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Speciation and historical invasions of the Asian black-spined toad (*Duttaphrynus melanostictus*)

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Animal translocations provide striking examples of the human footprint on biodiversity. Combining continental-wide genomic and DNA-barcoding analyses, we reconstructed the historical biogeography of the Asian black-spined toad (*Duttaphrynus melanostictus*), a toxic commensal amphibian that currently threatens two biodiversity hotspots through biological invasions (Wallacea and Madagascar). The results emphasize a complex diversification shaped by speciation and mitochondrial introgression that comprises two distinct species. One species (true *D. melanostictus*) is distributed in the Indian subcontinent and is invasive in Wallacea. The other species, whose nomenclature remains unsettled, diverged from *D. melanostictus* in the Miocene era (~7 Mya) and diversified across Southeast Asia, from where it was introduced to Madagascar. Remarkably, the Indonesian population of *D. melanostictus* was recently established from India, which suggests historical, possibly human-assisted dispersal across the Bay of Bengal, reflecting the centuries-old connection between these regions.

Artificial movements of animals are hallmarks of the human footprint on biodiversity. Retracing the evolution of commensal species thus offers a window to examine certain aspects of human history, such as the cultural relationships between civilizations (ethnozoology)[1,2] and the effect of human activities on their distribution, especially in the context of biological invasions.

Amphibians hold fascinating prospects for ethnozoological and biological invasion research[3]. Translocations of anurans (frogs and toads) have been documented both in ancient and recent times, associated with e.g., religious rituals[4], food harvest[5–7], pest insect control[8], horticulture[9], and passive stowaway in trucks or ships[10,11]. One way to understand these amphibian movements is to reconstruct the phylogeographic history of species, which is, however, full of pitfalls. Widespread species often consist of species complexes in which closely related lineages have abundantly hybridized throughout their diversifications[12–15], which can render single-gene inferences such as mitochondrial DNA (mtDNA) barcoding analyses inadequate for species identification, distributions, and delimitation[16,17].

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The Asian black-spined toad *Duttaphrynus melanostictus* (Schneider, 1799)[18] (Anura: Bufonidae) functions as a human commensal[19] that lives alongside half of the world's inhabitants, from Pakistan to southeastern China and Indonesia[20]. Bufonids are poisonous through the production of a highly potent cardiotoxic cocktail[21–23] which, for *D. melanostictus*, was specifically shown to interfere with the physiology of human blood cells, leading to their membrane disruption and rapid lysis[24]. Accordingly, the species is considered a risk for food poisoning to people who include amphibians in their diet – the ingestion of their skin has been suspected to cause serious illness and even death[25,26]. With an active poison and other therapeutical properties, toads like *D. melanostictus* are of interest for pharmacological research[27] and have been part of the traditional pharmacopeia of Asian countries like India[28,29] and China for thousands of years[23]. In parts of India, large anurans including *D. melanostictus* are central to folk culture, for instance, through the ritual ceremonies of Bhekuli Biya (i.e., frog marriage)[30]. In parallel, the vast distribution of *D. melanostictus* encompasses hotspots of ethnic diversity such as Southeast Asia[31], where human populations have a complex genetic and cultural heritage that reflects multiple migration waves and trading routes from neighboring regions, notably India[32,33]. The shared history of *D. melanostictus* with Asian people thus makes the species a potential bearer of past human movements and connections[34].

Today, *D. melanostictus* illustrates the world's globalization. Often thriving in rural and urban areas[35], the species can venture into shipping containers or even planes and can inadvertently become a stowaway[11,36,37]. Numerous incursions outside its native range are known, e.g., from Madagascar[38,39], the Maldives[3], the Middle East[40], Australia[37], New Zealand[36], southern Africa[41], Wallacean islands including Maluka, New Guinea, Sulawesi, and Timor-Leste[42,43], and potentially the Andaman-Nicobar archipelago[44]. It is considered a problematic amphibian by wildlife authorities worldwide[45,46] due to its ability to travel and its potential environmental impact, notably through the threats posed by its toxicity to naïve autochthonous predators[47–49], which might soon include iconic species like the Komodo dragon[50,51]. The invasions in Madagascar and Wallacea, two of Earth's biodiversity hotspots, have received substantial interest by the academic community[43,52]. Australia is also concerned about a future establishment of *D. melanostictus*, which has been intercepted at seaports and airports multiple times[37], and measures are in place to prevent a second toxic toad invasion after the devastating introduction of the cane toad *Rhinella marina* (Linnaeus, 1758)[53].

Informed research and management of *D. melanostictus* can benefit from a better understanding of its complex evolutionary history. Previous studies reported multiple genetic lineages and intricate phylogeographic patterns across the enormous range of the Asian black-spined toad, putatively including several cryptic species[19,39,54–57]. However, these analyses essentially focused on two mtDNA barcoding genes (16S, *ND3*) and may be inadequate given the propensity of mitonuclear discordances in amphibians, particularly in Bufonidae[58,59]. Additionally, different geographic regions were studied with different genes by different researchers (e.g., *ND3* in Southeast Asia, 16S in India), with little correspondence between their respective sampling, thus making the diversity and distributions of the reported lineages even more difficult to comprehend.

Here we revisited the phylogeography of *D. melanostictus* by carrying out phylogenetic and population genetic analyses of double digest Restriction Associated DNA sequencing (ddRAD-seq) genomic data, genome size data, and mitochondrial barcoding gene sequences taken from the whole range. We demonstrate the existence of two species hiding under the name *D. melanostictus* in South and Southeast Asia, whose respective distributions are blurred by pervasive mitochondrial introgression over thousands of kilometers, and establish a recent Indian origin for the Indonesian populations, suggesting historical overseas dispersal.

Results

Phylogenomics and population genomics

Phylogenomic analyses of 88 specimens from five *Duttaphrynus* species recover two clades, one including *D. dhufarensis* (Parker, 1931)[60], *D. olivaceus* (Blanford, 1874)[61] and *D. stomaticus* (Lütken, 1864)[62], and one including *D. himalayanus* (Günther, 1864)[63] and *D. melanostictus* (Fig. 1a, Supplementary Fig. 1). Our focal species *D. melanostictus* comprises two distinct nuclear lineages (Fig. 1a, Supplementary Fig. 1), one occurring in western ranges (South Asia: India, Nepal, Pakistan) and Indonesia (orange in Fig. 1a), and one occurring in eastern ranges (Southeast Asia; green in Fig. 1a). Timetree analyses indicate that these lineages diverged during the Late Miocene ~7.2 Mya (95% Highest Probability Density (HPD): 5.6–8.7 Mya, Fig. 1a, Supplementary Fig. 2).

Population genomic analyses of *D. melanostictus* ($n = 69$ specimens from 68 localities), namely Bayesian clustering with STRUCTURE and a Principal Component Analysis (PCA), recover the two nuclear lineages as distinct genetic groups, with little trace of mixed ancestry (Fig. 2a, Supplementary Figs. 3–4). Analyses of each lineage separately reveal phylogeographic structure with up to four STRUCTURE clusters in each lineage (Fig. 2a, Supplementary Fig. 3). These are also distinguished on the PCAs (Supplementary Fig. 4) and the phylogenetic trees (Supplementary Figs. 1–2) and networks (Fig. 2b). In the western lineage, the clusters correspond to populations from Indonesia/South India (yellow), most of India (orange), Pakistan (light brown), and Nepal (dark brown) (Fig. 2a). In the eastern lineage, the clusters correspond to China/North Vietnam (red), South Vietnam/Cambodia/South Thailand/Madagascar (dark green), North Thailand/NorthVietnam (light green), and Myanmar/Malaysia (purple) (Fig. 2b). In each lineage, mixed ancestry coefficients, consistent with recent admixture, are retrieved between clusters (Fig. 2a, b). Some populations featured unexpected genomic backgrounds in respect to their geographic origins: our southernmost Indian sample shares alleles with the Indonesian samples; one sample from South Vietnam clusters with the Chinese/North Vietnamese samples (Fig. 2b); samples from peninsular and insular Malaysia are genetically related to the Burmese samples (Fig. 2b).

Genome size analyses

Genome size measurements ($n = 90$ specimens from 32 localities) revealed diagnostic differences between the two *D. melanostictus* lineages. The genome of the eastern lineage is on average 17% larger (11.8 pg, range: 11.5–12.4 pg) than the genome of the western lineage (10.1 pg, range: 9.4–10.4 pg), and the estimates do not overlap (Fig. 1, Supplementary Data 1). As inferred from genome size (triangles in Fig. 1a), the respective distributions of the two lineages are consistent with the ddRAD-seq results. These confirm the eastern lineage across mainland Southeast Asia, and the western lineage across the Indian subcontinent (including Sri Lanka) as well as Indonesia (Fig. 1a).

mtDNA analyses

Phylogenetic trees built for the mtDNA genes 16S (558 bp, $n = 382$ sequences; Supplementary Fig. 5) and *ND3* (469 bp, $n = 494$ sequences; Supplementary Fig. 6), overall representing 876 *Duttaphrynus* specimens, recover seven deep mitochondrial lineages (thereafter mitogroups) assigned to *D. melanostictus*. Spatially-explicit network analyses of the *D. melanostictus* sequences (16S: $n = 159$ from 99 localities; *ND3*: $n = 483$ from 191 localities) reveal strong geographic associations (Fig. 3). Mitogroups are labelled A–G, following previous studies[39].

The western nuclear lineage of *D. melanostictus* corresponds to mitogroups A and B (Fig. 3). Mitogroup A is found across Indonesia, Wallacea and South Asia and is subdivided into two subgroups noted A1 (most sequences) and A2 (two Indian sequences lacking locality information, and one Nepalese sequence). All Indonesian 16S sequences are identical to a sequence from South India (Supplementary Data 1,

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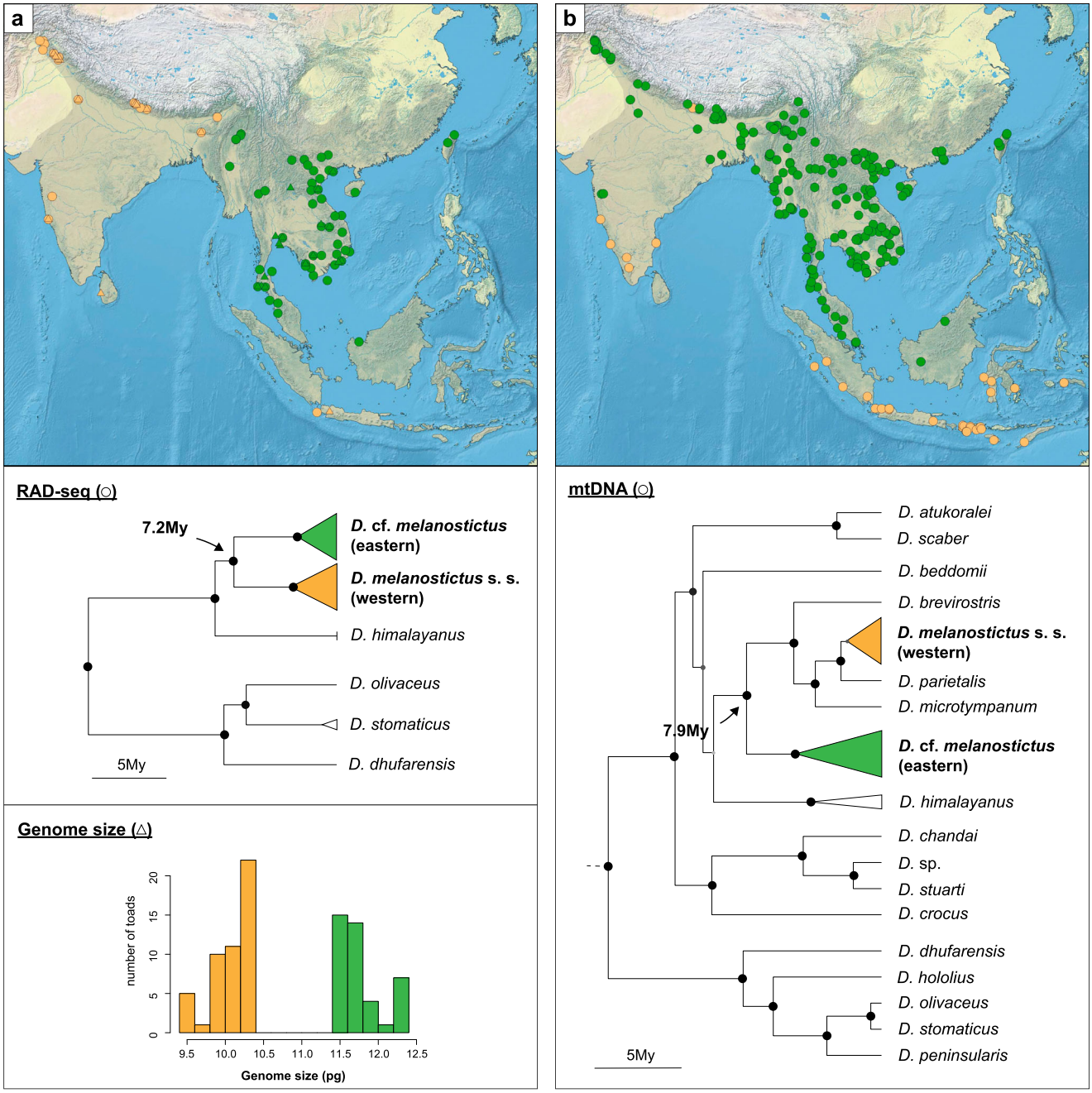

Fig. 1 | Phylogeny of *Duttaphrynus* and distribution of the two main lineages of the *D. melanostictus* complex. a Nuclear DNA assessment. The map combines population assignments based on ddRAD-seq (circles) and genome size (triangles). The tree represents a time-calibrated Bayesian phylogeny for 83,652 bp of concatenated ddRAD-seq markers. The barplots show genome size variation. **b** Mitochondrial DNA assessment. The map combines 16S and *ND3* lineage assignment. The tree represents a time-calibrated Bayesian phylogeny for up to 16,844 bp of mitochondrial sequences. On the trees, node circle size and darkness are proportional to branch support. Green: eastern lineage designated as *D.* cf. *melanostictus*; orange: western lineage designated as *D. melanostictus* s. s. The data used in the graphs are provided in the Source Data. Trees were visualized with FigTree 1.4.3. Maps were generated with QGIS 3.24.3.

Supplementary Fig. 5). The Indonesian *ND3* sequences consist of two closely related haplotypes, including one that is identical to a sequence from India (Supplementary Data 1, Supplementary Fig. 6). Mitogroup B is found mostly across North India and adjacent regions and is also subdivided in two subgroups, noted B1 (most of the range plus Malaysia) and B2 (Nepal and Northeast India) (Fig. 3, Supplementary Figs. 5–6).

The eastern nuclear lineage of *D. melanostictus* corresponds to the Southeast Asian mitogroups C–G, whose distributions partly associate with the structure retrieved from the ddRAD-seq data (Figs. 2–3). Specifically, the purple cluster in Myanmar corresponds to mitogroup E; the light green cluster in North Vietnam/North Thailand corresponds to mitogroup F; the red and dark green clusters found in the rest of the Indochinese Peninsula correspond to mitogroup G, which is also found in the introduced populations of Madagascar and the Persian Gulf (Fig. 3). Mitogroups C and D were not investigated with nuclear markers.

Built from 27 full or partial mitogenomes representative of 17 species, the mitochondrial phylogeny of *Duttaphrynus* is generally robust (Fig. 1b). The *D. melanostictus* mitogroups form two distinct clades: one comprising mitogroup A and samples from three Western Ghats endemic species, namely *D. brevirostris* (Rao, 1937)[64], *D. microtympanum* (Boulenger, 1882)[65], and *D. parietalis* (Boulenger, 1882)[65]; the other comprising mitogroups B–G. According to the

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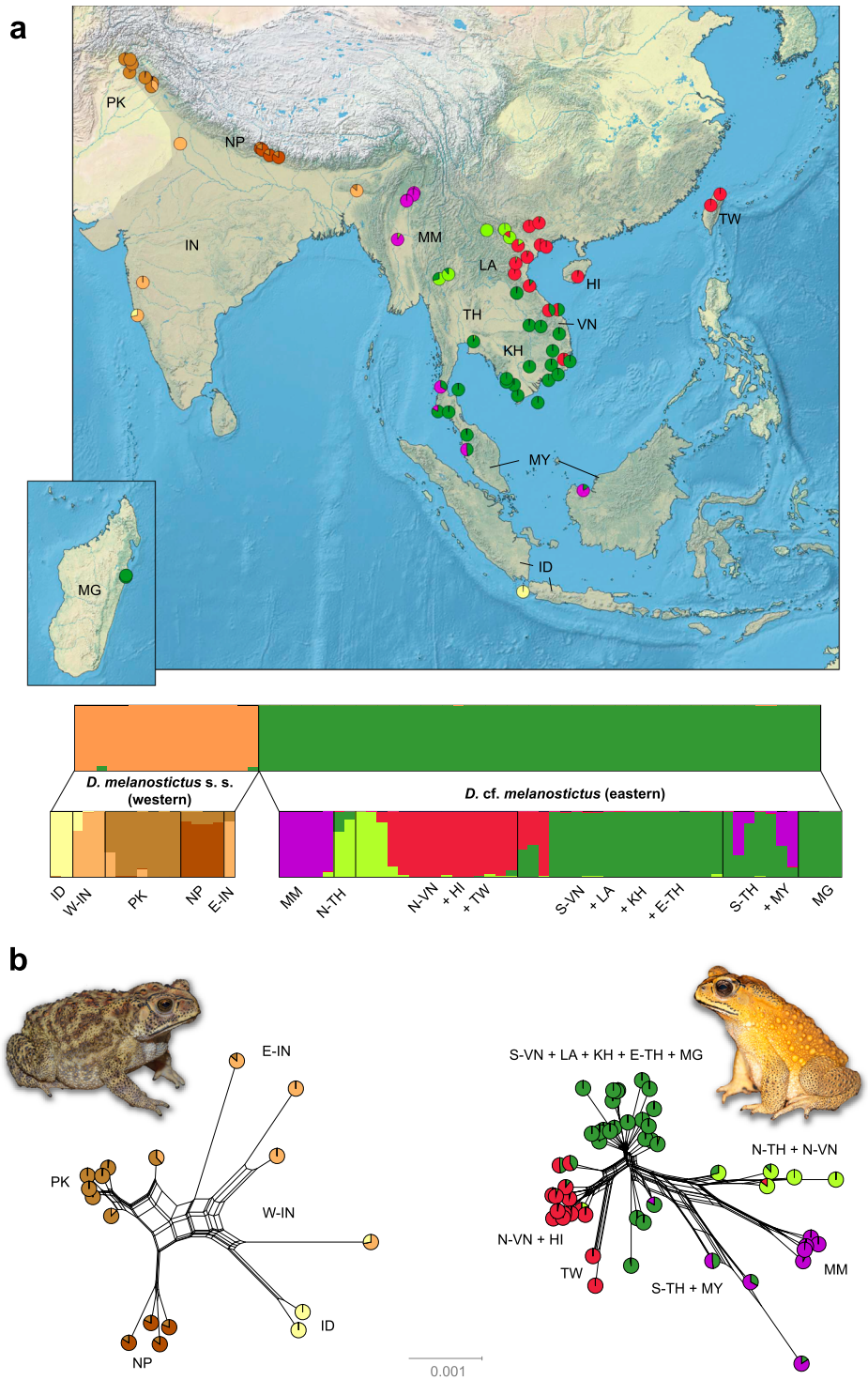

Fig. 2 | Nuclear genetic structure and divergence in the *D. melanostictus* complex. a Bayesian clustering of SNP datasets. The barplots show the ancestry estimates obtained for 851 SNPs genotyped in the whole complex (two clusters: green and orange); of 3364 SNPs genotyped in the western nuclear lineage *D. melanostictus* s. s. (four clusters: yellow, orange, brown and light brown); of 4782 SNPS genotyped in the eastern nuclear lineage *D.* cf. *melanostictus* (four clusters: purple, red, green and light green). Ancestries to the intraspecific clusters are reported on the map. **b** Phylogenetic networks of sequence datasets. The left network is built from 482,648 bp (western lineage). The right network is built from 628,293 bp (eastern lineage). Ancestries to the intraspecific clusters are reported on the networks. Acronyms indicate countries and regions, HI: Hainan; ID: Indonesia; IN: India; KH: Cambodia; LA: Laos; MG: Madagascar; MM: Myanmar; MY: Malaysia; NP: Nepal; PK: Pakistan; TH: Thailand; TW: Taiwan; VN: Vietnam; N-, S-, E- and W-: northern, southern, eastern, western, respectively. Photographs: *D. melanostictus* s. s. from Pakistan (credits: D.J.) and *D.* cf. *melanostictus* from Thailand (credits: C.D.). The data used in the graphs are provided in the Source Data. The map was generated with QGIS 3.24.3. Barplots were generated with Distruct 1.1. Networks were generated with SplitsTree 4.18.3 and overlayed by ancestry coefficients with the *R* code provided in Supplementary Code 1.

mitochondrial timetree, these clades diverged 7.9 Mya (95% HPD: 6.2–9.5 Mya; Supplementary Fig. 7).

The mitochondrial and nuclear phylogeographies thus have one major discrepancy: mitogroup B, which is predominantly found in the western nuclear lineage, is phylogenetically more closely related to mitogroups of the eastern nuclear lineage (C–G). In other words, populations from Pakistan, Nepal, and North India carry mtDNA derived from Southeast Asia, while being most closely related to the South Indian and Indonesian populations at the nuclear level (Fig. 1a, b).

Discussion

Speciation in Asian black-spined toads

Our study reports molecular evidence for a species complex in *D. melanostictus*, which is composed of at least two candidate species. The nuclear and mitochondrial timetree estimation agreed that the western and eastern lineages of *D. melanostictus* diverged during the Late Miocene (7.2/7.9 Mya), prior to the emergence of many other *Duttaphrynus* species (Fig. 1b). Deep genomic divergence is a reliable indicator of amphibian speciation when it can be related to reproductive isolation[66,67] and in this respect, the documented species complex is older than most nascent species of Eurasian amphibians for which reproductive barriers have been demonstrated[68,69]. For instance, Palearctic anuran lineages (including Bufonids) that diverged more than 6 Mya invariably evolved genetic incompatibilities that prevent or significantly restrict gene flow in parapatric ranges[66]. These observations are corroborated in the few Asian toads studied, e.g., the oldest lineages of the *Bufo praetextatus* Boie, 1826[70] complex, which diversified ~5.7 Mya[71], barely admix and accordingly underly distinct species[72]. From these observations, the contact zone between the western and eastern lineages of *D. melanostictus* is thus expected to feature little to no admixture, which is preliminarily suggested by our samples from the putative transition zone in Myanmar and Northeast India (Fig. 2). In contrast, the phylogeographic structure documented within each lineage is much younger (≤ 3 Mya) and involves traces of admixture over large distances that are rather consistent with reproductive compatibility and conspecificity.

Naming the western (South Asia/Indonesia) and eastern (Southeast Asia) species requires examining the nomenclatural history of *D. melanostictus*. The oldest taxon, *Bufo melanostictus* Schneider, 1799[18] was described from modern-day India[18,73] and thus corresponds to the western species *D. melanostictus* sensu stricto (s. s.). In Southeast Asia, three available names could apply to the eastern species[74]. The oldest one, *Bufo gymnauchen* Bleeker, 1858[75], was described from "Bintang" (currently Bintan Island in Indonesia), which is located ~20 km south of the Malay Peninsula where the eastern species is expected to occur. Genetic analysis of type or topotypical specimens will be necessary to confirm this hypothesis. Otherwise, the next (younger) names to consider are *Bufo longecristatus* Werner, 1903[76], with type locality given as "Borneo Island", and *Bufo tienhoensis* Bourret, 1937[77], with type locality given as "Lang Son Province, Northeast Vietnam". For now, we leave the eastern species unnamed and provisionally refer to it as *D.* cf. *melanostictus*.

From a biogeographical perspective, speciation between the western and eastern populations of *D. melanostictus* sensu lato (s. l.) is not unexpected. The herpetofauna of South and Southeast Asia are separated by the Indo-Burman mountains that mark the biogeographic transition between the Ganges-Brahmaputra lowlands and the Indochinese Peninsula and are accordingly composed of different species[78]. Climate cooling and monsoon regimes, which have intensified since the Late Miocene, are frequently invoked to explain amphibian diversifications between and within these environmentally heterogenous regions[79]. The timing of divergence also corresponds to the final uplift of the Himalayan Mountain system, and the associated shortening, compression and clockwise rotation along the eastern Himalayan syntaxis[80]. Moreover, given their distinct ranges, speciation in *D. melanostictus* s. l. may involve ecological divergence and thus potential phenotypic differentiation, which remains to be examined. Given the enormous distribution of these species, parts of their range have not yet been studied at the molecular level, and may thus hide additional diversity, e.g., Central India, Sri Lanka, or the Laccadive archipelago.

Genetic discordances and taxonomic imbroglio

Previous studies reported multiple mitochondrial lineages in *D. melanostictus*, but the different geographic scopes and genes sequenced obscured the overall picture. In particular, candidate species lineages were emphasized between Pakistan/India and northern Indochina based on 16S[54,56], as well as between Southeast Asia and Indonesia based on *ND3*[19], some of which turned out to be the same (mitogroup A/*D. melanostictus* s. s. in Indonesia and South India). The incomplete sampling also led to biogeographic misinterpretations, e.g., mitogroup A was initially considered as an Indonesian endemic that evolved by insular vicariance[34].

Besides study design, the deep cyto-nuclear discordance in the sub-Himalayan ranges was a major source of confusion. Our nuclear data assign these populations to *D. melanostictus* s. s., which implies that their mtDNA (mitogroup B) has a foreign origin, i.e., derived from *D.* cf. *melanostictus*. Without this knowledge, it was concluded that mitogroup B corresponds to a separate candidate species for which the name *D. bengalensis* (Daudin, 1802)[81] was recently resurrected[57] and employed[82]. The shallow nuclear differentiation between S-Asian populations argues against the recognition of *D. bengalensis* which we herein formally consider to be a junior subjective synonym of *D. melanostictus*.

The divergence and distribution of mitogroup B remain puzzling as this mitogroup does not seem to correspond to any extant nuclear cluster. One possible origin is mitochondrial transfer from Southeast Asian's *D.* cf. *melanostictus* to the sub-Himalayan population of *D. melanostictus* s. s. several million years ago at times when these species remain permeable to gene flow. Alternatively, mitogroup B might be a ghost mitochondrial lineage, i.e., the remnant of an extinct lineage (e.g., an ancestral Himalayan population of *D.* cf. *melanostictus*) for which the nuclear genome has been entirely assimilated through genetic introgression (lineage fusion)[83].

The potential unreliability of mtDNA casts doubt on other *Duttaphrynus* taxa. For instance, the weak mitochondrial divergence between *D. olivaceus* and *D. stomaticus* (<1 Mya, Supplementary Fig. 7), as previously retrieved[55], does not reflect their nuclear divergence, which we found to be 10 times older in our ddRAD-seq timetree (6.3 Mya, Supplementary Fig. 2). In India, *D. melanostictus* s. s. branches within the mitochondrial diversity reported in the Western Ghats[54], which has been associated with the young *D. brevirostris*, *D. microtympanum*, *D. parietalis* (diverged 2–5 Mya, Supplementary Fig. 7). In the Himalayas, *D. himalayanus* may also represent a species complex, as it shows two deeply diverged mtDNA lineages that segregates in the Indian and Nepalese ranges[84] that we estimated to have emerged 4.1 Mya (Supplementary Fig. 7). These populations/taxa thus deserve a genomic evaluation.

Duttaphrynus toads add to an alarming number of cases in which cyto-nuclear discordances, in the form of ghost lineages or mitochondrial captures, blur phylogeographic and systematic inferences, as seen also in e.g., butterflies[85], reptiles[86] and other amphibians[58]. Our results thus bring direct empirical support to recent calls for caution regarding taxonomic revisions based essentially on (mt)DNA barcodes[17,87,88] and illustrate how such revisions can be made more reliable using genomic approaches.

Two invaders in one

Asian black-spined toad invasions involve two distinct species, namely *D. melanostictus* s. s. in Wallacea, introduced from the main Indonesian islands[43] and *D.* cf. *melanostictus* in Madagascar, introduced from

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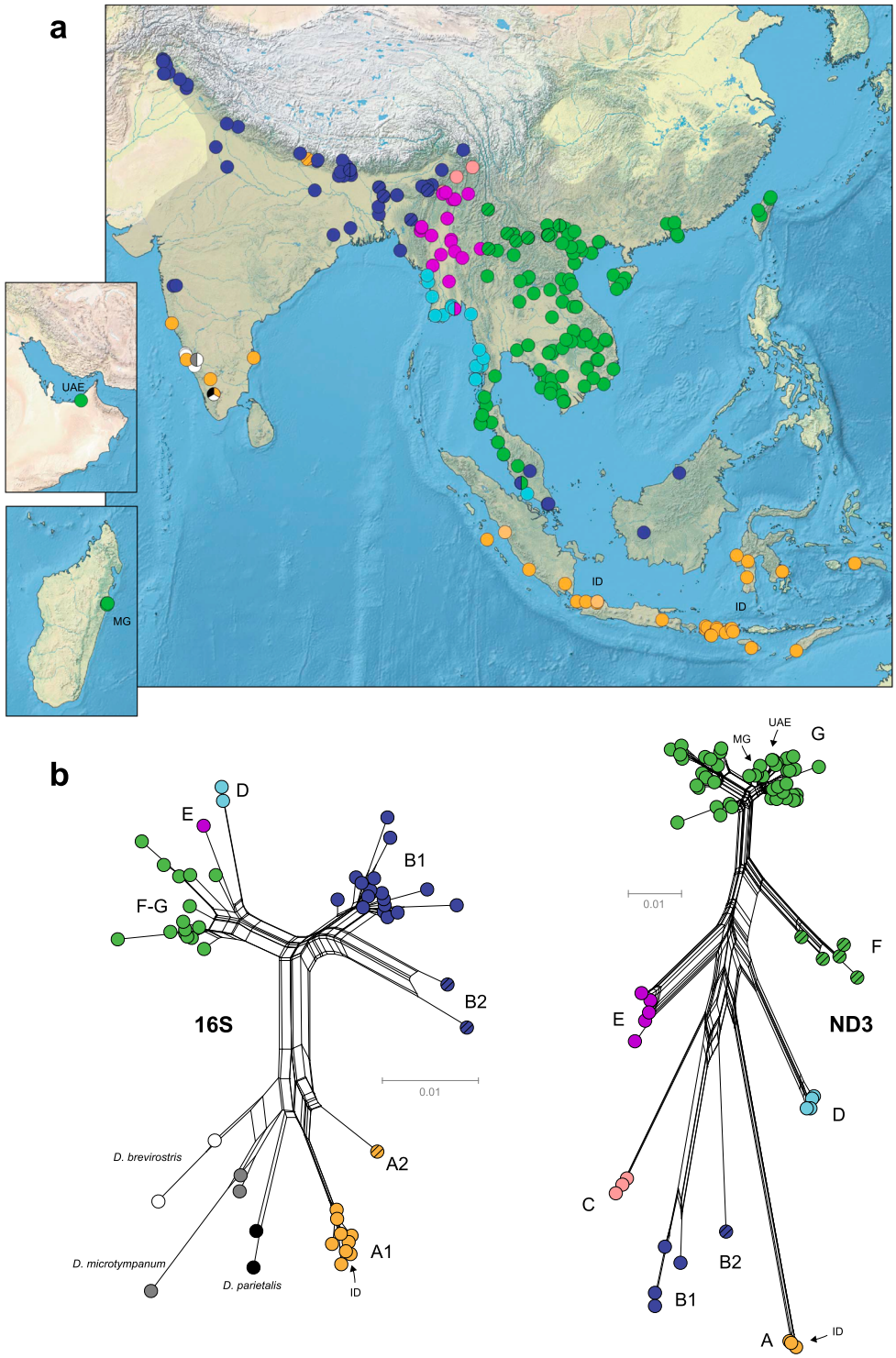

Fig. 3 | Mitochondrial genetic structure and divergence in the *D. melanostictus* complex. a Geographic distributions of mitogroups. **b** Phylogenetic networks of 16S and *ND3*. Colors distinguish the different lineages identified in the complex. Acronyms indicate introduced populations and haplotypes: Madagascar (MG), United Arab Emirates (UAE), Indonesia (ID). The data used in the graphs are provided in the Source Data. The map was generated in QGIS 3.24.3. Networks were generated with SplitsTree 4.18.3.

South Vietnam[39]. Their evolutionary divergence should encourage separate risk assessments, notably when documenting life-history traits (dispersal abilities, reproductive output, diet) and ecological requirements (climatic tolerance) to predict potential spread and impact on native wildlife[11]. Toxicity levels may also deserve a species-specific reconsideration, as bufotoxin composition shows variation between closely related bufonids[58,89]. So far, most invasion research on Asian black-spined toads come from the Malagasy invasion by *D.* cf. *melanostictus*[49,90–95] and the insights might thus not all be transposable to the Wallacean invasion.

Furthermore, the phylogeography of *D.* cf. *melanostictus* revealed surprising patterns that can potentially reflect translocations within native ranges. In South Vietnam, one toad opportunistically sampled in a highly frequented mountain pass (Hon Giao Mountain in Bidoup-Nui Ba

National Park) where *Duttaphrynus* is supposedly absent, clustered with samples from North Vietnam, and could have been transported there by a vehicle. The intricate mixture of various lineages among the Malaysian populations also raises questions about their native origin[57]. Their mitochondrial and nuclear diversity is shared with the geographically distant Indo-Burman populations, as well as with the proximate populations from Thailand. One explanation could be that the Malaysian populations represent a natural enclave of Indo-Burman lineages, if they formerly expanded southward from the Himalayas to Borneo and have since experienced introgressive replacement following hybrid zone movement[96]. Alternatively, the Indo-Burmese lineages could have been artificially added to the Malaysian diversity. Extensive movement of goods and people from the Bengal region (notably Calcutta [Kolkata] and Dhaka) to Malaysia during the 19th century is well documented, especially when both areas belonged to the Bengal Presidency division of British India. Human transport of toads to this area since colonial times is therefore not unexpected[97]. This case emphasizes the difficulty of ascertaining translocations within native ranges when their genetic signature confounds that of dynamic historical biogeography[7].

Ethnozoology of South-Southeast Asian connections

The peculiar presence of *D. melanostictus* s. s. in Indonesia, despite little genetic divergence from South India, implies a recent connection that cannot be reconciled with the biogeography of the Indo-Malayan realm. Most of the terrestrial fauna of Indonesia is intimately related to nearby Southeast Asia, as these regions form a single landmass (Sundaland) that was exposed during past sea-level subsidence, enabling exchanges between Peninsular Malaysia, Borneo, Sumatra and Java, and smaller islands[98,99]. A natural colonization of Indonesia from South India would imply a former distribution of *D. melanostictus* s. s. around the Bay of Bengal, namely along the western Indochinese coastline and/or the Andaman-Nicobar ridge via land-bridges. However, several arguments run against this scenario. First, the continental lands that separate Indonesia from S-India are presently inhabited by several competing lineages that diversified in situ (Himalayan *D. melanostictus* s. s. and *D.* cf. *melanostictus*). The colonization and subsequent replacement of *D. melanostictus* s. s. in Southeast Asia by these lineages should have left some phylogeographic traces such as relic populations or genetic introgression[96], which we did not detect. Second, the *Duttaphrynus* toads that inhabit the Andaman-Nicobar archipelago[100] are believed to have arrived there by boat[44], which would imply that the toads did not use this pathway between South Myanmar and Northwest Indonesia during the Quaternary, an hypothesis that could be clarified by a molecular identification of the Andaman-Nicobar population. Third, the genetic homogeneity across the Indonesian archipelago[19], as well as between Indonesia and India (including identical mtDNA haplotypes), indicates a more recent origin of the Indonesian populations than the last natural connections. While the Sunda shelf was never submerged prior to the last hundreds of thousands of years, sea level variations hampered movements of terrestrial organisms thereafter, at least during interglacial periods[99]. The disconnection between the Indian and Indonesian ranges following the retraction of glacial land bridges and the expansion of *D.* cf. *melanostictus* over mainland Southeast Asia, would have been expected to generate genetic structure between these areas, as well as between the Indonesian islands. Finally, a recent transmarine rafting event could in principle produce a phylogeographic pattern like the one we observe. Overseas dispersal has been documented in several amphibian groups, but it remains exceedingly rare over the course of their evolution, with known examples involving landmasses separated by a few hundred kilometers (e.g., Africa, Madagascar and surrounding islands)[101]. Toad rafting across the Bay of Bengal, which consists of 2000 km of open ocean or 3500 km of coastal waters, thus seems highly improbable.

In the light of the above, one potential explanation for the Indian origin of the Indonesian population of *D. melanostictus* s. s. is human-mediated dispersal. Asian black-spined toads are prone to being stowaways in ships cruising the Indian ocean. For instance, they have been transported to Mauritius and the Maldives during or before the 19th century[102,103]. Given their importance in folk culture[30], toads may have even been deliberately brought on board by Indian sailors for good fortune. Moreover, the extensive ranges of the species in Indonesia and the fact that it was already present in the country a century ago[104] suggest an arrival of *D. melanostictus* at least hundreds or thousands of years ago. This timeframe corresponds to important cultural and economic links between Indonesia and India during the last millennia.

Indian culture had an important influence upon Southeast Asia, and its spread is believed to have occurred through trade[105]. In particular, Indonesia seems to have had early, direct and privileged connections with South India and Sri Lanka[106], at least since 2000 years before present (BP)[107], as illustrated by archeological evidence from the late prehistoric period (200 BC–AD 500) in Sembiran and Pacung (northern coast of Bali). Pottery and later fabrics from Sembiran correspond to those from the same era in Arikamedu (Puducherry, Southeast India) and other Indian sites[108], while gold and carnelian beads point to additional links with northern India[109]. This indicates at least two trading routes, a mainland one through western Southeast Asia and a maritime one across the Indian ocean. The latter, sometimes dubbed the maritime silk road[110,111], is also suggested by South Indian rouletted potteries recovered in Java[112,113] and by glass beads typical of Arikamedu[114] found in Bali (Pangkung Paruk)[115] and Sumatra[116] from the first centuries AD. Additionally, analyses of the human Y chromosome inferred gene flow from Indian to Indonesian human populations between 2600 and 3100 years BP[32], thus confirming recurrent contacts since at least this epoch. An ancient translocation of *D. melanostictus* s. s. from India to Indonesia thus corresponds with evidence for this trans-Asiatic maritime route.

The vast naturalized range of *D. melanostictus* s. s. in Indonesia emphasizes the enormous colonization potential of the species, which might have benefited from further transportation by humans at the regional scale. In several amphibians and reptiles, phylogeographic studies suggested recent, putatively human-mediated dispersal across the Lesser Sundas Archipelago, where populations similarly lack genetic structure[117,118]. Whether these long-term invasions have impacted past faunal communities remains to be established. The current invasions of the Wallacean biodiversity hotspot[43] by *D. melanostictus* s. s. may thus be the follow up of a wider invasion that was already initiated centuries ago, and that will continue to expand without a strong international management response.

Methods

This study adheres to the ethical guidelines of the Institutional Animal Care and Use Committee (IACUC) and the Ethics Committee of Nanjing Forestry University and is approved under IACUC permit number 2023007. Fieldwork was authorized by the Department of Forests and Environment, Government of Meghalaya, India (FWC/G/173/Pt-II/295 dt. 08.05.2014); the Department of Environment, Forest and Climate Change, Government of Nagaland, India (CWL/Gen/97/102-105 dt. 02.05.2013; CWL/Gen/97/659-661 dt. 04.02.2014); the Department of Environment, Forests and Climate Change, and Principal Chief Conservator of Forests (Wildlife), Government of Bihar, India (643/27.09.2023); the Chief Conservator of Forests (Wildlife), Government of Jharkhand, India (582,17); the Principal Chief Conservator of Forests (Wildlife), Government of Maharashtra, India (22/8/Research/CR-25/2225/23-24); the Principal Chief Conservator of Forests (Wildlife) and Chief Wildlife Warden, Uttarakhand State Forest Department (1014/5-6/2024 and 2701/5-6/2023); the Principal Chief Conservator of Forest (Wildlife), Karnataka State Forest Department (KFD/WL/E2(RE)/46/2023/1143364); the Department of National Park and Wildlife Conservation, Nepal (DNPWC), the Department of Forestry of the Ministry of Agriculture and Forestry, Lao PDR (511–271/08 dt. 05.05.2009; 299/

DoF dt. 01.08.2019; 009/DoF dt. 23.06.2020; 3820/DoF dt. 10.08.2023); the University of Mandalay, Ministry of Education, Myanmar (005 dt. 07.08.2019); the Institute of Animals for Scientific Purpose Development (IAD), Bangkok, Thailand (U1–01205–2558 dt. 01.04.2022; UP–AE59–01–04–0022; UP–AE64–02–04–005; UP–AE59–01–04–712–0022); Bu Gia Map National Park, Binh Phuoc Province, Vietnam (137/HD NCKH dt. 23.06.2010); Cat Tien National Park, Dong Nai Province, Vietnam (37/HD dt. 23.06.2010); the Department of Forestry, Ministry of Agriculture and Rural Development of Vietnam, Vietnam (170/TCLN–BTTN dt. 07.02.2013; 831/TCLN–BTTN dt. 05.06.2013; 400/TCLN–BTTN dt. 26.03.2014; 547/TCLN–BTTN dt. 21.04.2016; 822/TCLN–BTTN dt. 01.06.2016; 432/TCLN–BTTN dt. 30.03.2017; 142/SNgV-VP dt. 11.04.2017; 712/TCLN–BTTN dt. 17.05.2017; 1735/TCLN–DDPH dt. 25.10.2017; 1539/TCLN–DDPH dt. 19.09.2018); the Forest Protection Department of the Peoples' Committee of Ba Ria–Vung Tau Province, Vietnam (769/CNPN dt. 03.12.2020; 14449/UBND–VP dt. 21.12.2020), of Bac Giang Province, Vietnam (1743/UBND–NgV dt. 29.05.2017), of Cao Bang Province, Vietnam (1659/UBND–NC dt. 02.06.2017; 513/SNN–KHTC dt. 31.03.2021), of Da Nang City, Vietnam (97/TTNDVN–STND dt. 09.01.2023; 645/UBND–SNN dt. 16.02.2023), of Dak Lak Province, Vietnam (1567/UBND–TH dt. 06.04.2011; 995/SNN–CCKL dt. 12.04.2019; 388/SNgV–LS dt. 24.04.2019), of Dak Nong Province, Vietnam (209/UBND–NGV dt. 13.01.2021), of Gia Lai Province, Vietnam (1951/UBND–NV dt. 04.05.2016; 530/UBND–NC dt. 20.03.2018; 1103/UBND–NC dt. 31.05.2022), of Ha Giang Province, Vietnam (109/SNgV–LS dt. 15.03.2022; 182/SNgV–LS dt. 03.04.2023; 574/SNN–KL dt. 06.04.2023), of Ha Tinh Province, Vietnam (2358/UBND–NL4 dt. 11.05.2022), of Khanh Hoa Province, Vietnam (5565/UBND–KT dt. 08.06.2023; 522/SngV–TTDN&HTQT dt. 13.06.2023), of Lam Dong Province, Vietnam (5832/UBND–LN dt. 22.10.2022; 3369/UBND–NV4 dt. 17.05.2022), of Lao Cai Province, Vietnam (1148/UBND–TNMT dt. 26.03.2019; 5110/UBND–NC dt. 27.10.2022; 2099/UBND–NC dt. 10.05.2023), of Nghe An Province, Vietnam (1700/UBND.VX dt. 22.03.2018; 2089/UBND.VX dt. 03.04.2019), of Ninh Thuan Province, Vietnam (317/UBND–VXNX dt. 03.02.2023), of Phu Tho Province, Vietnam (2394/UBND–TH3 dt. 16.06.2016), of Phu Yen Province, Vietnam (05/UBND–KT dt. 04.01.2021), of Quang Binh Province, Vietnam (776/UBND–KT dt. 10.05.2022), of Quang Nam Province, Vietnam (308/SNgV–LS dt. 01.04.2019; 320/SNgV–LS dt. 22.04.2021; 370/SNgV–LS dt. 25.04.2022), of Thanh Hoa Province, Vietnam (3532/UBND–THKH dt. 27.03.2019; 562/GP dt. 01.06.2022), of Thua Thien-Hue Province, Vietnam (755/SNgV–HTQT dt. 04.05.2023) and of Yen Bai Province, Vietnam (738/TTNDVN–ST dt. 23.03.2021; 3535/UBND–NV dt. 20.10.2022); the Pakistan Museum of Natural History, Islamabad, Pakistan (PMNH/EST1[89]/05); Ministère de l'Environnement et du Développement Durable, Madagascar (021/14/MEF/SG/DGF/DCB.SAP/SCB; N°113N–EA04/MG15). Rules for conducting scientific research using animals in Russia, as regulated by orders of the Presidium of the USSR Academy of Sciences No. 12000-496 (02.04.1980), and the USSR Ministry of Education No. 22 (13.09.1984), have been followed.

Sampling

A total of 197 ethanol-preserved samples of *D. melanostictus* and other *Duttaphrynus* species, including *D. himalayanus*, *D. stomaticus*, *D. olivaceus*, and *D. dhufarensis*, were gathered for genetic analyses during fieldwork trips (Supplementary Data 1). Most samples originate from specimens curated in herpetological collections, namely of the Muséum National d'Histoire Naturelle in Paris, France (MNHN), the Institute of Cytology of the Russian Academy of Science in St. Petersburg, Russia (INCRAS), the Zoological Museum of Lomonosov Moscow State University in Moscow, Russia (ZMMU), and the Bombay Natural History Society in Mumbai, India (BNHS). A few samples originate from live adults released after capture. For ddRAD-seq and mitochondrial sequencing, DNA was isolated from thigh muscles (vouchered specimens) or buccal swabs (live adults) using the Qiagen Blood & Tissue kit. Archival DNA (archDNA) from two geographically important specimens collected in the early 20th century (MNHN-RA.1902.0132 and MNHN-RA.1902.0133) were processed in a separate batch. For genome size measurements, blood samples were obtained from live anaesthetized toads (immersion in 1% MS222) prior to their curation.

ddRAD-seq analyses

A genomic library was prepared for 88 *Duttaphrynus* individuals (including 74 *D. melanostictus* from 73 localities, Supplementary Data 1) by adapting a ddRAD-seq protocol[119] that consisted of the following steps. (1) Enzyme restriction was carried in 9 μL reaction volumes containing 6 μL of template DNA, 0.1 μL of *MseI* (10,000 U/ml), 0.1 μL of *SbfI* (20,000 U/ml), 0.9 μL of Cutsmart (10×) and 1.9 μL of ultrapure water. Products were incubated at 37 °C for 3 h followed by 65 °C for 20′ for enzyme inactivation. (2) Adaptor ligation was carried in 11.6 μL reaction volumes containing the 9 μL of digested products, 1 μL of *MseI* adaptors (10 μM), 1 μL of individually-barcoded *SbfI* adaptors (0.1 μM), 0.26 μL of Cutsmart (10×), 0.12 μL of ATP (100 mM), 0.17 μL of T4 DNA Ligase (400,000 U/ml) and 0.05 μL of ultrapure water. Products were incubated at 16 °C for 3 h. (3) Purification of ligated produced was carried by the addition of 11.6 μL of AMPure (Agencourt), followed by incubation at room temperature for 5′, bead attraction with a magnetic device for 10′, two consecutive washes of 30″ each in 100 μL of 70% ethanol, and elution in 45 μL of ultrapure water. (4) Amplification was performed by two replicate polymerase chain reactions (PCRs) with the TruSeq Illumina primers D7-D5 (index D701), each carried in 15 μL reaction volumes containing 4.5 μL of purified product, 1.0 μL of each primer (5 μL), 0.12 μL of dNTP (25 mM), 0.15 μL Q5 hot start high-fidelity polymerase (2000 U/ml), 3 μL of Q5 buffer, 3 μL of High GC enhancer and 2.23 μL of ultrapure water. The thermocycling program consisted of an initial denaturation at 98 °C for 30″, 20 cycles of 98 °C for 20″, 60 °C for 30″, 72 °C for 40″, and a final elongation at 72 °C for 2′. Replicate products were pooled, amplicons were checked individually on a 1.5 % agarose gel (120 V for 30′), and all were subsequently pooled into a single library. (5) Library concentration was achieved by purifying 800 μL of library with 800 μL of AMPure (Agencourt) as in step (3), except for the wash volumes (1 ml of 70% ethanol) and the elution volume (31 μL of ultrapure water). The obtained concentration was estimated by fluorometric quantification of 1 μL using Qubit (Thermo Fisher Scientific), and the library was diluted to 100 ng/μL. (6) Size selection of 400–500 bp fragments was performed with a Pippin Prep (Sage Science) using 30 μL of the concentrated library, following the manufacturer's instructions. A tutorial (including a video) of this protocol is available at https://doi.org/10.17504/protocols.io.kxygx3nzwg8j/v1. Oligonucleotide sequences are provided in Supplementary Data 2.

The final library was sequenced on a NextSeq 550 (Illumina) with the 2×75 bp kit at the Max Planck Institute for Evolutionary Biology (Plön, Germany), which yielded 794 million reads. Paired-end reads were demultiplexed with STACKS 2.59[120] using the *process-radtags* function, removing uncalled bases (*–c*), discarding reads below the default quality phred score (*–q*), rescuing barcodes and RAD tags (*–r*), filtering adapter sequences (*–adapter-1* and *–adapter-2*) with up to two mismatches allowed (*–adapter-mm* 2) and trimming final reads to 65 bp (*–t* 65). The denovo_map.pl pipeline was applied for RAD loci construction, assembly, and cataloging (default *–m*, *–n*, and *–M* values) with removal of PCR duplicates (*–rm-pcr-duplicates*). The final catalog contained 410,773 loci with a mean effective per-sample coverage of 18.2×. For downstream population genomics and phylogenomic analyses, the module *population* of STACKS was used to obtain SNP datasets (*–structure*) and supermatrix alignments (*–phylip-var-all*), as follow.

The data was preliminarily explored by a maximum-likelihood analysis with PhyML 3.0[121] based on an alignment of 130,792 bp that

concatenates the RAD tags present in at least 80 (–*p* 80) of the 88 samples (other parameters left as default). The analysis was run on a dedicated server (http://www.atgc-montpellier.fr/phyml/), using the smart selection algorithm[122] and 100 bootstrap replicates.

Samples assigned to *D. melanostictus* s. l. were examined further after excluding five samples with high proportions of missing data in the previous analysis. First, clustering analyses were performed on SNP datasets encompassing the whole range ($n = 69$; 851 SNPs), the western lineage *D. melanostictus* s. s. ($n = 17$; 3364 SNPs), and the eastern lineage *D.* cf. *melanostictus* ($n = 52$; 4782 SNPs). These datasets were obtained by calling the RAD tags sequenced in all samples (–$p = n$), keeping only one SNP per tag (–*write-random-snp*) to avoid physically linked loci, and leaving other parameters as default. Analyses were performed with STRUCTURE 2.3.4[123], using the admixture model, uncorrelated allele frequencies, and 10 replicate runs from $K = 1$–10, each with 10,000 iterations after a burn-in of 100,000. Second, Principal Component Analyses (PCAs) were conducted on the three datasets with *adegenet* 2.1.10[124] in the statistical environment *R* 4.1.3. Third, phylogenetic networks were computed for the western *D. melanostictus* s. s. and the eastern *D.* cf. *melanostictus* lineages with SplitsTree 4.18.3[125] using default settings, based on alignments of 482,648 bp and 628,293 bp that concatenate the RAD tags present in the corresponding sample sets (–$p = n$; other parameters left as default).

A time-calibrated phylogeny was reconstructed for a subset of 25 unadmixed *Duttaphrynus* samples representative of available species and their identified phylogeographic lineages, based on an alignment of 83,652 bp that concatenates the RAD tags present in all samples (–*p* 25; other parameters left as default). The analysis was performed in BEAST 2.6[126] with the Birth Death tree prior, a strict clock, the GTR + G + I substitution model, and time constraints to three most recent common ancestors (MRCAs). In the absence of fossil-based calibrations relevant to our species, and given the drastically different timeframes retrieved for the diversification of *Duttaphrynus* in previous studies (from ~27 Mya[55] to ~2 Mya[34]), we implemented normally distributed MRCA priors reflecting median times and their confidence intervals taken from timetree.org[127] as of February 2023: (1) 15.7 Mya ($\sigma = 2.6$) for the early split of the *D. stomaticus* clade; (2) 8.5 Mya ($\sigma = 1.6$) for the early split of *D. dhufarensis* in the *D. stomaticus* clade; (3) 10.2 Mya ($\sigma = 1.8$) for the split of *D. himalayanus* from the branch leading to *D. melanostictus* s. l. Preliminary runs (few million iterations) were conducted to optimize operator size and scaling factors. The final analysis was run for 50 million iterations (sampling every 5000), monitored in Tracer 1.7[128] and DensiTree 3.0.2[129]. A maximum-clade credibility tree (MCCT) was built using the TreeAnnotator module of BEAST (discarding the first 10% of sampled trees as burnin).

Genome size analyses

Closely related amphibian species often differ in their genome size, which allows genetic identification without sequencing[130]. For this purpose, the raw DNA content was measured by DNA flow cytometry for 90 *D. melanostictus* s. l. specimens from 32 localities scattered across the range, following a published methodology[130] as follows. (1) Blood cell samples (~1 million cells/ml) were suspended in PBS with EDTA (0.7 mM), lysed by 0.1% of Triton X-100 (Ferak), and stained with a mixture of olivomycin (20 μg/ml), ethidium bromide (40 μg/ml) and $MgCl_2$ (15 mM). Blood cells of *Rana temporaria* Linneaus, 1758[53], which genome size has been determined by the same technique[130], were used as a reference standard. (2) Samples were stored at 4 °C for 24 h and measured by a microscope-based (Lyumam I-1, Lomo, St. Petersburg) flow fluorimeter with a mercury arc lamp constructed at the Institute of Cytology, Russian Academy of Sciences, St. Peterburg (https://patents.google.com/patent/SU1056008A1/ru). DNA-histograms were acquired with a multichannel analyzer at a rate of 100–200 cells per second. For each sample, four runs were performed to reach a total number of cells per sample above 10,000. (3) The peaks of DNA histograms were approximated to Gaussian curves by the least-square technique using BARS 1.0[131], and their mode were transformed into absolute genome size values (picograms) based on their ratio with the modes of the peaks of the reference standard.

mtDNA analyses

The mtDNA lineages (mitogroups) of 876 *Duttaphrynus* individuals were inferred from sequences of 16S ($n = 382$) and/or *ND3* ($n = 494$), combining the published sequences of 72 sources from GenBank[132] with new sequences from 71 of our samples (Supplementary Data 1). To this end, we first amplified a ~570 bp fragment of 16S with the primers 16SA–L and 16SB–H[133] in 60 samples. Second, archival DNA from the two historical vouchers were targeted for a shorter 16S fragment (~290 bp) with the eDNA primer pair Vert-16S-eDNA[134]. Third, for nine samples (series RGK in Supplementary Data 1), a ~1500–2000 bp fragment encompassing 12S-tVal–16S was amplified using the primers pairs LX12SN1 and LX16S1R, and 12SAL and 16S2000H[135]. Finally, for eight of the RGK samples (Supplementary Data 1), we amplified a ~530 bp fragment encompassing *ND3* using the primers L-COXIII and Arg-HND3III[136]. Oligonucleotide sequences are provided in Supplementary Data 3. PCRs were conducted in 12.5 μl reaction volumes containing 8.05 μl of water, 2.5 μl of PCR buffer, 0.25 μl of dNTPs (10 μM), 0.3 μl of each primer (10 μM), 0.1 μl of Taq polymerase and 1 μl of DNA template. The thermocycling program consisted of an initial denaturation of 94 °C for 1'30", 35 cycles of 94 °C for 45", 53 °C for 45", and 72 °C for 1', followed by a final elongation of 72 °C for 1'. Amplicons were sequenced in the forward direction, except for the 12S-tVal–16S fragments, which was sequenced in both directions. Raw sequences were quality-checked with Geneious Prime 2022.0.1 (Biomatters) or MEGA X[137].

For each gene, new and published sequences were manually aligned and trimmed to 558 bp (16S) or 469 bp (*ND3*) in Seaview 5[138]. Exploratory phylogenetic analyses were run with PhyML as above, using *Bufotes turanensis* (Hemmer, Schmidtler & Böhme, 1978)[139], a closely related bufonid[140], as outgroup. For *D. melanostictus* and three closely related Western Ghats species, phylogenetic networks were produced and visualized with SplitsTree (default settings), and mitogroup distributions were mapped from georeferenced sequences (16S: $n = 159$ from 99 localities; *ND3*: $n = 483$ from 191 localities). Mitogroup correspondence between genes was assessed directly (samples sequenced for both genes) or indirectly based on distributions (samples sequenced for one gene).

To reconstruct the mitochondrial phylogeny of the genus, a supermatrix of 27 full or partial (1–9 genes) mitogenomes (16,844 bp) was designed from newly available and published sequences. These covered the main mitogroups of 17 *Duttaphrynus* species and the outgroup *Bufotes turanensis* (Supplementary Data 4). Some mitogenomes were composite, i.e., they combined sequences of different genes from different individuals that carried the same mitogroup. A time-calibrated Bayesian phylogenetic analysis was performed in BEAST with the same models and priors as for the ddRAD-seq timetree.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Raw ddRAD sequencing reads have been archived on NCBI SRA under BioProject PRJNA949685 and mtDNA sequences have been deposited on GenBank; accessions are listed in Supplementary Data 1. Genome size estimates are provided in Supplementary Data 1. The genotype matrices and sequence alignments analyzed in this study have been deposited on Zenodo (https://doi.org/10.5281/zenodo.14044209). The data used in the graphs are provided in the Source Data. Source data are provided with this paper.

Code availability

Bioinformatic commands and *R* scripts used are provided in Supplementary Code 1.

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Competing interests

The authors declare no competing interests.

Additional information

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