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Author for correspondence:
Guinevere O. U. Wogan
e-mail: gwogan@berkeley.edu

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Deep genetic structure and ecological divergence in a widespread human commensal toad

Guinevere O. U. Wogan[1], Bryan L. Stuart[2], Djoko T. Iskandar[3] and Jimmy A. McGuire[1]

[1]Museum of Vertebrate Zoology, University of California, Berkeley, CA 94720, USA
[2]North Carolina Museum of Natural Sciences, Raleigh, NC 27601, USA
[3]School of Life Sciences and Technology, Institut Teknologi, Bandung, Indonesia

The Asian common toad (*Duttaphrynus melanostictus*) is a human commensal species that occupies a wide variety of habitats across tropical Southeast Asia. We test the hypothesis that genetic variation in *D. melanostictus* is weakly associated with geography owing to natural and human-mediated dispersal facilitated by its commensal nature. Phylogenetic and population genetic analyses of mitochondrial and nuclear DNA sequence variation, and predictive species distribution modelling, unexpectedly recovered three distinct evolutionary lineages that differ genetically and ecologically, corresponding to the Asian mainland, coastal Myanmar and the Sundaic islands. The persistence of these three divergent lineages, despite ample opportunities for recent human-mediated and geological dispersal, suggests that *D. melanostictus* actually consists of multiple species, each having narrower geographical ranges and ecological niches, and higher conservation value, than is currently recognized. These findings also have implications for the invasion potential of this human commensal elsewhere, such as in its recently introduced ranges on the islands of Borneo, Sulawesi, Seram and Madagascar.

1. Introduction

The ecology of organisms plays an important role in shaping their genetic structure. Species that are commensal with humans usually tolerate a wide variety of habitat types and tend to be highly vagile, either by their own dispersal capabilities through the extensive human-modified landscapes that are now available, or by intentional or accidental human-mediated transport. Such high vagility usually results in genetic admixture among populations and a weak association between geography and genetic variation [1].

Southeast Asia has the highest rate of deforestation of any major tropical area in the world as a result of its large human population and rapidly growing economies. *Duttaphrynus melanostictus* (Schneider, 1799), the Asian common toad, thrives in human-modified environments throughout tropical Southeast Asia. The species naturally occurs in open habitats such as grasslands and deciduous dipterocarp savannahs, but has benefited from human activities and expanded its range into agricultural lands, villages, towns and cities. There, it commonly lives around human dwellings and, in urban areas, feeds on insects that are attracted to artificial lights. The species is a human commensal, an attribute that allows it to easily disperse, including over previously impermeable saltwater barriers to colonize islands, and it possesses the 'optimal range-expansion phenotype' associated with highly dispersive bufonid species with large distributions [2]. The species recently invaded Borneo [3], Sulawesi and Seram (JA McGuire, DT Iskandar 1974, 1998, personal
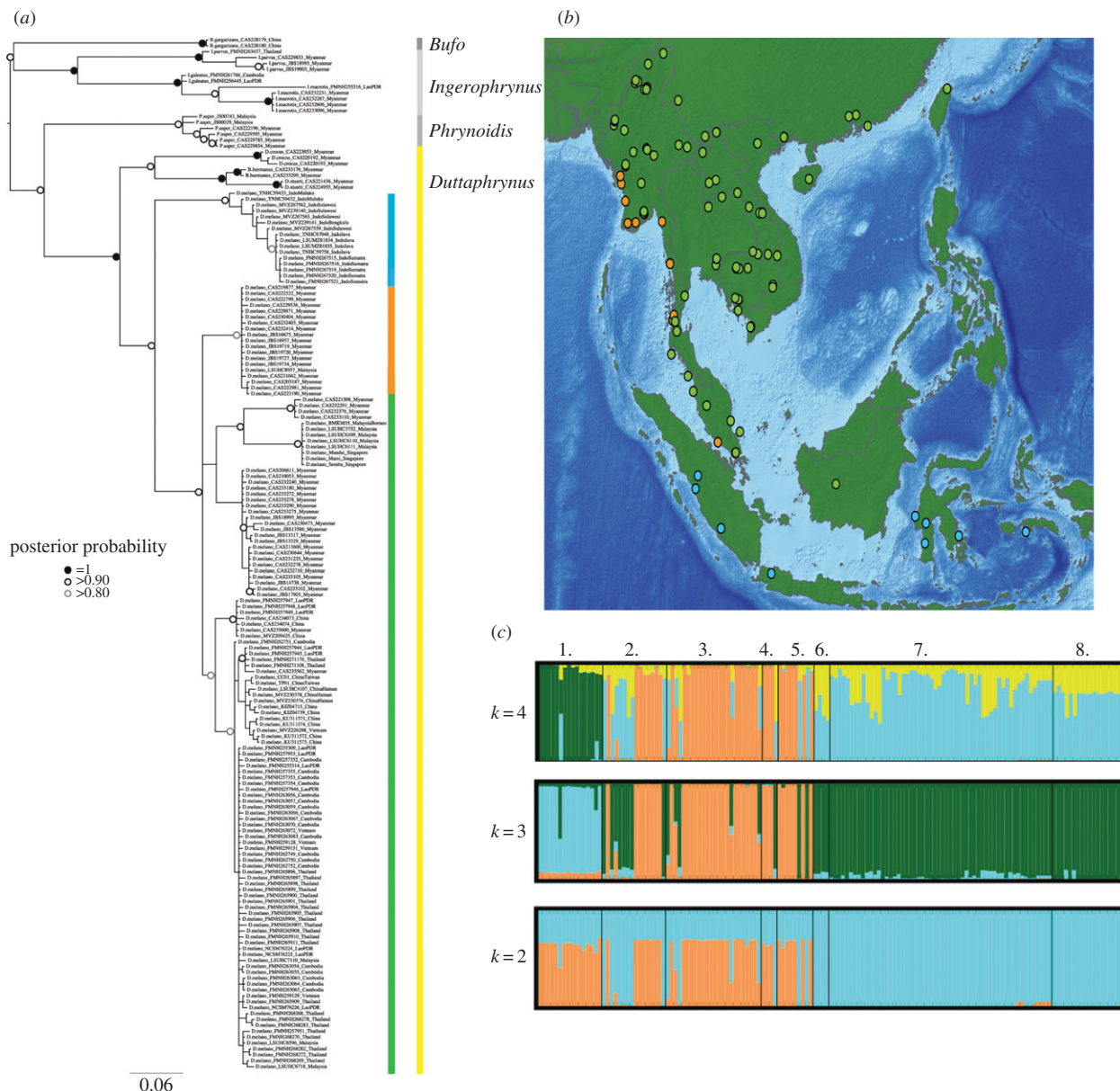
Figure 1. (a) *Duttaphrynus melanostictus* phylogenetic tree based on mtDNA and nuclear loci inferred from mixed-model Bayesian inference. (b) Distribution of the three major clades; green, mainland; orange, coastal; blue, island. (c) Results from population clustering for $k = 2, 3, 4$ (based on nuDNA).

observation), additional Indonesian islands [4] and Madagascar [5], probably via shipping containers [5], as its current range on the most recently invaded islands is primarily restricted to the vicinity of seaports [5]. In Borneo, where it has been documented for over a century, it has moved into the interior around villages, towns and cities [3], likewise in Bali, which was invaded in 1958 [4]. There is urgent concern of its spread on these islands, and elsewhere in the world, as the species poses a threat, like its relative *Rhinella marina*, the cane toad, to native amphibians and naive predators from competition, predation and disease introduction [5].

Many new species of amphibians continue to be discovered in tropical Southeast Asia [6], in part from recent fieldwork in previously unexplored areas, but also from increased use of genetic and bioacoustics tools in systematic research [7]. Most geographically widespread species of Southeast Asian amphibians, upon closer examination, have proven to actually consist of multiple, cryptic species (two or more species hidden under a single name) [1,7,8]. However, these analyses have mostly focused on species having

Table 1. Genetic distances. On the diagonal are within-group mean distances (mtDNA). Estimates of evolutionary divergence between groups are above (mtDNA) and below (nuDNA) the diagonal.

	mainland	coastal	island
mainland	3.9% (0.005)	0.2% (0.001)	1.4% (0.004)
coastal	7.1% (0.011)	0.1% (0.001)	1.4% (0.004)
island	11.9% (0.018)	11.2% (0.019)	0.1% (0.001)

restricted ecological requirements, such as closed-canopy forest and lotic streams, and therefore have limited dispersal capabilities (but see [9,10]). Here, we carry out the first well-sampled phylogeographic analysis for *D. melanostictus* encompassing mainland and insular Asian populations. We test the hypothesis that genetic variation in *D. melanostictus* is weakly associated with geography owing to natural and human-mediated dispersal facilitated by its high dispersive ability and commensal nature. Alternatively, a strong
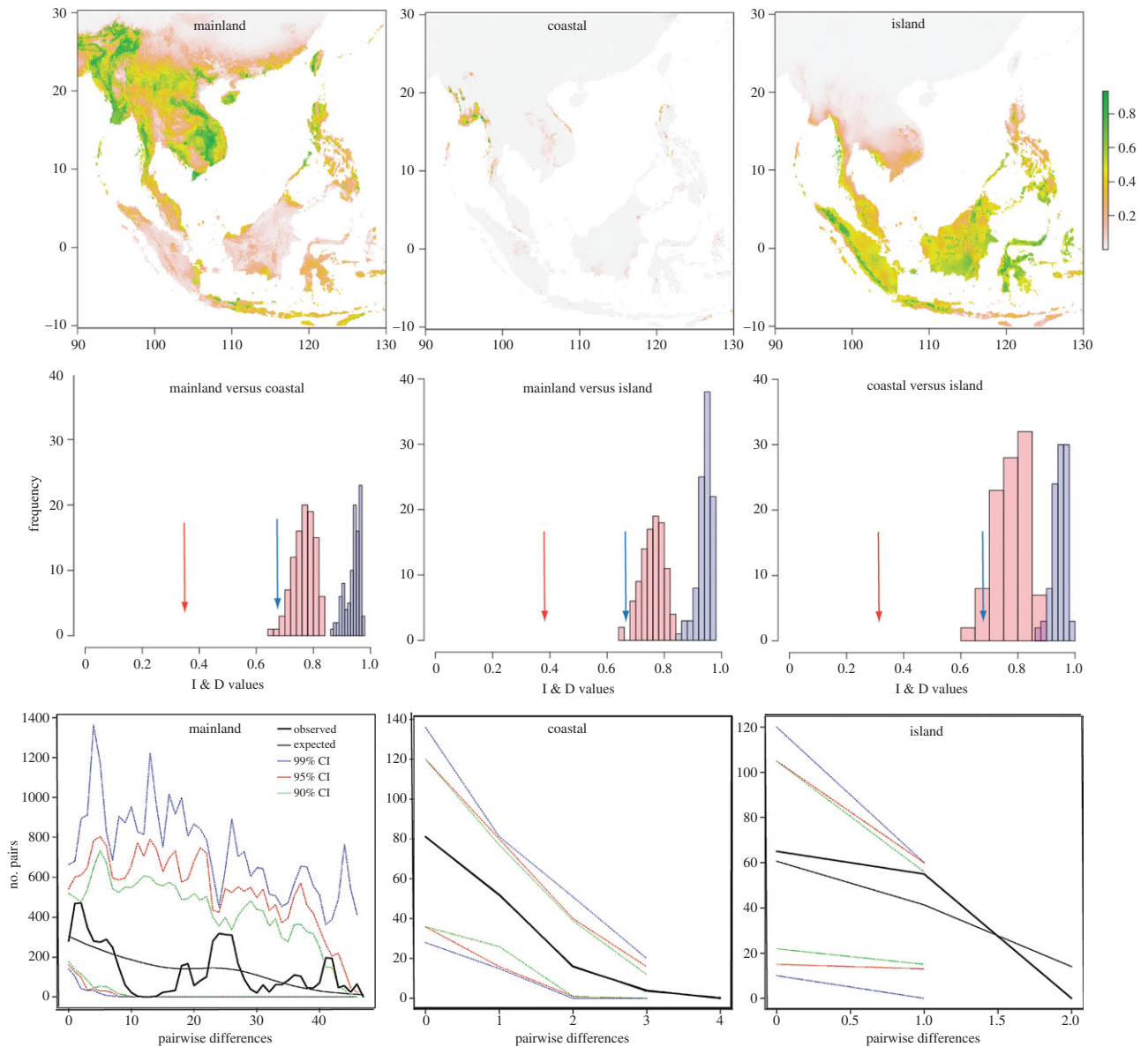
Figure 2. Top: ENMs for the three major clades of *Duttaphrynus melanostictus*. Middle: results from niche equivalency tests for each clade pair; observed Schoener's *D* (red arrow) and Hollinger's *I* (blue arrow) plotted against a null distribution of Schoener's *D* (red) and Hollinger's *I* (blue). Bottom: mismatch distributions reflecting the demography within each major clade.

Table 2. Diversity statistics. The number of haplotypes (n_{haplo}) and nucleotide diversity (π) indicate the high diversity found in the mainland clade. Non-significant *p*-values for Tajima's *D* indicate a failure to reject neutrality and demographic stability. Significant *p*-values and highly negative values for Fu's *F* indicate recent spatial or demographic expansion. SSD and Harpending's *R* relate to the mismatch distributions; non-significant *p*-values indicate a failure to reject a model of population expansion.

	n	n_{haplo}	π	Tajima's D	Fu's F	SSD	Harpending's R
mainland	113	51	17.40076	−0.18346 (0.482)	−7.5022 (0.001)	0.01252 (0.012)	0.00557163 (0.910)
coastal	18	4	0.62745	−1.38107 (0.075)	−3.4 × 10 (0.000)	0.00003 (1.000)	0.09812465 (0.750)
island	16	2	0.45833	1.03439 (0.888)	−3.4 × 10 (0.000)	0.01419 (0.990)	0.21701389 (0.710)

association between geography and genetic structure, despite these characteristics, suggests the possibility of local adaptation and cryptic speciation. We also include samples from the invaded Southeast Asian islands of Borneo, Sulawesi and Seram to test for genetic signatures that indicate the origins of those populations.

2. Material and methods

Genomic extractions of 152 *D. melanostictus* and nine outgroup species were amplified and sequenced for one mitochondrial DNA fragment (ND3: 467–489 bp) and two nuclear DNA fragments (POMC: 601 bp and SOX9: 604–688 bp). We selected the partitioning scheme and models of sequence evolution using

PARTITIONFINDER [11], and inferred phylogenies using mixed-model Bayesian analysis in MRBAYES v. 3.2 [12]. We phased the nuclear markers and then tested the association between genetic structure and geography in STRUCTURE [13]. We performed tests of selective neutrality, demographic expansion and mismatch distributions to check for population expansion, and estimated the ancestral range. We then generated environmental niche models (ENMs), and assessed niche equivalencies among major clades. See the electronic supplementary material for voucher information and Genbank accession numbers (table S1), sequencing protocols, and detailed analytical methods.

3. Results

Combined analyses of mitochondrial and nuclear DNA recovered three deeply divergent clades within the natural range of *D. melanostictus*, which correspond to the Southeast Asian mainland, the Sundaic islands of Sumatra and Java, and the coastal regions of Myanmar (figure 1 and electronic supplementary material, figures S1 and S2). Genetic divergence among the three clades is substantial (uncorrected pairwise differences from 7.1% to 11.9% in mtDNA, 0.2–1.4% in nuDNA; table 1). Shared mtDNA haplotypes identify the source populations of the invasive Indonesian populations of *D. melanostictus*. The Bornean haplotype matched that of samples from Peninsular Malaysia in the mainland clade, whereas those of the Sulawesi and Seram populations matched those from within Indonesia, by way of either Sumatra or Java.

Population structure analyses also strongly inferred these three clusters within the *D. melanostictus* data. Tajima's D was not significant in any of the populations, suggesting neutrality (table 2). Fu's F was negative and significant in all three populations, providing evidence of population expansion (table 2). Mismatch distributions and associated statistics inferred a stable population size with significant population structure within the mainland clade, and spatial or demographic expansions within both the island and coastal clades (figure 2 and table 2). The ancestral range reconstruction identified two ancestral areas: the primary on the Myanmar–China border and a secondary on Sumatra (electronic supplementary material, figure S3).

ENMs of the three major clades identified largely non-overlapping predicted distributions with non-equivalent niches (figure 2). Schoener's D values ranged from 0.651 to 0.684, whereas Hollinger's I ranged from 0.355 to 0.395, with the greatest disparity between mainland and insular clades (electronic supplementary material, tables S2 and S3).

4. Discussion

Our findings reject the hypothesis that genetic variation in *D. melanostictus* is weakly associated with geography owing to natural and human-mediated dispersal. Instead, we unexpectedly found that *D. melanostictus* contains at least three distinct evolutionary lineages that differ genetically and ecologically. Deep genetic structure within one of the most common amphibian species in Asia suggests that Asia's widespread, lowland amphibian species warrant additional attention, as they may be particularly informative for assessing patterns of contemporary and historical landscape connectivity. The ancestral range for the *D. melanostictus* clade is estimated to lie on the Myanmar–China border,

where other ancient Asian bufonid lineages are also found [14], reflecting its role as an important biogeographic crossroads between South Asian, Southeast Asian, Eastern Asian and Malayan faunas [15]. While these toads appear to have a mainland origin, as recently as 11 kya there was ample opportunity for dispersal before sea levels rose to near their current levels isolating the Sundaic landmasses from mainland Southeast Asia [16]. We did not recover a break at a well-documented biogeographic barrier, the Isthmus of Kra, but did at the seawater barrier that separates mainland Southeast Asia from the Sundaic islands. The coastal clade is restricted to tidal mud flats and represents a case of micro-allopatry with the mainland clade. We found a lack of genetic structure between Sumatran and Javan samples, conversely to some studies across this region [17], and despite the existence of many Javan endemics. This suggests the possibility that *D. melanostictus* has been introduced to one of these islands from the other; however, additional sampling is needed to definitively determine if this is indeed the case.

Our finding of three distinct evolutionary lineages that differ genetically and ecologically within *D. melanostictus* has at least two important implications for conservation. First, *D. melanostictus* probably consists of more than one species, suggesting that tropical Asia's 'weediest' amphibian species may not be as common, with as large a geographical range, as it has been treated for more than two centuries. Micro-allopatry or maintenance of isolation of mainland and coastal clades, despite opportunity for contact, provides further evidence for species distinction. We note that genetic assessment of South Asian populations is needed to fully resolve *D. melanostictus* species boundaries, because these populations have historically been treated as conspecifics. Second, a lack of detectable genetic admixture between the Asian mainland and Sundaic islands (with the exception of Borneo), despite the great opportunity for human-mediated dispersal, suggests that toads of one clade may not be able to persist within the range of the other clade, perhaps limited by climatic barriers. This accords with our findings that the ecological niches of the three clades have diverged and are narrower than is currently recognized for the 'species' as a whole. Genome-wide markers would provide higher resolution for detecting admixture if it exists.

Our assessment of genetic variation within *D. melanostictus* in Asia provides an opportunity to determine the Asian provenance of the invasive population in Madagascar, far outside of the species' native range. Those findings, in conjunction with a comparison of the ecological niches of Madagascar with the niche of the membership clade of *D. melanostictus* in Asia, will provide us a basis for inferring the invasive potential of *D. melanostictus* within Madagascar, a hotspot of global amphibian diversity.

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