

Working at the interface of phylogenetics and population genetics: a biogeographical analysis of *Triaenops* spp. (Chiroptera: Hipposideridae)

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Abstract

New applications of genetic data to questions of historical biogeography have revolutionized our understanding of how organisms have come to occupy their present distributions. Phylogenetic methods in combination with divergence time estimation can reveal biogeographical centres of origin, differentiate between hypotheses of vicariance and dispersal, and reveal the directionality of dispersal events. Despite their power, however, phylogenetic methods can sometimes yield patterns that are compatible with multiple, equally well-supported biogeographical hypotheses. In such cases, additional approaches must be integrated to differentiate among conflicting dispersal hypotheses. Here, we use a synthetic approach that draws upon the analytical strengths of coalescent and population genetic methods to augment phylogenetic analyses in order to assess the biogeographical history of Madagascar's *Triaenops* bats (Chiroptera: Hipposideridae). Phylogenetic analyses of mitochondrial DNA sequence data for Malagasy and east African *Triaenops* reveal a pattern that equally supports two competing hypotheses. While the phylogeny cannot determine whether Africa or Madagascar was the centre of origin for the species investigated, it serves as the essential backbone for the application of coalescent and population genetic methods. From the application of these methods, we conclude that a hypothesis of two independent but unidirectional dispersal events from Africa to Madagascar is best supported by the data.

Keywords: biogeography, coalescent theory, Madagascar, phylogenetics, population genetics, *Triaenops*

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Introduction

Biogeography is an inherently interdisciplinary field, drawing from the realms of ecology, evolutionary biology, systematics, geology, climatology, and geography. Over the past several decades, researchers (Emerson 2002; Raxworthy *et al.* 2002; Evans *et al.* 2003) have recognized the need to incorporate patterns of relatedness among taxa into biogeographical studies, overlaying phylogenetic trees on

geographical maps to determine the direction of movement within lineages and patterns of connection between areas. The resulting field of cladistic biogeography was organized around the central assumption that common branching patterns among areas of endemism for multiple codistributed species groups will reveal patterns of vicariance (Nelson & Platnick 1981). More recently, an explicitly historical perspective has been advocated, which would attach dates to trees and directly compare the timing of cladogenic and geological events to test hypotheses of causality (Donoghue & Moore 2003; Yoder & Yang 2004).

While methods for dating phylogenetic trees are not without their critics (Heads 2005), dated phylogenies can

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be powerful tools for testing biogeographical hypotheses of dispersal and vicariance (Yoder *et al.* 2003; Near & Keck 2005). For example, if an inferred taxon cladogram is topologically and temporally consistent with its relevant area cladogram, then a hypothesis of vicariance is inferred, whereas cladogenic events that postdate the establishment of a barrier are presumed to result from dispersal (Donoghue & Moore 2003).

However, even the most accurately dated phylogenies may not suffice for discriminating among biogeographical hypotheses. If an observed phylogenetic topology is consistent with multiple dispersal scenarios, then a phylogenetic approach may be of limited value. Moreover, there are numerous cases in which phylogenetic resolution is weak, further limiting the power of phylogenies to reveal the biogeographical history of a species group. Poor resolution can result from conflict among gene trees, rapid bursts of speciation, or patterns of reticulation that are characteristic of recent speciation (Givnish *et al.* 2004; Echelle *et al.* 2005; Wickström *et al.* 2005). In such situations, a synthetic approach combining coalescent and population genetic with phylogenetic analyses can be informative for discriminating among competing biogeographical hypotheses.

In the best-case scenario, wherein a phylogeny is completely resolved and (for the sake of argument) known with certainty, an investigator can accept or reject a single-origin hypothesis. Similarly, if the organismal group of interest is completely sampled both taxonomically and geographically, dispersal hypotheses can be identified and supported with high degrees of confidence. These are the undeniable powers of phylogenetic analysis for biogeographical study. Unfortunately, however, many biogeographers find themselves in situations in which phylogenies are poorly resolved, species radiations are incompletely sampled, and/or phylogenetic results are ambiguous with regard to directionality of geographical movements. In the latter case, coalescent and population genetic analyses offer auxiliary and complementary information for discriminating among hypotheses. This is particularly relevant for recently diverged species radiations. A distinct strength of coalescent analysis is its ability to harness information contained among alleles that reflect incomplete lineage sorting, a situation typical of young species. Coalescent-based methods have been developed to estimate ancestral population sizes, divergence times, and migration rates (Wakeley 1996; Rannala & Yang 2003; Hey & Nielsen 2004; Kuhner 2004). Layered upon these analyses, traditional population genetics can estimate patterns of gene flow, selection, and population growth, and thus evaluate assumptions implicit in many coalescent analyses. Therefore, it is via the synthetic application of phylogenetic, coalescent, and population genetic analyses that an investigator can derive and compare hypotheses from deep divergence events up through present population dynamics, including the various stages along

this continuum. Here, we illustrate the utility of this approach with an example from *Triaenops*, a genus of Chiroptera distributed in eastern Africa, southwestern Asia, and Madagascar.

Study organism

Despite the potential for comparative studies among animals with varying degrees of vagility, the bats of Madagascar have largely been ignored by historical biogeographical studies. The geological history of Madagascar precludes vicariance as a biogeographical hypothesis for bats, given that the isolation of the island from Africa [165 million years ago (Ma); Storey *et al.* 1995] and from India (88 Ma; Coffin & Rabinowitz 1992) considerably predates the evolution of Chiroptera (71–58 Ma; Teeling *et al.* 2005). Biogeographical hypotheses for Malagasy bats therefore must involve scenarios of dispersal. The capacity for flight in bats might be expected to increase the likelihood of long-distance dispersal. Chiroptera have experienced some of the longest known dispersal events among mammals, and several isolated island groups such as the Galapagos (McCracken *et al.* 1997), Hawaii (Stone & Pratt 1994), and New Zealand (King 1990) have native terrestrial mammalian faunas consisting largely or exclusively of bats. Even so, long-distance flight is more likely in some chiropteran taxa than in others. Families with large wings such as Pteropodidae, or long, narrow wings (high wing-aspect ratio) such as Molossidae, are better adapted for long-distance dispersal than those with short, broad wings (low wing-aspect ratio) such as Hipposideridae (Ahmad 1984).

The genus *Triaenops* (Chiroptera: Hipposideridae) consists of four species (Fig. 1a), of which three are endemic to Madagascar, and a fourth is distributed in eastern Africa and southwestern Asia (Peterson *et al.* 1995; Eger & Mitchell 2003; Ranivo & Goodman in press). On the basis of the modern geographical distribution of *Triaenops* species, we can propose various hypotheses regarding the biogeographical history of the genus (Yoder *et al.* 2005). Either the Malagasy *Triaenops* species *T. rufus*, *T. furculus*, and *T. auritus* form a clade that originated via a single dispersal event from Africa (Fig. 1b); or, they do not form a clade, meaning that the biogeographical history of the genus may have involved multiple independent dispersal events. Within the nonmonophyly category, the centre of origin could have been either Africa or Madagascar. If Africa was the centre of origin, one would hypothesize two independent but unidirectional dispersal events (Fig. 1c) that most probably occurred at different times. If Madagascar was the centre of origin, one would hypothesize an initial colonization of Madagascar by a proto-*Triaenops* ancestor, with a subsequent back dispersal to Africa (Fig. 1d). Given that these are hipposiderid bats with low wing-aspect ratios, one might assume that their proclivity for long-distance

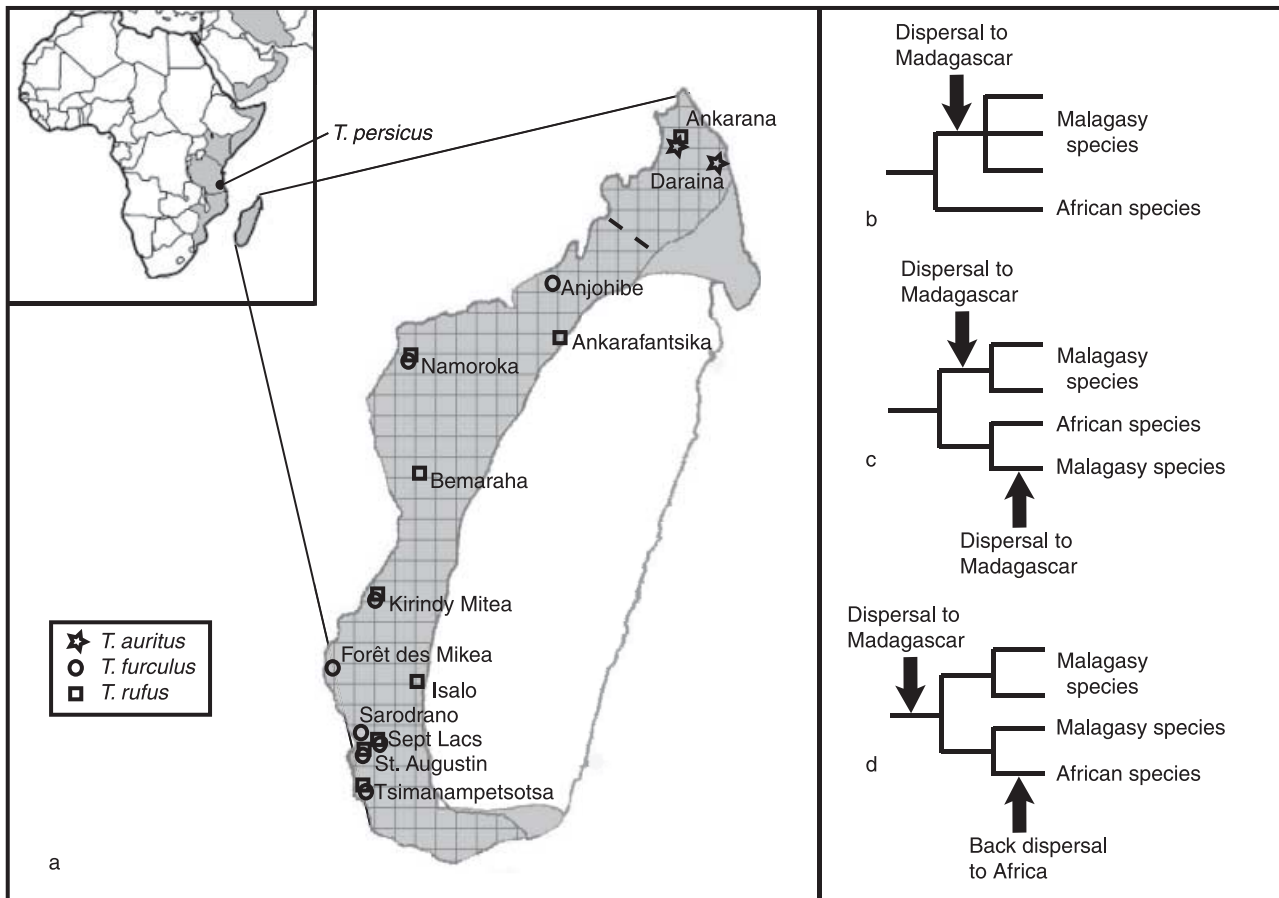


Fig. 1 (a) Geographic distributions and sampling sites of *Triaenops* species. The range of *Triaenops persicus* in mainland Africa and Asia is shown in the inset map. Ranges of Malagasy species are indicated in the blow-up of Madagascar, with the *Triaenops rufus* range indicated by shading and the *Triaenops auritus* (north of the dashed line) and *T. furculus* (south of the dashed line) ranges indicated by hatch marks. The dashed line is meant to indicate the approximate location of the geographical division between *T. auritus* and *Triaenops furculus*. The right panel illustrates biogeographical hypotheses proposed for *Triaenops* species. (b) Single dispersal hypothesis, all Malagasy species form a monophyletic clade that derives from a single dispersal event from mainland Africa. (c) Multiple dispersal hypothesis, Malagasy *Triaenops* are paraphyletic with respect to mainland African *Triaenops*. This scenario differs from that described by (d) in the direction of dispersal events.

dispersal would be diminished relative to other Malagasy bats, thereby setting a null expectation that Madagascar was colonized only once within the *Triaenops* lineage.

Phylogenetic analyses can be sufficient to reject the single-dispersal hypothesis. If the Malagasy species are found to constitute a clade, then the hypothesis is supported (Fig. 1b). If the Malagasy lineage is shown to be polyphyletic, then additional approaches are required to differentiate among dispersal scenarios. In the case of the two most parsimonious multiple-dispersal hypotheses (Fig. 1c, d), we would expect to see significant variation in the ages of lineages resulting from different dispersal events. We use population genetic analyses to compare patterns of population substructuring and population growth among *Triaenops* lineages, with the expectations that (i) older lineages will have had time to develop significant genetic differentiation among geographically distinct populations, and that (ii) species resulting

from more recent dispersal events may still retain the signal of population growth following their immigration and founding of a new population. We employ mitochondrial DNA (mtDNA) sequence data to compare these biogeographical hypotheses and to explore their evolutionary implications using a combination of phylogenetic, coalescent, and population genetic approaches.

Methods

Sample collection

In the context of a broad geographical survey of bats occurring in the dry habitats of the island, a considerable number of sites were visited (Table 1; Goodman *et al.* 2005). Animals were trapped at each site using various methods, including harp traps and mist nets placed along trails, over

Species	Location	Sample size	Haplotype diversity	Nucleotide diversity (in %)
<i>T. auritus</i>	RS d'Ankarana	23	0.52	0.08
	Daraina	1	—	—
	Total	24	0.56	0.09
<i>T. furculus</i>	Anjohibe	9	0.56	0.09
	RNI de Namoroka	6	0.60	0.14
	PN de Kirindy-Mitea	1	—	—
	Forêt de Mikea	3	0.00	0.00
	Sarodrano	2	1.00	0.14
	Sept Lacs	4	0.50	0.14
	St Augustin	7	0.48	0.07
	PN de Tsimanampetsotsa	11	0.60	0.10
	Total	43	0.70	0.15
<i>T. rufus</i>	RS d'Ankarana	2	0.00	0.00
	PN d'Ankarafantsika	2	1.00	1.31
	RNI de Namoroka	3	1.00	1.05
	PN de Bemaraha	8	0.96	0.99
	PN de Kirindy-Mitea	8	0.89	0.95
	PN de l'Isalo	4	1.00	0.93
	Sept Lacs	2	1.00	1.29
	St Augustin	9	0.94	0.63
	PN de Tsimanampetsotsa	16	0.98	0.87
	Total	54	0.97	0.87
<i>T. persicus</i>	Pugu Hills, Tanzania	12	1.00	0.67

Table 1 Sample size and diversity measures for each *Triaenops* species, given by site and as totals. We consider the two northern sites from the *Triaenops furculus* range to represent a distinct species, *Triaenops auritus* (see text for details). Total diversity measures are calculated by considering the entire species as a single gene pool.

water, and at cave entrances. Voucher specimens were collected, and pectoral muscle samples were saved in a tissue storage buffer containing NaCl-saturated 25% DMSO and 250 mM EDTA within 30 min after an animal was sacrificed. These specimens and associated tissue samples are housed in the Field Museum of Natural History, Chicago, and the Département de Biologie Animale, Université d'Antananarivo, Antananarivo.

Specimens of *Triaenops rufus* were collected at sites distributed relatively evenly throughout the range of the species (Fig. 1a), while specimens of *Triaenops furculus* were collected from sites that were more regionally clustered. We refer to these clustered regions of sampling sites as the central (Anjohibe and Namoroka) and southern regions (Kirindy Mitea, Forêt de Mikea, Sarodrano, Sept Lacs, St Augustin, and Tsimanampetsotsa; see Fig. 1a). *Triaenops auritus* is represented by the northernmost sampling sites (Ankarana and Daraina) in Fig. 1(a). *Triaenops persicus* samples were collected in the Pugu Hills, a cave site near Dar es Salaam, Tanzania.

DNA isolation, amplification, and sequencing

Total genomic DNA was isolated using a DNeasy DNA isolation kit (QIAGEN), and stored in the provided elution buffer. We used primers L14724: 5'-CGAAGCTTGATA TGAAAACCATCGTTG-3' and H15506: 5'-AGTGGRT

TRGCTGGTGTRTARTTTC-3' (Kocher *et al.* 1989) to amplify approximately 750 bp of the mitochondrial cytochrome *b* gene. Polymerase chain reactions (PCRs) were performed in 50 µL reaction volumes, each containing 2.25 mM MgCl₂, 0.25 mM dNTPs, 2.5 U *Taq* DNA polymerase, 5 µL Promega 10X buffer, 5 µL genomic DNA, and 20 pmol of each primer. The amplification involved an initial denaturation at 94 °C for 2 min, followed by 40 cycles of 94 °C for 45 s, 52 °C for 45 s, and 72 °C for 1 min, with a final elongation step at 72 °C for 4 min. The target fragment was then purified using either gel band excision (Gel Excision kit, QIAGEN) or PCR purification (PCR Purification Kit, QIAGEN).

The purified DNA fragment was sequenced from both directions using the BigDye terminator cycle sequencing kit version 3.0 (Applied Biosystems) in a 20-µL reaction volume containing 2 µL of the BigDye solution, 1.2 µL ABI 5X sequencing buffer, 5 pmol of primer, and 100–200 ng of purified PCR product. The sequencing reaction consisted of 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min, with temperatures changing at 1 °C per second. The sequencing reactions were cleaned of unincorporated nucleotides using genCLEAN dye terminator removal plates (Genetix), and analysed on an MJ BaseStation automated sequencer.

Each individual was sequenced four to 10 times from multiple PCR reactions to resolve any ambiguities present

in single sequencing passes. We used SEQUENCHER version 4.2 (Gene Codes) to create consensus sequences for each individual, and aligned the sequence data by eye using MACCLADE version 4.0 (Maddison & Maddison 2000), yielding a final data set of 732 sites. All sequences were submitted to GenBank (Accession nos DQ005772–DQ005850; see Appendix).

Phylogenetic analyses

We used both Bayesian and maximum parsimony phylogenetic analyses to determine the number of independent lineages present in the data, and to examine the relationships among those lineages. We included all *Triaenops* sequences in these analyses, as well as sequences (Kingston *et al.* 2001; Sakai *et al.* 2003; Wang *et al.* 2003) from other species in the families Hipposideridae and Rhinolophidae (following the classification of Simmons 2005) as outgroups (see Appendix).

We performed parsimony analyses with PAUP* version 4.0b10 (Swofford 1998) using heuristic searches with tree-bisection–reconnection (TBR) branch swapping, and setting the maximum number of trees saved to 1000. Parsimony bootstrap analyses were performed with PAUP* using heuristic searches, TBR branch swapping, and 100 replicates of the random addition search option.

We used MRBAYES version 3.0b4 (Huelsenbeck & Ronquist 2001) to perform a Bayesian analysis with flat priors. We ran four chains of 10 million generations each, with sampling every 500 generations. The chains were heated using the temperature scaling factor $T = 0.2$. We specified an HKY + Γ model with four rate categories and the gamma shape parameter $\alpha = 0.1231$, which was estimated using MODELTEST version 3.06 (Posada & Crandall 1998). After examining the likelihood profile using TRACER version 1.1 (<http://evolve.zoo.ox.ac.uk/software.html?id=tracer>), we discarded the first 5000 trees as a burn-in and constructed a 50% majority consensus tree from the remaining 15 000 trees in PAUP* (Swofford 1998). Branch lengths on the Bayesian consensus tree were estimated by maximum likelihood in PAUP* with the tree topology fixed.

Coalescent analyses

Using the species tree topology recovered by both the parsimony and Bayesian analyses (Fig. 2), we used MCMCOAL version 1.0a (Yang 2002; Rannala & Yang 2003) to estimate the parameters $\theta = 2N_g\mu$, where μ is the mutation rate in substitutions/site/year and τ , the divergence time scaled by the mutation rate μ . This analysis estimates θ both for tips (current species) and nodes (hypothesized ancestral species) in the tree, as well as τ for all relevant nodes; for this data set, the analysis resulted in the estimation of 10 parameters ($\theta_A, \theta_F, \theta_P, \theta_R, \theta_{AF}, \theta_{PR}, \theta_{AFPR}$

Table 2 Evolutionary model parameters for *Triaenops* species. Alpha is the shape of the gamma distribution describing the among-site rate variation.

	<i>T. auritus</i>	<i>T. furculus</i>	<i>T. persicus</i>	<i>T. rufus</i>
Model	HKY	HKY	HKY	HKY + Γ
–ln L	1037.756	1063.038	1170.694	1412.517
π_A	0.286	0.289	0.269	0.268
π_C	0.299	0.293	0.296	0.289
π_G	0.136	0.141	0.170	0.172
π_T	0.280	0.277	0.265	0.271
Ti/Tv ratio	6.012	8.473	10.525	18.251
Alpha	–	–	–	0.243

τ_{AF}, τ_{PR} , and $\tau_{AFPR} - \tau_{AF}$, where A = *T. auritus*, F = *T. furculus*, P = *T. persicus*, R = *T. rufus*, and multiple subscripts refer to the most recent common ancestor of the specified species). Using the default priors ($\theta = 0.001 \pm 0.00071$; $\tau_{AF} = 0.0016 \pm 0.0008$; $\tau_{PR} = 0.005 \pm 0.00112$; $\tau_{AFPR} - \tau_{AF} = 0.0074 \pm 0.00272$), we first ran several short runs (100 000 samples) to fine-tune the algorithm parameters $\epsilon_1 - \epsilon_5$ until the acceptance ratios of the proposals fell within the interval (0.1, 0.7). We then estimated the parameters of interest from a long run (1 000 000 samples), which was then repeated with a different random seed to verify the accuracy of the results via convergence.

Population genetic analyses

We used population genetic methods to test predictions of population expansion and genetic structure within the four *Triaenops* species. Because a fundamental assumption of these methods is that the data are not significantly influenced by selection, we first tested for evidence of selection using the McDonald–Kreitman test (McDonald & Kreitman 1991) on pairs of sister species as implemented in DNASP version 4.0 (Rozas *et al.* 2003). Given the negative results of those tests, indicating a lack of significant selective pressure, we proceeded with further population genetic analyses.

For each species, we used MODELTEST version 3.06 to determine the best-fitting model of evolution, and to estimate evolutionary parameters such as nucleotide frequencies and transition/transversion ratios (see Table 2). Using these species-specific parameter values, we employed ARLEQUIN version 2.001 (Schneider *et al.* 2001) to calculate descriptive measures such as haplotype diversity (h) and nucleotide diversity (π). We also examined patterns of genetic structuring among sampling sites using an analysis of molecular variance (AMOVA), pairwise F_{ST} measures, and Mantel tests of correlations between genetic and geographical distances (Slatkin 1991; Excoffier *et al.* 1992).

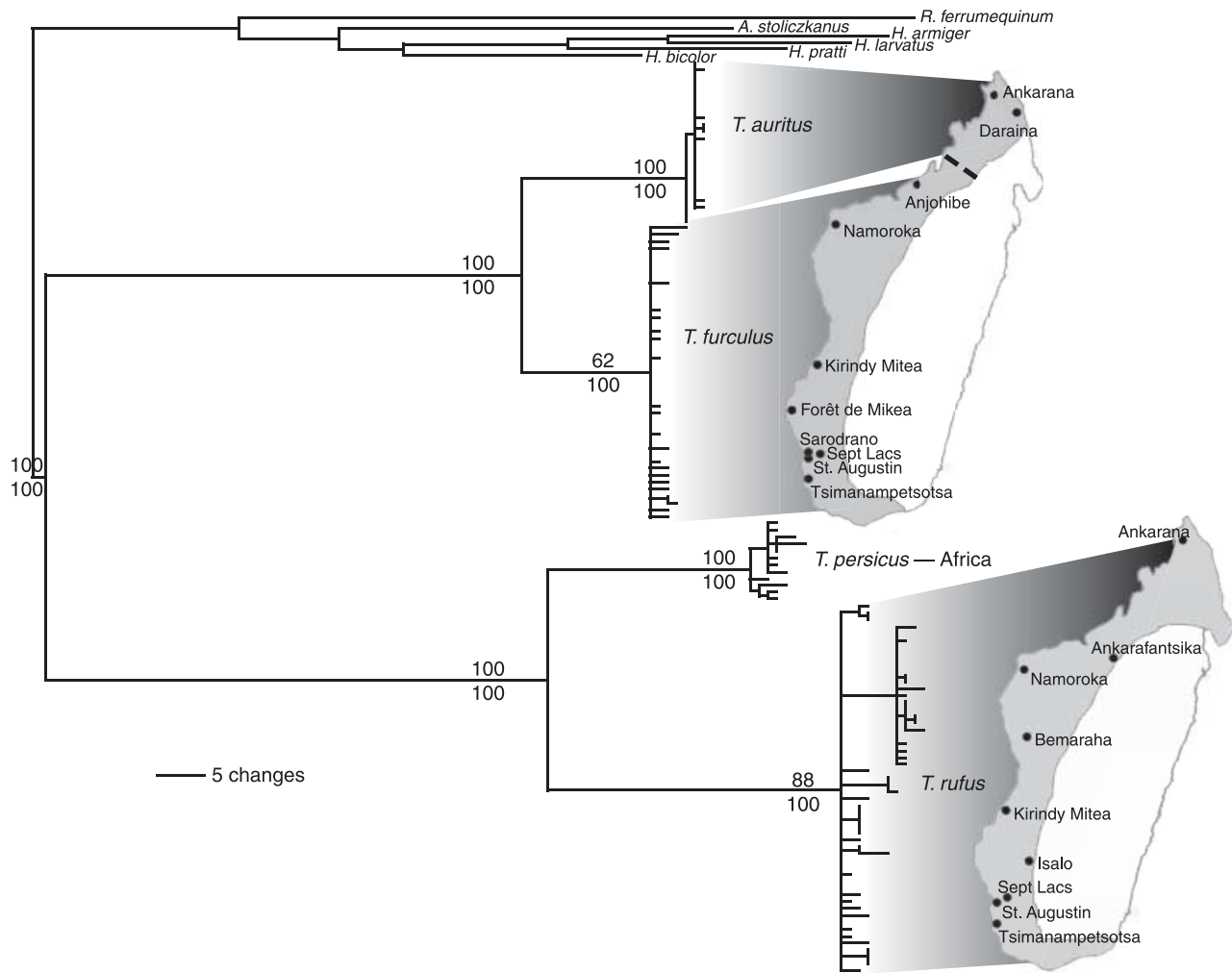


Fig. 2 Bayesian phylogeny of *Triaenops* species, with geographical distributions of Malagasy lineages. Support measures of species-level lineages are given as Bayesian posterior probabilities (%) below each branch and maximum parsimony bootstrap support values above each branch. Samples of *Triaenops persicus* represent a single population in Tanzania.

We tested hypotheses of population expansion using comparisons of the diversity statistics π and h , neutrality tests such as Fu's (1997) F_S , Fu & Li's (1993) F^* and D^* , Ramos-Onsins and Rozas's (2002) R_2 , and von Haeseler *et al.*'s (1995) expansion coefficient S/d , as implemented in the software package DNASP. Under a model of sudden population expansion, haplotype diversity (h) is expected to be high as most new haplotypes are maintained in the population; concomitantly, because these new haplotypes are all recently derived, the nucleotide diversity (π) of the population is predicted to be low. A similar relationship is described by von Haeseler *et al.*'s (1995) expansion coefficient, S/d , or the ratio between the number of polymorphic sites (S) and the mean pairwise sequence difference (d). Fu (1997) showed that comparisons of statistical neutrality tests could be used to infer the demographic history of a population. If Fu's (1997) F_S is significant but Fu & Li's

(1993) F^* and D^* are not, then a history of population growth or range expansion is indicated; the reverse situation suggests the influence of background selection. Ramos-Onsins & Rozas (2002) recently described another powerful statistic for detecting population growth, R_2 .

As a more rigorous analysis of population growth, we used LAMARC version 2.0.2 (Kuhner 2006) to estimate the population growth parameter g for each of the three Malagasy species. For each species, we ran three independent runs of a Bayesian search. Each run consisted of three replicates of one short and one long chain. The short chains were 10 000 steps long, with 500 trees sampled every 20 steps and a burn-in of 1000 trees. The long chains were 1 000 000 steps long, with 10 000 trees sampled every 100 steps and a burn-in of 1000 trees. Priors for the growth rate were set at the maximum allowable range of -500–15 000.

	Prior	Posterior \pm SD	Parameter estimate \pm SD
θ_A	0.001 \pm 0.00071	0.00202 \pm 0.00081	$N_e = 5050 \pm 2025$
θ_F	0.001 \pm 0.00071	0.00278 \pm 0.00096	$N_e = 6950 \pm 2400$
θ_P	0.001 \pm 0.00071	0.00555 \pm 0.00131	$N_e = 13\,875 \pm 3275$
θ_R	0.001 \pm 0.00071	0.01257 \pm 0.002	$N_e = 31\,425 \pm 5000$
θ_{AF}	0.001 \pm 0.00071	0.00284 \pm 0.001	$N_e = 7100 \pm 2500$
θ_{PR}	0.001 \pm 0.00071	0.00180 \pm 0.001	$N_e = 4500 \pm 2500$
θ_{AFPR}	0.001 \pm 0.00071	0.01293 \pm 0.00198	$N_e = 32\,325 \pm 4950$
τ_{AF}	0.0016 \pm 0.0008	0.00229 \pm 0.00086	57 250 \pm 21 500 BP
τ_{PR}	0.005 \pm 0.00112	0.00556 \pm 0.00116	139 000 \pm 29 000 BP
$\tau_{AFPR} - \tau_{AF}$	0.0074 \pm 0.00272	0.00509 \pm 0.00209	127 250 \pm 52 250 BP
τ_{AFPR}	—	0.00738	184 500 BP

Table 3 Estimates of population size parameters (θ_i) and ages of nodes (τ_i) in the *Triaenops* species tree, scaled by mutation rate. Estimates of θ are given for each species and for ancestral species indicated by the nodes in the species tree. Parameter estimates (N_e , absolute ages of nodes) were derived assuming $\mu = 4 \times 10^{-8}$ substitutions/site/year (Hulva *et al.* 2004) and a generation time of 5 years.

Results

Phylogenetic analyses

Both parsimony and Bayesian phylogenetic analyses reveal a phylogeny that is consistent with four reciprocally monophyletic species lineages. Because the Malagasy endemic *Triaenops rufus* is supported as a lineage separate from, but sister to, the mainland African species *Triaenops persicus*, a single origin hypothesis is not supported (Fig. 2). Furthermore, a separate species lineage, *Triaenops auritus*, is supported in northern Madagascar and is genetically and geographically distinct from, but sister to, *Triaenops furculus*. Measures of HKY-corrected genetic diversity further illustrate the differentiation of the previously subsumed *T. auritus* species. Intraspecific diversity measures range from 0.28% for *T. auritus* to 2.19% for *T. rufus*, while diversity measures between sister-species pairs are 4.57% for *T. auritus*-*T. furculus* and 8.19% for *T. rufus*-*T. persicus*. These measures correspond well with those typically observed at cytochrome *b* between sister species within Chiroptera (mean = 6.83%, range = 2.50–16.42%; Bradley & Baker 2001).

Coalescent analyses

We used a coalescent-based analysis (Rannala & Yang 2003) to estimate the divergence time parameter τ , as well as the population diversity parameter θ , for each node (and tip, in the case of θ in our *Triaenops* species tree (Table 3). Using a mutation rate from the cytochrome *b* gene of *Pipistrellus* bats (Hulva *et al.* 2004) and assuming a generation time of 5 years (S.M.G., personal observation), we calculated N_e and the absolute ages of nodes from the mutation rate-scaled parameters θ and τ , respectively. The species tree was constrained to the observed topology [(*T. auritus*, *T. furculus*), (*T. persicus*, *T. rufus*)] for these analyses.

Estimates of the population diversity parameter θ indicate significant differences in the sizes of current and ancestral

species. The clade containing *T. auritus* and *T. furculus* is distinguished by small population sizes for both current ($\theta_A = 0.00202 \pm 0.00081$; $\theta_F = 0.00278 \pm 0.00096$) and ancestral populations ($\theta_{AF} = 0.00284 \pm 0.001$). Conversely, while the current population sizes for *T. persicus* and *T. rufus* are much larger ($\theta_P = 0.00555 \pm 0.00131$; $\theta_R = 0.01257 \pm 0.002$), our analyses indicate that this species pair evolved from a much smaller ancestral population ($\theta_{PR} = 0.00180 \pm 0.001$). The earliest inferred species in the *Triaenops* lineage, represented by the root node, is estimated to have had a larger effective population size, similar to that of modern *T. rufus* ($\theta_{AFPR} = 0.01293 \pm 0.00198$).

Divergence age estimates indicate that the sister species *T. auritus* and *T. furculus* diverged much more recently ($\tau_{AF} = 0.00229 \pm 0.00086$) than the sister species *T. rufus* and *T. persicus* ($\tau_{PR} = 0.00556 \pm 0.00116$) (Fig. 3). The root node for *Triaenops* is estimated as being not much older than the divergence of *T. rufus* and *T. persicus* ($\tau_{AFPR} = 0.00738$). These analyses place the root node of *Triaenops* at 184 500 BP. The divergence event separating *T. rufus* and *T. persicus* dates to no earlier than 139 000 \pm 29 000 BP, and the divergence of *T. auritus* and *T. furculus* dates to 57 250 \pm 21 500 BP.

Population genetic analyses

Hierarchical analyses of molecular variance (AMOVA) and pairwise F_{ST} tests reveal different patterns of genetic structuring between *T. furculus* and *T. rufus*. *T. furculus* is characterized by significant genetic structuring between the central (Anjohibe and Namoroka) and southern (Kirindy Mitea, Forêt de Mikea, Sarodrano, Sept Lacs, St Augustin, and Tsimanampetsotsa) geographical regions (see Fig. 1a), but high levels of genetic mixing within those regions. An AMOVA shows significant structure between the two regional groups ($\Phi_{CT} = 0.608$, $P = 0.038$), though populations within regional groups are thoroughly mixed (central region: $\Phi_{ST} = 0.049$, $P = 0.253$; southern region: $\Phi_{ST} = -0.115$, $P = 0.818$). A remarkably different pattern is observed in *T. rufus*.

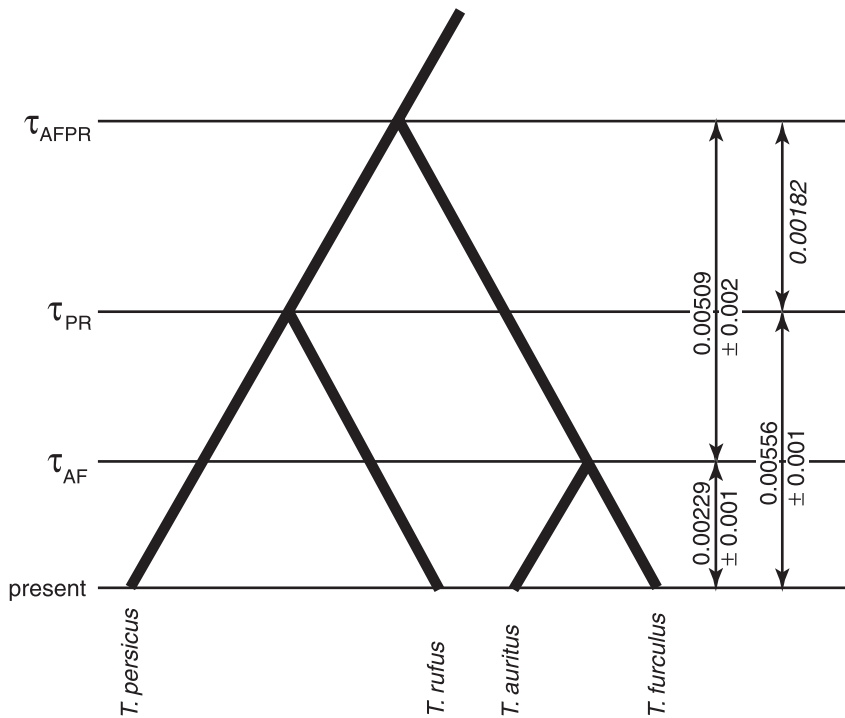


Fig. 3 Mutation rate-scaled divergence times for nodes in the *Triaenops* species tree. Internode intervals inferred from direct estimates (e.g. $\tau_{AFPR} - \tau_{PR}$) are given in italics.

Table 4 Results of demographic analyses of Malagasy *Triaenops* species, including the expected results under a scenario of population expansion. Excepting coalescent estimates of population growth, results for *Triaenops furculus* are presented separately for the structured central and southern regions.

	<i>T. auritus</i>	<i>T. furculus</i> (central)	<i>T. furculus</i> (south)	<i>T. rufus</i>	Expansion
Haplotype diversity (<i>h</i>)	0.590	0.562	0.468	0.966†	High
Nucleotide diversity (π), %	0.091	0.139**	0.083	0.872**	Low
Expansion coefficient (<i>S/d</i>)	7.463	6.565	5.848	8.144	High
Fu & Li's (1993) <i>F</i> *	-0.563	-2.181	-0.464	-2.294	Not significant
Fu & Li's (1993) <i>D</i> *	-0.189	-1.956	-0.240	-2.408	Not significant
Fu's (1997) <i>F</i> _S	-1.895	-1.754	-1.208	-19.268**	Significant
Ramos-Onsins & Rozas's (2002) <i>R</i> ₂	0.085	0.133	0.096	0.054*	Significant
Population growth (<i>g</i>)	13301.39	4715.58		949.31	
95% CI of <i>g</i> parameter	(905.41, 14971.63)	(269.35, 14726.08)		(467.45, 2024.40)	

P* < 0.05; *P* < 0.01; †observed value > upper 95% CI.

Analyses of population structuring reveal no significantly distinct gene pools within *T. rufus*, indicating instead that the species represents a single deme over its entire range. We found no evidence of genetic structuring among populations in an AMOVA ($\Phi_{ST} = -0.028$, *P* = 0.725), and all pairwise *F*_{ST} estimates were insignificant (*F*_{ST} = -0.496–0.192). Over a geographical distribution that spans the entire 1200 km length of the island, we found no evidence of isolation by distance (*r* = 0.212, *P* = 0.133). To test whether the structure present among *T. furculus* sites reflects a real segregation of gene pools and not just a sampling artefact, we performed an AMOVA among *T. rufus* sites selected to mirror the gaps in our sampling of *T. furculus* (Ankarafantsika

and Namoroka vs. Kirindy Mitea, Isalo, Sept Lacs, St Augustin, and Tsimanampetsotsa). This analysis further illustrates the real lack of genetic structure within *T. rufus* ($\Phi_{ST} = -0.07\%$; *P* = 0.334) as compared with *T. furculus*.

We used diversity statistics, neutrality tests, and coalescent estimates of the population growth rate *g* to look for signals of population growth in each of the three Malagasy *Triaenops* species (Table 4; Fig. 4). Analyses of *T. auritus* strongly indicate a demographic history of a small but constant population size, with insignificant results for all statistical tests and a nearly flat posterior probability distribution for *g*. Demographic analyses of the structured central and southern populations of *T. furculus* also support

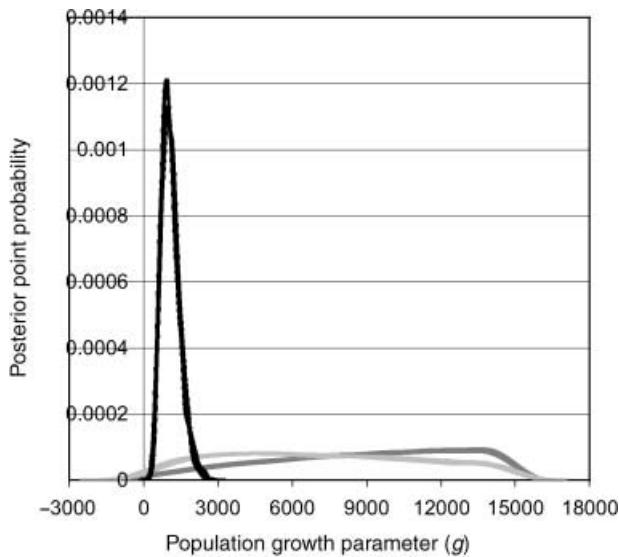


Fig. 4 Posterior probability distributions of population growth parameter (g) for Malagasy species. Each species is represented by three independent runs. *Triaenops rufus* is shown in black, *Triaenops auritus* in dark grey, and *Triaenops furculus* in light grey.

a model of a small but constant population size. The nucleotide diversity for the central population of this species is significantly low, but the haplotype diversity is well within the 95% CI (upper 95% confidence limit = 0.886). Notably, the two tests shown to be the most powerful at detecting population growth, Fu's (1997) F_S and Ramos-Onsins & Rozas's (2002) R_2 , are not significant. Correspondingly, estimates of g have a nearly flat posterior probability distribution. In contrast, a history of population growth or range expansion is strongly indicated for *T. rufus*. This species is characterized by very high haplotype and significantly low nucleotide diversities, and the neutrality tests F_S and R_2 are significant, but F^* and D^* are not. Furthermore, estimates of g reveal a strong signal of population growth in this species with $g = 949.31$ (CI = 467.45, 2024.40), averaged across three runs.

Discussion

Historical biogeography of *Triaenops*

Parsimony and Bayesian phylogenetic analyses reveal a phylogeny that is consistent with four reciprocally monophyletic species lineages (Fig. 2). Because the Malagasy endemic *Triaenops rufus* is supported as a lineage separate from, but sister to, the mainland African species *Triaenops persicus*, a single origin hypothesis is not supported. Previous work had suggested that *T. rufus* and *T. persicus* be synonymized (Hill 1982; Garbutt 1999), but the long branches separating the two clades, reflecting considerable genetic

differentiation between them, indicates that the proposed synonymy be rejected. Furthermore, our data support Ranivo & Goodman's (in press) morphological analyses in recognizing a species lineage in northern Madagascar, *Triaenops auritus*, which is genetically and geographically distinct from, but sister to, *Triaenops furculus*.

Using the *Triaenops* phylogeny, we can address the competing biogeographical hypotheses concerning the origin of the Malagasy species *T. auritus*, *T. furculus*, and *T. rufus*. Our data clearly reject a single-dispersal hypothesis (Fig. 1b), which predicts that lineages endemic to Madagascar form an exclusive clade. Instead, we find that Malagasy *Triaenops* are paraphyletic with respect to the mainland African species *T. persicus*, indicating that the distribution of Malagasy and African *Triaenops* involved at least two independent dispersal events (Fig. 1c, d). The phylogenetic results in isolation, however, cannot detect the direction of dispersal events, and are therefore unable to discriminate between the dispersal hypotheses depicted in Fig. 1(c, d). In other words, the phylogeny does not allow us to determine whether Madagascar or Africa was the geographical centre of origin for this species radiation.

Timing of dispersal events

The multiple-dispersal models suggested by our phylogenetic analyses suggest an analysis of the timing and direction of these events. A population genetic model incorporating parameters of population substructuring might posit that, given rough equalities in geographical range and dispersal ability, older lineages will tend to be more geographically structured than younger lineages (Sawyer 1977; Castric & Bernatchez 2003). Under the unidirectional dispersal hypothesis, we would then expect to detect significant differences between the extent of population substructuring in the Malagasy lineages, with the extent of substructuring positively correlated with the age of the lineage. Under the bidirectional hypothesis, we expect to find equivalent patterns of substructure within the two Malagasy lineages, while the mainland species *T. persicus* would lack significant population substructure.

We used a coalescent-based analysis (Rannala & Yang 2003) to estimate the divergence time parameter τ , as well as the population diversity parameter θ , for each node (and tip, in the case of θ) in our *Triaenops* species tree (Table 3). Divergence age estimates indicate that the sister species *T. auritus* and *T. furculus* diverged much more recently than the sister species *T. rufus* and *T. persicus* (Fig. 3). The root node for *Triaenops* is not much older than the divergence of *T. rufus* and *T. persicus*. These divergence age estimates are very similar to the prior settings, possibly indicating a lack of signal in the data for estimating time parameters; therefore, we advocate extreme caution in interpreting these results. Additional error may result from bias in the

estimation method. Using data from great apes, Rannala & Yang (2003) found that their analyses consistently underestimated divergence dates. To alleviate this problem, the authors used a highly informative prior based on fossil information for the chimp-human node, which resulted in more reasonable estimates of τ throughout the tree. Unfortunately, such fossil evidence is lacking in African hipposiderid bats, and the absolute dates presented here must be taken as preliminary estimates to be verified with additional data.

Implications from population genetic analyses

Although we are not able to date nodes in the species tree with a great deal of confidence, our population genetic model does predict significant differences in the extent of population structuring within species. Specifically, if the unidirectional dispersal hypothesis is correct and if intra-island dispersal capacities are roughly equivalent between species, we expect to detect significant differences in patterns of population substructuring between the two largely sympatric Malagasy lineages, *T. rufus* and *T. furculus*, with the extent of substructuring positively correlated with the age of the lineage. Alternatively, if the bidirectional hypothesis is correct, we expect to find equivalent patterns of substructure within the two more geographically widespread Malagasy species, *T. rufus* and *T. furculus*, while the mainland species *T. persicus* would lack significant population substructure. Unfortunately, our sampling of *T. persicus* comes from only a single location; therefore, tests of this model will be limited to comparisons of Malagasy species.

Hierarchical AMOVAS and pairwise F_{ST} tests reveal markedly different patterns of genetic structuring between *T. furculus* and *T. rufus*. *T. furculus* is characterized by significant genetic structuring between the central and southern geographical regions (see Fig. 1a), but high levels of genetic mixing within those regions. A notably different pattern is observed in *T. rufus*. Analyses of population structuring, including AMOVAS, pairwise F_{ST} s, and Mantel tests, reveal no significantly distinct gene pools within *T. rufus*, indicating instead that the species represents a single deme over its entire range. Under a population genetic model incorporating population substructure and assuming that differences in population structure do not reflect any current differences in intra-island dispersal behaviour, these results support the unidirectional dispersal hypothesis, and furthermore suggest that the dispersal event in the *T. auritus*/*T. furculus* lineage predated that in the *T. rufus* lineage.

While it seems clear that multiple dispersal events between Madagascar and mainland Africa have occurred in the history of *Triaenops*, we expect that these events have been rare and that an evolutionarily successful dispersal

would involve only one or a few individuals. Furthermore, theory predicts that significant population growth should follow the successful immigration of a small founding population (Carson & Templeton 1984); however, the genetic signal of that demographic growth will dissipate over time. If we are correct that the dispersal event in the *T. auritus*/*T. furculus* lineage predated that in the *T. rufus* lineage, then we expect to detect a signal of population growth following immigration more clearly in *T. rufus* than in *T. furculus* or *T. auritus*. We used diversity statistics, neutrality tests, and coalescent estimates of the population growth parameter g to test this prediction (Table 4; Fig. 4). Analyses of both *T. auritus* and the structured central and southern populations of *T. furculus* strongly indicate a demographic history of a small but constant population size, with insignificant results for nearly all statistical tests. Furthermore, while maximum posterior estimates of g are positive, the posterior probability distributions for both species are nearly flat and do not yield a clear signal of growth. In contrast, both coalescent and summary statistical analyses strongly indicate a history of population growth or range expansion in *T. rufus*. While it is possible that these analyses reflect a population bottleneck in Madagascar that is not associated with the colonization process, we consider that an unlikely scenario, as such an event probably would have had similar demographic effects on *T. auritus* and *T. furculus*. In summary, these analyses appear to support the unidirectional dispersal hypothesis, with *T. rufus* having undergone population expansion following a relatively recent dispersal event and the *T. auritus*/*T. furculus* lineage deriving from an older dispersal event with subsequent speciation and intraspecific population structuring.

While relatively simplistic, the population genetic model we consider here illustrates how the conclusions drawn from genetic analyses depend to a large extent on the complexity of the model being used. A model incorporating population structure appears to support the unidirectional dispersal hypothesis, with *T. rufus* representing the earlier dispersal event. The topology of the phylogeny appears to support the conclusions of this model, with *T. rufus* being the most closely related Malagasy species to the presumed source species, *T. persicus*. However, the range of *T. persicus* covers a much larger geographical area than is represented in our sample, from Mozambique north to Iran and southwestern Pakistan, and is categorized into two subspecies (Hill 1982). A more thorough sampling of *T. persicus* might indicate that the *T. rufus* and *T. auritus*/*T. furculus* lineages differ substantially in their areas of origin, or that Tanzanian *T. persicus* do indeed represent a back migration to mainland Africa while other populations of *T. persicus* have served as populations of origin for Malagasy *Triaenops*. Indeed, the signature of population growth in our sample of *T. persicus* makes the latter scenario an intriguing possibility (results not shown).

Conclusions

We have shown how coalescent and population genetic approaches may potentially discriminate among competing biogeographical hypotheses of dispersal posed by phylogenetic analyses. In general, we advocate initial analyses in which a rigorously dated phylogeny, preferably incorporating information from fossil samples, is compared with a corresponding area cladogram to distinguish between hypotheses of dispersal and vicariance. In cases wherein the data reject vicariance, and if the topology of the phylogeny is consistent with more than one hypothesis of dispersal, coalescent and population genetic methods can then be used to test expectations regarding measures of genetic diversity, population structuring, and population growth in light of the relative timing of dispersal events. We emphasize the importance of using population genetic analyses to test assumptions implicit in hypotheses comparing levels of genetic diversity. Population genetic forces may increase diversity in a species, as in the case of balancing selection or population structuring, or they may decrease diversity, as in the case of directional selection or a population bottleneck. Furthermore, forces such as selection and recombination will tend to affect diversity at a specific locus, while variation in population size or patterns of structure will affect diversity throughout the genome equally. Therefore, specific hypotheses at any level within this methodological framework will be strongly dependent on the system being examined.

We have used this synthesis of phylogenetic, coalescent, and population genetic approaches to address the historical biogeography of trident bats (*Triaenops* spp.). These analyses clearly reject a single origin hypothesis, and indicate a history of multiple dispersal events in this species group. The most parsimonious hypotheses involving multiple dispersals include either two dispersals from Africa to Madagascar or an early dispersal to Madagascar followed by a back dispersal to Africa. Coalescent-based estimates of divergence times are not conclusive for distinguishing between these hypotheses, but observed population genetic patterns, including relative measures of genetic diversity, patterns of genetic structuring, and patterns of population growth, suggest the highest support for two diachronic dispersal events from Africa to Madagascar, given the assumptions of a relatively simplistic population genetic model. We prefer this hypothesis for the reasons outlined here; however, there are a number of alternative models that are consistent with our observed results, including biogeographical models involving more than two dispersal events.

The naivety of the population genetic model considered here points to a need for quantitative phylogeographical

models that explicitly incorporate geography, climatology, dispersal, and coalescent-based population genetics in a unified analytical framework. For example, for most of the year, prevailing winds in the Mozambique Channel flow from east to west, towards mainland Africa (Hastenrath & Greischar 1991). A unified analysis of the biogeography of *Triaenops* might attach a higher likelihood to the bidirectional rather than the unidirectional dispersal hypothesis, given this fact. We anticipate that future work will increasingly utilize an approach testing predictions at multiple biological levels to reach informed conclusions regarding evolutionary and biogeographical history. We hope that the abundance of multidisciplinary data used to answer such questions will stimulate the development of much-needed analytical methods and models.

Further exploration of the biogeography of *Triaenops* should focus on better sampling of *T. persicus* to give a broader perspective on the diversity and historical demography of mainland African populations. Additionally, the inclusion of multiple unlinked loci is necessary to verify patterns observed at the cytochrome *b* locus and to yield more rigorous estimates of species divergence dates. Our study highlights ecological questions that remain to be answered for Malagasy *Triaenops*; namely, whether *T. auritus* and *T. furculus* differ significantly in their diet and echolocation characteristics, how Malagasy *Triaenops* disperse across the landscape, and whether differences in genetic structuring can be explained by differences in patterns of regional dispersal.

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