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Genetic Confirmations of Dwarf Lemurs Across Madagascar Highlights Complex Biogeographic Patterns

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ABSTRACT

Conservation action, vital in biodiversity hotspots, depends on reliable data on endangered taxa. This is especially important for species that are morphologically cryptic and difficult to distinguish in the field. The dwarf lemurs of Madagascar (*Cheirogaleus* spp.) exemplify these challenges: They are widespread but endangered, nocturnal, small-sized, and cryptic. The genus is diverse, with nine described species and as many candidate species, falling into four distinct lineages ("medius," "sibreei," "crossleyi," and "major"). Many protected areas in Madagascar harbor dwarf lemur populations that await genetic species confirmation. Here, we collated tissue samples from 30 individual dwarf lemurs in 10 forest sites collected by a network of collaborators over a decade. We sequenced the simple but diagnostic cytochrome-b marker gene and placed samples into a curated database of > 200 published sequences. Except for an individual from the "sibreei" lineage in Marojejy that separated from close relatives farther south, our samples fell into described or candidate species, supporting geographic clustering within lineages. We identified two sites in the northeast that harbor different assemblages of species from three distinct lineages in sympatry.

Marina B. Blanco and Lydia K. Greene are the co-first authors of this study.

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We confirmed the presence of *C. crossleyi* at sites across Madagascar's central highlands, considerably extending its known range, and posit that this is the dominant dwarf lemur of the highlands north of the Mangoky river. Although our results are limited in resolution and call for in-depth genomics, we advocate for foundational field work, natural history exploration, and survey/sampling expeditions to generate the very data on which sophisticated genomics and computational methods rely.

1 | Introduction

Madagascar is one of Earth's biodiversity hotspots (Antonelli et al. 2022; Ganzhorn et al. 2001; Jones et al. 2022; Myers et al. 2000) and contains more than 15% of all primate species globally, that is, the lemurs that are endemic only to the island (Mittermeier et al. 2023). In the face of ongoing anthropogenic habitat degradation and fragmentation, considerable attention has focused on safeguarding local ecosystems and endemic species (Ralimanana et al. 2022). Most notably, in 2003, Marc Ravalomanana, the then-president of Madagascar, pledged through the “Durban Vision” to triple the number of protected areas, a goal which was reached and then surpassed in the following two decades (Gardner et al. 2018; Kappeler et al. 2022; Ralimanana et al. 2022; Waeber et al. 2020). Many of these “new” protected areas are co-managed by local associations and NGOs and engage communities in conservation action (Eklund et al. 2016; Goodman et al. 2018; Jones et al. 2022; Ralimanana et al. 2022; Rasoamanana et al. 2023). Nevertheless, many contain small and fragmented forests, are isolated from large forest blocks, are difficult to reach by road from large towns, and are challenged by historic and continued degradation (Gardner et al. 2018; Goodman et al. 2018). Many also lack complete lists of inhabiting lemur species, which has implications for managers developing action plans and for island-wide assessments of species' true ranges and extinction risks (Gardner et al. 2018; Rafanoharana et al. 2024; Ralimanana et al. 2022).

Importantly, research teams have conducted biodiversity inventories in dozens of previously established and newly designated protected areas; however, many of these inventories lack resolution for species that cannot be identified by morphology alone, that is, cryptic species (Chenuil et al. 2019), which hinders conservation assessments. The challenge of identifying cryptic species is heightened in lineages that are small, nocturnal, difficult to see and sample, and for those that have recently undergone (or are undergoing) taxonomic revision (Christidis et al. 2014; Everson et al. 2018; Markolf et al. 2011; Tattersall 2007; Yoder et al. 2005). Even when biological samples are collected, local research teams rely on international collaborations and funding for the genetic sequencing to confirm species, because there are no readily available sequencing facilities in Madagascar (Hauff et al. 2025; Helmy et al. 2016). Often, such species confirmations are prioritized when there is potential for describing new species, or when they coincide with the specific agenda or research interests of international collaborators (Waeber et al. 2016). As a result, there can be a decoupling between field teams that identify wildlife populations and laboratory/computational teams that can confirm/describe species (Yoder et al. 2005).

The dwarf lemurs (*Cheirogaleus* spp.) exemplify all these challenges. Dwarf lemurs are small and nocturnal animals that forage high in the canopy and are the only obligate hibernators among primates (Blanco 2022). Sample collection is all the more difficult as the hibernation season coincides with the dry season, which is the best time of year for biological surveys due to road accessibility. To a highly trained eye and on close encounter, dwarf lemurs can be morphologically assigned in the field to one of four lineages that broadly match different habitat types. Dwarf lemurs within the “medius” lineage are small (~150 g) with gray fur, and typically occupy low-elevation, dry-deciduous forests. Dwarf lemurs within the “sibreei” lineage are larger (~250 g) with gray-fawn colored fur, are confined to high-elevation rainforests above 1500 m, and are rarely seen or much less studied. Dwarf lemurs within the “crossleyi” lineage are bigger still (~350 g), with reddish-brown fur, a pointy snout, and dark, furry ears, and are broadly distributed in montane forests, including in degraded environments (Blanco and Godfrey 2014; Petter et al. 1977). And dwarf lemurs within the “major” lineage are the largest (~450 g), with gray-brown fur and a rounded face, and are found in low-elevation rainforests in the east (Blanco 2022). Nevertheless, there is significant interindividual morphological variation within these lineages that makes field identifications tentative at best.

There is also significant species diversity within these lineages that can only be identified through genetic diagnostics. Currently, we recognize nine species across the four lineages (Frasier et al. 2016; Lei et al. 2014, 2015; McLain et al. 2017). This number is projected to double, given the number of candidate species that have been proposed based on genetic data (Lei et al. 2014, 2015; McLain et al. 2017). Many of the described and proposed species are known from a few individuals and/or localities, and we do not know the true extent of their geographic occurrence. Indeed, many of the protected areas established since the “Durban Vision” initiative are known to harbor dwarf lemurs but lack species confirmations, given that current data are limited to field observations. This is also the case for many better-known protected areas, including some historic National Parks. In searching the three volumes on “The Terrestrial Protected Areas of Madagascar” (Goodman et al. 2018), we realized that more than 55% of the listed sites (42/76) report dwarf lemurs that await genetic species confirmation.

To begin filling these gaps, we amassed 30 biological samples from dwarf lemur populations at 10 field sites across Madagascar, collected by a network of researchers conducting field work over the past decade (Table 1). We sequenced a reliable marker gene, the full mitochondrial cytochrome-b gene (Louis and Lei 2014, 2016), and confirmed identifications by placing sequences into a large repository of 217 published sequences that span all described and candidate dwarf lemur species. Our samples encompass individuals tentatively assigned in the field to all four lineages based on morphology. Whereas some of the field sites sit in the middle of known geographic ranges with clear predictions for species confirmations, other sites are far removed from any known ranges with less obvious species

Summary

Species are the unit by which we count biodiversity and assess extinction risk. We therefore need to know which species live where. Assigning species in the wild is challenging when individuals look alike, and when they are small-sized and nocturnal. This is the case for the dwarf lemurs of Madagascar. There are currently nine known species and several candidate species of dwarf lemurs that inhabit different forests in Madagascar. These lemurs are only identifiable by sequencing their DNA, and many sites in Madagascar await such genetic species confirmations. We use a diagnostic marker gene to confirm the dwarf lemur species at 10 sites for which prior data were sparse or inconclusive. We find that multiple dwarf lemur species inhabit the same forests in northeastern Madagascar. We also show that one species, the furry-eared dwarf lemur, is exclusively found in very small, fragmented forests across the central highlands. While acknowledging our limitations, we advocate for additional field surveys to better delineate true species geographic distributions. This information can help answer questions about how these lemurs got there in the first place and whether they may be resilient or tolerant to impending climate and anthropogenic changes.

- **Practitioner Points**
    - We confirmed the species identity of 30 dwarf lemurs inhabiting 10 protected areas across Madagascar using a mitochondrial marker gene, cytochrome-b.
    - We identified a large range expansion for the furry-eared dwarf lemur (*Cheirogaleus crossleyi*) across multiple sites in the central highlands and posit that this species is likely the dominant and only dwarf lemur in this understudied region.
    - We revealed centers of sympatry in the northeast, with different assemblages of three species of dwarf lemur inhabiting Marojejy National Park and the Tsihomanaomby Protected Area.

predictions (Figure 1). And still at other sites, morphological descriptions suggest that multiple dwarf lemur species exist in sympatry in locations with heterogeneous environments and wide elevational gradients, with confirmatory genetics clearly needed to clarify these predictions. Determining the size and contiguity of species' geographic distributions by filling these taxonomic gaps is necessary for accurately assessing conservation status for the dwarf lemurs (IUCN 2012).

2 | Results

We successfully amplified and sequenced the mitochondrial cytochrome-b marker gene from all 30 samples in the study and found that they clearly placed within the described or candidate species. See the Supporting Material for consensus sequences per sample and for the full phylogenetic tree depicting the location of each sample.

At Ambohidray, a montane rainforest in the east, we confirm the presence of *C. crossleyi*. Ambohidray sits within the known range of this species, but to the best of our knowledge, this is the first genetic confirmation of this species in this new protected area.

At Ambohijanahary, a medium-altitude subhumid forest on the western edge of the central highlands, we confirm the presence of *C. crossleyi*. This represents a major western range expansion for this species that is primarily known from the eastern montane forests.

At Anjajavy, a low-altitude dry deciduous forest in the northwest, we confirm the presence of Unconfirmed Candidate Species 2 (UCS2) from within the "medius" lineage. This candidate species is currently only known from the Anjiamangirana forest, which sits inland but within the same Inter River System (IRS III) between the Sofia river to the South and the Maevarano river to the North.

At Ankafobe, a medium-altitude subhumid forest in the central highlands, we confirm the presence of *C. crossleyi*. Although this species was genetically identified at this site already (McLain et al. 2017), we provide the first published cytochrome-b sequence for an individual from this population.

At Ankarabolava, a low-altitude rainforest in the southeast, we confirm the presence of Confirmed Candidate Species 4 (CCS4) from within the "major" lineage. This represents a minor range expansion for this candidate species and adds a new locality to its known distribution.

At Beanka, a tsingy (limestone) dry-deciduous forest in the west, we confirm the presence of *C. medius*. Beanka sits within the known range of this species, but to the best of our knowledge, this is the first genetic confirmation of this species in this protected area.

At Itremo, a mosaic habitat of medium-altitude forest fragments and grasslands characteristic of the central highlands, we confirm the presence of *C. crossleyi*. Like at Ambohijanahary, this site is a western range expansion for this species.

At Mahajeby, a mosaic habitat of medium-altitude subhumid forest fragments and grasslands that share characteristics with both the central highlands and western dry forests, we confirm the presence of *C. crossleyi*, representing a northwestern range expansion for this species.

At Marojejy, a large area of montane rainforest in the northeast, we sampled dwarf lemurs at three sites spanning an elevational gradient. At the lowest-altitude site (Camp Nord), we confirm the presence of Confirmed Candidate Species 2 (CCS2) from within the "crossleyi" lineage; at the medium-altitude site (Camp 2), we confirm the presence of both *C. major* and CCS2; at the highest-altitude site (Camp 4), we confirm the presence of *C. sibreei*, although we note the sequence from this individual clusters distinctly from central *C. sibreei* populations in our cytochrome-b phylogenetic tree (see Figure S1). *C. major* was previously confirmed in Marojejy using a different marker gene (Williams et al. 2020); however, to the best of our knowledge, these are the first published cytochrome-b sequences for any of these species at this site.

TABLE 1 | List of study sites from which we collected and sequenced dwarf lemur samples for species genetic confirmations.

Site	IUCN category, management	Year of inception	GPS coordinates, altitude	Collection date	# of samples
Ambohidray (AMB)	Not defined DBEV	2015	−18.599972° 48.301592°, 1000 m	November, 2023	2
Ambohijanahary (AMJ)	Cat IV, Special Reserve MPACT Madagascar	1958	−18.338889° 45.359056°, 800 m	October 3–9, 2024	2
Anjajavy (ANJ)	Cat V, Harmonious Landscape Private Management	2018	−15.008411° 47.250850°, 10 m	March 16–28, 2018 July 9–29, 2018 March 25–April 14, 2023	3
Ankafobe (ANK)	Undefined, Special Reserve MBG	2015	−18.106119° 47.187050°, 1470 m	November 1–7, 2016	1
Ankarabolava (ANR)	Cat VI, Natural Resources Reserve MBG	2015	−23.521306° 47.495694°, 50 m	October 17–29, 2023	1
Beanka (BEA)	Cat V, Harmonious Landscape BCM	2015	−18.031028° 44.508917°, 360 m	November 6–15, 2023	2
Itremo (ITR)	Cat V, Protected Harmonious Landscape RBG-Kew	2015	−20.539722° 46.404056°, 1430 m	November 23–December 3, 2023	3
Mahajeby (MAH)	Not defined, IMPACT Madagascar	Currently undergoing establishment	−18.041861° 45.973333°, 700 m	October 16–23, 2024	4
Marojejy (MAR)	Cat II, National Park MNP	1952	Camp 2: −14.434139° 49.762222°, ~700 m	October 17–November 3, 2013	2
			Camp 4: −14.444472° 49.742056°, 1550 m	February 9–28, 2014	1
			Camp Nord: −14.312139° 49.699222°, 200 m	October 7–25, 2014	1
Tsihomanaomby (TSI)	Cat VI, Natural Resources Reserve MBG	2015	−14.100417° 50.013083°, 260 m	February 23-March 16, 2016 February 13–25, 2017 July 11–16, 2017	8

Abbreviations: BCM, Biodiversity Conservation Madagascar; DBEV, Département de Biologie et Ecologie Végétales, Université d'Antananarivo; MBG, Missouri Botanical Garden; MNP, Madagascar National Parks; RBG-Kew, Royal Botanic Gardens, Kew.

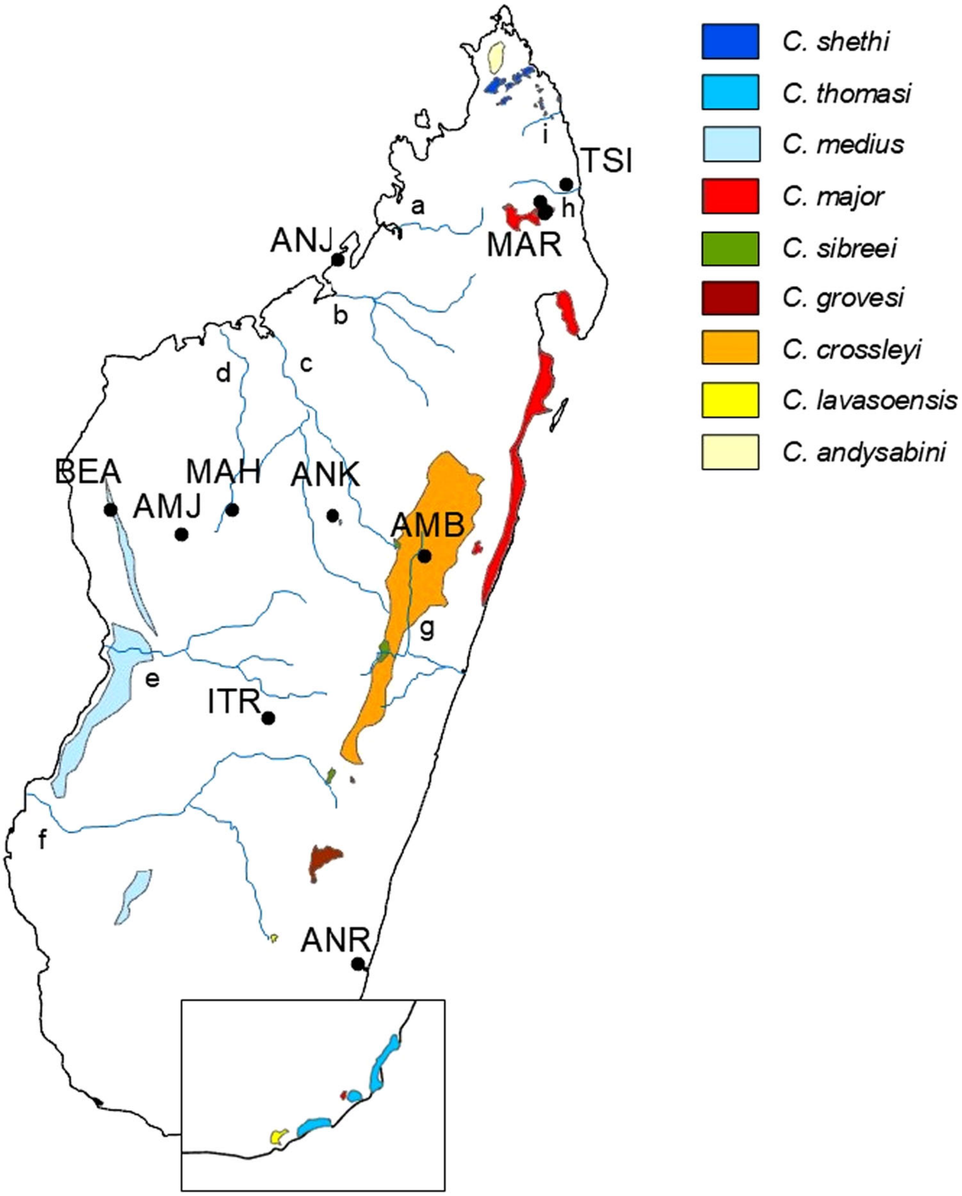

FIGURE 1 | Map of Madagascar depicting the current geographic distributions of described dwarf lemur species from the most-recent IUCN red-listings, not including candidate species. Also shown are the field localities with biological samples included in this study: AMB, Ambohidray; AMJ, Ambohijanahary; ANJ, Anjajavy; ANK, Ankafobe; ANR, Ankarabolava; BEA, Beanka; ITR, Itremo; MAH, Mahajeby; MAR, Marojejy; TSI, Tsihomanaomby. Major rivers are indicated by lower-case letters: a, Maevarano; b, Sofia; c, Betsiboka; d, Mahavavy Sud; e, Tsiribihina; f, Mangoky; g, Mangoro; h, Bemarivo; i, Manambato.

FIGURE 2 | Representatives of three distinctive dwarf lemur lineages found in the same forest fragment at Tsihomanaomby, namely "major" (left), "crossleyi" (center), "medius" (right). These lemurs were assigned to a morphotype in the field, and genetically confirmed to belong respectively to *C. major*, CCS2 ("crossleyi" lineage), and CCS7 ("medius" lineage). Photos by M. Blanco.

At Tsihomanaomby, a low-altitude subhumid fragmented forest in the northeast, we confirm the presence of Confirmed Candidate Species 7 (CCS7) that is sister to *C. shethi* and sits within the "medius" lineage, CCS2, and *C. major* in sympatry (Figure 2). Although these species (or members of their lineages) were previously purported to live in sympatry at this site (Williams et al. 2020), these are the first published cytochrome-b sequences for these particular populations.

3 | Discussion

In the present study, we sequenced a simple but diagnostic marker gene to identify 30 dwarf lemurs inhabiting 10 forest sites across Madagascar. Taken individually, each of these genetic confirmations enables the managers of protected areas to compile accurate species listings, which is important for local conservation planning (Gardner et al. 2018). Taken in aggregate, however, these confirmations illuminate intriguing biogeographic patterns that merit further study. First, except for the individual from the "sibreei" lineage sampled in Marojejy, all our samples fall neatly into described or candidate species of dwarf lemur and extend their known distributions (Lei et al. 2014). Second, we identify two sites in the northeast where different combinations of dwarf lemurs from three distinct lineages live in sympatry. And third, we confirm *C. crossleyi* is the dominant, and likely only, dwarf lemur species present in the central highlands north of the Mangoky river.

As expected, we found that the northern populations within the "major" and "crossleyi" lineages clearly separate in cytochrome-b sequences from their respective central and southern counterparts (Lei et al. 2014; Figure S1). We add that the single sample from the "sibreei" lineage in the north also clusters distinctly from counterparts in other localities, though its designation as a candidate species remains to be seen. Within the "medius" lineage, we note that the populations of CCS7 in the northeast at Tsihomanaomby cluster with those from Sambava, which lies on the opposite side of the Bemarivo river. These two populations then cluster distinctly from *C. shethi*, which is found farther north. Curiously, *C. shethi* was originally described north of the Manambato River using molecular markers (Frasier et al. 2016), with subsequent field surveys suggesting a range expansion south of the Manambato (Hending et al. 2017). Without confirmatory genetics, we lack resolution to know if these populations south of the Manambato cluster with *C. shethi* or CCS7, with implications for whether the Manambato river acts as a barrier. Both the Bemarivo and Manambato rivers have been proposed as possible barriers for lemur dispersal, with inconclusive results (Goodman and Ganzhorn 2004). Lastly, we note that the populations of UCS2 from Anjajavy and Anjiamangirana clearly separate from all other species within the "medius" lineage. If this dwarf lemur turns out to be a new species, it would be endemic to the northwest and restricted to IRS III, between the Maevarano River to the north and the Sofia River to the south. While showcasing this clustering, we advocate for more comprehensive genomics to identify which, if any, of these populations merit full species status.

Our results validate tentative field assignations that dwarf lemurs live in habitual sympatry in the northeast, in addition to known sympatric occurrences in the central and southeast forests (Blanco et al. 2009; Herrera et al. 2016; Thiele et al. 2013). In the rainforests of Marojejy that span a wide elevational gradient, we confirmed *C. major* and CCS2 ("crossleyi" lineage) below 1000 m, sometimes in the same location (i.e., Camp 2), and an individual from the "sibreei" lineage above 1500 m. In the subhumid forest fragment of Tsihomanaomby, we confirmed the presence of *C. major*, CCS2 ("crossleyi" lineage), and CCS7 ("medius" lineage), all caught in the same location (i.e., near the same camp area). Follow-up field work is needed to confirm the extent of dwarf lemur sympatry throughout Madagascar's north, including in the connected forests of Tsaratanana, COMATSA, and Anjanaharibe-Sud. Beyond such confirmations, our results call for more sophisticated population genomics applied to field-collected samples to reconstruct the evolutionary histories of these populations (Williams et al. 2020). Such an approach could elucidate the geographic factors and/or ecological forces that could have driven diverse dwarf lemur species to occur in sympatry.

Regarding biogeography, our results establish *C. crossleyi* as widely distributed in central Madagascar, including in montane eastern forests and across the highlands (Figure 3). Notably, our confirmations at Ambohijanajary, Mahajeby, and Itremo significantly expand this species' range to the west and validate sporadic anecdotal sightings dating back to the 1970s of larger and reddish dwarf lemurs in the Bongolava Region (Petter et al. 1977; Thalmann 2000). Our results call for a reassessment of this species' endangerment status. We posit that the Mangoky river, the tributaries of which lie just south of Itremo, may represent the southern-most boundary for this species (with *C. grovesi* and *lavasoensis* on the opposite side). We currently lack resolution from the field to delimit the northern boundary of *C. crossleyi* with its sister taxon, CCS2. Curiously, we identified little genetic variation (albeit in a single marker gene) between populations of *C. crossleyi* along an East-West gradient, from Ambohidray to Ankafobe, Mahajeby, Ambohijanahary, and from Ankazomivady to Itremo (Figure 3). In contrast, we find significant variation between species within the "crossleyi" lineage along a North-South gradient, from *C. andysabini* to CCS2, *crossleyi, grovesi*, and *lavasoensis*. These results suggest an earlier diversification within the lineage, that is, speciation events, along a North-South gradient, followed by a more recent westward expansion by *C. crossleyi*. Genomic analysis could clarify the timing of these events against the backdrop of geologic and habitat change in Madagascar, including the volcanism that uplifted mountain ranges in the north (Collins et al. 2022), the establishment of the rainforests along the island's eastern escarpment (Federman et al. 2015; Wells 2003), and the extent of the grassland-forest mosaics that alternated in dominance during the Pleistocene (Lehmann et al. 2022).

3.1 | Conservation Considerations

Ultimately, we showcase the value of using simple genetic diagnostics to confirm the populations of cryptic dwarf lemurs living in new and established, large and small, intact and fragmented protected areas across Madagascar's diverse ecosystems (Figure 4). Dwarf lemurs, as it turns out, are pervasive throughout remnant forests in Madagascar, which is counterintuitive given their specialized ecology as frugivores and obligate hibernators (Blanco et al. 2018; Lahann 2007), and their reported negative response to forest edges (Lehman et al. 2006). In particular, *C.*

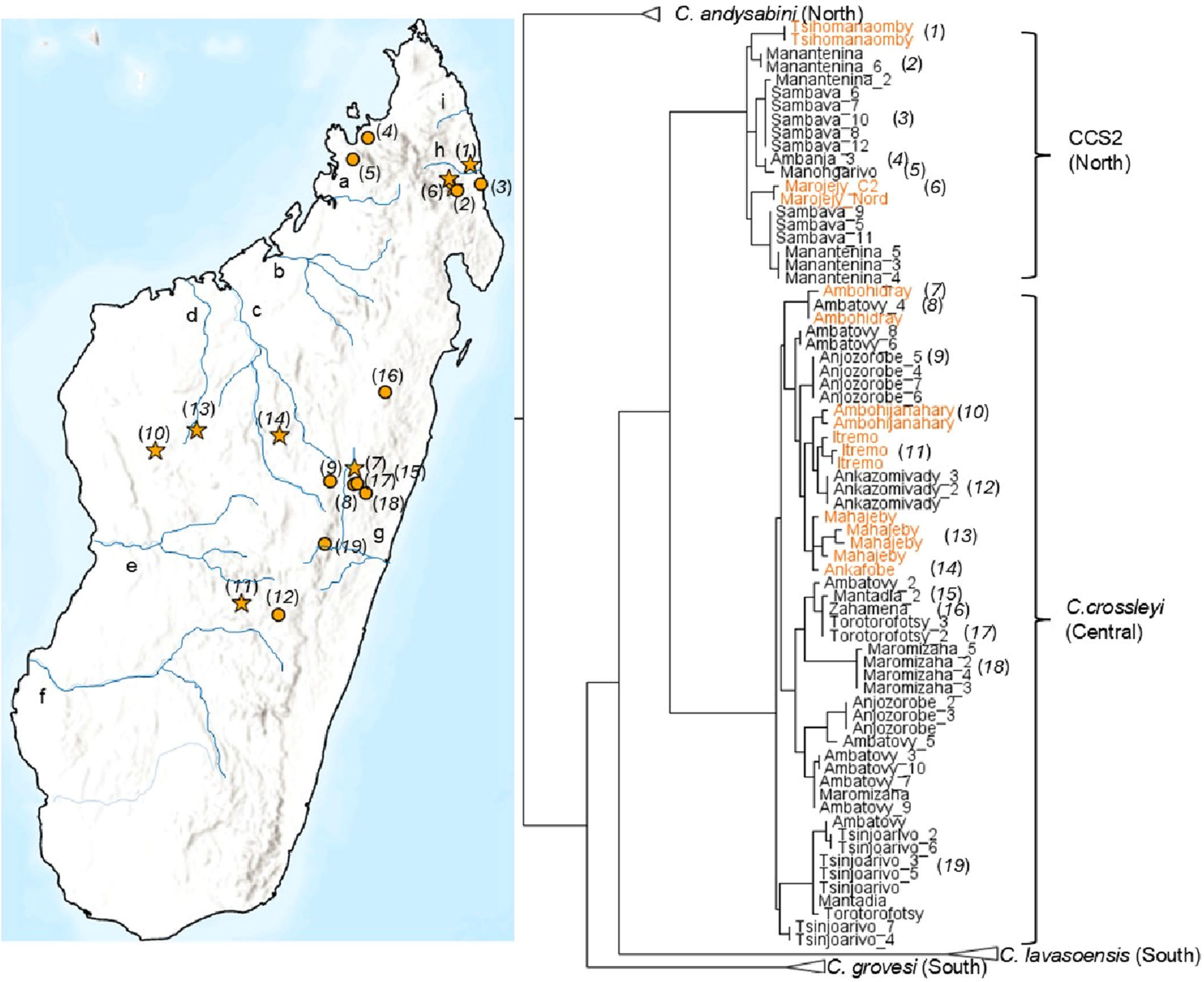

FIGURE 3 | (Left) topographical map of Madagascar showing known (circle) and new (star) localities of *C. crossleyi* and CCS2 ("crossleyi" lineage). Localities, by number, are further shown in the (right) cytochrome-b phylogenetic tree of all published sequences within the "crossleyi" lineage, spanning described and candidate species. Newly added samples from this study are in orange. Major rivers are indicated by lower-case letters with descriptions in Figure 1.

FIGURE 4 | Photo array of (top) *C. crossleyi* individuals spotted during nocturnal surveys in (bottom) forest fragments across the central highlands, including at (left) Ambohijanahary SR, (center) Mahajeby, and (right) Itremo. Photos by M. Blanco & L. Greene.

crossleyi has a greater range than previously thought. This species is found across numerous habitat types and is more ecologically flexible than congeners (Blanco et al. 2018; Blanco 2022). For this species, even greater sampling resolution is needed to delimit its northern and western geographic boundaries. When coupled with population genomics, such field expeditions to survey and sample could address when, why, and how this dwarf lemur was able to radiate west across the highlands when others did not.

In the future, we anticipate scaling up our approach and have already identified dozens of forest sites with dwarf lemurs awaiting similar confirmation. We regard improved sampling resolution across both the genus and the island as critically important to assess conservation risk due to habitat and climate change. Models built to predict lemurs' extinction risk are based on available input data (Brown and Yoder 2015) that are too often incomplete to make reliable predictions (e.g., Hending et al. 2023). Such data, typically resulting from surveys, are collected in the field by diverse teams but amassed by modelers who must vet sightings against an ever-updating taxonomy. As wildlife conservation continues to benefit from genetic, genomic, and computational methods that are ever more sophisticated, precise, powerful, and widespread (Hohenlohe et al. 2021; Supple and Shapiro 2018), we must not lose sight of the value of foundational field work, natural history exploration, and survey/sampling expeditions to generate the very data on which these methods rely.

4 | Materials and Methods

4.1 | Ethics Statement

Our research was conducted in compliance with Duke University Institutional Animal Care and Use Committee protocols (A040-12-02, A035-15-01, A263-17-12, A213-20-11, A194-23-09) and under research permits approved by the relevant national, regional, and local Governmental Offices in Madagascar (research permits: N°227/13/MEF/SG/DGF/DCB.SAP/SCB, N°229/14/MEEF/SG/DGF/DCB.SAP/SCB, N°225/15/MEEMF/SG/DGF/DAPT/SCBT, N°270/16/MEEF/SG/DGF/DSAP/SCB.Re, N°035/18/MEEF/SG/DGF/DSAP/SCB.Re, N°338/23/MEDD/SG/DGGE/DAPRNE/SCBE.Re, N°162/24/MEDD/SG/DGGE/DAPRNE/SCBE.Re. Our research complied with the American Society of Primatologists' Principles for the Ethical Treatment of Nonhuman primates (https://www.asp.org/2021/04/20/principles-for-the-ethical-treatment-of-non-human-primates/) and the Code for Best Practices in Field Primatology (https://www.asp.org/resources/docs/Code%20of_Best_Practices%20Oct%202014.pdf).

4.2 | Sites, Subjects, and Capture Protocols

The study subjects are 30 dwarf lemurs at 10 field sites collected by the authors between 2013 and 2024 and include 1–4 individuals per lineage per locality (Table 1). We captured dwarf lemurs using standard methods (Blanco et al. 2013; Blanco and Godfrey 2014). In brief, we set live Tomahawk traps (16”L x 5”W x 5”H) between 4-10 m high. Traps were baited with fermented fruit, opened around 16:00, and checked the following morning around 05:00. Captured lemurs were transported to the campsite, weighed, and briefly anesthetized using ketamine (10 mg/kg, IM). Once sedated, we tentatively assigned animals to one of the four lineages (i.e., “medius,” “sibreei,” “crossleyi,” “major”). We collected two small ear biopsies (one per ear, 2 x 2 mm) using standard ear punches for genetic analysis. All tissue samples were preserved in > 90% ethanol. We released all lemurs at capture sites later the same day, at sunset, when it was safe to do so.

4.3 | Biological Sample Preparation and Sequencing

Tissue samples (i.e., ear biopsies) were processed in different batches by different members of the author line based on collection era, but all were extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocols. These extractions were performed in the Yoder Lab at Duke University (USA) or in the Ratsoavina Lab at the University of Antananarivo (Madagascar) and later imported to the USA for storage and analysis.

We amplified samples from all but three field sites (Mahajeby, Ambohijanahary, and Anjajavy) between March and June 2024 in duplicate in the Yoder Lab at Duke University (NC, USA) using PCR and primers that capture the entire cytochrome-b gene (L14724/H15915). We amplified samples from Mahajeby and Ambohijanahary in December 2024 in duplicate in the Ratsoavina lab at the University of Antananarivo (Analamanga, Madagascar) using a modified PCR protocol with thermostable reagents from miniPCR Bio. We visualized PCR products in both labs on an agarose gel. Aliquots of PCR product amplified in Madagascar were hand-carried, on ice packs, to the Yoder Lab and stored at −20°C until sequencing. All PCR products were submitted for overnight Sanger sequencing in both the forward and reverse directions (Azenta Life Sciences, South Plainfield, NJ).

The remaining samples from Anjajavy underwent a different preparatory and sequencing pipeline. These samples were processed in conjunction with a different project testing a new sequencing platform that can be deployed in the field (Blanco et al. 2020). In 2018, the samples were extracted and amplified using the protocols described above, but sequenced using Oxford Nanopore's minION platform in Madagascar. For a description of these sequencing methods, see Blanco et al. (2020).

From all samples, raw sequences were processed using the Geneious software program (version 11.1.5). In brief, we reverse-complemented the reverse sequences, aligned forward and reverse reads to a reference sequence (*Cheirogaleus* sp., accession # EU825330, Sambava), trimmed any primers or other noise from either end, and generated a consensus sequence that spanned the entire cytochrome-b gene. We aligned all 30 consensus sequences to a reference database of 217 full cytochrome-b sequences that span all described and candidate species of dwarf lemurs, accessed through NCBI (see Table S1). We constructed a phylogenetic tree using RAxML (version 8.2.11) (Stamatakis 2014) implemented in Geneious. We used the General Time Reversible model with gamma-distributed rate variation (GTR + Γ), applying the Rapid Bootstrapping algorithm with 1000 bootstrap replicates to assess node support and the search for the best-scoring maximum likelihood tree.

Author Contributions

M.B.B. and L.K.G. contributed to the conception of the study. M.B.B., L.K.G., E.E.G., F.M.R., and A.D.Y. contributed to funding and infrastructure. M.B.B., L.K.G., A.A., J.B.A., H.A., A.A., J.M.D., A.M.M., M.F.R., T.H.R., L.S.R., E.R., Y.R., H.A.R., R.N.R., S.R.A., T.V.R., and H.R.T. contributed with sample collection for the study. R.A.,. L.B., C.B., F.R., J.R., and T.R. contributed to the field logistics. E.E.G. and R.C.W. contributed to the methods in the laboratory. M.B.B., L.K.G., and E.E.G. contributed to sample and data analysis. M.B.B. and L.K.G. wrote the first draft of the manuscript, and all authors contributed to the final version. All authors read and approved the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The sequences generated in our study are available in our Supporting Material.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.
Suppl Mat cyt b sequences. Suppl Mat Fig1.