

Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory

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Amphibians are in decline worldwide. However, their patterns of diversity, especially in the tropics, are not well understood, mainly because of incomplete information on taxonomy and distribution. We assess morphological, bioacoustic, and genetic variation of Madagascar's amphibians, one of the first near-complete taxon samplings from a biodiversity hotspot. Based on DNA sequences of 2,850 specimens sampled from over 170 localities, our analyses reveal an extreme proportion of amphibian diversity, projecting an almost 2-fold increase in species numbers from the currently described 244 species to a minimum of 373 and up to 465. This diversity is widespread geographically and across most major phylogenetic lineages except in a few previously well-studied genera, and is not restricted to morphologically cryptic clades. We classify the genealogical lineages in confirmed and unconfirmed candidate species or deeply divergent conspecific lineages based on concordance of genetic divergences with other characters. This integrative approach may be widely applicable to improve estimates of organismal diversity. Our results suggest that in Madagascar the spatial pattern of amphibian richness and endemism must be revisited, and current habitat destruction may be affecting more species than previously thought, in amphibians as well as in other animal groups. This case study suggests that worldwide tropical amphibian diversity is probably underestimated at an unprecedented level and stresses the need for integrated taxonomic surveys as a basis for prioritizing conservation efforts within biodiversity hotspots.

biodiversity estimates | new candidate species | phylogeny | DNA barcoding

The current biodiversity crisis demands the study of broad-scale spatial variation in species richness and endemism to identify areas that merit special conservation attention. Global efforts to minimize biodiversity loss have led to prioritizing biodiversity hotspots (1) which are defined as areas with high concentrations of endemic species and that are undergoing exceptional habitat loss (2–4). A second step is the efficient implementation of conservation measures at a local scale which requires an understanding of spatial patterns of richness and endemism within these hotspots (5). Assessments of such regional priority areas are often hampered by incomplete distributional and taxonomic information. The operational units used to assess conservation priority areas are described species, whereas estimates of undiscovered and undescribed species are usually ignored. Recently, many potential new species have been identified by DNA barcoding, but a taxonomic validation of these species will outdate the short time span left for efficient biodiversity conservation prioritization, and so far it is not clear how this undescribed diversity can nonetheless be considered. This undescribed diversity also bears relevance for understanding the tree of life: The completeness of taxon sampling is one of the major prerequisites for reliable phylogenetic analysis, reconstruction of character evolution, and inference of macro-evolutionary processes (6, 7).

Among terrestrial vertebrates, amphibians are characterized by a rapid rate of species discovery (8, 9), with an overall increase in the number of amphibian species globally of 19.4% during the last decade, reaching 6,449 currently recognized species (10). An important acceleration in the rate of new discoveries, mainly from tropical areas, is obvious from many recent studies (11–16). These discoveries are not the result of taxonomic inflation (9, 14, 17), but correspond to real divergent species (18, 19). Although high numbers of undescribed amphibians have been estimated to exist in poorly studied tropical regions (11, 15), these results remain unverified for complete, highly diverse amphibian faunas. In parallel, an increase of threatened amphibian species has been reported worldwide (8, 20, 21). Amphibians are of high conservation concern, with 43% of species being globally threatened (20), most of them in tropical regions with high amphibian diversity.

Madagascar is one of the top priority global hotspots for biodiversity conservation (1), affected by a high rate of habitat destruction (22). Its fauna and flora evolved largely in isolation (23), and many taxa are characterized by a high degree of microendemism within Madagascar (24–27). The native amphibian fauna is constituted by 5 endemic evolutionary lineages of frogs with 100% species-level endemism, 2 of which (the mantellids and the cophylinae and scaphiophryninae microhylids) are very species-rich. Large-scale taxonomic inventories conducted since 1991 have led to an increase from 133 to 244 described species, largely due to the exploration of new areas and the application of more efficient techniques. About 46 species were identified during the 1990s mainly based on bioacoustics; the application of combined methods, including molecular genetics, was crucial in the discovery of 51 new species since 2000 as well as in the resurrection of species formerly considered to represent synonyms.

Undescribed diversity may have an important impact on understanding the spatial patterns of endemic radiations on the island, but objective estimates of species numbers are not available so far. We report a comprehensive assessment of morphological, bioacoustic, and genetic variation of the anuran fauna of Madagascar. Our goals are (i) to provide a reliable estimate of the proportion of yet-undescribed amphibian species from Madagascar and their phylogenetic and geographic distribution, (ii) to discuss the impact of our findings for global estimates of amphibian diversity, and (iii) to propose a novel terminology to be better able to assess the increasing number of identified but taxonomically undescribed candidate species of animals.

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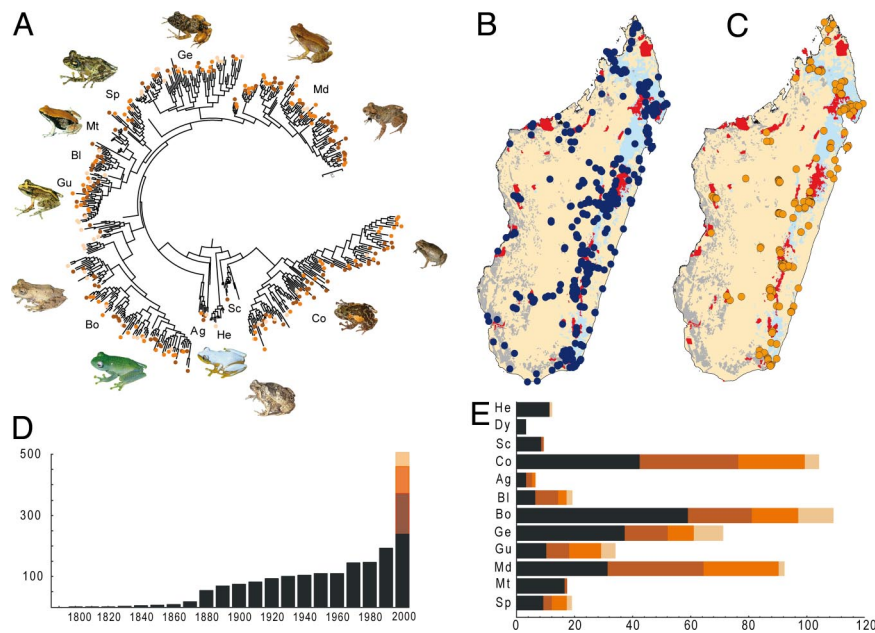


Fig. 1. Phylogenetic, geographic, and historical patterns of undescribed amphibian diversity in Madagascar. (A) Bayesian phylogenetic tree of 236 (out of 244) described species and 258 deeply divergent genealogical lineages of Malagasy frogs (among them 129 CCS and 92 UCS and 37 DCL with $>3\%$ genetic divergence to nearest described neighbor) based on a fragment of the mitochondrial 16S rRNA gene. Circles represent CCS (brown), UCS (orange), and DCL (light orange). Inset photos show some of these forms (see *SI Appendix*). He, *Heterixalus* (Hyperoliidae); Dy, *Dyscophus*; Sc, Scaphiophryninae; Co, Cophylinae (Microhylidae); Sp, *Spinomantis*; Md, *Mantidactylus*; Mt, *Mantella*; Gu, *Guibemantis*; Ge, *Gephyromantis*; Bo, *Boophis*; Bl, *Blommersia*; Ag, *Aglyptodactylus* (Mantellidae). (B and C) Maps of collecting localities of described species (B) and of CCS and UCS (C) of Malagasy frogs, and remaining primary vegetation (evergreen forests, blue; non-evergreen forests, gray). Current protected area network is shown in red. (D) Cumulative number of species currently considered as valid per 10-year intervals (2001–2008 for the current decade), and the CCS, UCS, and DCL identified in the present paper. (E) Total numbers of described species and CCS, UCS, and DCL in major clades of Malagasy frogs.

Results

From our integrative analyses of morphological, bioacoustic, and genetic data, we identified many more species of Malagasy frogs than there are names available. The number of described species of Malagasy frogs has slowly increased during the 20th century, reaching 100 described species around 1940, and 133 at the time of the last monographic account in 1991 (28) (Fig. 1). The current number of 244 valid native species indicates an increase of 83.5% since 1991.

We classify frog lineages from Madagascar in 3 categories (Table 1): (i) confirmed candidate species (CCS) are those differing clearly by morphological and bioacoustic characters and usually showing high genetic differentiation that we hypothesize are distinct, undescribed species; (ii) unconfirmed candidate species (UCS) are deep genealogical lineages (15)—bioacoustically and morphologically unstudied and usually derived from geographically distant populations—for which general indications exist that they are distinct, undescribed species; and (iii) deep conspecific lineages (DCL) are deeply divergent genealogical lineages, studied but not having clear morphological or bioacoustic differences with described species. Our data provided an additional 129 CCS, 92 UCS, and 37 DCL. If CCS plus UCS are assumed to represent distinct undescribed species, a 90.6% increase from the current 244 to 465 frog species is projected. This projection would constitute an overall increase of 250% since 1991.

Undescribed diversity is phylogenetically widespread in Malagasy frogs (Fig. 1A and *SI Appendix*). We found candidate species in most clades except in a few monospecific or species-poor genera (e.g., *Wakea*, *Dyscophus*). Genera such as the colorful *Mantella* or *Heterixalus* are rather well studied and consequently contain only a few candidate species, whereas in the 3 most speciose and diverse clades (*Mantidactylus*, *Boophis*, and Cophylinae), the number of candidate species is close to or even exceeds the number of described species (Fig. 1E). In general, in most genera the average differentiation in the 16S rRNA gene of CCS and UCS is $>4\%$. Most described species of Malagasy frogs show genetic distances of 6–8% to their closest relatives depending on the period of discovery (9), but these values refer to distances corrected by the Kimura-2-parameter-model (K2P) which are higher than the uncorrected distances we report. The molecular differentiation of CCS and UCS is thus at similar levels as between described species of Malagasy

frogs, indicating that they are not the result of taxonomic inflation (e.g., elevating subspecies to specific status).

New discoveries are also geographically ubiquitous. They occur throughout Madagascar, both in poorly explored and in better-studied areas. Described species are known from 451 sites and 87.3% are found in protected areas, whereas CCS and UCS are known from 168 sites and only 66.4% are found in protected areas (Fig. 1B and C). Even in 2 of the best-studied sites for amphibians in Madagascar, Mantadia/Analamazaotra and Ranomafana National Parks, harboring a total of 94 and 112 species of frogs, we found 10 and 31 CCS and UCS (see *SI Appendix*). Most described species are known from one or a few localities and have not been found elsewhere. This pattern is consistent among the undescribed species; only 6 of the 219 CCS and UCS are widespread, whereas 63 are currently known from small ranges and 154 from single localities.

Discussion

The Concept of Candidate Species and Their Delimitation. By integrating molecular, morphological, and bioacoustic data, we have identified a large number of undescribed species of amphibians in Madagascar. The remarkable increase in estimated species numbers is independent of the species concept applied. Almost all described species are well defined as reciprocally monophyletic and strongly differentiated units by molecular datasets and by morphological and/or bioacoustic evidence. The only exceptions are *Mantella milotympanum*, *Mantella nigricans*, *Dyscophus antongilii*, *Heterixalus andrakata*, *Heterixalus variabilis*, and *Heterixalus tricolor*, which are not clearly diagnosable by molecular data and require further study (29). The same combination of character sets defines all CCS (e.g., Fig. 24), and, based on our ongoing studies, we expect the same for most UCS. Many of these taxa occur in syntopy with their nearest relatives without any signal of admixture, corroborating their species status under biological, evolutionary, and phylogenetic species concepts. In contrast, many DCL correspond to genetically differentiated allopatric populations of widespread species for which the application of a biological species concept is inherently difficult. If criteria such as those for Malagasy primates (30) were applied to these DCL, many of them would be considered evolutionary species as well. Because the process of species formation in amphibians is an active and controversial area of research (31), we currently discourage such conclusions.

Table 1. Definitions of proposed categories of candidate species

Category	General definition	Definition used in Malagasy frogs
Unconfirmed Candidate Species (UCS)	Default category for deep genealogical lineages of unknown status. The genetic differentiation must be above a threshold value typical for comparisons among closely related species in the group of animals under study. Data deficient for morphology, ecology, and distribution.	Uncorrected pairwise genetic divergences in 16S rRNA gene >3% to all other described species. No data on morphology and bioacoustics due to unavailability of voucher specimens or immature state of vouchers.
Confirmed Candidate Species (CCS)	Specimens or populations characterized by a detectable genetic differentiation to all described species, not necessarily above any threshold, but in concordance with at least one of the following criteria: (i) a distinct differentiation in a character that mediates a pre mating reproductive barrier, (ii) a diagnostic morphological difference in a character that in the respective group of animals is known to be of low intraspecific variability and of high value to discriminate species, (iii) sympatric occurrence without admixture, and with at least one phenotypic character state difference, even if subtle, strictly correlated to the genealogy inferred from a neutral molecular marker.	Uncorrected pairwise genetic divergences in 16S rRNA gene to all other described species in most cases >3%, sometimes only 1–2%. Concordance of this molecular differentiation with one of the following: (i) a qualitative difference in advertisement calls, (ii) diagnostic difference in at least one morphological character known to be generally species-specific in Malagasy frogs: e.g., presence and extent of dermal spines, ridges and tubercles, extension of terminal finger tips, morphology of femoral glands, relative tympanum size, iris color, or tadpole mouthparts.
Deep Conspecific Lineage (DCL)	Deep genealogical lineages above a threshold value typical for comparisons among closely related species in the group of animals under study. One or several of the following must apply: (i) morphological and chromatic data reveal no differences to topotypic populations of a described species, or only subtle differences in characters that are known to show intraspecific variability, or only quantitative differences (in characters such as body size), (ii) if characters relevant for pre mating isolation are known, then these show no or only quantitative differences to topotypic populations of described species, (iii) phylogeographic studies are available and show indications for genetic admixture with other genealogical lineages within a species.	Uncorrected pairwise genetic divergences in 16S rRNA gene > 3% to all other described species in combination with one or several of the following: (i) no morphological differences, or differences only in characters known to show intraspecific variability: e.g., size, relative hindlimb length, body color and pattern, (ii) no difference in advertisement calls, or only differences in variables known to show intraspecific variability, (iii) co-occurrence with other deeply divergent haplotypes within the same populations, not concordant with any distinct or subtle morphological difference among individuals.

Recent progress in molecular techniques leads to acquiring newly determined DNA sequences at a faster pace than taxonomists are able to follow up with results. In many cases, divergent DNA sequences are observed that probably correspond to distinct, undescribed species, but a taxonomic analysis of these data are impracticable in the short term, especially in morphologically cryptic and highly diverse groups (32, 33). The term “candidate species” is rarely used by zoologists, but has recently been proposed for newly discovered units that probably correspond to undescribed species (31), and we advocate using it in a less formal way than the *Candidatus* status in microbiology (34–36).

Delimiting species is a resurgence issue in biology for which various explicit procedures have been proposed (37, 38). Most of these procedures require a relatively good state of taxonomic knowledge. For tree-based methods, organisms usually need to be sampled from several populations and to have their phylogenetic relationships well resolved. Many nontree methods require extensive datasets as well. These datasets are usually not available for those genetically divergent individuals for which we propose the category of candidate species. Also, species are known to be often paraphyletic in their mitochondrial haplotypes (39). Even more importantly, phylogenies based on single DNA fragments, such as used in DNA barcoding approaches, are often not sufficiently resolved. We therefore do not recommend explicit tree-based delimitations of candidate species, although such methods are certainly a great improvement for the eventual taxonomic description of species.

Distance-based DNA barcoding methods based on divergence thresholds are prone to 2 kinds of errors. Because there is no

fixed time span needed for speciation, there should be a continuum of pairwise genetic divergences of sister species, down to 0% in cases of rapid adaptive speciation. Any threshold will therefore miss a proportion of very young species (false negatives). The second kind of error is to wrongly identify intraspecific genealogical lineages as species (false positives). The accuracy of the method depends on the so-called barcoding gap between intraspecific and interspecific divergences, but previous work on amphibians has shown a wide overlap of these values and absence of a distinct barcoding gap (40). Because DNA barcoding can only be a preliminary tool for a first and rough identification of candidate species, we favor a conservative approach that minimizes the error probability of false positives. This approach will miss species of recent origin, but it will more efficiently help taxonomists to focus on those genealogical lineages likely to be undescribed species. The categories CCS, UCS, and DCL constitute an advance over uncritical approaches to DNA barcoding because they incorporate this useful molecular tool but emphasize the need of complementary data to understand biological reality.

Following our definitions, assignment of a genealogical lineage to the CCS category has the highest reliability of the 3 categories proposed here, and the number of 129 CCS thus provides a minimum estimate of undescribed species in Madagascar. Many DCL are largely based on the lack of evidence for CCS status. Some of these genealogical lineages may thus be upgraded to CCS when more extensive phylogeographic data and integration of nuclear genetic markers are available (e.g., *Boophis luteus*, Fig. 2B), although in many cases the DCL status is well assessed by comprehensive datasets (e.g., in *Guibemantis*

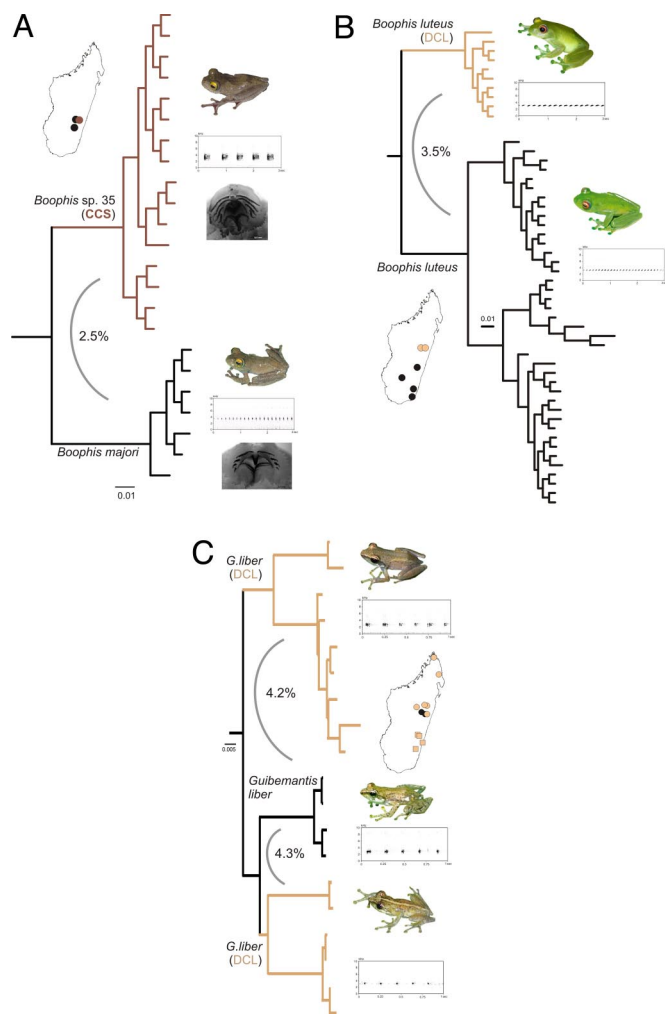


Fig. 2. Examples of combined phylogeographic, bioacoustic, and morphological evidence used to classify divergent mitochondrial lineages as CCS or DCL. (A) *Boophis majori* and *B. aff. majori* are sympatric CCS; despite their only low genetic divergence, they have distinct and constant differences in tadpole morphology and qualitative differences in advertisement calls, without signal of genetic admixture. (B and C) *B. luteus* (B) and *Guibemantis liber* (C) consist of deeply divergent genealogical lineages classified as DCL, because the call differences only affect quantitative parameters such as note-repetition rate in *B. luteus*, and there are no morphological or ecological differences between the populations. Furthermore, genetic admixture of the lineages was detected in *G. liber* at one locality.

liber, Fig. 2C). Finally, the UCS status is, by definition, unreliable and is used to accommodate those genealogical lineages with insufficient data.

These category definitions are not restricted to amphibians. In Madagascar and elsewhere, molecular data have been used to detect unexpected levels of cryptic diversity in other animal groups (32, 33, 41–43), and in many cases the biological identity of the previously undescribed genealogical lineages remains largely unassessed. Our definition of CCS (Table 1) relies on genetic divergence combined with a distinct difference in either morphology or in a character that mediates precluding isolation (advertisement calls in frogs). Characters and levels of morphological divergence will be different in each animal group, but in general we expect a wide applicability of this approach. More work must focus on understanding conditions under which the concordance of mitochondrial and nuclear gene genealogies can become a defining standard for CCS (15, 44, 45).

Molecular Data in Integrative Taxonomy. Integrative taxonomic analyses (46) are boosting the discovery and description of amphibians worldwide (17). The global biodiversity crisis requires these kinds of taxonomic studies (21) because they are the prerequisite for understanding diversity patterns and thus for the identification of priority areas for conservation. Despite the fact that recent efforts in amphibian systematics significantly increased the number of species, inventories—especially from tropical areas—are far from complete. These inventories are also needed to complete our knowledge of the tree of life of amphibians.

Amphibians tend to have conservative morphological evolution (47–49), which makes their species-level identification difficult. We here demonstrate the efficiency of an approach that first identifies genealogical lineages with high genetic divergences (40) and in a second step classifies these as CCS, UCS, or DCL by integrating other datasets. This approach does not preclude detailed morphological and bioacoustic studies in cases where biological species show low genetic differentiation. In fact, our approach is close to current taxonomic practice in herpetology: We propose diagnostic character-state differences in morphology or bioacoustics as a central prerequisite for CCS status, agreeing with the fact that most descriptions of new amphibian species are today still based only on morphology, despite the availability of more sophisticated methods (37, 38). To extend current standards in amphibian taxonomy, we strongly recommend the routine inclusion of DNA sequences in descriptions of new species. Such a practice would simplify the subsequent identification of divergent genealogical lineages and their integrative taxonomical study (40), even if this sequence information would not be sufficient to assess phylogenetic relationships of the new species.

Amphibian Diversity Patterns in Madagascar and Conservation Priorities. Although Madagascar's fauna has been studied extensively during the last century, the increase in species discoveries is making diversity estimates tentative in most cases. Our data suggest that current diversity figures largely underestimate the real diversity of frogs, and probably of other terrestrial vertebrates as well. This underestimation has direct consequences on our understanding of the pattern of biodiversity in Madagascar. Most of the Malagasy amphibian species are known from <10 localities, and many from single localities only (50), making spatial diversity analyses difficult. In many cases, this analysis certainly does not represent species actual distribution ranges, but indicates a real lack of information on species spatial distribution patterns in Madagascar whereas a considerable number of species are probably indeed restricted to very small and isolated ranges.

Important increases in species numbers have also characterized other groups of Malagasy vertebrates during the last 2 decades (30, 51). Numerous undescribed species of reptiles, mammals, and especially freshwater fishes are known to exist but are usually not included in conservation assessments. Probably by far the largest proportion of species of invertebrates remains undescribed as is usual for most tropical regions. For amphibians as well as many other groups of animals, the ongoing discovery of undescribed diversity is being triggered by several factors, including the increase of taxonomic work in Madagascar, incorporating new molecular tools with traditional methods, and an increase in field exploration efforts. Some larger patches of rainforest in Madagascar have not been properly explored yet and probably harbor numerous additional new candidate species. However, many of the amphibians we have identified here are found in some of the best-studied areas of Malagasy rainforests, such as Ranomafana and Analamazaotra/Mantadia National Parks (Fig. 1 B and C; *SI Appendix*). Taxonomic exploration should thus not be limited to unexplored areas but should include putatively well-known plots as well. The combination of phylogenetic datasets with spatial modeling of species or clades

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Supplementary Information S1.

Extended methods on DNA sequencing and analysis.

A fragment of the mitochondrial 16S rRNA gene was amplified and sequenced using primers 16SraL and 16SrbH using standard procedures (1). Altogether, ca. 1700 adult specimens and 1150 tadpoles of Malagasy frogs were sequenced in 2000-2007. We used the FFT-NS-2 algorithm in the software MAFFT v. 6.624 (2) for a multiple alignment of these sequences, and manually verified the resulting alignment. Based on Neighbor-joining trees obtained with PAUP* (3) we identified sequences showing high divergences from reference specimens of described species. Selected sequences were used to compute a tree using Bayesian inference (4) after determining the appropriate substitution model (5). This tree (see below), largely agrees with published phylogenetic knowledge, although a few well-established clades are not recovered due to the limited phylogenetic information in the short gene fragment. Phylogeographic analyses of three complexes of deep genealogical lineages (*Boophis majori* / *B. sp. 35*, *B. luteus*, *Guibemantis liber*; Fig. 2) were computed using the same general methods.

While we use the tree based on the MAFFT alignment to show the distribution of undescribed diversity across major clades, possible alignment artefacts in the distribution of indels in this non-coding DNA fragment can affect the computation of pairwise distances between sequences. To avoid this problem we used the software TaxI (6) to compute uncorrected pairwise distances between all species pairs of Malagasy frogs. This software first aligns each possible pair of sequences and then calculates the number of differences between them, not counting positions with indels. From the distance matrices obtained, we extracted the shortest distance of each included taxon to the most similar sequence overall, and to the most similar sequence of an already described species.

Further definitions of categories of Candidate species.

In Microbiology, a *Candidatus* status is regularly ascribed to distinct prokaryotic entities for which more than just a sequence is available but characteristics required for description according to the Bacteriological Code (1990 Revision) are lacking. Although we see the need for standardized terms to refer to such distinct but yet undescribed animal species, we do not propose to recognize candidate species as done by the microbiological community, where an official *Candidatus* list exists. One reason is that the requirements to define and describe a new species are less strict in zoology than in microbiology where minimum standards are defined by official committees.

In our study, when genealogical lineages were not sister to those species that are most similar morphologically or bioacoustically, we used this as additional confirmation for CCS status, which however we primarily determined through a combination of distance- and character-based methods. In this concordance approach, divergent individuals are first identified by distance-based DNA barcoding. If the divergence to described species is above a certain threshold, which will differ among groups of organisms, there is a high probability that the individual belongs to an undescribed species which warrants its inclusion in the UCS category. However, it cannot be emphasized enough that the DNA barcoding approach alone is insufficient to reach

CCS status. The second mandatory step is the search for diagnostic characters in morphology and, especially, in advertisement calls. If the genetically divergent frog individual differs convincingly in such characters, or if slight but consistent differences are detected in sympatry, it is categorized as CCS. If the differences are only slight and it occurs in allopatry, it is to be considered as DCL.

Criteria of reciprocal monophyly of haplotype lineages are extremely useful in species delimitation. To assess monophyly or non-monophyly with statistical support, however, often requires longer DNA sequences than usually become available from DNA barcoding studies, and therefore was used only peripherally in our approach to define candidate species.

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