutata	ZMUC 28229	Vondrozo, Madagascar	Museum tissue
Terpsiphone mutata singetra	ZMUC 28258	Ranpotaka, Madagascar	Museum tissue
Terpsiphone bourbonnensis bourbonnensis	NRM 553920	Réunion	Museum tissue
Terpsiphone bourbonnensis desolata	NRM 556022	Mauritius	Museum tissue
Terpsiphone atrochalybeia	ZMUC 59966	São Tomé	Museum tissue
Terpsiphone corvina	UMZC 27/Mus/51/f/6	Praslin	Museum tissue
Terpsiphone atrocaudata atrocaudata	NRM 68533	North China	Museum tissue
Tterpsiphone cyanensis	ZMUC 105237	Palawan	Museum tissue
Terpsiphone paradisi floris	RMNH 85100	Flores	Museum tissue
Terpsiphone batesi	RMCA 107459	DR Congo	Museum tissue
Terpsiphone paradisi leucogaster	ZMUC 28233	Afghanistan	Museum tissue
Terpsiphone paradisi paradisi	ZMUC 28237	India	Fresh tissue
Hypothymis azurea	MNHN 5 40 4 1997	Laos	Fresh blood

Museum samples were provided by the respective museums as listed under sample ID number.
[a] All fresh blood samples were collected from mistnetted individuals that were then released unharmed.

mus is the sister genus to *Terpsiphone* (Coates et al., 2006; Fabre et al., 2012).

2.2. DNA extraction, PCR, sequencing and alignment

All DNA extractions from blood samples were carried out using the Ammonium Acetate method following Nicholls et al. (2000). Fragments from the following six loci were amplified and sequenced: cytochrome-b (cyt-*b*) (888 bp), NADH dehydrogenase subunit 3 (*ND*3) (467 bp), NADH dehydrogenase subunit 2 (*ND*2) (1030 bp), control region (888 bp), glyceraldehyde-3-phosphodehydrogenase intron 11 (*G*3*PDH*) (398 bp), and the melanocortin-1 receptor gene (*MC*1*R*) (758 bp).

Loci were amplified by Polymerase chain reaction (PCR) and sequenced using the primers listed in Table 3. Each PCR reaction comprised the following reagents; 1–4 μl template DNA, 5 μl NH4 reaction buffer (10×), 1.5 μl $MgCl_2$ (50 mM), 8 μl dNTP's, 1 μl of each of the forward and reverse primers, 0.4 μl of 5 U/μl Biotaq DNA polymerase (Bioline) and UV sterilised DNA grade distiled water to mix to a total volume of 50 μl. PCR thermal cycling conditions were as follows for all genes: an initial denaturing step of 94 °C for 4 min followed by 30 cycles of [94 °C for 30 s, 49–63.4 °C (specific to primer pair) for 45 s, 72 °C for 60 s,] ending with 10 min extension at 72 °C. The annealing temperatures used for each primer pair are listed in Table 3.

PCR products were purified using the GENECLEAN Turbo kit (MP Biomedicals, LLC). Purified PCR product was sequenced by Macrogen-South Korea, Macrogen-Europe and Eurofins MWG Operon–Germany. Sequence reads were manually checked and then aligned and edited using the programme FinchTV 1.4 (Geospiza). Consensus sequences were aligned using the programme ClustalX 2.1.12 (Larkin et al., 2007) and GeneDoc 2.7.000 (Nicholas and Nicholas, 1997).

For some of the outgroup taxa for which we did not have fresh blood samples, we extracted DNA from museum specimens. For the museum samples we amplified and sequenced DNA for four loci (cyt-*b*, *ND*3, *ND*2, *G*3*PDH*). For extractions, amplifications, and sequencing procedures from museum skin samples we followed the methods described in Irestedt et al. (2006). However, 20 μl of DTT (dithiothreitol) was added in the lysis phase during the extractions and we amplified shorter fragments (around 250 bp including primer sequence lengths) in order to increase the ratio of successful amplifications. The additional primers used for museum skin samples can be found in Fabre et al. (2012).

Table 3
List of primers used to amplify and sequence the genes used in this study.

Loci	Primer name/sequence (5′–3′)	Source	Ta (°C)
cyt-*b*	F: TerpCytb_F (CCCCCAACCTACGTAAAAATC) R: TerpCytb_R (TTTGTGATAGGGGTCGGAAG)	Designed for this research from existing *Terpsiphone paradisi* sequence (GenBank Accession Number EF081356)	60.0
ND3	L10755 H11151	Chesser (1999)	49.0
ND2	L5216 H6313	Sorenson et al. (1999)	55.0
Control region (PCR)	F: TerpCRF (GGACTTTCTCCAAGATCTATGGC) R: TerpCRR (GCAACCATGACACTATTAGCTAC)	Rebecca Kimball, pers. comm.	59.0
Control region (internal sequencing primers)	F: TerpCRIntSeq20_F (CCCCATGTTTTTACATGGTTT) F: TerpCRIntSeq400_F (TCGTGTTTCTCACGCTACCC)	Designed for this research	
G3PDH	G3P13b G3P14b	Fjeldså et al. (2003)	60.0
MC1R	F: *MC1R*_F (TGGACATTCCCAACGAGCTG) R: *MC1R*_R (AGATGAGGGGGTCAATCACTG)	Designed for this research from chestnut-bellied and melanic monarch sequences provided by Albert Uy, pers. comm.	63.4

Ta, annealing temperature.

2.3. Phylogenetic analysis

2.3.1. Maximum Likelihood analyses on the single and concatenated dataset

Phylogenetic tree inferences were computed on each single gene matrix and on the concatenated datasets using the Maximum Likelihood (ML) criterion. The MODELTEST 3.07 software (Posada and Crandall, 1998) was employed in order to determine the best fitting model for the DNA sequence evolution using the Akaike Information Criterion (AIC). This method can implement partitioned analyses by appropriating to each partition either a GTR (general time reversible) model with rate heterogeneity accommodated with a gamma (Γ) distribution (GTR + Γ), or a GTR + CAT model (general time reversible model with rate heterogeneity accommodated with a number of discrete rate categories). For the partitioned datasets [6 gene partitions and 3 codon partitions (for cyt-*b*, *ND2* and *ND3*) for coding genes], we used the GTR + MIX option of RAxML, which assumes the faster GTR + CAT model for topological tree searches, but assumes the GTR + Γ model when computing the likelihood value of each topology. We used RAxML default parameters and specified 1000 tree search replicates. Node stability on partitioned supermatrices was computed with 1000 non-parametric bootstrap replicates (Felsenstein, 1985). Bootstrap percentages (BPs) were calculated using RAxML under a GTR + MIX model. ML searches for the best trees were performed using the PAUP* program (Swofford, 2002), version 4b10. We conducted our analyses in two steps. Firstly, we used a heuristic search to estimate ML model parameters on a neighbour-joining (NJ) starting tree. Secondly, the previously estimated parameters were entered in a new search with tree bisection reconnection (TBR) branch swapping. The robustness of nodes was estimated by ML bootstrap percentages after 100 replicates using previously estimated parameters, NJ starting tree and TBR branch swapping.

2.3.2. Bayesian analyses on the partitioned supermatrices

Phylogenetic tree inferences were performed on each single gene matrix and on the concatenated datasets using Bayesian methods. We applied a partitioned strategy to the supermatrices in which each gene was assigned to its own partition (“gene partitioned”). Bayesian analyses used MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) which allows different models for each gene partition. Models for the partitioned Bayesian analyses were identified using the MrModeltest 2.2 software (Nylander et al., 2004), and models preferred by the AIC were implemented. All parameters except topology were unlinked across partitions and two independent runs (with one cold and three heated chains) were computed simultaneously, with trees sampled every 100 generations. The MrBayes analyses were run for 5×10^7 generations. Majority rule consensus was constructed, with burn-ins of 5×10^5 generations. Support for different clades was calculated by posterior probabilities.

In order to test hypotheses regarding monophyly of the Indian Ocean lineages and several other potential scenarios inferred by our phylogenetic trees, we employed the approximately unbiased (AU) test (Shimodaira, 2002) and the Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) as implemented in CONSEL (Shimodaira and Hasegawa, 2001). The six gene supermatrix dataset was used for these tests and Programme PAUP* version 4.0b10 (Swofford, 2002) was used to calculate the site likelihoods for each of the test topologies with the gene partitioning scheme assumed and the appropriate model for each partition specified using the output from Modeltest. The CONSEL analyses employed 10 batches of 1×10^6 bootstrap replicates.

2.3.3. Molecular dating and DNA based species delimitation

We used Beast v1.6 (Drummond et al., 2002; Drummond and Rambaut, 2007) to estimate the divergence dates within Indian Ocean *Terpsiphone*, applying the best fitting model, as estimated by Modeltest 2.0, to each of the partitions. We assumed a Yule Speciation Process for the tree prior and an uncorrelated lognormal distribution for the molecular clock model (Ho et al., 2007). We used default prior distributions for all other parameters and ran MCMC chains for 200 million generations. The program Tracer (Rambaut and Drummond, 2007) was used to assess convergence diagnostics. Because no fossil data are available for this group, we used a molecular clock approach in order to estimate the diver-

gence dates among Indian Ocean *Terpsiphone* species. Using the Tajima's relative test (Tajima, 1993) implemented in pegas package (Paradis, 2010) of the R software we tested if the molecular clock hypothesis could be applied to our dataset. Because a molecular clock hypothesis could not be rejected, we applied both a strict and a relaxed molecular clock to our matrix using partitions by genes and codon positions. The Hawaian honeycreeper rate of evolution of 1.6% sequence divergence per million years (Mya) was used to obtain the absolute date. This estimate is based on the geology of Hawaii and may be inaccurate as Hawaiian island emergence provides a maximum age for taxa inhabiting the particular island (Fleischer et al., 1998). Consequently, the date estimates generated with island age calibrations in this study can be regarded as maxima.

Geological calibration points have been applied to several avian groups of oceanic islands (e.g. Fuchs et al., 2008; Warren et al., 2003; and Moyle et al., 2009; Fabre et al., 2012; Tarr and Fleischer, 1993). Assumptions and criteria regarding geological calibration points (see Fleischer et al., 1998; Warren et al., 2003; Heads, 2011; Emerson et al., 2000) are generally fulfilled for the Indian Ocean islands of Mauritius and Réunion. We therefore used the split between *Terpsiphone bourbonnensis desolata* from Mauritius (age c.7.8 Mya; McDougall and Chamalaun, 1969) and *T. b. bourbonnensis* from Réunion (age c.2.1 Mya; Chevallier and Vatin-Perignon, 1982) in the Indian Ocean as a geological calibration point. We assume that the divergence between the lineages on Mauritius and Réunion cannot be older than the younger of the two islands (Réunion, 2.1 Mya). Thus, to obtain a calibration point based on the split between these two species, we applied a lognormally distributed prior at 1.5 Mya ± 0.25 standard deviations (95% confidence interval = 1.089–1.911 Mya). Finally, we used a 2%/Mya rule to corroborate the dates resulting from the island calibrations.

In order to delineate Indian Ocean taxa and to discuss the importance of our phylogenetic results for conservation purposes, we employed the Pons et al. (2006) method. This likelihood approach detects the switch in the rate of lineage branching to intraspecific short budding branching and identifies clusters of specimens corresponding to potential taxonomic units. Two models can be applied to account for different branching processes within the phylogeny. Within the null model, the sample grows from a single population following a coalescent process. The other model follows a general mixed Yule coalescent (GMYC) model which takes into account branching at the population level (coalescent process) and at the macro-evolutionary level (with extinction and speciation rate inferred from the Yule process). The GMYC model optimised a threshold (T) from which we could consider the species number estimation and then delineate taxonomic units. The fit of both models were compared using Likelihood-ratio test (LRT). We used the package SPLITS (Pons et al., 2006) within R version 2.10.1 (R Development Core Team, 2009). In addition, uncorrected pairwise distances between each island population were calculated using our four gene mitochondrial DNA dataset in the programme PAUP* (Swofford, 2002).

3. Results

3.1. Phylogenetic results

The results from the phylogenetic analyses are displayed in Fig. 2. Both Maximum Likelihood (ML) and Bayesian (BI) trees converged to produce very congruent topologies, so only the ML tree is shown in Fig. 2 however both the ML and BI values for each node are given on the ML tree, the ML value above and the BI value below. The full BI tree can be found in Supplementary Information. The single gene and concatenated datasets produced congruent results (see Supplementary Information), although the nuclear gene trees provided little resolution, reflecting the recent diversification of the group. Thus the species trees are largely driven by the mitochondrial data. Six main biogeographic monophyletic lineages are supported by our analyses: (i) a *Terpsiphone bourbonnensis* clade (Mascarenes), (ii) a *Terpsiphone corvina* clade (Seychelles), (iii) a *Terpsiphone mutata mutata* + *T. m. singetra* + *T. m. pretiosa* clade (Madagascar and Mayotte), (iv) a *Terpsiphone mutata vulpina* + *T. m. voeltzkowiana* clade (Anjouan and Moheli), (v) a *Terpsiphone mutata comorensis* clade (Grande Comore) and (vi) a *Terpsiphone viridis* + *batesi* clade (central Africa). Of these, five are Indian Ocean lineages which are nested alongside African *Terpsiphone* clades but within an Asian clade (see also Fabre et al., 2012). However, due to an absence of branch support for divergences between these lineages in both analyses, their relationships remain uncertain. Within the Mascarene clade the subspecies *T. bourbonnensis bourbonnensis* and *T. bourbonnensis desolata* constitute two distinct clades, however the relationship of *T. bourbonnensis* to the other western Indian Ocean taxa remains unresolved. Within the *T. mutata* lineages, our analyses strongly support (*cf.* PP = 1) the monophyly of each of the Comoros subspecies (*T. m. comorensis*, *T. m. voeltzkowiana*, *T. m. vulpina* and *T. m. pretiosa*), however there appears to be no separation of the two *T. mutata* subspecies from Madagascar (*T. m. mutata and T. m. singetra*). The occurrence of several poorly supported nodes separated by short internodal branches in our analyses may indicate a case of simultaneous dispersal and/or rapid diversification or possibly gene flow between populations. AU tests allow us to reject a hypothesis of monophyly of Indian Ocean taxa ($P = 4.00 \times 10^{-15}$). However, we are unable to reject the monophyly of a clade including *T. atrochalybeia* and the Indian Ocean taxa ($P \geqslant 0.341$), the monophyly of *T. atrochalybeia* and *T. corvina* ($P \geqslant 0.398$), and the monophyly of *T. mutata* ($P \geqslant 0.440$). The five biogeographically different monophyletic Indian Ocean lineages revealed by our analyses are well-supported and provide a valuable delineation of *Terpsiphone* evolutionary history across the Indian Ocean.

3.2. Molecular dating and species delimitation

A time scale for the evolution of the Indian Ocean *Terpsiphone* derived from the Bayesian dating analysis is shown in Fig. 3. Divergence times of Indian Ocean *Terpsiphone* clades with high nodal support (see Fig. 2) (Mya) with 95% highest posterior densities obtained using a relaxed clock rate and island constraints are listed in Table 4. Using the calibration point provided by the islands of Mauritius and Réunion, we provide maximum estimates for several key phylogenetic events in the diversification of the *Terpsiphone* in order to delineate ESUs for conservation purposes. The arrival of *Terpsiphone* in the Indian Ocean dates back to the Pliocene, around 2 Mya (see Fig. 3A for detail).

The analyses of the branching rate pattern revealed the existence of six lineages within the Indian Ocean islands (Fig. 3). The lineage-through-time plot derived from the BEAST ultrametric tree displayed an increase in branching rate towards the present, which corresponds to intraspecific splitting events. To delineate between older interspecific and more recent intraspecific lineage splitting, the Pons et al. (2006) methodology was applied to our dated phylogeny (Fig. 3). Both GMYC models showed a significantly better fit compared to the null model of uniform branching rates; with respectively the multiple threshold model ($\log L = 105.51$, compared to the null model $\log L = 96.91$; $2\Delta L = 17.20$, χ^2 test, d.f. = 3, $p < 0.0006$) and the single threshold model ($\log L = 106.29$, compared to the null model $\log L = 96.91$; $2\Delta L = 18.75$, χ^2 test, d.f. = 3, $p < 0.002$). The GMYC multiple threshold model was a slightly better fit than the single threshold model but was only marginally significant (χ^2 test, d.f. = 6, $p = 0.95$). The GMYC single threshold model delineated the switch in the branching pattern around

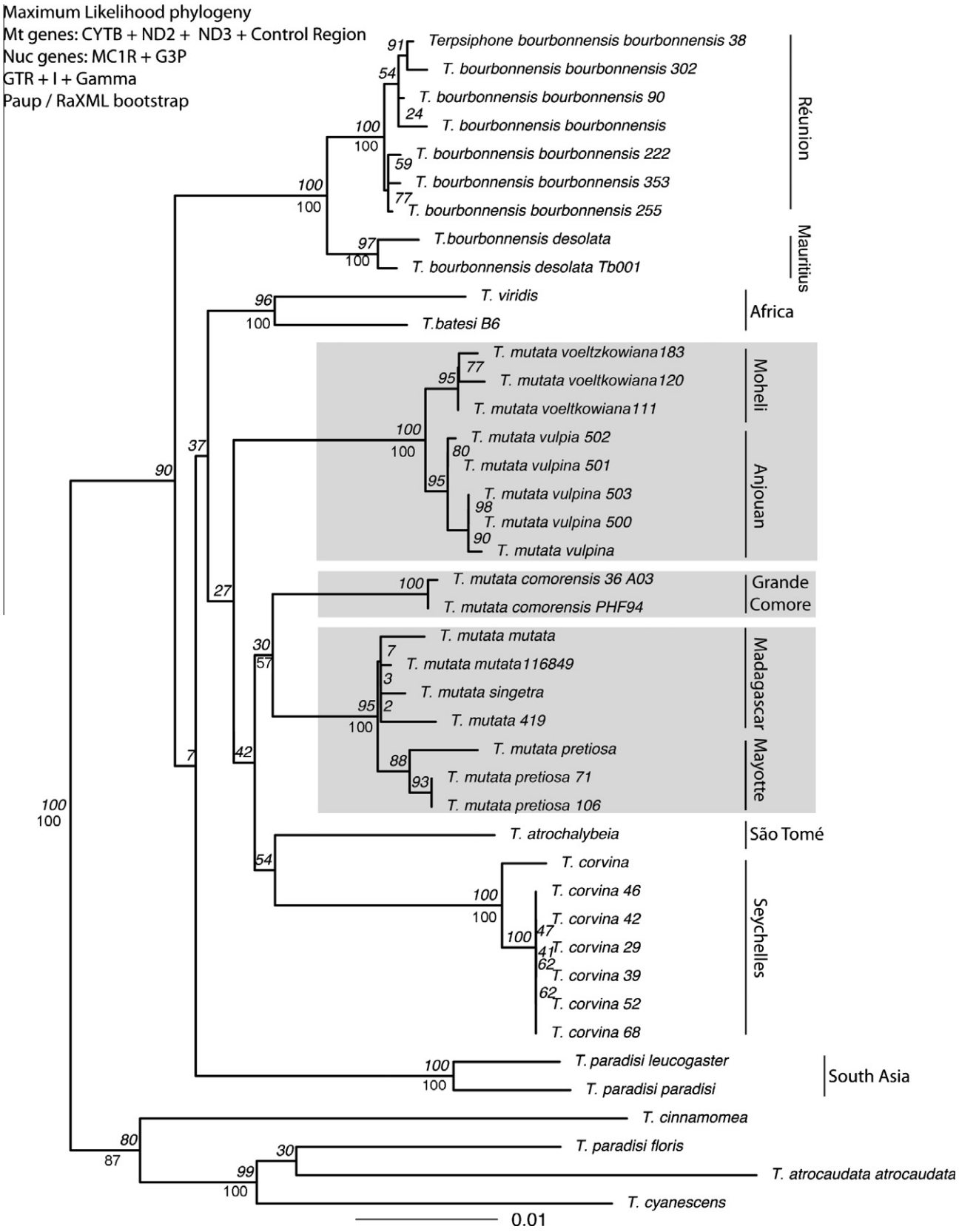

Fig. 2. Maximum Likelihood topology of Indian Ocean paradise flycatcher produced from the mito-nuclear supermatrix analyses. Biogeographic origins are shown on the side of the phylogeny. BP = Bootstrap proportion issued from the PAUP analysis. Voucher numbers are indicated for each specimen used for this study in Table 2. *T. mutata* clades indicated by grey shading. The posterior probabilities from the partitioned Bayesian analysis are shown below the Maximum Likelihood Bootstrap values for each node.

−0.35 Mya leading to an estimate of six putative Indian Ocean *Terpsiphone* lineages (estimated number of species ranged from 6 to 7; see Fig. 3, lineages are highlighted in red). One *T. corvina* lineage (IOL1), three *T. mutata* lineages (IOL2, IOL3, IOL4) and two *T. bour-*

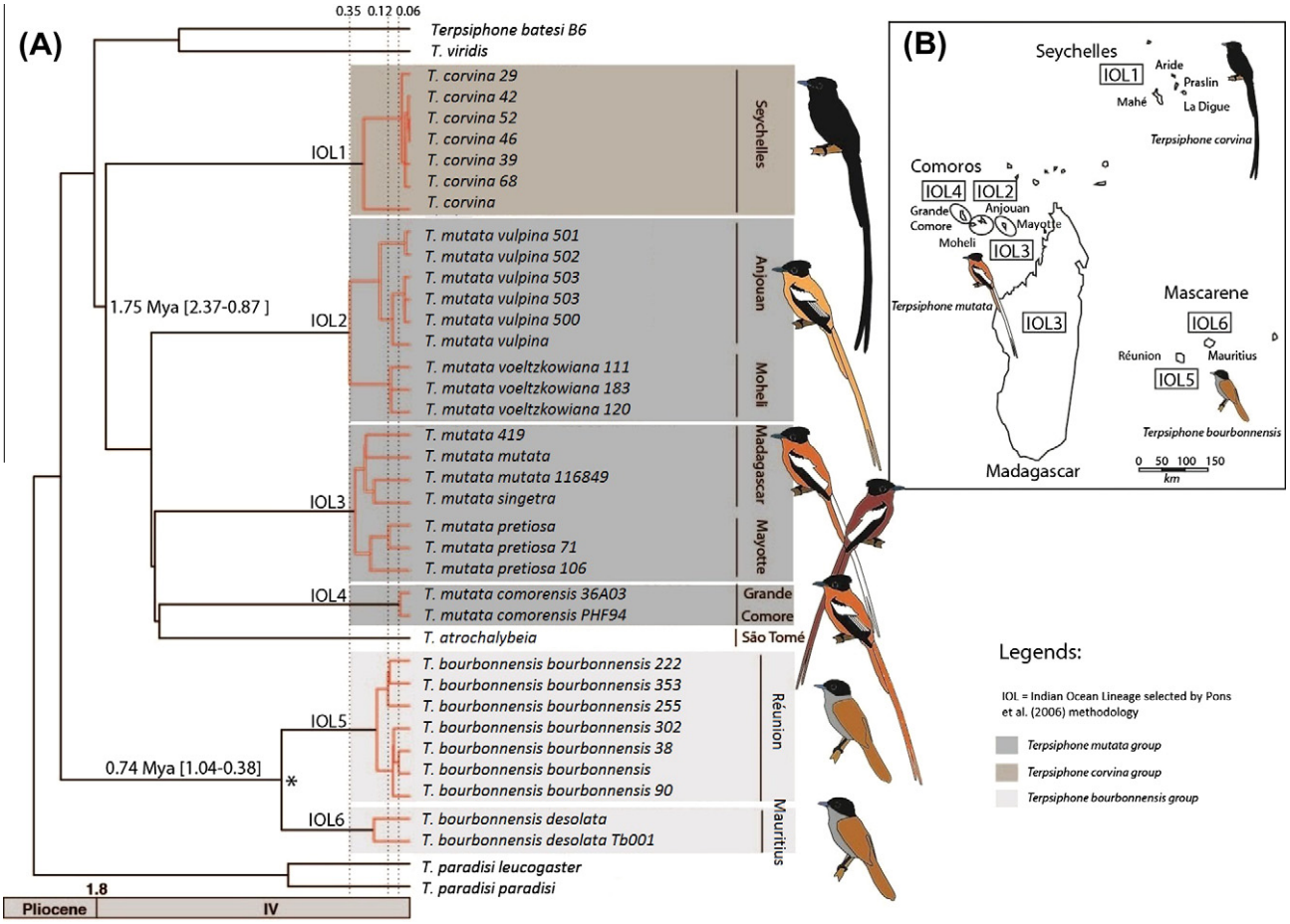

Fig. 3. Indian Ocean *Terpsiphone* dated tree with Indian Ocean geographical map. (A) Indian Ocean *Terpsiphone* ultrametric tree obtained with BEAST and cluster of specimens as putative species by the methods of Pons et al. (2006). Genetic cluster recognised as a putative species are coloured in red. The vertical bars group all sequences within each significant clusters, labelled IOL1–IOL6. (B) Map of Indian Ocean with significant clusters mapped. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Divergence times of Indian Ocean *Terpsiphone* clades with high nodal support in million years ago (Mya) with 95% highest posterior densities obtained using a relaxed clock rate and island constraints. IOL, Indian Ocean Lineages as described in Fig. 3 and Section 3.2 of the text.

	Divergence times (Mya)	
Clade	Mean	[Min–Max]
Terpsiphone clade	4.26	[3.22–5.44]
South East Asian clade	3.66	[2.53–4.85]
Node *T. bourbonnensis*/*T. paradisi*/*T. mutata*	2.92	[2.09–3.79]
Terpsiphone corvina IOL1	0.35	[0.13–0.64]
Terpsiphone mutata	1.75	[2.37–0.87]
IOL2	0.58	[0.29–0.91]
IOL3	0.53	[0.23–0.89]
IOL4	0.14	[0.10–0.42]
Terpsiphone bourbonnensis	0.74	[0.38–1.04]
IOL5	0.35	[0.02–0.43]
IOL6	0.24	[0.05–0.47]

bonnensis lineages (IOL5, IOL6) are identified (as indicated in Fig. 3) corresponding to six putative species following Pons et al.'s (2006) approach. The GMYC multiple threshold model indicated the three thresholds ranged from 0.35/0.12/0.06 Mya and the estimated number of species ranged from five to seven (i.e. estimates falling within 2 log-likelihood units of the ML solution).

Mitochondrial DNA mean uncorrected pairwise distances between the three species-level taxa within the Indian Ocean region are as follows: 3.71% (range 2.85–4.49%) between *T. corvina* and *T. bourbonnensis*; 3.60% (range 2.94–4.49%) between *T. corvina* and *T. mutata*; and 3.77% (range 2.06–4.95%) between *T. bourbonnensis* and *T. mutata*. Mean uncorrected pairwise distances observed between the different Indian Ocean Lineages (IOL) (delineated as described above) are as follows: 1.38% (range 1.12–1.81%) between the two Mascarene flycatcher subspecies, IOL5 (*T. b. bourbonnensis* on Réunion) and IOL6 (*T. b. desolata* on Mauritius), (c.f. mean within population pairwise distances of 0.29% and 0.49% respectively); 3.46% (range 2.64–4.24%) between IOL2 (*T. m. vulpina* on Anjouan and *T. m. voeltzkowiana* on Moheli) and IOL3 (*T. m. singetra* + *T. m. mutata* on Madagascar and *T. m. pretiosa* on Mayotte); 3.27% (range 2.50–3.90%) between IOL2 and IOL4 (*T. m. comorensis* on Grande Comore); and 2.92% (range 2.24–3.41%) between IOL3 and IOL4. The uncorrected pairwise distance matrix for our four gene mtDNA dataset is provided as Supplementary Information.

4. Discussion

4.1. Previously undetected taxonomic diversity

Within the Indian Ocean islands, *Terpsiphone corvina* on the Seychelles and *T. bourbonnensis* on the Mascarenes are clearly divergent, reflecting their taxonomic status as different species. Remarkably, however, our analyses have revealed a high degree of divergence within the Madagascar paradise flycatcher *T. mutata* species found on Madagascar and the Comoros. Our molecular

phylogeny shows three distinct clades within *T. mutata* that are almost as diverged from each other as they are from the Seychelles paradise flycatcher *T. corvina* and from the Mascarene paradise flycatcher *T. bourbonnensis*, two species which are both morphologically more divergent and geographically more distant. Interspecific uncorrected pairwise distances between full species of closely related taxa reported in other studies are within the range of our findings for our Indian Ocean lineages; Johnson and Cicero (2004) report mtDNA mean uncorrected pairwise distances of 1.86% (range 0–8.2%) amongst 39 pairs of sister species of North American birds, whilst Lovette and Bermingham (1999) report interspecific distances of 0.9–1.7% between sister species of *Dendrocia* warblers. Results from our study indicate the three different *T. mutata* lineages (mtDNA mean uncorrected pairwise distances of between 2.92% and 3.46%) are more genetically differentiated from each other than are some other avian taxa with full species status (see Johnson and Cicero, 2004; Lovette and Bermingham, 1999). Our analysis has revealed a similar pattern of clearly diverged island subspecies within the Mascarene paradise flycatcher, with mtDNA mean uncorrected pairwise distance of 1.38% between *T. bourbonnensis desolata* on Mauritius *and T. b. bourbonnensis* on Réunion. Given these levels of genetic differentiation we observe within *T. bourbonnensis* and *T. mutata*, our findings suggest that the two island lineages on Mauritius and Reunion and the three island lineages within *T. mutata* (Madagascar + Mayotte; Anjouan + Moheli; and Grande Comore) should be considered as separate ESUs and that they should be managed separately for conservation.

Within *T. corvina* on the Seychelles, sequence from one sample was obtained from a historical museum specimen collected in 1888 and stands out as divergent from the six other samples collected from modern specimens on the Seychelles. Careful examination of the DNA sequence traces showed the nucleotide differences to be authentic. The museum specimen was collected on Praslin Island, where the species is now extinct, whereas the six modern samples were all collected from La Digue Island, the only remaining population of this critically endangered species. This result is an example of loss of genetic diversity as a result of the extinction of *T. corvina* on Praslin (Authors unpublished data.).

4.2. Prioritizing conservation effort based on evolutionary distinctiveness

Our results show the Mascarene clade (encompassing Mauritius and Réunion taxa) to be the most deeply diverging Indian Ocean clade, and likely the earliest colonisation of the Indian Ocean islands. Within this clade the Mauritius and Réunion populations are sufficiently diverged to warrant management as separate ESUs. This information is likely to be important because the population of *T. b .desolata* on Mauritius, consisting of 100–223 pairs, is considered to be under threat from habitat degradation, fragmentation and impacts of invasive species (Safford, 1997). Currently, due to the sub-specific status afforded to the flycatcher population on Mauritius, and the fact that the Réunion population is still fairly widespread and common, the population on Mauritius has struggled to attract conservation resources, despite local efforts to obtain funds for basic survey and ecological studies of this island form. Our findings may help to improve the conservation attention that this island population receives.

The Seychelles paradise flycatcher *T. corvina* is highly evolutionarily distinct and forms its own monophyletic clade dating back to the early Pleistocene. Given its critical conservation status (IUCN, 2011), the current conservation efforts to improve this species' long term survival prospects are supported by our findings and should be continued.

One of the most unexpected findings from this study is the considerable evolutionary diversity amongst the Madagascar paradise flycatcher *T. mutata*. The three *T. mutata* lineages (IOL2, IOL3 and IOL4; see Fig. 3). While *T. mutata* is currently divided into subspecies, our molecular reconstruction has revealed that there is a strong evolutionary case for, at minimum, treatment of these three lineages as separate ESUs, warranting conservation management as if they were separate species. This information is likely to be important for conservation efforts on the Comoro islands as little conservation work is currently undertaken on their *T. mutata* subspecies due to the species' wide range and healthy overall numbers. Knowledge that there are three highly diverged lineages amongst these nearby islands may encourage baseline survey work to determine in more detail population sizes and distributions of these unique lineages and allow this novel phylogenetic diversity to be conserved.

4.3. Biogeography and chronology of dispersal and colonisation

Our phylogeny agrees with the results of Fabre et al. (2012), supporting an Asian origin of the *Terpsiphone* species' on the Indian Ocean islands and the African continent. It does not, however, resolve whether the Indian Ocean was colonised directly from Asia or via Africa, or whether the Indian Ocean was colonised independently from both Africa and Asia. A characteristic of this phylogeny is the short internal branch lengths and low branch support for nodes separating the major western Indian Ocean lineages, meaning that the phylogeny is less able to determine the precise chronology of dispersal and island colonisation within the Indian Ocean. Other phylogenetic studies have reported patterns of hard polytomies for several taxonomic groups and attributed this occurrence to rapid radiation (Jønsson et al., 2012; Lara et al., 1996; Leite and Patton, 2002; Rabosky and Lovette, 2008), or the extinction of some taxa (eg Marshall and Baker, 1999).

That the Indian Ocean taxa are not recovered as monophyletic in our study indicates it is likely that more than one colonisation event between the continent and western Indian Ocean occurred to explain the distribution and phylogenetic placement of taxa. The most likely scenario would appear to be two or more independent colonisations of the western Indian Ocean. However, we cannot rule out the possibility of a single colonisation of the western Indian Ocean, followed by a colonisation (or back colonisation) from the Indian Ocean to other landmasses. Likewise, our inability to reject the monophyly of a clade containing *T. atrochalybeia* and the Indian Ocean taxa is most likely explained by the independent colonisation of the Indian Ocean and São Tomé by a common ancestor on Africa that has either become extinct on Africa since these colonisations, or has not been sampled. An alternative scenario is the colonisation of the Indian Ocean from Africa or Asia, followed by (back-) colonisation of Africa from the Indian Ocean, and colonisation of São Tomé thereafter. While our data do not allow us to rule out the latter scenario, it requires more steps and therefore seems less likely. Since the Mascarene clade (encompassing Mauritius and Réunion taxa) is the most deeply-diverging Indian Ocean clade, it was likely an early colonisation of the region, either from Asia or Africa.

The estimated divergence times based on island calibrations and the pairwise genetic distances generated from our study are broadly consistent with a rate of 2% per Mya, an observation that adds confidence to our date estimations. Our estimation of maximum divergence times implies that the *Terpsiphone* genus colonised the Indian Ocean relatively recently (approximately 2 Mya) and that the genus has subsequently rapidly expanded its range and diversified across the region. The Seychelles paradise flycatcher (*T. corvina*) appears to have been isolated for c. 1.75 Mya and the three *T. mutata* lineages have all had continuous evolutionary independence for c. 1.5 Mya. Sea level low stands of 70–80 m below present levels (bpl) occurred at approximately 1.9 and 1.5 Mya (Miller et al., 2005) and these events may have facilitated

range expansion by flycatchers. Elsewhere in the region, shallow-water plateaus exist that would have been exposed during particular low stands (e.g. Saya da Malha, 40,000 km^2, 8–150 m bpl and Nazareth, 7000–20,000 km^2 and 30–150 m bpl lying between India, the Seychelles and the Mascarenes, with additional shallow areas between Madagascar and the Comoros archipelago). These would have resulted in (i) much larger landmasses in the western Indian Ocean including the granitic Seychelles and other islands along the Mascarene bank between the Seychelles and the current Mascarene islands, (ii) a chain of islands extending from India to the Seychelles, and (iii) additional islands between Madagascar and Mayotte, creating stepping stones from Asia through the Indian Ocean islands to Africa. These additional islands would have greatly reduced distances across large expanses of ocean from one landmass to another. The timing of these sea level low stands aligns well with our estimates of species divergence times, and is therefore consistent with an island hopping scenario for the rapid range expansion and divergence shown in the Indian Ocean *Terpsiphone*. Sea level rises between these times would have reduced the number and size of landmasses and may have prevented dispersal between islands. During this time, effects of genetic drift and evolutionary adaptation to island life may have reduced the resulting species tendencies for dispersal, a phenomenon displayed by many island taxa (Bennett and Owens, 2002). More recent sea level low stands during the last glaciation c. 18–23 thousand years ago (Siddall et al., 2003; Rohling et al., 1998) may have facilitated dispersal of *T. mutata* between Madagascar and Mayotte (c.250 km apart) where at least two additional stepping stone islands would have been present at this time. It is not surprising that the Moheli and Anjouan island populations are so similar as the islands are only 42 km apart; what is surprising is how diverged the Grande Comore Island population is given that Moheli is only 35 km away. The monophyly of lineages on Madagascar and Mayotte allows us to rule out a simple conveyor belt 'volcanic islands colonised as they emerge' scenario. Additionally, at least within *T. mutata*, evolutionary affinity does not correlate with geographical distances.

4.4. Plumage as an indicator of phylogeny

The Seychelles paradise flycatcher *T. corvina* has very similar plumage to the São Tomé flycatcher *T. atrochalybeia*. Males of both species are entirely black and possess elongated central tail feathers while the females of both species also possess very similar black, rufous and white plumage. Maximum Likelihood analyses revealed a possible relationship between *T. corvina* and *T. atrochalybeia* but without strong support. Since we could not reject monophyly of *T. corvina* and *T. atrochalybeia*, it is possible that the phenotypic similarities observed in these two species results from a shared common ancestor where males were black with long tails. However given the lack of branch support (bootstrap = 54, PP = 0.55) for their monophyly, convergent or parallel evolution of their phenotype is also a possibility; melanin deposition obscuring ancestral plumage patterns is a common occurrence particularly in island populations, and the tri-colour plumage of the females of both species (black head, rufous wings and tail and light under parts), is thought to be the ancestral *Terpsiphone* plumage type (Fabre et al., 2012).

The Mascarene paradise flycatcher is the only species in the western Indian Ocean lacking elongated central tail feathers, aligning with our phylogenetic reconstruction indicating that this species is the most diverged of the Indian Ocean taxa, and likely the result of a separate earlier colonisation of the region.

4.5. Summary and conclusion

Our phylogenetic reconstruction shows relatively recent colonisation of the western Indian Ocean by *Terpsiphone* flycatchers, that greatly postdates the formation of the older islands of the region. A minimum of two colonisations between the continent and Indian Ocean must have occurred to explain current *Terpsiphone* distribution. Subsequent radiation has not followed a stepwise succession of populations on older islands colonising newer islands as they emerge, but rather appears to have involved rapid range expansions. The resulting lineages, however, are well diverged following relatively long periods of isolation. Within *T. mutata*, only one of the three most diverged lineages corresponds to a current taxonomic unit (*T. mutata comorensis*), while the other two lineages group two or more subspecies. Surprisingly, the phylogenetic placement of *T. mutata* subspecies are neither consistent with a distance-based nor island age-based model of colonisation. The Seychelles paradise flycatcher *T. corvina*, the only Critically Endangered species in the genus, is highly diverged and worthy of the conservation attention it receives. *Terpsiphone bourbonnensis* is the most diverged of the Indian Ocean *Terpsiphone* taxa and likely results from an earlier colonisation of the region.

This phylogenetic reconstruction highlights the importance of sub-specific molecular phylogenies in complex island archipelago settings in clarifying phylogenetic history and evolutionary significant units that may otherwise be overlooked and inadvertently lost. The ability of the Pons et al. (2006) GMYC method to objectively delimit species units based on DNA sequence data makes it a powerful tool to assist conservation planners with the difficult task of objective allocation of finite conservation resources. Our phylogenetic reconstruction has provided a valuable platform upon which to identify hidden pockets of evolutionary distinctiveness, and re-evaluate investment of conservation resources within the *Terpsiphone* flycatchers of the Indian Ocean.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.01.019.

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