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Small body size increases the regional differentiation of populations of tropical mantellid frogs (Anura: Mantellidae)

M. PABIJAN*†, K. C. WOLLENBERG‡§ & M. VENCES*

*Division of Evolutionary Biology, Zoological Institute, Technical University of Braunschweig, Braunschweig, Germany
†Department of Comparative Anatomy, Institute of Zoology, Jagiellonian University, ul. Gronostajowa 9, 30-387, Kraków, Poland
‡Department of Organismic and Evolutionary Biology & Museum of Comparative Zoology, Harvard University, Cambridge, MA, USA
§Department of Biogeography, Trier University, Trier, Germany

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Abstract

The processes affecting species diversification may also exert an influence on patterns of genetic variability within species. We evaluated the contributions of five variables potentially influencing clade diversification (body size, reproductive mode, range size, microhabitat and skin texture) on mtDNA divergence and polymorphism among populations of 40 species of frogs (Mantellidae) from two rainforest communities in Madagascar. We report an inverse association between body size and nucleotide divergence between populations but find no influence of other variables on genetic variation. Body size explained ca. 11% of the variation in nucleotide divergence between populations and was coupled with high F_{ST} levels and an absence of haplotype sharing in small-bodied and medium-sized frogs. Low dispersal ability is likely the proximate mechanism producing higher population differentiation in small mantellids. The lack of genetic cohesion among populations establishes regional genetic fragmentation which in turn has the potential to accelerate rates of allopatric speciation in small frogs relative to large species. However, there is little evidence of increased speciation rates in these or other small-bodied organisms. We reconcile these contradictory observations by suggesting that lower dispersal ability also curbs colonization of new areas, decelerating diversification in weak dispersers. Our results imply that the intermediate dispersal model also applies to amphibians and may explain inconsistent previous results on the correlation of body size and speciation rate.

Introduction

The processes shaping population genetic structure within species are central to studies of speciation (Palumbi, 1994; Fitzpatrick *et al.*, 2009). The relative magnitude of neutral population genetic divergence can be interpreted in terms of the permeability of physical barriers to dispersal (e.g. Xiang *et al.*, 2000), past and present demography (Avise, 1995; Knowles & Richards, 2005), and clade-specific factors determining potential mobility (Avise, 2004). Clade- or species-specific factors include life-history and morphological attributes (e.g. Hamrick & Godt, 1996; Moyle, 2006; Duminil *et al.*, 2007) that are likely to interact with landscape features and either increase or decrease divergence and gene flow between populations (Gomez-Uchida *et al.*, 2009). Typically, high levels of divergence (or strong genetic structure in allele frequencies) imply little gene flow between populations and in time may result in the partitioning of an ancestral lineage into incipient species (Coyne & Orr, 2004). Conversely, low divergence (or weak population structure) is indicative of extensive gene flow and species cohesion (Coyne & Orr, 2004) and/or fast range expansion (Excoffier *et al.*, 2009). However, these population-level processes

Correspondence: Maciej Pabijan, Department of Comparative Anatomy, Institute of Zoology, Jagiellonian University, ul. Gronostajowa 9, 30-387 Kraków, Poland.
Tel.: +48 12 6645056; fax: +48 12 6645101;
e-mail: maciej.pabijan@uj.edu.pl

cannot be directly extrapolated to rates of species diversification. Indeed, dispersal ability (and traits determining it) has been shown to have a multifarious influence on cladogenesis. If dispersal ability is high, speciation is inhibited because of high gene flow among populations (Claramunt *et al.*, 2012). In taxa with low dispersal capacity, speciation can occur at smaller spatial scales (Kisel & Barraclough, 2010) but is not necessarily accelerated (Orme *et al.*, 2002a), leading to highest species diversity (and presumably highest speciation rates) for species of intermediate dispersal ability which probably corresponds to intermediate body size (Etienne & Olff, 2004; Agnarsson & Kuntner, 2012; Claramunt *et al.*, 2012).

A little explored means of gaining insight into the factors determining levels of gene flow and patterns of genetic variation is the simultaneous examination of entire assemblages of related taxa. Assemblage approaches such as that of Papadopoulou *et al.* (2009, 2011) have the advantage of keeping the environmental setting of the populations constant, thereby decoupling the influences of extrinsic and intrinsic factors shaping genetic variation across taxa. Comparisons of multiple species that inhabit the same locality therefore permit a test of variables other than physical barriers, such as ecological and life-history characteristics, which may also influence divergence (Whiteley *et al.*, 2004). However, it remains logistically challenging to obtain sufficient samples and data from homologous markers from a large number of species at the same sites for a meaningful analysis. Here, we take advantage of a speciose tropical anuran assemblage inhabiting the eastern rainforest zone of Madagascar, the mantellid frog radiation (Amphibia, Anura, Mantellidae). Mantellids colonized Madagascar during the Late Cretaceous or Paleocene (Crottini *et al.*, 2012; reviewed in Samonds *et al.*, 2012) and are currently one of the richest groups of tropical frogs in terms of number of species and diversity of morphology, ecology and reproductive modes. Some typical mantellid ecotypes include arboreal and semi-arboreal treefrogs, semi-aquatic frogs and numerous cryptically coloured or aposematic terrestrial leaf litter species (Glaw & Vences, 2007). Representatives of each of these ecological types differ in body size, reproductive mode and taxonomically important morphological traits.

Mantellids have recently served as a model to propose the existence of a microendemic phenotype in frogs (Wollenberg *et al.*, 2011) which combines two highly correlated traits: small body size and small range size. Small body size appears to be linked both to small range sizes and to higher rates of nucleotide substitution in mantellid frogs (Wollenberg *et al.*, 2011). This partly contrasts with another case study in amphibians, in which a dispersal-prone phenotype characterized by large body and range size triggered the radiation of bufonid toads (Van Bocxlaer *et al.*, 2010). Moreover, it is striking (although statistically untested) that almost all very widespread anuran species are comparatively large sized while the wide majority of frogs have smaller body sizes. Besides body and range sizes, several other traits have been hypothesized to influence species diversification in amphibians such as disparate reproductive modes and stable vs. unstable breeding habitats with associated variation in clutch size (Inger *et al.*, 1974; Vences *et al.*, 2002; Dubois, 2005; Chan & Zamudio, 2009), skin texture (Van Bocxlaer *et al.*, 2010; Gonzalez-Voyer *et al.*, 2011) and mass-specific active metabolic rate (Santos, 2012).

In this contribution, we extend the hypotheses developed for species diversification in clades to genetic variability within species. On the basis of the idea that processes such as drift, immigration and selection exert similar influences on both species diversity and within-species genetic diversity at the same location (Vellend, 2005; Vellend & Geber, 2005; Papadopoulou *et al.*, 2011), we expect that the same factors influencing species diversification in amphibians also determine intraspecific genetic variation. To do this, we examine genetic variation between and within populations of 40 mantellid species based on mitochondrial DNA (mtDNA) sequences of over 1000 individuals from two sites in central eastern Madagascar. The selected localities contain highly diverse batrachofaunas (around 100 species each) with considerable overlap of species (Vieites *et al.*, 2009). Altogether, we evaluate the contributions of five potential explanatory variables on the degree of intra-populational polymorphism and inter-populational divergence: body size, reproductive mode, range size, microhabitat and skin texture. We report a link between small body size, a probable surrogate for low dispersal ability in frogs, and increased levels of population divergence in mantellids, and propose that the intermediate dispersal model may explain inconsistent previous results on the association of body size and speciation rate in mantellids.

Materials and methods

Focal species and sampling sites

Frogs were sampled from two sites in central-eastern Madagascar: Andasibe–Mantadia National Park (AMNP: 48°23′ – 48°26′ E and 18°54′ – 18°57′ S) and Ranomafana National Park (RNP: 47°18′ – 47°37′ E and 21°02′ – 21°25′ S). For some species, we included individuals found in private reserves adjacent to the parks and in a few cases, from forests up to ca. 20 km away from the parks. AMNP and RNP are located in the same general forest biome, span a similar elevation (ca. 600–1250 m. a.s.l. in AMNP and 500–1500 m.a.s.l. in RNP), and are in the same bioclimatic zone (Schatz, 2000). Both parks are located on the east-facing escarpment of Madagascar's central high plateau characterized by a very

rugged topography. Until the arrival of humans on Madagascar less than 2000 years ago (Dewar & Richard, 2012), a corridor of mid-altitude rainforest provided habitat continuity between the sites. Deforestation has reduced this habitat into a patchwork of semi-isolated small forest fragments along the escarpment. The distance between AMNP and RNP (about 250 km) precludes the direct migration of individual amphibians between the sites. About 95 anuran species and candidate species are known from AMNP and 110 from RNP; see Vieites *et al.* (2009) for species lists. Approximately 50 frog species are shared between the two sites.

Table 1 Morphological traits, life-history attributes and statistics summarizing nucleotide variation in the mitochondrial 16S rRNA gene in 40 frogs (Mantellidae) from two localities in east-central Madagascar. SVL, maximum snout-vent length in males; range, range size in 10^3 km^2; *n*, number of individuals from Andasibe (AMNP) and Ranomafana (RNP); *h*, number of haplotypes in AMNP and RNP, respectively; D_{xy}, the average number of nucleotide substitutions between populations (Nei, 1987); π, the average number of nucleotide substitutions within Andasibe (AMNP) and Ranomafana (RNP) populations; F_{st}, according to Hudson's sequence-based formula (Hudson *et al.*, 1992); na, not applicable; see main text for explanations of reproductive mode, microhabitat and skin texture.

Species	SVL	Range	Repr. mode	Habitat	Skin texture	n_{AMNP}	n_{RNP}	*h*	D_{xy}	π_{AMNP}	π_{RNP}	F_{st}
Aglyptodactylus madagascariensis	41	78.8	Pond	Terrestrial	Smooth	25	22	6, 4	0.012	0.002	0.003	0.772
Blommersia blommersae	21	102.2	Pond	Semi-arboreal	Smooth	20	20	2, 3	0.052	0.001	0.007	0.932
B. grandisonae	23	102.0	Stream	Semi-arboreal	Smooth	1	1	1, 1	0.047	na	na	na
Boophis albilabris	73	153.1	Stream	Arboreal	Smooth	6	20	2, 6	0.006	0.002	0.003	0.577
B. elenae	46	4.3	Stream	Arboreal	Smooth	0	25	na, 6	na	na	0.003	na
*B. goudoti**	70	186.2	Stream	Semi-arboreal	Smooth	6	11	4, 7†	0.028	0.006	0.009	0.737
B. luteus	40	79.2	Stream	Arboreal	Smooth	14	25	7, 14	0.053	0.004	0.015	0.818
B. madagascariensis	65	143.6	Stream	Arboreal	Smooth	25	25	5, 5	0.012	0.005	0.007	0.508
B. pyrrhus	32	49.9	Stream	Arboreal	Smooth	20	20	2, 1	0.006	0.001	0.000	0.955
B. tasymena	23	5.2	Stream	Arboreal	Smooth	13	24	6, 6	0.032	0.006	0.002	0.879
B. tephraeomystax	42	174.9	Pond	Arboreal	Smooth	5	13	2, 1†‡	0.001	0.001	0.000	0.000
B. boehmei§	29	22.7	Stream	Arboreal	Smooth	7	21	1, 3	0.109	0.000	0.001	0.995
B. bottae	24	4.5	Stream	Arboreal	Smooth	6	14	2, 4	0.033	0.001	0.003	0.933
B. guibei	40	10.2	Pond	Arboreal	Smooth	9	18	5, 7†‡	0.033	0.033	0.013	0.295
B. mandraka	26	1.9	Stream	Arboreal	Smooth	1	1	1, 1	0.030	na	na	na
B. pauliani	23	18.6	Pond	Arboreal	Smooth	3	14	1, 2	0.086	na	0.002	0.991
B. marojezensis	27	78.8	Stream	Arboreal	Smooth	17	12	2, 1	0.099	0.000	0.000	0.998
B. picturatus	33	8.2	Stream	Arboreal	Smooth	7	12	2, 2	0.040	0.001	0.001	0.978
B. rappiodes	25	17.0	Stream	Arboreal	Smooth	10	16	5, 4	0.051	0.003	0.004	0.926
B. reticulatus	35	38.7	Stream	Arboreal	Smooth	2	23	1, 1	0.027	na	0.000	na
B. viridis	30	45.9	Stream	Arboreal	Smooth	16	1	4, 1	0.036	0.003	na	na
Gephyromantis asper	30	42.4	Non-water	Semi-arboreal	Granular	10	7	2, 1	0.097	0.002	0.000	0.992
G. aff. *boulengeri*	30	28.3	Non-water	Terrestrial	Granular	12	7	1, 1	0.071	0.000	0.000	1.000
G. decaryi	23	2.5	Non-water	Terrestrial	Smooth	0	11	na, 2	na	na	0.002	na
G. enki	21	0.6	Non-water	Terrestrial	Smooth	0	28	na, 4	na	na	0.001	na
G. redimitus	53	61.7	Non-water	Semi-arboreal	Smooth	11	13	5, 5†	0.049	0.004	0.010	0.858
G. sculpturatus	43	12.3	Non-water	Semi-arboreal	Smooth	10	23	1, 4	0.051	0.000	0.003	0.975
G. tschenki	36	0.7	Non-water	Semi-arboreal	Smooth	4	21	2, 2	0.019	na	0.003	0.702
Guibemantis depressiceps	45	10.8	Pond	Arboreal	Smooth	1	21	1, 1	0.027	na	0.000	na
G. liber	29	204.6	Pond	Arboreal	Smooth	11	21	6, 4	0.049	0.022	0.004	0.739
G. pulcher	25	67.3	Non-water	Arboreal	Smooth	5	17	2, 4	0.007	0.001	0.001	0.842
G. tornieri	51	21.6	Pond	Arboreal	Smooth	12	17	3, 4†	0.028	0.001	0.006	0.877
Mantidactylus aerumnalis	27	18.6	Stream	Terrestrial	Smooth	14	20	2, 3	0.096	0.002	0.001	0.984
M. aff. *betsileanus*	24	17.0	Stream	Semi-aquatic	Granular	16	25	2, 3	0.009	0.005	0.002	0.655
M. betsileanus	24.3	21.6	Stream	Semi-aquatic	Granular	20	11	5, 1†‡	0.002	0.003	0.000	0.256
M. femoralis	37	189.0	Stream	Semi-aquatic	Granular	6	10	1, 3	0.036	0.000	0.002	0.976
M. grandidieri	90	117.4	Stream	Semi-aquatic	Granular	7	13	2, 2	0.004	0.001	0.001	0.742
M. melanopleura	40	106.1	Stream	Terrestrial	Smooth	11	25	3, 5	0.016	0.002	0.003	0.858
M. mocquardi	40	3.1	Stream	Semi-aquatic	Granular	1	1	1, 1	0.009	na	na	na
M. opiparis	26	171.1	Stream	Terrestrial	Smooth	12	25	3, 2	0.026	0.004	0.004	0.857

*Includes sibling species *Boophis goudoti* (AMNP) and *B. obscurus* (RNP).
†RNP population contains a low-frequency haplotype present in the AMNP area.
‡AMNP population contains a low-frequency haplotype present in the RNP area.
§Includes sibling species *Boophis boehmei* (AMNP) and *B. quasiboehmei* (RNP).

Of these, we sampled populations of 37 species inhabiting both sites. Intra-populational data were gathered for a further three species present only in RNP. Sample sizes for all species are shown in Table 1.

Nucleotide sequence data

We sequenced a highly variable fragment of the mitochondrial 16S rRNA gene from 40 mantellid species present in AMNP and/or RNP (Table 1). This mtDNA fragment has been extensively used in DNA barcoding and taxonomy of Malagasy amphibians (Vences *et al.*, 2005; Vieites *et al.*, 2009; Strauß *et al.*, 2010). PCR amplification and 16S rRNA sequencing followed standard procedures outlined in Vences *et al.* (2005) and are available in the Supporting Information (Table S1). We supplemented the newly obtained molecular data with published sequences from GenBank (Table S1), especially from an extensive barcoding study of amphibian larvae inhabiting 29 different streams of RNP (Strauß *et al.*, 2010). Because the alignment of loop regions in the 16S rDNA is often ambiguous across mantellid species, we discarded loop regions altogether. The final alignment consisted of a fully homologous 330–333 bp fragment of the mitochondrial 16S rRNA gene for 40 mantellid species.

Response and explanatory variables

For each species, we calculated the average number of nucleotide substitutions between AMNP and RNP populations (D_{xy}; Nei, 1987), and the average number of nucleotide substitutions within populations (π_{AMNP}, π_{RNP}; Nei, 1987) in DNAsp (Librado & Rozas, 2009). It should be noted that D_{xy} was originally intended as a measure of between-species divergence; however, we use this measure as an inter-population summary statistic because in many of the analysed mantellid species, mean D_{xy} between populations approached species level divergence (cf. Vieites *et al.*, 2009). D_{xy} and π were used as response variables in all subsequent linear models. Because of a departure from a normal distribution (Fig. 1a,b), both response variables were box-cox transformed before analysis. We also calculated F_{st} according to Hudson's sequence-based formula (Hudson *et al.*, 1992) for species sampled at both localities.

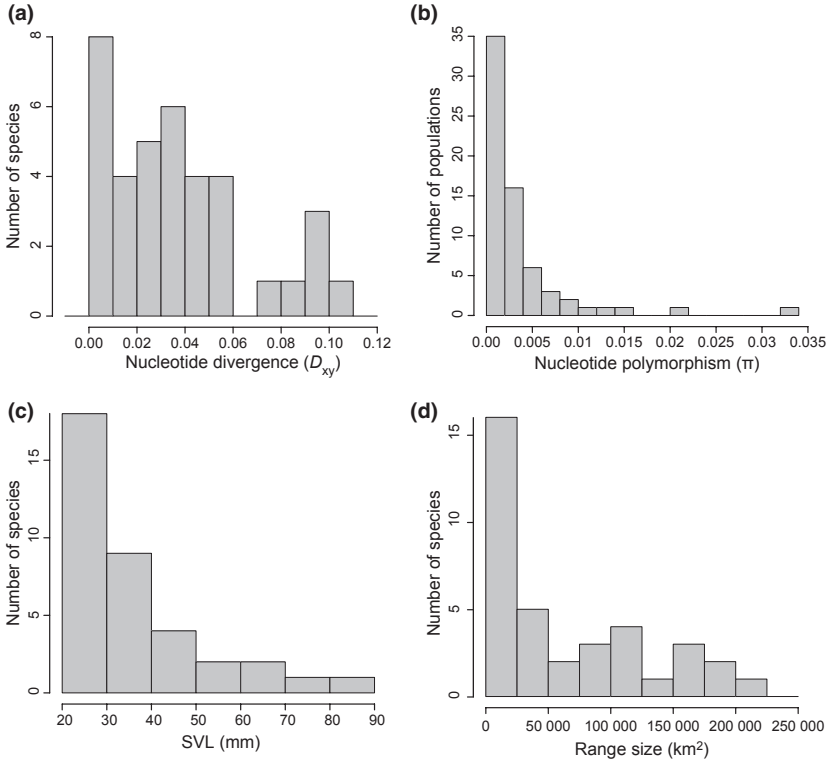

Fig. 1 Histograms representing the distributions of four continuous variables used in this study. Response variables included (a) nucleotide divergence (D_{xy}, calculated for 37 species) and (b) nucleotide polymorphism (π, calculated for 67 populations of 37 species). Body size, measured as (c) maximum snout-vent length [snout-vent length (SVL), for 40 species] and (d) range size in km^2 (calculated for 40 species) were used as explanatory variables.

We analysed the effect of five explanatory variables on the response variables D_{xy} and π. Two explanatory variables were coded as continuous (body and range size) and three were categorical (reproductive mode, microhabitat and skin texture). In line with the response variable transformations, body and range size (both had right skewed, non-normal distributions, Fig. 1c,d) were box-cox transformed to ensure linearity in further analyses. The rationale behind the selection of these variables is given below.

1 The first predictor variable, body size, is positively correlated with range size in mantellids (Wollenberg *et al.*, 2011) and thus may be associated with dispersal capability in these anurans, that is, smaller body size may lead to lower movement capabilities, whereas larger species may be more vagile. Small body size should therefore lead to higher levels of population differentiation. We used maximum snout-vent length (SVL, Table 1) for males (data source: Glaw & Vences, 2007; Wollenberg *et al.*, 2011) as a proxy for body size, an approach consistent with other recent studies (Moen & Wiens, 2009; Gonzalez-Voyer *et al.*, 2011; Wiens *et al.*, 2011). Female body size was not used because of the small numbers of females known for some species.

2 Range size. We expected a negative correlation between range size and population divergence because widespread species may be inherently more mobile than species with restricted ranges and therefore more frequently exchange genes between populations (Phillips *et al.*, 2010). At least some microendemic species show very high between-population divergence (e.g. Vences *et al.*, 2005; Blackburn, 2008; Tolley *et al.*, 2010), attesting to a lack of gene flow between populations even at very small spatial scales. Range sizes (in km^2) for each mantellid species were taken from Wollenberg *et al.* (2011) in which point locality information was used to plot and calculate the area of minimum convex polygons connecting all known localities for each species from an extensive GIS-referenced database (Glaw & Vences, 2007).

3 Microhabitat. The microhabitat most often frequented by amphibians poses various limitations to their dispersal, for example, movement in the three-dimensional canopy inhabited by many treefrog species is probably more complex than the two-dimensional landscape of terrestrial species. Dispersal in semi-aquatic species associated with streams is configured by the habitat itself (Hughes *et al.*, 2009). For example, dispersal and gene flow in a stream-adapted salamander was high within streams but low between streams in the same catchment (Mullen *et al.*, 2010). Moreover, in the context of climate induced changes in forest cover as thought to have occurred through the Pliocene and Pleistocene in Madagascar (Wilmé *et al.*, 2006), some microhabitat specialists may have been more reluctant to disperse across a treeless or more arid landscape than others (e.g. arboreal vs. semi-arboreal and terrestrial species). We included microhabitat as a categorical variable with the following levels: arboreal (n = 21), semi-arboreal (n = 7), terrestrial (n = 7) and semi-aquatic (n = 5). Mantellid species were assigned to microhabitat categories based on habitat preferences described in Glaw & Vences (2007) and sometimes the original species descriptions (e.g. Vences *et al.*, 2010).

4 Reproductive mode. Anurans possess a wide variety of reproductive modes that can be differentiated primarily by egg deposition site (arboreal, terrestrial, aquatic), larval development site (pond, stream, terrestrial, endotrophic) and by the presence or absence of congregations of breeding individuals (Wells, 2007). At present, nine distinct reproductive modes are known in mantellids (Glaw & Vences, 2007). The transient character of lentic breeding sites may select for high dispersal capabilities in pond breeders and hence for low levels of population divergence (Inger *et al.*, 1974; Vences *et al.*, 2002; Chan & Zamudio, 2009). In contrast, in water-independent species, low mobility and small effective population sizes may interact to produce low within-population polymorphism but high between-population divergence. Explosive breeding species that use ponds as mating and larval development sites are expected to show high levels of within-population nucleotide polymorphism on account of larger effective population sizes. Another hypothesis (Dubois, 2005) suggests that anurans producing small egg clutches (e.g. terrestrial species with endotrophic larvae) will more often lose their entire reproductive output on account of predation than species with large clutches and free-swimming larvae (e.g. explosive pond breeders). Lower effective population sizes and lower within-population genetic variation are expected outcomes of this type of sweepstakes mortality effect correlated with family survival (Hedrick, 2005). We simplified the diversity of reproductive modes in mantellid frogs (data source: Glaw & Vences, 2007) into three general categories emphasizing egg deposition site and larval development site: pond breeders (n = 8), stream breeders (n = 24) and water-independent species (n = 8).

5 Skin texture. Amphibian skin is heavily vascularized allowing for considerable exchange of gases with water or air and playing a considerable (but species-specific) role in osmoregulation and respiration (Wells, 2007). A more aerolate ventral skin was positively correlated with species richness in New World direct developing frogs (Gonzalez-Voyer *et al.*, 2011). A granular and thick skin is also characteristic of widespread bufonids (Van Bocxlaer *et al.*, 2010). The variety of skin textures present in mantellids is

representative of anurans in general, varying from completely smooth to strongly granular. The studied mantellid frogs were divided into two groups differing in skin texture: smooth-skinned (n = 33) and granular-skinned (n = 7) species.

Statistical analysis

Closely related species tend to have similar ecologies and life histories; therefore, they may also partition genetic variation in similar ways. Consequently, we first assessed how well the mantellid phylogeny predicts similarity among species in any of the genetic variables (D_{xy}, π_{AMNP}, π_{RNP}). The K statistic of Blomberg *et al.* (2003) gives the proportion of phylogenetic signal in a trait compared to that expected under Brownian motion. The significance of this signal is then assessed according to a permutation-based procedure as implemented in the R package Picante (Kembel *et al.*, 2010). Phylogenetic relationships among mantellid species were based on a comprehensive, multi-gene phylogeny (Wollenberg *et al.*, 2011) pruned to include only the taxa examined in this study (Fig. S1). Phylogenetic signal was also measured for the predictor variables SVL, range size, reproductive mode, microhabitat and skin texture. We found that the genetic variables were not influenced by phylogeny, whereas many of the predictor variables had a moderate to strong phylogenetic signal (see Results). Because we are mainly interested in the relative influence of the various predictors on the genetic variables, regardless if phylogeny contributes to this signal or not, we argue that the use of non-phylogenetic analysis is acceptable in this situation. This is supported by Revell (2010) who simulated phylogenetic signal in the independent (predictor) variable and uncorrelated residual error in Y (the response variable), and found that a phylogenetic regression was not necessary to correctly fit the regression model. Hence, subsequent to tests of phylogenetic independence, the data were analysed using two conceptually different approaches (Anderson & Burnham, 2002) without phylogenetic correction, that is, conventional regressions and an Akaike Information Criterion (AIC)-based model selection technique. In this way, we exploited goodness-of-fit measures to judge the explanatory power of simple univariate models and at the same time, examined both univariate and multivariate patterns of causality with more suitable information-theoretic methods (Stephens *et al.*, 2005). Regressions were performed using linearized data; multiple-state categorical variables were 0.1 dummy coded to yield semiquantitative variables. All calculations were performed in R 2.14.1 (R Development Core Team., 2009).

We used an information-theoretic framework derived from Kullback–Leibler information (Burnham & Anderson, 2002) measuring the strength of evidence for all competing models from a meaningful candidate set. We ranked the models according to the Akaike Information Criterion corrected for small sample size (AICc), where the lowest values indicate the most parsimonious models of the set. Model uncertainty was assessed by comparing Δ AICc values, Akaike weights and evidence ratios (Burnham & Anderson, 2002). We considered models with Δ AICc $\leq$ 3.0 as supported by the data and used model averaging (Burnham & Anderson, 2002) to calculate regression coefficients, standard errors and 95% confidence intervals for the parameters. Calculations were carried out in the AICcmodavg package v. 1.17 for R (Mazerolle, 2006, 2011).

The variation in D_{xy} between mantellid species was modeled by multiple linear regression with normally distributed errors fitted by maximum likelihood. The proportion of variability in D_{xy} that is accounted for by our global model (including all our predictor variables and specified interactions) is moderate (adjusted R^2 = 0.37). We constructed a set of candidate models based on the rationale given for predictor variables in Material and Methods. Because of the relatively small number of observations (n = 37), we limited the number of terms in our models to a maximum of three. Moreover, preliminary univariate results showed that SVL and range size are correlated in our study species (Spearman's r_s = 0.31; P = 0.0501, Fig. S2) as they are in mantellids overall (Wollenberg *et al.*, 2011); therefore, we excluded range size from all models but two: (i) range size as the only explanatory variable and (ii) a model that included SVL, range size and their interaction. We also included other selected interactions between variables: SVL × reproduction, SVL × skin structure and SVL × microhabitat. All candidate models are presented in Table S2.

Models for π were equivalent to those for D_{xy} (Table S3). However, the number of observations of π amounted to 67 because most species were represented by two populations (one from AMNP and one from RNP, see Table 1); consequently, pairs of π values per species are non-independent. We therefore nested 'species' within 'population' to avoid pseudoreplication and treated these as random factors in linear mixed effects models constructed in the nlme package in R (Pinheiro *et al.*, 2011). Random factors were included in each candidate model.

Results

Variation in response and explanatory variables

We collected 16S rRNA sequences from a total of 1030 individuals for 40 mantellid species. In five cases (*Blommersia grandisonae*, *Boophis mandraka*, *B. viridis*, *Guibemantis depressiceps* and *Mantidactylus mocquardi*), we calculated nucleotide divergence between populations (D_{xy}) based on a single specimen from one or the other locality. Sample sizes of $\geq$ 5 individuals per population

were used for calculating within population nucleotide polymorphism (π) values. A total of 13 population samples from 10 different species (listed in Table 1) did not meet this requirement and were therefore discarded from analyses involving π. There was no association between sample size and either D_{xy} ($r_s = -0.09$, $P = 0.589$) or π values ($r_s = 0.19$, $P = 0.127$), showing that the small sample sizes for populations of some species did not influence our results.

We measured between-population nucleotide divergence (D_{xy}) in 37 mantellid species (Table. 1, Fig. 1a). Mean D_{xy} equalled 0.038 ± 0.0296 and ranged from 0.001 (in *Boophis tephraeomystax*) to 0.109 (*B. boehmei*/*B. quasiboehmei*). Haplotype sharing between RNP and AMNP was detected in six species (Table 1). All but one of these frogs were large (> 40 mm), smooth-skinned, arboreal and semi-arboreal species representing all three reproductive modes (three pond breeders, one stream breeder, one water-independent). The single exception was *Mantidactylus betsileanus*, a small, granular-skinned, stream breeding, semi-aquatic species. However, this species exhibits a very wide ecological tolerance and is also common in secondary habitat. The majority of F_{st} values were very high (> 0.7), indicating substantial population structure at the regional scale. There was a strong and positive association between D_{xy} and F_{st} ($r_s = 0.70$, $P = 0.0001$, $n = 31$; calculations based on linearized values for populations with over five sequenced individuals).

Nucleotide polymorphism within populations (π) was assessed in 67 populations from 37 species (Table 1, Fig. 1b). Mean π attained a value of 0.003 ± 0.0053 and ranged from 0 (for 12 populations of 11 species) to 0.033 (*B. guibei* from AMNP). High π values could be attributed to the presence of appreciable frequencies of two divergent haplotypes per population because of haplotype sharing between AMNP and RNP (e.g. in *B. guibei*, *B. goudoti* and *Gephyromantis redimitus*) or the presence of a third mitochondrial lineage (i.e. clearly distinct from both AMNP and RNP haplotypes) in *B. luteus* (RNP) and *Guibemantis liber* (AMNP). For 30 species in which we could obtain sufficient sample sizes ($\geq$ 5 individuals) from both sampling sites, mean π did not differ between the two sites (0.004 ± 0.0069 and 0.004 ± 0.0039 for AMNP and RNP, respectively; paired Wilcoxon signed rank test, $V = 186$, $P = 0.502$). There was a significant correlation between π values for AMNP and RNP populations of the same species (Fig. S3). We used Spearman's rank correlation to check for a relationship between D_{xy} and levels of π but did not find a consistent or significant association between them when all populations were included in the analysis ($r_s = -0.07$, $P = 0.597$, $n = 64$), after the exclusion of the above-mentioned outliers with unusually high π values ($r_s = -0.13$, $P = 0.319$, $n = 60$), only in AMNP ($r_s = -0.31$, $P = 0.104$, $n = 29$, no outliers) or only in RNP ($r_s = 0.00$, $P = 0.981$, $n = 31$, no outliers).

Mean SVL for males was 36.5 ± 15.62 mm (range: 21–90 mm) and was right-skewed because of a greater number of small species than large species (Fig. 1c). Mean range size was 60 583 km^2 ± 62 905.5 (range: 571–204 598 km^2) and was also right-skewed (Fig. 1d).

Phylogenetic signal, as measured by the Blomberg *et al.* (2003) K statistic, was low and random in all measured genetic variables and range size (D_{xy}: $K = 0.559$, $Z = -0.51$, $P = 0.333$; π_{AMNP}: $K = 0.581$, $Z = 0.15$, $P = 0.579$; π_{RNP}: $K = 0.509$, $Z = 0.61$, $P = 0.747$; range size: $K = 0.375$, $Z = 1.82$, $P = 0.948$). We detected a significant phylogenetic component for SVL ($K = 0.841$, $Z = -2.02$, $P = 0.004$) and for all of the categorical predictor variables (reproductive mode: $K = 1.606$, $Z = -3.25$, $P = 0.001$; microhabitat: $K = 2.608$, $Z = -3.93$, $P = 0.001$; skin texture: $K = 1.347$, $Z = -2.87$, $P = 0.001$). Because our primary goal was to evaluate the determinants of the non-phylogenetically structured genetic variables, we based our analysis on univariate and multivariate tests without phylogenetic correction.

Univariate results

Most predictors of D_{xy} had negligible explanatory power (Table 2), however, body size (as measured by maximum SVL) explained 11% of the variation in D_{xy}, and was negatively correlated with D_{xy} (Table 2, Fig. 2). Semi-aquatic microhabitat was the only categorical variable explaining a significant portion of the variance in D_{xy} (17%). Semi-aquatic frogs had lower values of D_{xy}. Nearly all tested variables were poor predictors of π, explaining usually less than 4% of the variation (Table 2), with the exception of skin texture in RNP (12%) but not in AMNP (3%).

Model selection

The five best-supported information-theoretic models (within three AICc units of the top model) explaining between population nucleotide divergence (D_{xy}) in mantellids included body size (SVL), microhabitat and skin texture as explanatory variables (Tables 3 and S2). Support for our highest ranked model (SVL × microhabitat) was rather low (0.24 Akaike weight) in the candidate model set. Nonetheless, this model explained ca. 8.7 times more variation in D_{xy} than the null model. We used model averaging to extract information on the magnitude and direction of explanatory variables within our set of five best-supported models. Model-averaged parameter estimates showed that SVL had an appreciable negative effect on D_{xy} (-3.54 ± 1.69; 95% CI: -6.85, -0.24); 95% confidence intervals overlapped with zero for all other variables (Table S4) which can therefore be considered as uninformative. However, between-population divergence tended to be less pronounced in semi-aquatic frogs (Fig. 2).

Table 2 Conventional regressions between response variables (D_{xy}, π) and morphological traits/life-history attributes in 40 frog species (Mantellidae) from east-central Madagascar. *n*, number of species used for each regression; sign, indicates the slope of the regression; other abbreviations as in Table 1.

	D_{xy}				π_{AMNP}				π_{RNP}			
Variable	*n*	Sign	R^2	*P*	*n*	Sign	R^2	*P*	*n*	Sign	R^2	*P*
Body size	37	–	0.11	0.04	29	–	0.00	0.77	34	–	0.03	0.34
Range size	37	–	0.02	0.37	29	+	0.00	0.73	34	–	0.01	0.55
Microhabitat	37											
Arboreal	20	+	0.00	0.87	14	–	0.00	0.88	18	+	0.00	0.69
Semi-arboreal	7	+	0.06	0.14	6	+	0.01	0.57	5	–	0.04	0.24
Terrestrial	5	+	0.01	0.61	5	+	0.00	0.83	7	–	0.00	0.96
Semi-aquatic	5	–	0.17	0.01	4	+	0.00	0.82	4	+	0.02	0.46
Reproductive mode												
Pond breeder	8	–	0.00	0.87	4	–	0.02	0.51	8	+	0.04	0.25
Stream breeder	23	–	0.01	0.54	19	+	0.03	0.36	19	–	0.00	0.90
Water-independent	6	+	0.03	0.32	6	–	0.01	0.61	7	–	0.04	0.29
Skin texture												
Smooth	30	+	0.03	0.32	23	+	0.03	0.34	28	+	0.12	0.04

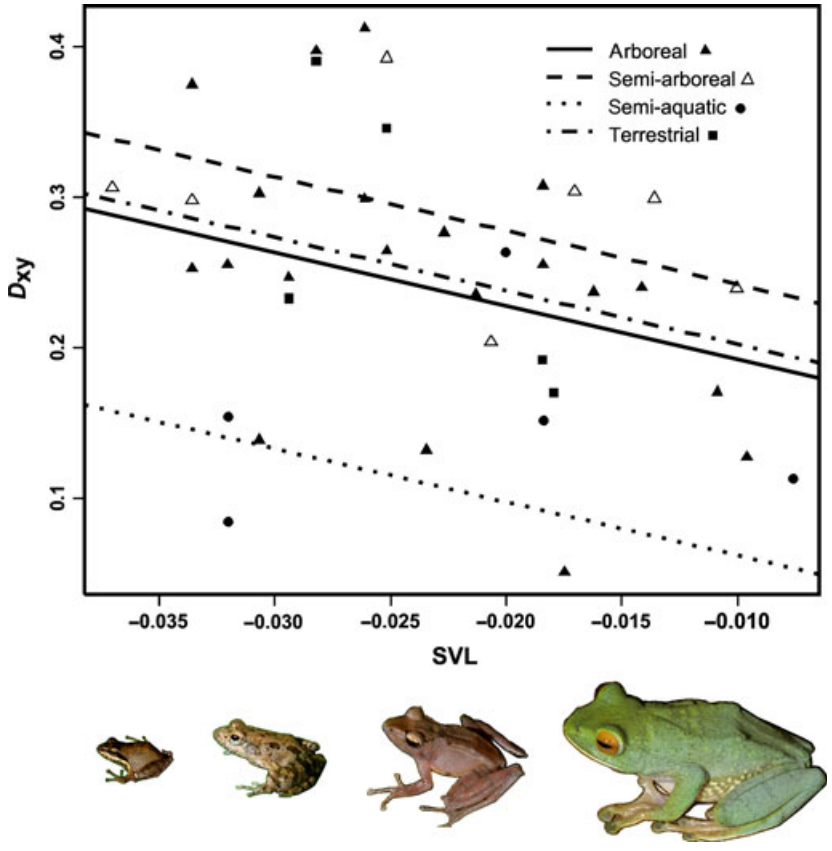

Fig. 2 Relationship between inter-population nucleotide divergence (D_{xy}) and body size shown for 37 mantellid frogs. Regression lines show differences between frogs living in four microhabitats. Based on linearized data. Body size increases towards the right of the scale, as approximated by photographs underneath. Photographs illustrate (from left to right) the following: *Gephyromantis enki*, a small, smooth-skinned, terrestrial and water-independent mantellid exemplifying a microendemic phenotype; *Mantidactylus betsileanus*, a small, semi-aquatic, stream breeding frog with low mitochondrial divergence between populations; *G. sculpturatus*, a medium-sized, semi-arboreal species breeding outside of water and characterized by high genetic divergence between populations; *Boophis albilabris*, a large, arboreal, smooth-skinned treefrog breeding in streams with low genetic divergence between populations.

Table 3 Best-supported information-theoretic models explaining nucleotide divergence (D_{xy}) and polymorphism (π) in the mitochondrial 16S rRNA gene from populations of 40 frog species found in east-central Madagascar. For each model: K, the number of parameters (including intercept and variance); ΔAICc, the difference in AIC units between the best model and the model under examination; AICc wt, Akaike weights representing the ratio of the ΔAICc of a given model relative to the whole set of candidate models which must sum to one; ER, the evidence ratios calculated as the ratio of Akaike weights of the best model and competing models. Nested random effects (population, species) for models explaining variation in π are not shown.

Model	K	ΔAICc	AICc wt	ER
D_{xy} ~ SVL x microhabitat*	6	0.00	0.24	1.00
D_{xy} ~ SVL + microhabitat + skin texture	7	0.34	0.20	1.18
D_{xy} ~ SVL + microhabitat	6	0.70	0.17	1.42
D_{xy} ~ SVL	3	2.41	0.07	3.33
D_{xy} ~ microhabitat	5	2.98	0.05	4.45
π ~ skin texture	6	0	0.323	1
π ~ intercept (NULL)	5	0.749	0.222	1.45
π ~ SVL + skin texture	7	1.922	0.124	2.62
π ~ SVL	6	2.616	0.087	3.70

*'x' denotes only the interaction between snout-vent length (SVL) and microhabitat.

Four best-supported information-theoretic models explaining within population nucleotide variation included only two variables: body size and skin texture (Table 3; see Table S3 for a ranking of all tested models). However, the null model was equally well supported; therefore, we conclude that the candidate models are weak predictors of π and refrain from conducting parameter estimates.

Discussion

Lack of predictive power for most analysed variables

A number of intrinsic properties of taxa, such as life-history, ecology and certain key morphological traits, are thought to influence species diversification (Marzloff & Dial, 1991; Barraclough *et al.*, 1998; Cardillo *et al.*, 2003; Isaac *et al.*, 2005; Phillimore *et al.*, 2006) and may concurrently shape genetic diversity within species (Vellend, 2005; Vellend & Geber, 2005). In this study, we evaluated several traits known to influence species diversification in amphibians but found that relatively little of the genetic variation could be accounted for by the explanatory variables. We conclude that with the sole exception of body size, none of the proposed factors (range size, adult habitat preference, skin texture and reproductive mode as defined herein) correlate with regional differentiation or levels of genetic variation within populations of mantellid frogs, although all seem to exert an influence on species diversification on at least some parts of the amphibian tree of life (Vences & Wake, 2007; Van Bocxlaer *et al.*, 2010; Gonzalez-Voyer *et al.*, 2011; Santos, 2012). This lack of explanatory power for most studied variables could be attributed to inadequate sample sizes in our analysis or insufficient variability in the applied 16s rRNA gene fragment. However, the amount of explained variation, according to the univariate results (Table 2), is close to nil for most variables, and it is therefore unlikely that an increase in number of studied species would substantially alter our conclusions. Alternatively, because a number of factors may determine differences between two populations, sampling more populations per species may improve the explanatory power of our analyses.

Snout-vent length as a predictor of regional differentiation in mantellids

We provide two measures of among population differentiation in mantellids: sequence-based divergence between populations (D_{xy}) and a measure of population subdivision (F_{st}) that is more sensitive to differences in allele frequencies between populations. Both measures were correlated (r_s = 0.70) and can be viewed as representing the degree of genetic fragmentation among populations. In both univariate and multivariate analyses, we most consistently found body size (measured as maximum male SVL) predicting nucleotide divergence between populations: large-bodied species tend to have lower levels of population divergence than small-bodied species. Although this correlation is relatively weak, (ca. 11% of the explained variation in D_{xy}), we detected deep conspecific genetic differences between the two study sites coupled with high F_{ST} levels and in most cases a complete lack of haplotype sharing in the majority of small-bodied and medium-sized mantellids in this regional assemblage. This implies substantial population subdivision and low levels of gene flow. Most small-sized AMNP and RNP frog lineages are probably already evolving along independent evolutionary trajectories. Mantellid taxonomy reflects this assertion to some extent: some sister lineages found in AMNP and RNP are currently considered as distinct sibling species that besides high genetic variation have evolved some morphological dissimilarity (e.g. *Boophis boehmei* and *B. quasiboehmei*; probably also populations of *Gephyromantis asper, G. boulengeri* and *Mantidactylus aerumnalis*). At least in the case of *B. boehmei* and *B. quasiboehmei,* the lineages are also separated at the level of nuclear genes, without haplotype sharing (Vences *et al.*, 2010). In most large species, however, low genetic differentiation and evidence of haplotype sharing strongly advocate shallow coalescence, probably through conspecific populations inhabiting the mid-elevation rainforest biome that until recently was continuously distributed between the two sampling sites.

Mechanisms leading to stronger divergence in small-bodied frogs

Lower dispersal capacity in small-bodied frogs may explain the size-specific differences in regional differentiation observed in mantellids. If the rate at which individuals move between subpopulations is high, the entire fragmented population will behave as a single unit (Amos & Harwood, 1998). At the other extreme, if movement is negligible, then the sub-populations become isolated from one another. The necessarily smaller effective population sizes in fragmented populations may lead to the fixation of different rare alleles in these subpopulations, ultimately increasing differentiation between them. This is particularly true for organellar genomes with smaller effective population sizes and lower numbers of migrants than nuclear genes (Birky *et al.*, 1989). Amphibians are poor dispersers in general: meta-analyses of population structure have revealed such low levels of gene flow ($N_em < 1$) that differentiation at neutral loci through drift or local adaptation is inevitable in many species (Ward *et al.*, 1992; Morjan & Rieseberg, 2004). Field measurements have also shown that large-bodied frogs tend to move farther than small-bodied frogs (Pilliod *et al.*, 2002). Moreover, small-sized amphibian species as well as juveniles are more vulnerable to desiccation (e.g., Ray, 1958; Chelgren *et al.*, 2006; Becker *et al.*, 2007; Tracy *et al.*, 2010) and therefore less likely to successfully disperse through unfavourable (exposed) habitat. On the other hand, large body size has been shown to be a correlate of a pioneering phenotype in some anuran clades (reviewed in Wells, 2007; Van Bocxlaer *et al.*, 2010) encompassing a suite of traits leading to the evolution of greater dispersal capacity, enabling the colonization of large areas during which vagility might even increase through spatial phenotype assembly (Shine *et al.*, 2011).

Nonetheless, gene flow via dispersal is only one of many factors that contribute to the amount and distribution of genetic variation among populations (Whitlock & McCauley, 1999). The higher sequence divergence observed between populations of small vs. large mantellids could also potentially be caused by variation in mitochondrial mutation rate (Nabholz *et al.*, 2009) because of size-specific differences in metabolic rate (e.g. Martin & Palumbi, 1993). However, the very low resting metabolic rate of amphibians (Weathers & Snyder, 1977) and a positive correlation with body mass (Fig 5.1, pg. 185 in Wells, 2007) makes this unlikely and contradictory to our findings (but see Santos, 2012). Organisms with shorter generation times may have higher DNA replication rates per unit of time and hence a higher mutation rate (Kohne, 1970; Nabholz *et al.*, 2008a,b, 2009). Basic life-history data are unavailable for most mantellid frogs but based on age structure data of a few species (Guarino *et al.*, 1998, 2010; Andreone *et al.*, 2002, 2011; Jovanovic & Vences, 2010), it is likely that small-sized species (20–30 mm) attain sexual maturity within the first post-metamorphic year, whereas larger frogs (SVL 40–80 mm) attain maturity only in their second or third year of life. Generation time is therefore probably attenuated in small compared to large mantellids which could accelerate mtDNA mutation rate in the former. A confounding factor may involve the interaction between generation time and dispersal ability in frogs. Mobility may be greater for species that take several years to mature (e.g. larger species) than for those that mature early (small species), simply because juveniles of the former will have had more time for broader dispersal (Palo *et al.*, 2004). Larger, slowly maturing species would therefore be subject to both lower mutation rates and the homogenizing effect of greater juvenile dispersal.

Genetic diversity as a species-specific trait

The second response variable in our data set, within population genetic diversity (π), was not correlated with any of the explanatory variables nor with phylogeny, suggesting either that other predictors need to be invoked to explain π or that intra-populational mitochondrial diversity of a species is largely stochastic. Although the 16s rRNA gene fragment that we applied is highly informative at the inter-population and interspecific levels (Vences *et al.*, 2005), it is less revealing at the level of within-population polymorphism. Nevertheless, we detected large differences in 16s rRNA variability among species. For example, many populations contained only a single haplotype, whereas others had up to 14. Interestingly, the relationship between population divergence and polymorphism in our data was inconsistent and insignificant, suggesting that fragmentation does not increase the rate at which variability is lost because of genetic drift. This lack of a definitive population size effect on mitochondrial variation was recently emphasized by an extensive meta-analysis (Bazin *et al.*, 2006). Despite considerable interspecific variability, π values were remarkably similar in populations of the same species (Fig. S3). The data therefore provide an indication that mitochondrial genetic diversity is relatively constant across populations within species. The evolutionary significance of this correlation is unclear and needs to be tested using other markers to judge whether it is representative of the overall nucleotide variability within a population and to understand whether mitochondrial intra-population diversity is a proxy for overall (nuclear) genetic diversity.

Implications for species diversification

The higher regional differentiation in small-sized mantellid frogs attests to a lack of or low levels of gene

flow at the regional scale. Their distributions are most probably composed of smaller demographic units evolving independently from one another. Without population cohesion provided by gene flow, the differentiation of these local demes is inevitable because of the action of genetic drift and directional selection with reproductive isolation emerging as a by-product of both processes (Coyne & Orr, 2004). Given enough time, the ranges of small-bodied mantellids will tend to subdivide into genetically isolated, separate demes, providing the potential for incipient speciation. Empirical evidence for this scenario stems from the correlation between body size and range size in mantellids and from the observation that the youngest mantellid sister species typically occur in close spatial proximity (Wollenberg *et al.*, 2011). More generally, simulation studies have shown that under limited individual dispersal, a widespread gene pool will inherently subdivide into smaller units solely because of an isolation by distance effect and that these subpopulations will accumulate mutations to the point of becoming reproductively incompatible even in the absence of environmental barriers or preferential mating (Hoelzer *et al.*, 2008). Given time, the outcome of this asymmetric accumulation of lineages across a spectrum of body sizes would result in the right-skewed distribution of body size (and range size) shown in Fig. 1. There is some evidence of such mechanisms operating in other tropical amphibian radiations. For example, a combination of low vagility, high philopatry, small home range size and direct terrestrial development has been implicated in high levels of regional population subdivision and consequent elevated diversification rates in Costa Rican plethodontid salamanders (García-París *et al.*, 2000). In tropical tree-frog radiations, a large diversity in body size has arisen independently at least seven times (Moen & Wiens, 2009) with high species richness in local hylid assemblages consistently associated with a predominance of small- and medium-sized species and long-term sympatry (Wiens *et al.*, 2011).

Despite our findings of increased genetic fragmentation in small-bodied mantellids, and a potentially straightforward mechanism explaining levels of divergence between species of different sizes, it is striking that the evidence for a correlation between body size and speciation rate in mantellids is at best weak (Wollenberg *et al.*, 2011) and indeed may not apply to other taxa (Orme *et al.*, 2002a,b). Hence, it seems clear that different processes are operating at the micro- and macroevolutionary levels that possibly favour speciation in intermediate dispersers (Etienne & Olff, 2004; Agnarsson & Kuntner, 2012; Claramunt *et al.*, 2012), and our data facilitate their discernment.

The intermediate dispersal model foresees that the rate of speciation increases with decreasing dispersal ability, and thus with decreasing body size, at the population or phylogeographic levels. Assuming a prevalence of vicariant speciation, our results support this hypothesis in mantellid frogs: the degree of genetic divergence and thus probability of genetic incompatibility and vicariant speciation (Sasa *et al.*, 1998) are inversely correlated with body size. However, our results are not indicative of an 'intermediate dispersal' phenotype effect (Etienne & Olff, 2004; Claramunt *et al.*, 2012) which would predict D_{xy} values to peak at intermediate body sizes: the respective regression graphs show no tendency for higher D_{xy} values at intermediate body sizes (Fig. 2). In contrast to the outcome of the analyses of population divergence, Wollenberg *et al.* (2011) found a non-significant negative relationship between clade diversity and body size in mantellid frogs, signifying that the population-level effect does not extend to phylogenetic speciation rate in mantellids.

These results suggest that at larger spatial and temporal scales, small body size may in fact negatively influence speciation rate in an indirect way, because the ability to disperse also determines the chances of colonizing new suitable habitats and of radiating therein (Rosenzweig, 1995; Owens *et al.*, 1999; Van Bocxlaer *et al.*, 2010). Species with dispersal ability below a certain threshold might persist within their microendemic ranges, unable to overcome large-scale environmental barriers (Kisel & Barraclough, 2010). In effect, lower dispersal capacity may inhibit their propensity for allopatric diversification, outweighing the higher probability of small species diversifying within their established ranges.

The interplay of these two components of the model predicts that species of intermediate body size may have highest overall speciation rates, but also identifies as speciation-prone such clades in which body size (or other traits determining dispersal ability) undergoes multiple evolutionary changes: in these clades, large-sized ancestors could disperse to and colonize new areas, diverge and evolve into small-sized species which then undergo rapid allopatric speciation. Apart from mantellid frogs in the rainforests of Madagascar, this model may help explain recurrent patterns of microendemicity in other biodiversity hotspots, for example, caddisflies and other taxa in New Caledonia (Espeland & Johanson, 2010) or amphibians in Central or South America (García-París *et al.*, 2000; Wiens *et al.*, 2011). Species-rich temperate taxa may show a similar pattern, for example, Knouft & Page (2003) inferred first dispersal, and then diversification in isolated streams as a mechanism explaining a trend of decreasing body size through time in North American freshwater fishes.

In conclusion, we suggest that although lower dispersal ability may potentially accelerate species diversification, it simultaneously reduces the chances of colonization of new areas, resulting in a lower propensity for allopatric speciation in small amphibians as exemplified by the mantellids studied herein. The

counteraction of these two consequences of small body size in anurans seems in line with recently proposed intermediate dispersal models (Etienne & Olff, 2004; Claramunt *et al.*, 2012).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Time-calibrated Bayesian phylogeny (Wollenberg *et al.*, 2011) pruned to include only the taxa examined in this study

Figure S2 Correlation ($r_s = 0.31$, $P = 0.050$) between maximum male snout vent length (a measure of body size) and range size (in km^2) in 40 mantellid species

Figure S3 Significant positive correlation between pairwise values of intrapopulation nucleotide polymorphism (π) across 30 species of mantellid frogs from two sites in east-central Madagascar ($r_s = 0.56$, $P = 0.0012$)

Table S1 GenBank accession numbers and sources of sequences

Table S2 Candidate models explaining between population divergence (D_{xy}) in 37 mantellid species ranked according to the Akaike information-theoretic criterion

Table S3 Candidate models explaining within population nucleotide polymorphism (π) in 37 mantellid species ranked according to the Akaike information-theoretic criterion

Table S4 Model-averaged coefficients and unconditional standard errors for variables present in the best supported models explaining between population divergence (D_{xy}) across 37 mantellid species.

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