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Tracing a toad invasion: lack of mitochondrial DNA variation, haplotype origins, and potential distribution of introduced *Duttaphrynus melanostictus* in Madagascar

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Abstract. The black-spined toad, *Duttaphrynus melanostictus*, is widespread in South and South-East (SE) Asia, although recent molecular analyses have revealed that it represents a species complex (here called the *D. melanostictus* complex). Invasive populations of this toad have been detected in Madagascar since at least 2014. We here trace the origin of this introduction based on mitochondrial DNA sequences of 340 samples. All 102 specimens from Madagascar have identical sequences pointing to a single introduction event. Their haplotype corresponds to a lineage occurring in Cambodia, China, Laos, Thailand, Vietnam, and some locations of eastern Myanmar and northern Malaysia, here named the SE Asian lineage. Within this lineage, specimens from one location in Cambodia and three locations in Vietnam have the same haplotype as found in Madagascar. This includes Ho-Chi-Minh-City, which has a major seaport and might have been the source for the introduction. Species distribution models suggest that the current range of the Madagascan invasive population is within the bioclimatic space occupied by the SE Asian lineage in its native range. The potential invasion zone in Madagascar is narrower than suggested by models from localities representing the full range of the *D. melanostictus* complex. Thus, an accurate taxonomy is essential for such inferences, but it remains uncertain if the toad might be able to spread beyond the potential suitable range because (1) knowledge on species-delimitation of the complex is insufficient, and (2) the native range in SE Asia might be influenced by historical biogeography or competition.

Keywords: amphibia, Anura, black-spined toad, Cambodia, invasive species, Madagascar, Maxent, mitochondrial DNA, risk assessment, species distribution model, Vietnam.

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Introduction

Invasive, allochtonous species play a major role in the global biodiversity conservation crisis (Clavero and Garcia-Berthou, 2005; Crowl et al., 2008; Keller et al., 2011; Simberloff et al., 2013). The introduction of non-native species to naïve environments has led to the extinction of many native taxa, especially on islands (Reaser et al., 2007). Impacts have involved direct competition and predation, as well as the indirect consequence of non-natives serving as vectors of new parasites and infectious diseases (Conn, 2014). Although amphibians are not generally ranked among the most hazardous invasives (Hatcher et al., 2012; Roy, 2016), in some cases the introduction of a non-native amphibian has harmed endemic faunas. The most prominent case is the cane toad, *Rhinella marina*, which still endangers endemic amphibians and possible predators in Australia, although its effects on the native fauna are mixed and not always detrimental (Shine, 2010).

Another representative of the true toads (family Bufonidae), the black-spined toad (*Duttaphrynus melanostictus*), is also an invasive species. According to current taxonomy, this toad is widespread in South and South-East (SE) Asia (AmphibiaWeb, 2016) and has been introduced to Borneo, Sulawesi and Seram, and additional Indonesian islands (Church, 1960; Inger and Stuebing, 2005; Wogan et al., 2016).

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It has also been introduced to Madagascar where its presence has been recorded since 2014, but might date back at least to 2010 (Andreone et al., 2014; Crottini et al., 2014; Kolby, 2014; Kull et al., 2014; Moore et al., 2015). Recent molecular analyses (Hasan et al., 2014; Wogan et al., 2016) have revealed a deep genetic structure within *D. melanostictus*, suggesting that this species is, in fact, a largely unresolved complex of several allopatric species (herein termed the *D. melanostictus* complex). As the various species of this complex inhabit distinct bioclimatic niches, their potentials of invasion likely differ. Thus, understanding the identity and source of invasive populations is critical for risk assessment and management.

The introduction of *D. melanostictus* to Madagascar has received much attention from the conservation community because it may constitute a serious threat to the unique biota of this island (Andreone et al., 2014; Crottini et al., 2014; Kolby, 2014; Moore et al., 2015; Pearson, 2015). Madagascar harbors a unique native amphibian fauna, with over 300 described species in four families (Hyperoliidae, Mantellidae, Microhylidae, Ptychadenidae) and 100% endemism at the level of native species (Glaw and Vences, 2007; AmphibiaWeb, 2016). Currently, habitat destruction is the main factor threatening numerous species of Madagascan frogs (Andreone et al., 2005). The amphibian chytrid fungus has been recorded from a small proportion of specimens (Bletz et al., 2015), yet no known disease-related declines like those affecting amphibians in other parts of the globe are known (Berger et al., 1998; Vredenburg et al., 2010; Fisher et al., 2012). Threats to the unique Madagascan amphibian fauna through the introduction of foreign pathogens via invasive toads, as well as the threats of competition or predation, are of serious concern (Brown et al., 2016). Competition for reproduction sites seems to be an issue as well, given that this species has a high rate of fecundity with up to nine thousand eggs per clutch (Cai, 1979; Van Leeuwen, personal observation; sup-

plementary table S1). Further, it uses all available water bodies including ponds and streams (Ngo and Ngo, 2013) that native amphibians usually inhabit. Given the toxicity of the secretions of bufonid toads, *D. melanostictus* might also pose threats to native Madagascan predators, including the many frog-eating snakes, birds and mammals (Glaw and Vences, 2007). As far as known, this invasion involves the area around the city of Toamasina only (Moore et al., 2015), but further spread is to be expected as the species has already been observed among bananas transported by trucks (R. Dolch, personal observation).

The current risk assessment is based on knowledge from the complete distribution area of the *D. melanostictus* complex (e.g., McClelland et al., 2015; Moore et al., 2015), and the available species distribution model (Pearson, 2015) estimates the potential distribution range in Madagascar using records from the entire range of the complex. For a more precise understanding of the geographical invasive potential of the species, it is crucial to identify the source lineage (species) of the Madagascan invasion and then to base invasion predictions on knowledge of its ecological requirements.

Herein, we identify the origins and potential spread of *D. melanostictus* in Madagascar as part of the risk assessment of this toad invasion. We (1) use mitochondrial DNA (mtDNA) sequences to assess the extent of genetic variation of the toad in Madagascar and exclude the possibility of independent introductions from multiple sources, (2) compare the mtDNA data with an expanded data set of homologous sequences from SE Asia to identify the origin of the introduction, and (3) apply species distribution models to assess the bioclimatically suitable range and the invasive potential of the toad in Madagascar.

Methods

Sample collection

We analysed DNA sequences of 340 samples of *Duttaphrynus melanostictus*. We first performed an exploratory phylogenetic analysis (supplementary fig. S1) of some sequences of Madagascan samples combined with the comprehensive set of sequences of Wogan et al. (2016), plus a comparison with a few sequences from India. Together these samples were representative of a large portion of the entire range of the *D. melanostictus* complex. The Madagascan samples were nested within a particular mitochondrial clade, marked in green color in the figures of Wogan et al. (2016). Within this clade, the Madagascan samples belonged to a major subclade occurring in SE Asia, which we herein call the 'SE Asian lineage' and on which we focused further sampling and analysis. The rationale of restricting the analysis to this subclade is given in supplementary fig. S1.

Our sampling of the SE Asian lineage included 81 samples from Wogan et al. (2016), one additional sequence (without precise locality) from GenBank, and 258 newly generated sequences. The new sequences were obtained from samples collected in Cambodia (9 samples), China and Taiwan (19), Malaysia (1), Thailand (4), Vietnam (123), and Madagascar (102). Most of the Madagascan samples (100) were collected at eight localities in the vicinity of the port and city of Toamasina, which represented most of the current range of the species in March 2015. Two additional samples came from the same general area but with no precise locality information.

Molecular analyses

DNA was extracted from tissue samples or buccal swabs using a standard salt protocol (Bruford et al., 1992). We amplified an approximately 480 bp fragment of mitochondrial DNA that encodes part of the genes encoding complete cytochrome oxidase c subunit III (*COIII*), tRNA glycine (tRNA-Gly), NADH dehydrogenase subunit 3 (*ND3*), and part of tRNA arginine by PCR (94°C 45 s, 49°C 30 s, 72°C 1 min) for 35 cycles, using the primer pair L-COXIII (5′-CCGCATGATACTGACACTT-3′) and Arg-HND3III (5′-AACTGTCTTTTTTGGACTAGC-3′) of Stuart et al. (2006). Chromatograms were checked and sequences corrected where necessary by using CodonCode Aligner (CodonCode Corp.). Alignment used MEGA7 (Kumar et al., 2016). After trimming sequences on both sides, we retained 347 bp corresponding to 69 bp of tRNA-Gly and 278 bp of *ND3*; this fragment was subsequently referred to as ND3. All new sequences were submitted to GenBank (accession numbers KY823030-KY823289; see supplementary table S2 for a detailed list). We reconstructed a maximum-likelihood tree in MEGA 7 under a general-time-reversible substitution model with gamma-shape parameter and a proportion of invariable sites, and used the obtained tree as a basis for building a haplotype network in HaploViewer (Salzburger et al., 2011).

Species distribution models

Species distribution models (SDMs) were generated in MaxEnt 3.3.3k (Phillips et al., 2006), as implemented in SDMtoolbox 1.1c (Brown, 2014). We used museum records (downloaded from GBIF.org), data from collaborators, and

data from our own fieldwork to compile occurrence records, which then were vetted for spatial and taxonomic accuracy. The list of GBIF localities was curated and we excluded localities outside the known range of the species or without precise coordinates, and pre-1950 records without recent confirmation. We randomly selected one of multiple occurrence records per species within a 10-km radius using SDMtoolbox (Brown, 2014). All models used the 19 standard bioclimatic variables representing spatial patterns of precipitation and temperature, at a 30 arc-second resolution (available at www.worldclim.org) (Hijimans et al., 2005; supplementary table S3). Models were built with occurrence points based on two datasets: (1) a large portion of the entire range of *D. melanostictus* (comprising various candidate species; Wogan et al., 2016) and (2) a subset consisting of the spatial locations of members of the SE Asian lineage, including only genetically verified occurrence records. Background points were sampled from an adaptive convex hull with a 100-km buffer drawn around the occurrence localities ($\alpha = 3$, done in SDMtoolbox). The final dataset for species distribution modelling was composed of 324 and 90 records for the full dataset and the SE Asian lineage dataset, respectively.

To parameterize the SDMs, we evaluated the performance of various combinations of five feature classes (linear; linear and quadratic; hinge; linear, quadratic and hinge; and linear, quadratic, hinge, product and threshold), and 10 regularization multipliers (from 0.5 to 5, in increments of 0.5) (Shcheglovitova and Anderson, 2013). We evaluated the performance of SDMs built under each combination of model parameters through a geographically structured k-fold cross-validation. Thus, the occurrence records were partitioned into k-equal geographically clustered subsamples, where $k = 3$, and the models were trained with two of the groups and then evaluated with the excluded group until all group combinations were run. Model fit was assessed through measurement of the omission rate, area under the curve (AUC), and model feature class complexity (Brown, 2014). After optimum model parameters were determined (those leading to the lowest omission rate, highest AUC, and lowest complexity, in the order listed), a final SDM was built with all occurrence sites. The best-fit SDM built on the entire dataset was parameterized with a linear feature-class and a regularization multiplier of 3. The best-fit model for the SE Asian lineage dataset was parameterized with a regularization multiplier of 3 and linear, quadratic, hinge, product and threshold features classes. The best resulting SDMs for the full dataset and SE Asian lineage dataset (of 150 SDMs with alternative parameter combinations for each dataset) showed high predictive accuracy (average omission rates 0.211 and 0.087, respectively) and average AUCs of 0.987 and 0.767, respectively.

Assessing shared climate space in different parts of the species' distribution

We measured shared climate space using the method and R scripts of Broennimann et al. (2012). This entailed generating principal component analyses of all climate variables from study areas and corresponding occurrence localities.

Specifically, we plotted the climate space occupied by three groups of *D. melanostictus*: (1) all known localities, (2) localities of the SE Asian lineage in its native (Asian) range, and (3) Madagascar localities of the SE Asian lineage. In addition, we characterized the climate space of two study areas: the entire climate space of Madagascar and the climate space accessible to all known localities of *D. melanostictus* (light grey outer shape in fig. 2D-F) depicted by climate within a 200-km buffer drawn around an adaptive convex-hull (ACH) of all the occurrence localities ($\alpha = 3$, done in SDMtoolbox), which resulted in separate buffered polygons for localities in Madagascar and SE Asian localities. For the three groups of *D. melanostictus*, we calculated Warren's D statistic and tested for niche equivalency (n of sims = 100) and niche similarity (n of sims = 1000) among groups to assess how the three groups shared climate. Warren's D statistic was used to assess an overall match between the occupied climate space and to determine if an inference of the climate characteristics of one distribution could have been made from the other. The niche equivalency measure statistically tested if the climate space occupied among the two groups' ranges were equivalent in terms of specific climate space and total breadth of climates shared by each distribution. Lastly, the niche similarity tested if the overlap between two ranges differed from the climate space in one range and the climate space selected at random from the other range. The niche similarity test addressed whether or not the environmental space occupied in one range was more similar to the one occupied in the other range than would be expected by chance. If the value was significant this meant a majority of the climate space occupied in one range was identical to the climate space, or a subset of it, occupied by the other distribution. This directional test was performed in both ways.

Results

All new sequences of ND3 from the target region in SE Asia belonged to the SE Asian lineage of *D. melanostictus*. The haplotype network for the 340 sequences of this lineage (fig. 1) contained 63 distinct haplotypes. For convenience and easier graphical representation, we divided these into *ad hoc* haplogroups H1-H4, which were colored differently in fig. 1. These main haplogroups were differentiated from each other by at least three mutational steps, but H4 (blue) was more distinct, differing by at least 14 steps from all other haplogroups. All 102 samples from Madagascar had identical sequences and we assigned them to a unique subcategory H1a, although they differed by only a single mutational step from the most common haplotype in H1.
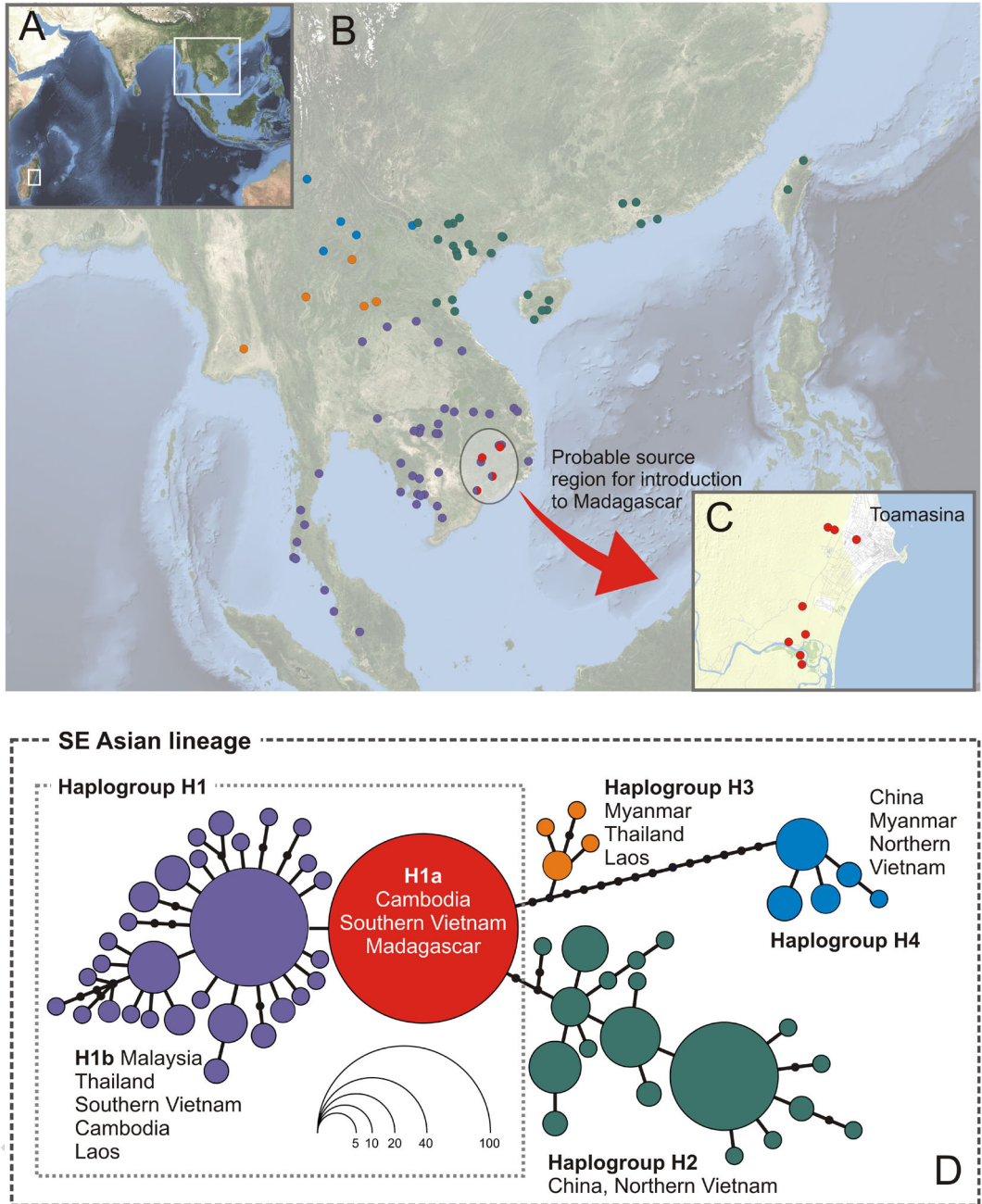
Figure 1. Sampling localities and mitochondrial haplotype network of *Duttaphrynus melanostictus*. (A) Overview map showing in white rectangles the areas highlighted in map (B) showing South-East Asia, and map (C) showing a part of eastern Madagascar where the invasive toad is currently present. Dots on the map are collection localities for samples used in the genetic analysis. Colors of the dots correspond to the haplotype network (D). The network was reconstructed from 347 bp of the mitochondrial region encoding ND3 from 340 samples, all corresponding to a single mitochondrial subclade of Wogan et al. (2016), herein called the SE Asian lineage. Haplogroups H1-H4, including subcategories H1a and H1b, belong to the SE Asian lineage; they were *ad hoc* defined for convenience, and do not correspond to equally differentiated units. The circle in map B defines the area in Cambodia and Vietnam where specimens with H1a were found, which is the only haplotype detected in Madagascar.
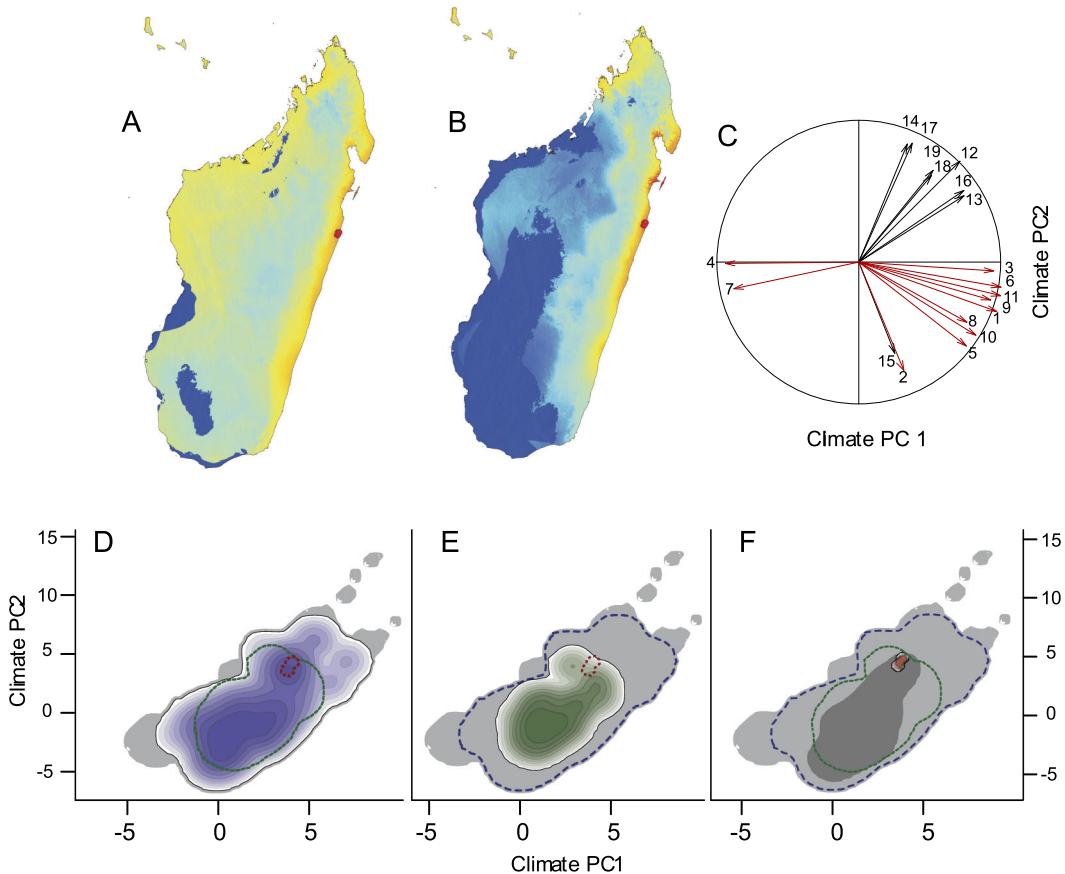
Figure 2. Potential distribution of *Duttaphrynus melanostictus* in Madagascar as predicted by species distribution models derived from (A) a large portion of the entire Asian range of the *D. melanostictus* complex in Asia, and (B) the range of the SE Asian lineage only. Warmer colors indicate a higher prediction. Red dots show the currently known range in the Toamasina area. (C) Correlation circle depicting the relationships among the 19 bioclimatic variables (supplementary table S3) throughout SE Asia and Madagascar. Variables representing temperature are in reddish. PC1 and PC2 axes of a principal component analysis explain 52.2% and 25.6% of the variation, respectively. Lower row of graphs shows the climate space accessible to *D. melanostictus* (light grey), and represents climate sampled within a 200-km buffer drawn around an adaptive convex-hull (ACH) of all the occurrence localities ($\alpha = 3$, done in SDMtoolbox), which resulted separate buffered polygons for localities in Madagascar and SE Asia. The climate space occupied by (D) all Asian localities (purple), (E) Asian localities of the SE Asian lineage (green), and (F) the Madagascar localities (red); dark grey shape in F is climate space occupied by all of Madagascar. Dotted green line represents the bioclimatic space shaded green in graph E; purple dashed line represents the purple shaded space in graph D; red dotted line represents the space of the Madagascar samples shaded red in graph F.

Haplogroups H1-H4 had distinct geographic distributions (fig. 1). Haplogroup H1 (including H1a) occupied all of the southern part of the range (Cambodia, southern Vietnam, parts of Laos and Thailand, and ranging into northern Malaysia). Haplogroup H2 mostly occurred in southeastern China and northern Vietnam. Haplogroup H3 occupied the northern parts of Laos and Thailand and Myanmar. The strongly divergent haplogroup H4 was from localities in Myanmar, China, as well as northern Laos and northern Vietnam.

The SDM based on localities of the entire *D. melanostictus* complex predicted a wide range of bioclimatically suitable habitat in Madagascar (fig. 2A and supplementary fig. S2), including all eastern and northern coastal lowlands of Madagascar as well as vast areas in the west and north-west of the island. Moderately suitable bioclimate was also present in

parts of the eastern mid-elevation rainforest. In contrast, an SDM based only on localities in the native range of the SE Asian lineage (fig. 2B and supplementary fig. S3) resulted in a distinctly more restricted distribution of climatically suitable areas in Madagascar. The suitable habitat was confined to coastal lowland areas in eastern and northern Madagascar, with low prediction values for the south-eastern and western parts of the island, and for mid- and high-elevations.

Our principal component analysis of climate data accessible by *D. melanostictus* characterized 77.8% of the climatic variation in the first two components (explaining 52.2% and 25.6% of the variation, respectively). A correlation circle (fig. 2C) showed the relative loadings of each bioclimatic variable on the first two principal components and suggested that many of the bioclimatic variables characterize very similar spatial patterns and climatological information. Caution was taken in assuming they were biologically equivalent, as principal components maximized variance among all input variables, which was likely driven by coarse spatial patterns associated with elevation and latitude. In other words, the 22.2% variation not characterized in the first two components was likely largely comprised of the unique portion of climate space restricted to individual bioclimatic variables.

All tests of niche equivalency were significant ($p < 0.01$). They rejected the null hypothesis of niche equivalence among the different groups of localities included in the SDM analyses (supplementary table S4). Values of Warren's D statistic ranged from moderately high (0.59) between all populations against Madagascar and SE Asian lineage to being very low between Madagascar and the two larger datasets (0.03, in both instances) and between SE Asian lineage and the other two dataset (0.02, in both instances). These values characterized niche overlap, with complete overlap being represented by 1 and no overlap being 0. The tests of niche similarity confirmed that climates occupied by the entire dataset and the SE Asian lineage dataset were very similar (but not identical) and differences were significant in both directions ($p = 0.002$); these calculations excluded localities from Madagascar. These tests also revealed that the climate occupied by the Madagascan population was similar to that of the native SE Asian lineage ($p = 0.046$), but not vice versa ($p = 0.563$). In part, this was due to the very small climatic space occupied by Madagascan populations, which nested well within climate space of SE Asia (fig. 2D-F). A similar pattern was observed between the Madagascan population and the complete dataset ($p = 0.012$ and $p = 0.559$, respectively). Thus, the two larger datasets represented similar, broad climates (fig. 2D, E) whereas the current Madagascan localities represented a climate space similar to a small portion of the climates occurring in the native range of the SE Asian lineage.

Discussion

Our study uses a conservation genetic approach to assess the diversity and origin of populations of *D. melanostictus* introduced into Madagascar. Our molecular analyses, based on mitochondrial DNA gene sequences, allow for two main conclusions: (1) introduced Madagascan toad populations are genetically uniform for mtDNA and are likely to have originated from an introduction of a few individuals from a single source population; (2) the mitochondrial data pinpoint the source population for the introduction to a limited region in Cambodia and Vietnam that includes Ho Chi Minh City, a town with a major seaport. Unfortunately, analyses do not include samples from the greater Bangkok area in Thailand, another seaport from which substantial trade with Madagascar takes place. However, given the distribution of haplotypes, it is unlikely that haplotype H1a occurs in or around Bangkok. Thus, Madagascan toads probably have their origin from Vietnam (or

Cambodia), and possibly from the greater Ho-Chi-Minh-City area. Future analyses can further test this hypothesis via fine-scale nuclear genetic markers.

The invasive toads sampled from Madagascar assign to a part of the *D. melanostictus* complex from SE Asia that probably represents a species distinct from other lineages of the complex that occur in Indonesia and Myanmar (Wogan et al., 2016). However, the taxonomy of this complex is far from being resolved. We here apply a very strict definition of this lineage and exclude from analysis other mitochondrial subclades of the *D. melanostictus* complex that might be conspecific with the SE Asian lineage (e.g., from Myanmar and Malaysia; Wogan et al., 2016). In addition, Wogan et al. (2016) did not include populations from South Asia (especially India) in their multigene study, and, thus, the identity of these requires future study. Although this taxonomic uncertainty might affect our geospatial modelling, it does not invalidate the identification of the source region for the invasive populations in Madagascar.

The haplotype network (fig. 1) serves to visualize the associations of the main haplotype groups within the SE Asian lineage, but it does not fully represent and objectively analyze the biogeographic history of the *D. melanostictus* complex in Asia. On one hand, the network is void of evolutionary direction (Kong et al., 2015), and on the other hand other lineages within the *D. melanostictus* complex that occur in the southern- and western-most parts of the area (Wogan et al., 2016) are not included in the network. In addition, our *ad hoc* haplotype groups contain geographically structured variation not considered herein.

The simplified representation shows that the haplotype H1a of Madagascar is part of a larger assemblage of other, distinct H1 haplotypes (fig. 1). Therefore, H1a is unlikely to occur naturally far outside its identified range that encompasses one Cambodian and three localities in southern Vietnam: (1) a logging concession in Cambodia, 1 sample; (2) the Saigon Zoo and Botanical Gardens (Ho-Chi-Minh-City, Vietnam), 8 samples; (3) Yok Don National Park (Vietnam), 1 sample; and (4) Cat Tien National Park (Vietnam), 1 sample (details in supplementary table S5).

The SDM based on the native distribution of the SE Asian lineage suggests that the potential range of the toad in Madagascar is restricted to the lowlands of the eastern and northern coasts (fig. 2B). This constitutes a more restricted area than suggested by models based on the entire Asian range of the *D. melanostictus* complex (Pearson, 2015; fig. 2A). SDMs based on bioclimate can provide useful information for a risk assessment of invasive species, but their spatial predictions must be interpreted with caution (e.g., Broennimann and Guisan, 2008; Sinclair et al., 2010; Elith et al., 2010). In a new environment, an invasive species might be able to occupy novel environmental niches that competitors or predators dominate in their native range, or are inaccessible due to geographical barriers. Using the analytical approach of Broennimann et al. (2012), our analyses ascertain that the bioclimatic space occupied by the toads in Madagascar fully overlaps with that of the SE Asian lineage and of all populations of the *D. melanostictus* complex; thus, it does not contradict our predictive SDM.

Discordance between the all-range SDM and the SDM of the SE Asian lineage is relevant because the favored model predicts a lower probability of invasion of the mid-elevation rainforest areas of eastern Madagascar, which harbor the highest diversity of endemic amphibian and reptilian species (Brown et al., 2016). Furthermore, analyses do not predict highly suitable habitat for the SE Asian lineage (fig. 2B) in several centers of diversity of threatened amphibians (Andreone et al., 2005) and reptiles (Jenkins et al., 2014), such as the highlands of southeastern and north-eastern Madagascar, as well as some massifs in the west. However, conservationists need to be prudent when translating these results into a risk assssessment. On one hand, our definition of the SE Asian lineage

might be too restrictive. Other populations of the *D. melanostictus* complex, especially from Myanmar and Malaysia, might be conspecific with the SE Asian lineage, and, thus, its climatic envelope might be wider than our models resolve. On the other hand, the environmental space of the toad in SE Asia might not be limited by bioclimate alone but also influenced by biogeographical barriers or competition, and the toad might thus invade bioclimates not occupied in its original SE Asian range when released from this constraints. Therefore, stochastic, spatially explicit, Individual Based Models (IBMs) based on data collected from spatial behavioural ecology, abundance estimates, survival rates, and dispersal capabilities of the Madagascan toads in their invasive area are now necessary to ascertain the reliability of our predictions. These should aim to complement the bioclimatic variables by including land cover and geology, as well as involve novel, mechanistic approaches that consider physiological parameters, and biotic and abiotic interactions (Kearney and Porter, 2009; Evans et al., 2015). Notwithstanding the potential for improvement, our model represents a plausible alternative hypothesis to that based on the entire range of the *D. melanostictus* complex (Pearson, 2015; fig. 2A), which probably includes multiple species (Wogan et al., 2016). Thus, our analyses highlight that a solid taxonomic assessment and a careful selection of localities for model-training are essential for accurately predicting SDMs and making risk assessments.

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