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A MULTIFACETED APPROACH TO REPTILE CONSERVATION FROM TAXONOMY AND SYSTEMATICS TO MICROBIAL ECOLOGY

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BIOLOGY

BY THE COMMITTEE CONSISTING OF

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Abstract

Roughly one in five reptile species globally is threatened with extinction as of 2022. Further, nearly 15% of described reptile species are considered Data Deficient by the IUCN and their conservation status has not yet been evaluated. Many of the world's 11,500+ known reptile species are in dire need of protection and others must first be described in the scientific literature to assess initial threats and potential conservation needs. For my dissertation research, I aim to contribute to ongoing reptile conservation efforts from several different angles. First, I help to better document the world's reptile biodiversity by describing novel species of geckos from the Philippines. Next, I examine gut microbial ecology in Philippine gekkonids to evaluate the roles of ecoevolutionary forces in shaping host-associated microbiomes. Finally, I study microbial compositions in two different reptile conservation translocation programs to determine how microbiomes respond following host introduction to novel, wild habitats. These studies expand our understanding of reptile biodiversity and help to improve methodologies used for applied reptile conservation.

In Chapter One, I use museum vouchered specimens to study a little-known group of geckos from the Philippines, Scaly-toed geckos of the genus *Lepidodactylus*. I use both morphological and molecular tools to analyze hundreds of gecko specimens collected throughout the Northern Philippines. In doing so, I identify four new species of Scaly-toed geckos and describe them formally in the scientific literature. I highlight remaining taxonomic uncertainties in the *L. yami-balioburius* clade and stress the need for additional studies to better understand *Lepidodactylus* diversity in the region. The description of these four species adds to our understanding of Philippine herpetofaunal biodiversity and lays the groundwork for future threat-assessments to evaluate the status of these previously unknown lizards.

In Chapter Two, I examine host-associated gut microbial communities in multiple gekkonid genera and species, with implications on the adaptive capacity of Philippine gekkonids to changing landscapes. I use cloacal samples collected in the field from nine gecko species to better understand the ecoevolutionary forces that influence gut microbial assemblages and whether historical or contemporary factors may shape such compositions. I identify microbial assemblages specific to each host species and note marked variation among conspecifics at distinct sampling sites, indicating that host locality influences gut microbiomes strongly. I document an interesting trend where individuals grouped as widespread and microendemic in their range tendencies regardless of host species identity display significant differences in alpha and beta diversity metrics examined. Such findings suggest certain species may have differing adaptive capacities to persist in novel or altered habitats.

In Chapter Three, I use these same microbial ecology tools to assess how gut microbial communities in both captive and wild Fijian crested iguanas (*Brachylophus vitiensis*) differ and how microbiomes respond in captive-reared hosts (e.g. those raised *ex-situ* in conservation-based facilities) after they are released into the wild. I use both cloacal swabs and fecal samples to establish an initial understanding of gut microbiomes in this IUCN Critically Endangered species. I find significant differentiation in gut microbial community composition and structure between captive and wild iguanas in both sampling schemes. Two months after captive-reared animals are released from captivity to native habitat, I show that microbial communities recovered from cloacal samples closely resemble wild counterparts. Microbial communities in fecal samples from these same individuals, however, remain significantly distinct from wild conspecifics. Interestingly, we also collected fecal samples from lizards reintroduced two years prior and which show microbiomes indistinguishable from those in wild lizards. These results

indicate that captive upbringings can lead to differences in microbial assemblages in captive-reared iguanas compared to wild individuals and such differences may persist for a time even after host reintroduction. This investigation highlights the necessity of continuous monitoring of reintroduced animals in the wild to ensure successful acclimatization after release.

Finally in Chapter 4, I again evaluate microbial compositions in reptiles from a reintroduction and translocation program. I analyze fecal samples from Texas horned lizards (*Phrynosoma cornutum*) raised at the Oklahoma City Zoo and Botanical Garden and released onto Tinker Air Force Base. I do this to identify differences in gut microbial communities between captive-reared and wild lizards and to assess whether gut microbiota in reintroduced Texas horned lizards shift to closely resemble wild counterparts following release. Within three months of reintroduction, translocated Texas horned lizard microbiomes were substantially more similar to wild counterparts than they were while housed in captivity. These results suggest reintroduced animals from captive-rearing and release programs have the capacity to rapidly alter gut microbiota to mirror microbiomes found in naturally occurring host populations. This study offers promising signs for the plasticity of microbiomes in reintroduced hosts and lays important foundations for continued evaluations of microbiomes and their impacts on an animal translocation program.

Chapter 1: Taxonomic Revision of Scaly-toed Geckos (Reptilia: Gekkonidae: *Lepidodactylus*) in the Northern Philippines, with Descriptions of Four New Species

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ABSTRACT: Recent higher-level phylogenetic analyses of gekkonid lizards of the genus *Lepidodactylus* uncovered an array of unrecognized species diversity, particularly within the Philippine archipelago. Novel phylogenetic analyses of multilocus datasets suggest as many as five previously undescribed, species-level lineages of Scaly-toed Geckos occur only in northern portions of the archipelago. Here, we evaluate *Lepidodactylus* species diversity in the *Lepidodactylus yami-balioburius* clade and describe four new forest species from Luzon Island and surrounding minor island groups. Interestingly, these species are the first endemic taxa described from Luzon proper and peripheral islands. In this first review of Philippine *Lepidodactylus* diversity in nearly half a century, we use a suite of morphological characters along with molecular data to delimit evolutionary lineages. All species described herein can be distinguished from congeners by an array of discrete external traits; all are also monophyletic groups, separated in our phylogenetic analyses of the mitochondrial ND2 gene. This study increases significantly the number of known Scaly-toed Geckos in the Philippines from seven to 11, which is likely still an underestimate of the species diversity in this understudied clade.

Key words: Biodiversity; Endemism; Luzon; Philippines; Species delimitation; Taxonomy

THE STRIKING diversity of gecko species found in the Philippines has been the subject of increased attention over the past decade (Brown et al. 2008, 2009, 2011a,b, 2020; Welton et al. 2009, 2010a,b; Linkem et al. 2010; Siler et al. 2014a, 2016a, 2017; Davis et al. 2015). Of the 58 gekkonid species now recognized from this Southeast Asian country, 18 have been described since 2009 (Uetz et al. 2020). Most recent phylogenetic studies have focused largely on three genera: *Cyrtodactylus* (Welton et al. 2009, 2010a,b), *Gekko* (Brown et al. 2008, 2009, 2011a; Linkem et al. 2010), and *Pseudogekko* (Siler et al. 2014a, 2016a, 2017; Davis et al. 2015; Brown et al. 2020), whereas the diversity within a number of other gekkonid genera in the Philippines (i.e., *Hemiphyllodactylus* and *Luperosaurus*) remains poorly understood (Brown et al. 2007, 2011b, 2012a; Grismer et al. 2013; Siler et al. 2014a). The genus *Lepidodactylus* Fitzinger 1843 is one such example of a group that has received limited taxonomic attention in the Philippines in recent years; the last comprehensive taxonomic revision was over 40 years ago (Brown and Alcala 1978). Recently, molecular phylogenetic studies have concluded that *Lepidodactylus* is closely allied with other Philippine gekkonid genera including *Gekko*, *Luperosaurus*, *Pseudogekko*, and *Ptychozoon*; all have even highlighted the paraphyletic nature of the genus with respect to the sister genera *Luperosaurus* and *Pseudogekko*, which are deeply embedded within *Lepidodactylus* (Brown et al. 2012a; Heinicke et al. 2012). However, a taxonomic reappraisal of *Lepidodactylus* species diversity in the Philippines is still lacking. Seven *Lepidodactylus* species are recognized from the archipelago with the most recent addition, *L. balioburius*, having been described 30 years ago (Duméril and Bibron 1836; Peters 1867; Stejneger 1905; Taylor 1915; Taylor 1917; Taylor 1923; Brown and Alcala 1978; Ota and Crombie 1989).

Scaly-toed Geckos of the genus *Lepidodactylus* are small-bodied species found across Southeast Asia and Oceania. Most species appear to have limited ranges along coastal habitats (Brown and Parker 1977; Brown and Alcala 1978; Bauer and Henle 1994), except for the wide-ranging *L. lugubris*, which, presumably due to its parthenogenetic reproductive mode, is thought to be native throughout most of insular Southeast Asia and the Pacific (Ota et al. 1995; Radtkey et al. 1995). With 41 species of *Lepidodactylus* recognized to date, the genus represents a diverse array of gekkonid species and the Philippines in particular, with six endemic species (*L. aureolineatus*, *L. balioburius*, *L. christiani*, *L. herrrei*, *L. labialis*, and *L. planicaudus*), and the widespread Southeast Asian taxon *L. lugubris* present, is home to one of the most varied assemblages of Scaly-toed Geckos in the world (Brown and Parker 1977; Brown and Alcala 1978; Ota and Crombie 1989; Uetz et al. 2020). Interestingly, nearly all species described to date occur in central or southern faunal regions or Pleistocene Aggerate Island Complexes of the island archipelago (PAICs; Brown and Guttman 2002; Brown et al. 2013), including Mindanao, Mindoro, and West Visayan PAICs (Brown and Parker 1977; Brown and Alcala 1978). The only exception to this is *L. balioburius* from the Batanes Island Group in the extreme northern extent of the country (Ota and Crombie 1989). Despite being the largest island in the Philippine archipelago, no species have been described from Luzon proper to date.

Over the last 15 years, our collaborative herpetofaunal surveys across the Philippines (Brown et al. 2013) have resulted in the gradual acquisition of *Lepidodactylus* specimens from the central and northern regions of the archipelago, including across Luzon Island. Assignment of such individuals to known species has proven difficult due to the morphologically conserved nature of taxa within the genus. Historical recognition of taxa based on morphological characters exclusively (Brown and Parker 1977; Brown and Alcala 1978) has led to some confusion

between specimens placed in *Lepidodactylus* and the closely related genus *Pseudogekko* (Kluge 1968; Brown and Alcala 1978; Siler et al. 2014a). To prevent further taxonomic inconsistencies, all *Lepidodactylus* specimens collected within the past 15 years have been assigned to *L.* cf. *lugubris* pending in-depth morphological and phylogenetic examination.

More recently, phylogenetic studies of Old World geckos have started to shed light on species-level relationships within *Lepidodactylus* as well as how the genus fits into the larger gekkonid tree of life (Radtkey et al. 1995; Heinicke et al. 2012; Oliver et al. 2018). As of 25 years ago, the first phylogenetic analysis inclusive of Philippine Scaly-toed Geckos provided support for the validity of *L. aureolineatus*, *L. herrei*, and *L. christiani* as distinct evolutionary lineages (Radtkey et al. 1995). Heinicke et al. (2012) included a single Philippine *L. lugubris* specimen collected in the Philippines in phylogenetic analyses focused on evolutionary relationships among multiple Asian gecko genera and found support for the widespread nature of this species.

Interestingly, it was not until a few years ago that Oliver et al. (2018) provided a comprehensive phylogeny including many of the recognized Philippine *Lepidodactylus* lineages. Although this most recent study omitted *L. labialis* due to a lack of genetic material, all other endemic *Lepidodactylus* species as well as a widespread sampling of *L. lugubris* were included. Despite the study's focus on higher-level relationships and biogeographic history of the genus, the results highlighted as many as six new, undescribed species may persist within the Philippines. Surprisingly, five of these divergent lineages are from the Luzon PAIC and were recovered as part of a clade with *L. balioburius* (Oliver et al. 2018) and *L. yami* from Lanyu Island, Taiwan, herein referred to as the *L. balioburius-yami* clade.

In this study, we examine all newly available vouchered specimens and genetic samples in

natural history collections to evaluate and revise the *L. balioburius-yami* clade in the Philippines. We employ morphological, molecular, and geographic datasets available for all Philippine *Lepidodactylus* specimens associated with the focal clade to describe four new species from Luzon Island, Lubang Island, and the Babuyan Island Group in the northern Philippines. In doing so, we provide the first in-depth investigation of the genus *Lepidodactylus* in the Philippines in almost 50 years and increase the country's diversity of Scaly-toed Geckos by over half. In contrast to past characterizations of the northern Philippines as a region without an endemic *Lepidodactylus* fauna (Brown et al. 1978) we demonstrate that this biogeographic province of the Philippines is home to a diverse, poorly-studied, highly distinct, endemic *in situ* radiation—composed of secretive forest species which may be imperiled by habitat destruction.

TAXONOMIC HISTORY

Historical Taxonomic Classifications

Lepidodactylus lugubris was first described by Duméril & Bibron (1836) as *Platydactylus lugubris* from Tahiti, Polynesia based on two female specimens. Shortly thereafter, Fitzinger (1843) described the monotypic genus *Lepidodactylus* for *L. lugubris* where the species remained until it was reassigned to *Amydosaurus lugubris* by Gray (1845) and the novel genus was sunk temporarily. The species was moved back to the genus *Platydactylus* by Cantor (1847) after examining a single *Lepidodactylus* male collected from the valley of Pinang, Malaysia. After another 20 years, Steindachner (1867) reassigned this species again from *Platydactylus* to *Gecko*.

That same year, Peters (1867) first mentioned what are now recognized as Scaly-toed Geckos in the Philippines in describing *Gecko labialis* from Mindanao Island based on a single

individual. Peters noted that the individual appeared closely related to *G. lugubris* from Tahiti (Peters 1867). The genus *Lepidodactylus* was resurrected in 1879 when *Platydactylus crepuscularis* was moved to *L. crepuscularis* in a report on geckos of New Caledonia (Sauvage 1879) and the genus expanded quickly thereafter. In an inventory of reptiles at the British Museum, Boulenger (1885) recognized the first Philippine *Lepidodactylus* when he moved both *Gecko labialis* and *Gecko lugubris* to the genus *Lepidodactylus*. At the time, only *L. labialis* was recognized from the Philippines, as *L. lugubris* was not documented officially in the country for another 45 years.

Lepidodactylus brevipes (Boettger 1897) from Samar Island and *L. planicaudus* Stejneger 1905 from Mt. Apo, Mindanao Island were both described from single specimens based on morphological distinction from known congeners and increased the number of Philippine *Lepidodactylus* to three around the turn of the 20th century.

In the early 1900s, E.H. Taylor described several additional *Lepidodactylus* species in the Philippines, including *L. aureolineatus* Taylor, 1915 from Bunauan, Mindanao Island, *L. christiani* Taylor, 1917 from Mt. Kanlaon, Negros Island, *L. divergens* Taylor, 1918 from Little Govenen Island, and *L naujanensis* Taylor, 1919 from Lake Naujan, Mindoro Island. Additionally, Taylor (1918) referenced a series of 17 specimens from Mindoro, Cancuman, Dipolod, Marongas, and Bubuan islands as the Solomon Island species *L. woodfordi* Boulenger, 1887 due to a lack of morphological differences between those specimens and the traits listed as belonging to *L. woodfordi*.

Taylor (1922) provided the first comprehensive examination of Philippine gekkonids and in this work he recognized eight species of *Lepidodactylus* at the time: *L. aureolineatus, L. brevipes, L. christiani, L. divergens, L. labialis, L. naujanensis, L. planicaudus*, and *L.*

woodfordi. The following year he described *L. herrei* Taylor, 1923 based on a single specimen from Luzurriaga, Negros Province, that was described as being closely related to *L. aureolineatus*, although different in having an apparently larger body size. Following this rise in descriptions of Scaly-toed Geckos by Taylor in the 1910s and 1920s, no new species of Philippine *Lepidodactylus* were described for over 60 years. Most work regarding the genus in the archipelago throughout the mid-20th century revolved around the validation of the nine species recognized by Taylor based on morphological features.

Specimens of *L. aureolineatus* and *L. divergens* collected by Taylor originally were re-examined by Smith (1935) and both species were collapsed into *L. lugubris* because Smith could not find a series of morphological characters by which to separate them from *L. lugubris*. As such, *L. lugubris* was considered present in the Philippines as of 1935 (Smith 1935). Another 30 years passed with no mention of *Lepidodactylus* in the Philippines before a checklist of amphibians and reptiles was released by Wermuth (1965) that agreed with these placements and recognized *L. lugubris* as a wide-ranging species in the Philippines.

Kluge (1968) reviewed the genus shortly after the release of this checklist and agreed with *L. divergens* being synonymous to *L. lugubris* but resurrected *L. aureolineatus* as a distinct lineage, citing that it was sufficiently distinct from *L. lugubris* to warrant its own species. The author went further and placed *L. christiani* as a *species inquirenda* and transferred *L. brevipes* to the genus *Pseudogekko*. Six species from the Philippines were retained by the end of the 1960s including: *L. aureolineatus*, *L. herrei*, *L. lugubris*, *L. naujanensis*, *L. planicaudus*, and *L. woodfordi*.

In the second exhaustive systematic review of Philippine gekkonids following Taylor's work in 1922, Brown and Alcala (1978) recognized four endemic species of *Lepidodactylus*: *L.*

aureolineatus, *L. christiani*, *L. herrei*, and *L. planicaudus*, as well as the widespread (non-endemic) *L. lugubris*. In this review, *Lepidodactylus naujanensis* was collapsed into *L. planicaudus* on the grounds of no morphological distinction between the two species and the presence of another distinct endemic species (*L. woodfordi*) in the Philippines was considered untenable. Brown and Alcala (1978) reassigned Taylor's (1918) series of specimens to *L. lugubris*. In addition to submerging previously recognized species, the authors reported on 30 additional specimens of the poorly understood taxon *L. labialis* and upon comparison to other *Lepidodactylus* species and *Pseudogekko brevipes*, the authors moved this species to the genus *Pseudogekko*. Brown and Alcala (1978) also named two subspecies of *L. herrei*: *L. h. herrei* and *L. h. medianus* and asserted that *L. h. medianus* possessed scale counts between *L. h. herrei* and *L. aureolineatus* and occupied a geographic area between these two congeners.

The late 1980s brought about the descriptions of the two most recent northern Philippine (and southern Taiwan) *Lepidodactylus* additions: *L. yami* Ota, 1987 from Lanyu Island, Taiwan, and *L. balioburius* from the Batanes Island Group. Though not from the Philippines, Ota (1987) considered *L. yami* a potential ancestral form that entered Lanyu Island from the Batanes and upon the description of *L. balioburius* the authors concluded that these two species may be closely related, based on shared morphological character states.

The most recent taxonomic revision to *Lepidodactylus* in the Philippines corroborated previously mentioned similarities between *Pseudogekko* and *Lepidodactylus* as Siler et al. (2014a) reverted the classification of *P. labialis* Brown and Alcala (1978), and placed the species back into the genus *Lepidodactylus*, as described originally by Boulenger (1885).

Morphological Groupings

Morphological differentiation has been the primary tool for distinguishing *Lepidodactylus* species in the Philippines for more than 150 years. Since the first account of *L. labialis* by Peters (1867), characters such as overall body size, digit size, head size, head shape, tail size, tail shape, scansor counts, supralabial and infralabial counts, and body coloration have all been used to separate species (Peters 1867; Stejneger 1905; Taylor 1922,23; Brown and Parker 1977; Brown and Alcala 1978).

Brown and Parker (1977) reviewed the entire genus *Lepidodactylus* where they recognized three species groups based primarily on morphological characters (namely scansor morphology). Group I (*L. pulmilis-oorti* group), from western Indonesia, New Guinea, islands in the Torres Straits, the Solomon and Fijian islands in the Pacific, and Christmas Island in the Indian Ocean, was considered the most primitive or "*Gekko*-like," and defined as containing only species with undivided toe scansors across the entirety of the toe. Group II (*L. guppyi-pulcher* group), from New Guinea, the Solomon, Admiralty, and Bismarck archipelagos, and Rotuma Island north of Fiji, was defined as species with undivided terminal toe scansors on all digits, but a varying number of divided subterminal scansors. Finally, Group III (*L. lugubris* group) contained *L. lugubris* and all members of the genus endemic to the Philippines. The authors recognized this group as the most derived, with divided terminal and subterminal toe scansors. In addition to scansor morphology, tail shape and the presence or absence of lateral flanges or spines were also described as diagnostic for group identification, with the tails on members of Groups I and II described as subcylindrical with no lateral flanges or spines, compared to flatter and broader tails with lateral flanges observed on members in Group III.

Brown and Alcala (1978) dove further into Group III from Brown and Parker (1977) in the second exhaustive systematic review of Philippine gekkonids following Taylor (1922) and split

Group III into two Sections: A and B. These sections are based primarily on scansor morphology again where Section A species exhibited high scansor counts (usually ≥ 12 scansors) across all digits, scansors covering most of each digit or at least the distal three-fourths of the digit, moderately to broadly dilated digits, webbing only at the base or basal one-fifth to one-fourth between Toes III and IV, and a tail that is slightly to moderately flattened without a broad flange of skin but often with modified, pointed scales on the lateral margin. In contrast, Section B contained species with a lower scansor count (usually < 10) across all digits, scansors usually covering the distal half of each digit only, broadly dilated digits, and strongly webbed about one-fourth to one-half between Toes III and IV, and a tail that was usually broad and strongly flattened with a marginal flange of skin. Based on their grouping system, Section A of Group III was comprised originally of *L. aureolineatus*, *L. herrei*, and *L. lugubris* while Section B consisted of *L. planicaudus* and *L. christiani*.

The late 1980s brought about the descriptions of the two newest species in Group III, *L.* yami from Lanyu Island, Taiwan and *L. balioburius* from the Batanes Island Group. Both species were assigned to Group III, Section A based on morphology though with some reservations in both descriptions (Ota 1987; Ota and Crombie 1989). These two species are most like each other morphologically compared to all other members of Group III and are so similar that, when describing *L. balioburius*, the authors used a suite of characters in principal component analyses to differentiate it from *L. yami* (Ota 1987; Ota and Crombie 1989).

Phylogenetic Analyses

The first phylogenetic analysis inclusive of Philippine Scaly-toed Geckos supported *Lepidodactylus aureolineatus*, *L. christiani*, and *L. herrei* as three valid, endemic species in the

archipelago (Radtkey et al. 1995). Interestingly, despite inferences based on analyses of the mitochondrial Cytochrome b gene only and limited taxonomic sampling, Radtkey et al.'s (1995) early phylogenetic study also supported some of the same morphological grouping system set forth by Brown and Parker (1977), with *L. guppyi* (Group II species) recovered as the sister lineage to the clade containing *L. lugubris* and three included Philippine species (Group III). Ota et al. (1998) in their description of *L. vanuatuensis* used the same *L. aureolineatus* sequences from Radtkey et al. (1995) and demonstrated marked genetic divergence (p-distance ~ 25%) between *L. guppyi* and *L. aureolineatus* for Cytochrome b, providing additional justification for the groups proposed by Brown and Parker (1977).

More recent studies including a greater diversity of Philippine gecko species have started to provide more resolution among relationships within *Lepidodactylus* as well as among other native genera (Heinicke et al. 2012; Siler et al. 2014b; Oliver et al. 2018; Wood et al. 2020). Heinicke et al. (2012) first recovered *Lepidodactylus* as a paraphyletic group inclusive of the genera *Pseudogekko* and *Luperosaurus*. More recently, Oliver et al. (2018) presented similar evidence of paraphyly within *Lepidodactylus*, *sensu lato*, through a robust phylogenetic analysis of six Philippine *Lepidodactylus* species and a host of other Southeast Asian gekkonids. Two clades are supported within Scaly-toed Geckos of the Philippines: 1) the *L. lugubris* clade, containing *L. lugubris*, *L. aureolineatus*, *L. herrei* (*L. h. herrei* and *L. h. medianus*), *L. planicaudus*, and an undescribed lineage referred to as *L.* sp. 5 from Zamboanga; and 2) the *L. balioburius-yami* clade, containing *L. balioburius*, *L. yami*, L. *christiani*, and five putative new lineages from the central and northern Philippines (Oliver et al. 2018). With the exception of the inferred phylogenetic placement of *L. planicaudus*, species groups defined by Brown and Alcala (1978).

MATERIALS AND METHODS

Field Work, Sample Collection, and Specimen Preservation

We conducted fieldwork on Babuyan Claro, Batan, Bohol, Calayan, Dalupiri, Leyte, Luzon, Mindanao, Negros, Polillo, and Sabtang islands in the Philippines. We collected specimens between 1600 and 0200 h and euthanized them via cardiac injection of nembutal or immersion in aqueous chloretone. We dissected specimens for genetic samples (liver or muscle preserved in 95% ethanol or flash frozen in liquid nitrogen), fixed them in 10% buffered formalin, and eventually transferred them to 70% ethanol. For all locality records, we used the WGS-84 datum. We used the museum acronyms of Sabaj (2016).

Morphological Data

We examined 196 fluid-preserved specimens for meristic, mensural, and qualitative characters using previous taxonomic revisions by Taylor (1922) and Brown and Alcala (1978) as well as phylogenetic results from Oliver et al. (2018) to guide our identification of recognized species versus novel lineages of *Lepidodactylus* (see Appendix). Sex was determined via the presence of precloacal/precloacofemoral pores and/or by gonadal inspection. For the purposes of mensural comparisons, we used sexually mature adults only. A 20% cutoff below max snout–vent length (SVL) was used to determine sexually maturity in each lineage. SJE took all measurements to the nearest 0.1 mm with Fowler Sylvac S 235 digital calipers.

We scored all characters on the left side of the body unless otherwise noted. Characters examined were based on features used in previous *Lepidodactylus* and Southeast Asian gekkonid literature (Taylor 1922; Brown and Alcala 1978; Ota 1987; Ota and Crombie 1989; Grismer et

al. 2013; Siler et al. 2014a; Eliades et al. 2019). We used a slash (/) to separate counts on the left from those on the right side of the same specimen. We used a dash for ranges of counts among specimens. Mensural characters measured include: snout–vent length (SVL, distance from tip of snout to vent); axilla–groin distance (distance between posterior edge of arm insertion and anterior edge of leg insertion); tail length (distance from posterior margin of vent to tip of tail); tail width (measured at widest section of tail posterior to hemipene bulge if present); tail depth (measured from ventral to dorsal surface of tail at the same point as tail width); snout–forearm length (distance from posterior edge of arm to a point in line with the snout tip); upper arm length (measured from arm insertion point to the elbow); forearm length (measured from elbow to base of palmar surface); arm length (sum of upper arm length and forearm length); thigh length; crus length (tibia length); leg length (sum of thigh length and crus length); Finger III length (measured from base of digit to end of digit exclusive of claw); Toe IV length (measured from base of digit to end of digit exclusive of claw); head length (from tip of snout to posterior tip of mandible); head width (widest measure of head width at middle of jaw articulations); head height (measured from ventral to dorsal surface of head at jaw articulations); eye–ear distance (from the anterior edge of auricular opening [external auditory meatus] to posterior edge of orbit); eye–nostril distance (distance from anterior margin of eye to posterior margin of nostril); snout length (distance from anterior border of orbit to tip of snout); interorbital distance (distance between midline of orbits from dorsal aspect); internarial distance (from dorsal aspect between most lateral edges of nares); ear diameter (measured at the widest diameter of the auricular opening); and eye diameter (at widest point).

Meristic characters counted include: midbody dorsal scales (number of scales running transversely across the midbody on the dorsal surface within one eye diameter), midbody ventral

scales (scales running transversely across the midbody on the ventral surface within one eye diameter), paravertebral scales (scales running longitudinally along the midbody on the dorsal surface within one eye diameter), and ventral scales (scales running longitudinally along the midbody on the ventral surface within one eye diameter); supralabials (number of enlarged supralabials, from first supralabial in contact with rostral to posterior most enlarged supralabial retaining distinct, square to rectangular shape); infralabials (number of infralabials posteriorly to the terminus of differentiation); circumorbitals (number of visible, small circumorbital scales encircling the eye); circumnasals (number of distinct scales surrounding the nostril exclusive of the rostral or supralabials); snout scales (number of scales bordering rostral excluding supralabials and including circumnasals); Finger III total scansors (number of enlarged, total scansor rows [divided and undivided] beneath Finger III, starting just distal to point where skin between digits ends exclusive of ungual scale); Finger III first divided scansor (first row of clearly divided scansors counted from basal end of digit to distal); Finger III last divided scansor (last row of clearly divided scansors along the digit); Finger III total divided scansors (total number of clearly divided scansors on the digit); Toe IV total scansors (number of enlarged, total scansor rows [divided and undivided] beneath Toe IV, starting just distal to point where skin between digits ends exclusive of ungual scale); Toe IV first divided scansor (first row of clearly divided scansors counted from basal end of digit to distal); Toe IV last divided scansor (last row of clearly divided scansors along the digit); Toe IV total divided scansors (total number of clearly divided scansors on the digit); precloacofemoral scales bearing pores (number of differentiated, enlarged, pore-bearing scales in series anterior to the cloaca and, in some specimens, extending onto femoral regions); enlarged precloacofemoral scales without pores (total number of differentiated, enlarged, scales in series anterior to the cloaca and, in some

specimens, extending into the femoral region onto femoral regions); and cloacal spurs (total number of enlarged scales on lateral sides of the base of tail).

Qualitative features examined include: pore series continuity (continuous vs. not); pore series shape (linear, v-shaped, etc.); position of the nostril (contacting the first supralabial, rostral); cleft of the rostral (cleft vs. not); ventral scale shape; degree of webbing between Toes II and III and Toes III and IV (coded from zero to five with zero being no webbing and five being webbing between entirety of digits); and body coloration in preservative and life when photos were available. To maximize utility and comparability of color descriptions, we use color terminology and referenced codes from Köhler (2012).

Morphological Analyses

We tested for sexual dimorphism within each species using Mann-Whitney U tests in R v. 3.6.2 (R Core Team 2019). For a single species comparison where no non-overlapping characters were identified, we used a principal component analysis in R to differentiate species in morphospace. We followed this analysis with a series of Mann-Whitney U tests in R examining particular morphological measures to provide statistical backing for noted differences between lineages.

Molecular Data

We extracted genomic DNA from liver samples of five *Lepidodactylus* specimens via high salt extraction (KU 331651, OMNH 46003, OMNH 44645, PNM 9874, PNM 9875). We amplified and sequenced a fragment of mitochondrial (mt) DNA using the Metf6 and CO1H primers from Macey et al. (1999) and PCR protocol from Welton et al. (2010a). Once sequenced, we trimmed the amplified region to 1,038 bp to encompass the NADH dehydrogenase subunit 2

(ND2) gene coding region only. Resulting sequences were deposited in GenBank (Accession Numbers: MW234407–11).

Phylogenetic Analyses

We used 61 additional gekkonid ND2 sequences from Oliver et al. (2018) representing six described Philippine *Lepidodactylus* species, all putative lineages suggested in Oliver et al. (2018), and sequences from *Pseudogekko brevipes*, *Lepidodactylus guppyi*, and *Gekko mindorensis* as outgroups following higher-level phylogenetic studies of gekkonid diversity (Heinicke et al. 2012; Siler et al. 2014b; Oliver et al. 2018). We trimmed all 65 total sequences to the same 1,038 bp sequence length and aligned all sequences using default parameters in MUSCLE (Edgar 2004). To identify the best-fitting model of sequence evolution for each codon position of the ND2 gene we used Akaike information criterion (AIC) in jModelTest v. 2.1.10 (Posada 2008). The model GTR + Γ was selected for each codon position. We used MrBayes v. 3.2.6 (Ronquist et al. 2012) on the Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway v. 3.3 (Miller et al. 2010) to perform partitioned Bayesian phylogenetic analyses. Two independent runs were performed for 30 million generations, both with four chains and default priors and a chain temperature set to 0.2. Trees were sampled every 3,000 generations and the first 25% of trees discarded as 'burn-in.' We viewed the resulting trace plots using Tracer v. 1.6 (Rambaut et al. 2014) to confirm stationarity. Nodes with posterior probabilities ≥0.95 we considered to have strong statistical support (Erixon et al. 2003). To support the Bayesian phylogenetic approach, we also used the Poisson Tree Processes (PTP) model to generate an additional tree modeling potential species delimitations (Zhang et al. 2013).

This tree was generated using default parameters with outgroups removed. We calculated uncorrected pairwise distances (p-distance) using PAUP* v. 4.0a (Swofford 2002).

Species Concept

Like many recent taxonomic revisions of gekkonids in the Philippines (see Siler et al. 2014a), we recognize the General Lineage Concept (de Queiroz 1998) as a continuation of the Evolutionary Species Concept (Simpson 1951; Simpson 1961; Wiley 1978). We consider lineages as distinct species based on a combination of diagnostic morphological characters, genetic distances, and in some cases, insular allopatry. Here, we have collected an extensive morphological dataset that includes representatives from all described Philippine *Lepidodactylus* and use these data in combination with phylogenetic estimates (Oliver et al. 2018) to recognize only diagnosable species of Scaly-toed Geckos of the *L. balioburius*-*L. yami* clade. Although additional phenotypic and genetic variation is apparent, we refrain from describing new species without agreement between both morphological and molecular data streams.

RESULTS

Morphology

Although we acknowledge that sample sizes are low for three of the four species described in this study, we have examined large series (≥ nine individuals) of all described Philippine *Lepidodactylus* for comparative purposes. Each of the three lineages with small sample sizes are identified readily based on suites of multiple, nonoverlapping differences in meristic and mensural characters from each other and all other known Philippine species (Table 1). No species examined in this study exhibited sexual dimorphism based on SVL. One lineage

represented with an expansive sample size from the Batanes Island chain can be separated from all Philippine species except *L. balioburius* based on non-overlapping morphological features. For this lineage, a principal component analysis (Fig. 1) weighted most heavily by SVL for PC1 and eye diameter for PC2 offers explicit distinction between its members and those of *L. balioburius* (see Ota and Crombie [1989] for previous use of PCA to distinguish species in *Lepidodactylus*). Additionally, we found that multiple body size measures including SVL, axilla–groin distance, total arm length and total leg length all differed significantly between the two lineages. Features used most commonly to discern *Lepidodactylus* species boundaries include body and head lengths, axilla–groin distance, and body, supra/infralabial, digital, and pore-bearing scale counts (Brown and Parker 1977; Brown and Alcala 1978; Siler et al. 2014a).

Phylogenetics

Discrete differences observed in ranges of morphological characters parallel results of Bayesian phylogenetic analyses (Fig. 2). The additional tree from PTP species delimitation procedures produced results similar to our analyses of morphological data and Bayesian phylogenetic analyses, although PTP proposed more extensive splitting than our most liberal interpretation based on morphology (Fig. 2). This additional delimiting is not unexpected given this model's basis on the phylogenetic species concept, and its explicit goal of inferring the smallest possible units of phylogenetic interrelatedness (Zhang et al. 2013).

Uncorrected pairwise sequence divergences show minimal variability within species described here (0.12–3.9% mtDNA divergence at ND2), whereas interspecific divergences are substantially higher (~ ≥ 10%). The exception to this general level of interspecific divergence lies within lineages found on island chains north of Luzon, including *Lepidodactylus yami, L.*

balioburius, and a novel lineage from the Babuyan Islands described herein. Although the novel species is differentiated readily from the two previously described species (6.2–7.7% mtDNA divergence), genetic divergence is less apparent between the Lanyu Island and Batanes Island chain lineages (1.7–2.7% mtDNA divergence). Interestingly, while describing *L. balioburius*, Ota and Crombie (1989) had some difficulty in identifying distinct diagnostic characters between it and *L. yami* and had to use a suite of characters in differentiation. Because we were only able to examine two juvenile *L. yami* specimens for comparisons of morphological character state differences, we are unable to definitively evaluate the validity of *L. balioburius* as distinct from *L. yami*; we hold evaluation of these two named species in abeyance and follow Ota and Crombie (1989) in recognizing both taxa until a comprehensive analysis of phenotypic variation is forthcoming.

Taxonomic Conclusions

Following examination of morphological data and consideration of molecular phylogenetic estimates, we recognize four lineages, unambiguously characterized by unique, non-overlapping suites of diagnostic morphological character state differences (Table 1) and which are distinct, genetically divergent clades (Fig. 2). All four novel lineages occur on Luzon Island, Lubang Island, or the Babuyan Island Group, all of which are part of (or geographically associated with) the Luzon PAIC in the northern Philippines.

Species Accounts

Lepidodactylus ***bisakol*** sp. nov.

(Figs. 3A, 4; Table 1)

Lepidodactylus sp. 1: Oliver et al. 2018:4.

Holotype.—PNM 9874 (formerly OMNH 46002; NAH Field No. 479), adult male, collected 17 March 2017 at 365 m on Mt. Mayon, Sitio Nagsipit, Barangay Mariroc, Municipality of Tabaco, Albay Province, Luzon Island, Philippines (13.30558°, 123.68898°), by N. A. Huron and J. B. Fernandez.

Paratype (Paratopotype).—OMNH 46003 (NAH Field No. 480), adult male, collected 14 March 2017 by N. A. Huron and J. B. Fernandez.

Other paratypes.—KU 331652 (RMB Field No. 13781), adult female, collected 16 January 2011 at 51 m in residential area on house walls in Barangay Tanawan, Municipality of Malinao, Albay Province, Luzon Island, Philippines (13.40534°, 123.67683°), by RMB. TNHC 62481 (RMB Field No. 4028), adult female, collected 23 November 2001 at 10 m in hilly and selectively logged primary rainforest near Bulusan Lake, on Mt. Bulusan, Barangay San Rogue, Municipality of Irosin, Sorsogon Province, Luzon Island, Philippines (12.752104°, 124.096736°), by RMB and B. R. Fernandez. KU 347921 (RMB Field No. 23234), adult male, and KU 348462 (RMB Field No. 23233), juvenile, collected 4 February 2017 at 260 m on Mt. Cawayan, Barangay Cawayan, Municipality of Irosin, Sorsogon Province, Luzon Island, Philippines (12.6968°, 124.0827°), by RMB and J. B. Fernandez. KU 346536 (RMB Field No. 24027), juvenile, collected 4 August 2017 at 643 m on Mt. Jormahan, Barangay Cogon, Municipality of Irosin, Sorsogon Province, Luzon Island, Philippines (12.76116°, 124.0036°), by RMB, J. B. Fernandez, M. Buehler, and C. Tracy. KU 346537 (RMB Field No. 24672), juvenile, collected 11 August 2017 at 82 m near Bayugin Falls, Barangay San Francisco, Municipality of Bulusan, Sorsogon Province, Luzon Island, Philippines (12.7586°, 124.11996°), RMB, J. B. Fernandez, M. Buehler, and C. Tracy.

Diagnosis.—*Lepidodactylus bisakol* can be distinguished from congeners by the following combination of characters: (1) body size moderate (SVL 34.5–39.2 mm); (2) thigh length moderate, 13.9–15.4% SVL; (3) total leg length moderate, 26.3–31.9% SVL; (4) head width moderate, 61.2–69.5% head length; (5) snout length short, 32.7–45.3% head length; (6) paravertebral scale count within one eye diameter 21–23; (7) midbody ventral scale count within one eye diameter 15–17; (8) ventral scale count within one eye diameter 15–18; (9) circumnasal scales 4; (10) precloacofemoral pores in males 23–27; (11) pore series shape linear; and (12) rostral scale not in contact with nostril.

Comparisons.—Characters distinguishing *Lepidodactylus bisakol* from all other species of Philippine *Lepidodactylus* are summarized in Table 1 and additional comprehensive comparisons are available in Supplemental Table 1. This new species most closely resembles *L. lugubris*; however, it differs in several characters, including having more circumnasal scales (4 vs. 3), fewer Toe IV total scansors (9–11 vs. 12–18), fewer precloacofemoral pores in males (23–27 vs. 32), a linear pore series shape (vs. v-shaped) and a rostral scale that does not contact the nostril (vs. in contact).

Considering all other Philippine congeners, *L. bisakol* can be distinguished readily from *L. aureolineatus*, *L. herrei herrei*, *L. herrei medianus*, *L. labialis*, and *L. planicaudus* by having a rostral scale separated from the nostril (vs. in contact); from *L. h. herrei* and *L. h. medianus* by havng more midbody dorsal scales (20–24 vs. < 15); from *L. babuyanensis*, *L. h. herrei*, *L. h. medianus*, *L. nakahiwalay*, and *L. bakingibut* by having more paravertebral scales (21–23 vs. < 21); from *L. aureolineatus*, *L. babuyanensis*, *L. christiani*, *L. h. herrei*, *L. h. medianus*, and *L. labialis* by having more midbody ventral scales (15–17 vs. < 15); from *L. aureolineatus*, *L. babuyanensis*, *L. h. herrei*, *L. h. medianus*, and *L. labialis* by having more ventral scales (15–18

vs < 15); from *L. bakingibut* by having more supralabial scales (13 vs. 11–12); from *L. aureolineatus*, *L. h. herrei*, *L. labialis*, and *L. planicaudus* by having more circumnasal scales (4 vs. 3); from *L. nakahiwalay* by having more total scansors on Finger III (9–12 vs. 7); from *L. h. herrei*, *L. h. medianus*, and *L. labialis* by having a moderate number of precloacofemoral pores (23–27 vs. < 14 [*L. labialis*], > 30 [*L. h. herrei*, *L. h. medianus*]); from *L. labialis* by having a linear pore series shape (vs. v-shaped), a rostral scale that is not cleft (vs. cleft), more webbing between Toes II and III (1–2 vs. 0) and more cloacal spurs (1–2 vs. 0); from *L. labialis* and *L. balioburius* by having more webbing between Toes III and IV (2–3 vs. > 2); from *L. nakahiwalay* by having a larger relative snout–forearm length (35.1–39.1% SVL vs. < 34.4%), relative crus length (12.3–16.5% SVL vs. 11.8%), and relative total leg length (26.3–31.9% SVL vs. < 26.2%); from *L. bakingibut* by having a smaller eye–nostril distance relative to head length (23.4–30.5% head length vs. > 32.3%); and from *L. aureolineatus*, *L. h. herrei*, and *L. h. medianus* by having a greater eye diameter relative to head length (21.8–25.3% head length vs. < 21.7%).

Description of holotype.—Adult male in good condition; small incision in ventral surface from retrieval of genetic sample. Body moderate, SVL 36.8 mm, axilla–groin distance 47.3% SVL; limbs well developed, moderately slender; tail original, wide, somewhat ornamental; margins of limbs smooth, lacking cutaneous flaps or dermal folds; trunk lacking ventrolateral cutaneous fold.

Head moderate in size, largely undifferentiated from neck due to hypertrophied temporal musculature; snout rounded in dorsal and lateral aspects; head length 29.6% SVL, 209.7% head height; head width 68.8% head length, 144.2% head height; snout length 37.6% head length, 54.7% head width; dorsal surfaces of head homogeneous, with only slight prefrontal and

interorbital concavities present; auricular opening large, ovoid, angled slightly anteroventrally and posterodorsally, positioned anterior to temporal swellings on either side of head; eye moderate; pupil vertical, margin straight; nostril contacting first supralabial, not contacting rostral; limbs and digits moderate in length, and moderately slender; legs longer than arms, thighs moderately thicker compared to brachium; thigh length 112.5% crus length; leg length 27.7% SVL, 136.0% arm length.

Rostral somewhat rectangular in anterior view, not cleft, bordered laterally by first supralabials, posterolaterally by anterior-most enlarged circumnasals, and posteriorly by two additional scales (= four snout scales); nostril surrounded by first supralabial and four equally sized enlarged circumnasals; supranasals separated by four heterogeneously sized median scales.

Total number of differentiated supralabials 13/12; total number of differentiated infralabials 11/12, bordered ventrally by slightly enlarged chin and undifferentiated gular scales; total number of chin scales between second and third infralabials eight; number of enlarged scale rows adjacent to chin scales one or two until fourth or fifth infralabials; patch of enlarged gular scales on anterior end of gular region continuing to a point in line with fifth infralabial on both sides.

Dorsal cephalic scales fairly homogeneous in size, shape, disposition, and distribution; cephalic scalation slightly convex, primarily round scales; prefrontal and interorbital depressions slight; undifferentiated posterior head scales slightly convex; gular and throat scales small, oval, rounded, and nonimbricate, making a moderately sharp transition in scalation towards posterior of end of neck on ventral surface, with enlarged rounded, hexagonal, non-overlapping scales; circumorbitals 38 (L). Dorsal body scales round, convex, juxtaposed, relatively homogeneous in size; dorsals gradually transition to sub-imbricate to non-overlapping ventrals along lateral body surface; midbody dorsal scales within one eye diameter 21; paravertebral scales within one eye

diameter 21; midbody ventral scales within one eye diameter 16; ventral scales within one eye diameter 16; scales on dorsal surfaces of limbs undifferentiated from dorsals; scales on dorsal surfaces of hands and feet similar to dorsal limb scales; ventral body scales flat, rounded, hexagonal, sub-imbricate to non-overlapping, much larger than dorsal body scales, relatively homogeneous in size. Enlarged precloacofemoral pore-bearing scales in a continuous, linear row 23; rectangular patch of moderately enlarged precloacal scales directly posterior to pore series and anterior to cloacal opening.

Digits moderately expanded and covered on palmar surface proximally with undivided bowed scansors and distally with divided scansors; total scansors on Finger III 10, first divided scansor on Finger III scansor eight, last divided scansor on Finger III scansor 10; total scansors on Toe IV 10, first divided scansor on Toe IV scansor eight, last divided scansor on Toe IV scansor 10; webbing between Toes II and III two, between Toes III and IV two.

Tail round, wide, length moderate, 113.9 % SVL; tail width 196.8% tail diameter; intermittent enlarged scales resembling spikes present along lateral sides; caudals slightly convex, much more sub-rectangular than dorsals, subcaudals much more rectangular than ventrals; ventrolateral ridge with intermittent, enlarged, imbricate scales present; cloacal spurs at base of tail two.

Coloration of holotype in preservative.—Dorsal surfaces of body, limbs, and tail Glaucous (Color 272) mottled with faint patches of Hair Brown (Color 277); posterior regions of head similar in color to body but transitions to Raw Umber (Color 280) anteriorly towards snout; ventral surfaces of head body and limbs Smoke Gray (Color 266) with small specks of Hair Brown (Color 277) present; ventral surface of tail shows similar coloration to body, however

with increased Hair Brown (Color 277) present; base coloration of tail gradually transitions form Smoke Gray (Color 266) to Sepia (Color 279) posteriorly.

Coloration of paratype in life.—Based on photograph of PNM 9874 in life (Fig. 3A). Dorsal surface of body Smoke Gray (Color 267) mottled with speckles of Smoky White (Color 261) to Sepia (Color 279) forming weak chevron patterning; dorsolateral coloration and dorsal surface of limbs darker than dorsal body surface, closer to Fuscus (Color 283) with speckles of Drab-Gray (Color 256) and Smoky White (Color 261); dorsal surface of head Fuscus (Color 283) with patches of Drab-Gray (Color 256) and Smoky White (Color 261); post orbital stripe Smoky White (Color 261) continues to a point in line with anterior edge of forelimb; faint stripe of Smoky White (Color 261) extending horizontally across head between center of eyes; dorsal surface of tail Fuscus (Color 283) with patches ranging from Smoky White (Color 261) to Smoke Gray (Color 267); tail coloration mostly Smoky White (Color 261) along lateral surfaces.

Measurements and scale counts of holotype (in mm).—Snout–vent length 36.8; axilla–groin distance 17.4; tail length 41.9; tail width 6.1; tail depth 3.1; snout–forearm length 14.4; upper arm length 3.0; forearm length 4.5; thigh length 5.4; crus length 4.8; Finger III length 2.6; Toe IV length 2.7; head length 10.9; head width 7.5; head height 5.2; eye–ear distance 3.3; eye–nostril distance 3.2; snout length 4.1; interorbital distance 1.2; internare distance 1.6; ear diameter 0.4; eye diameter 2.4; midbody dorsal scales 21; paravertebral scales 21; midbody ventral scales 16; ventral scales 16; supralabials 13; infralabials 11; circumorbital scales 38; circumnasals 4; snout scales 4; chin scales 8; Finger III total scansors 10; Finger III divided scansors 3; Toe IV total scansors 10; Toe IV divided scansors 4; precloacofemoral pores 23; cloacal spurs 2.

Variation.—Intraspecific variation among characters recorded is summarized in Table 1. Among the five specimens examined, we observed variation in the number of midbody dorsal, paravertebral, midbody ventral, ventral, infralabial, circumorbital, snout, and chin scales, Finger III total scansors, Finger III divided scansors, Toe IV total scansors, Toe IV divided scansors, and precloacofemoral pores in males (Supplemental Table 1).

Distribution.—*Lepidodactylus bisakol* is known from two sites approximately 80 km apart in southern portions of the Bicol Peninsula. We anticipate that additional populations exist in suitable habitat throughout Albay and Sorsogon Provinces. Future surveys could discover additional populations further north in the Bicol in Camarines Sur or Camarines Norte Provinces.

Ecology and natural history.—*Lepidodactylus bisakol* has been found in disturbed and secondary growth habitats. In some regions of its known range, substantial habitat degradation has occurred with agricultural fields fragmenting remaining forest patches. In Albay Province, individuals were observed on small tree branches and large shrubs along stream systems at low elevations on Mt. Mayon. Individuals of this species have also been found in residential areas on artificial buildings. This species is known to co-occur with the widespread *L. lugubris* in sympatry within the Municipality of Malinao, Albay Province and it may be sympatric with *L. lugubris* across most of its range. This is the first example of sympatric *Lepidodactylus* species on or near Luzon Island to date.

Etymology.—The specific epithet is chosen in reference to the biogeographically and culturally distinct Bicol Region of southern Luzon Island (Albay, Camarines Norte, Camarines Sur, Catanduañes and Sorsogon provinces). The cultural diversity on the peninsula is home to a diverse group of indigenous dialects, that are referred to collectively as the Bisakol languages.

This unique subfaunal region in the northern Philippines is home to a growing number of endemic vertebrates. Suggested common name: Bicol Scaly-toed Geckos.

Lepidodactylus ***bakingibut*** sp. nov.

(Figs. 3B, 5; Table 1)

Lepidodactylus cf. *lugubris*: Brown et al. 2013:52, their Fig. 50.

Lepidodactylus sp. 2: Oliver et al. 2018:4.

Holotype.—PNM 9875 (formerly KU 330066; RMB Field No. 14886), adult male, collected 15 July 2011 at 685 m on Mt. Cagua, Barangay Magrafil, Municipality of Gonzaga, Cagayan Province, Luzon Island, Philippines (18.236°, 122.104°), by J. B. Fernandez, L. J. Welton, C. H. Oliveros, and RMB.

Paratype.—KU 330065 (RMB Field No. 14765), adult female, collected 12 July 2011, at 785m on Mt. Cagua, Barangay Magrafil, Municipality of Gonzaga, Cagayan Province, Luzon Island, Philippines (18.219°, 122.111°), by J. B. Fernandez, L. J. Welton, C. H. Oliveros, and RMB.

Referred specimens.—PNM 7539 (RMB Field No. 4273), collected in Barangay Pancian, Municipality of Pagudpud, Ilocos Norte Province (on the boundary with Cagayan Province), Luzon Island, Philippines. PNM 8009 (ACD Field No. 1129), collected on Mt. Natib, Barangay Tala, Municipality of Orani, Bataan Province, Luzon Island, Philippines. ACD Field No. 3352, collected on Luzon Island (specific locality unknown; deposited at PNM). Although genetic data are available for these specimens (Fig. 2; Appendix; Oliver et al. 2018), morphological data were not available at the time of this investigation. As such, we assign these individuals to *L.* cf. *bakingibut* pending morphological data collection but designate them as paratypes.

Diagnosis.—*Lepidodactylus bakingibut* can be distinguished from congeners by the following combination of characters: (1) body size moderate (SVL 35.9–37.7 mm); (2) snout–forearm length moderate, 34.5–35.4% SVL; (3) forearm length moderate, 10.9–11.4% SVL; (4) thigh length moderate, 13.8–14.5% SVL; (5) crus length moderate, 13.8–14.2% SVL; (6) total leg length moderate, 27.6–28.7% SVL; (7) Finger III length long, 32.9–35.0% total arm length; (8) Toe IV length long, 35.0–39.4% total leg length; (9) head length moderate, 25.6–27.1% SVL; (10) head width moderate, 63.7–69.6% head length; (11) head height narrow, 36.3–37.0% head length; (12) eye–ear distance short, 23.5–26.1% head length; (13) snout length moderate, 41.2–42.4% head length; (14) eye diameter small, 21.6–22.8% head length; (15) midbody ventral scale count within one eye diameter 14–16; (16) ventral scale count within one eye diameter 16; (17) circumnasal scales 4; (18) Finger III total scansors 9; (19) Toe IV total scansors 9; (20) precloacofemoral pores in males 25; (21) rostral scale not in contact with nostril; and (22) webbing between Toes II and III minimal.

Comparisons.—Characters distinguishing *Lepidodactylus bakingibut* from all other species of Philippine *Lepidodactylus* are summarized in Table 1 and additional comprehensive comparisons are available in Supplemental Table 1. The new species most closely resembles *L. babuyanensis*; however, it differs in several characters, including having more midbody ventral scales (14–16 vs. 9–13), more ventral scales (16 vs. 8–12), more precloacofemoral pores (25 vs. 18–23), and a smaller thigh length relative to crus length (100.0–102.0% crus length vs. 103.8–120.8%).

Considering all other Philippine congeners, *L. bakingibut* can be distinguished readily from *L. aureolineatus*, *L. herrei herrei*, *L. herrei medianus*, *L. labialis*, *L. lugubris*, and *L. planicaudus* by having a rostral scale separated from the nostril (vs. contacting); from *L. h. herrei* and *L. h.*

medianus by having more midbody dorsal scales (19–22 vs. < 15); from *L. bisakol*, *L. h. herrei*, *L. h. medianus*, *L. labialis*, and *L. nakahiwalay* by having a moderate number of paravertebral scales (17 or 18 vs. < 16 [*L. h. herrei*, *L. h. medianus*], > 18 [*L. bisakol*, *L. labialis*, *L. nakahiwalay*]); from *L. h. herrei*, *L. h. medianus*, and *L. labialis* by having more midbody ventral scales (14–16 vs. < 13); from *L. aureolineatus*, *L. christiani*, *L. h. herrei*, *L. h. medianus*, *L. labialis*, *L. nakahiwalay*, and *L. lugubris* by having more ventral scales (16 vs. < 16); from *L. bisakol* and *L. labialis* by having fewer supralabials (11–12 vs. > 13); from *L. aureolineatus*, *L. christiani*, and *L. h. herrei* by having more circumorbital scales (32–35 vs. < 31); from *L. aureolineatus*, *L. h. herrei*, *L. labialis*, *L. lugubris*, and *L. planicaudus* by having more circumnasal scales (4 vs. 3); from *L. balioburius*, *L. h. herrei*, *L. h. medianus*, *L. labialis*, *L. lugubris*, *L. nakahiwalay*, and *L. planicaudus* by having a moderate number of precloacofemoral pores (25 vs. < 24 [*L. labialis*, *L. nakahiwalay*, *L. planicaudus*], > 30 [*L. h. herrei*, *L. h. medianus*, *L. lugubris*]); from *L. labialis* and *L. lugubris* by having a a linear pore series (vs. v-shaped); from *L. labialis* by having a rostral scale not cleft (vs. cleft) and more cloacal spurs (1 or 2 vs. 0); from *L. aureolineatus*, *L. christiani* *L. h. herrei*, *L. labialis*, and *L. planicaudus* by having minimal webbing between Toes II and III (1 vs. 0 [*L. labialis*], > 1[*L. aureolineatus*, *L. christiani*, *L. h. herrei*, *L. planicaudus*]); from *L. aureolineatus*, *L. balioburius*, and *L. labialis* by having more webbing between Toes III and IV (3 vs. < 3); from *L. aureolineatus*, *L. christiani*, and *L. h. herrei* by having a larger axilla–groin distance relative to head length (182.4–193.5% head length vs. < 177.9%); from *L. aureolineatus*, *L. balioburius*, *L. christiani*, *L. h. herrei*, *L. h. medianus*, *L. nakahiwalay*, and *L. planicaudus* by having a larger crus length relative to head length (51.0–55.4% head length vs. < 50.5%); and from *L. aureolineatus*, *L. balioburiu,s L. bisakol*, *L. christian,i L. h. herre,i L. h. medianus*, and *L.*

planicaudus by having a larger eye–nostril distance relative to head length (32.4–34.8% head length vs. < 31.7%).

Description of holotype.—Adult male in good condition; small incision in ventral surface from retrieval of genetic sample, hemipenes inverted from preservation. Body small, SVL 37.7 mm, axilla–groin distance 49.3% SVL; limbs well developed, moderately slender; tail regenerated, narrow; margins of limbs smooth, lacking cutaneous flaps or dermal folds; trunk lacking ventrolateral cutaneous fold.

Head moderate in size, largely undifferentiated from neck due to hypertrophied temporal musculature; snout rounded in dorsal and lateral aspects; head length 27.1% SVL, 275.7% head height; head width 63.7% head length, 175.7% head height; snout length 41.2% head length, 64.6% head width; dorsal surfaces of head homogeneous, with only slight prefrontal and interorbital concavities present; auricular opening large, slightly ovoid, elongated ventrally and dorsally, positioned towards posterior most point on either side of head; eye moderate; pupil vertical, margin straight; nostril contacting first supralabial, not contacting rostral; limbs and digits moderate in length, and moderately slender; legs longer than arms, thighs moderately thicker compared to brachium; thigh length 100.0% crus length; leg length 27.6% SVL, 130.0% arm length.

Rostral somewhat pentagonal in anterior view, not cleft, bordered laterally by first supralabials, posterolaterally by anterior-most enlarged circumnasals, and posteriorly by three additional scales (= five snout scales); nostril surrounded by first labial and four equally sized enlarged circumnasals; supranasals separated by four heterogeneously sized median scales.

Total number of differentiated supralabials 12/13; total number of differentiated infralabials 11/11, bordered ventrally by slightly enlarged chin and undifferentiated gular scales; total

number of chin scales between second and third infralabials 9; number of enlarged scale rows adjacent to chin scales one or two until sixth infralabials; patch of enlarged gular scales on anterior end of gular region continuing to a point in line with third infralabial on both sides.

Dorsal cephalic scales fairly homogeneous in size, shape, disposition, and distribution; cephalic scalation slightly convex, primarily round scales; prefrontal and interorbital depressions slight; undifferentiated posterior head scales slightly convex; gular and throat scales small, oval, rounded, and nonimbricate, making a somewhat gradual transition in scalation towards posterior of end of neck on ventral surface, with enlarged rounded, hexagonal, non-overlapping scales; circumorbitals 32 (L). Dorsal body scales round, convex, juxtaposed, relatively homogeneous in size; dorsals gradually transition to flat, sub-imbricate to non-overlapping ventrals along lateral body surface; midbody dorsal scales within one eye diameter 22; paravertebral scales within one eye diameter 17; midbody ventral scales within one eye diameter 14; ventral scales within one eye diameter 16; scales on dorsal surfaces of limbs undifferentiated from dorsals; scales on dorsal surfaces of hands and feet similar to dorsal limb scales; ventral body scales flat, rounded, hexagonal, sub-imbricate to non-overlapping, much larger than dorsal body scales, relatively homogeneous in size. Enlarged precloacofemoral pore-bearing scales in a continuous, linear row 25; triangular patch of moderately enlarged precloacal scales directly anterior to cloacal opening.

Digits moderately expanded and covered on palmar surface proximally with undivided bowed scansors and distally with divided scansors; total scansors on Finger III nine, first divided scansor on Finger III scansor seven, last divided scansor on Finger III scansor nine; total scansors on Toe IV nine, first divided scansor on Toe IV scansor six, last divided scansor on Toe IV scansor nine; webbing between Toes II and III one, between Toes III and IV three.

Tail round, narrow, regenerated, length short, 50.1 % SVL; tail width 170.0% tail depth; caudals slightly convex, similar in shape to dorsals, subcaudals fairly heterogeneous in shape, more rectangular than ventrals; ventrolateral ridge present but poorly defined; singular cloacal spur at base of tail.

Coloration of holotype in preservative.—Dorsal surfaces of body and limbs Glaucous (Color 272) with broken chevron patterning of Hair Brown (Color 277) running posteriorly from back of head to base of tail; dorsal surface of tail Sepia (Color 279) with small patches of Glaucous (Color 272); posterior regions of head similar in color to body but transitions to Raw Umber (Color 280) anteriorly towards snout; infralabial scale line Raw Umber (Color 280) with speckling of Smoke Gray (Color 266); ventral surface of head, body and limbs Smoke Gray (Color 266) with light speckling of Hair Brown (Color 277); ventral surface of tail Grayish Horn Color (Color 268).

Coloration of holotype in life.—Based on photograph of KU 330065 in life (Fig. 3B). Dorsal surfaces of body and limbs Smoke Gray (Color 267) with irregular chevron patterning of Grayish Horn Color (Color 268) on body from posterior end of head through to end of tail; dorsal surface of head Smoke Gray (Color 267) with speckling of Smoky White (Color 261) to Sepia (Color 286); post orbital stripe of Olive Clay Color (Color 85) runs to a point in line with the anterior edge of the forelimb; post orbital stripe has faint border of Smoky White (Color 261) above and Sepia (Color 286) below; three spots of Sepia (Color 286) present on dorsolateral surface just anterior to, even with, and posterior to forelimb insertion point; two similar dots of Sepia (Color 286) present even with, and just posterior to hind limb insertion point; intermittent patches of Sepia (Color 286) present on dorsolateral edges of tail.

Measurements and scale counts of holotype (in mm).—Snout–vent length 37.7; axilla–groin distance 18.6; tail length 18.9; tail width 5.1; tail depth 3.0; snout–forearm length 13.0; upper arm length 3.9; forearm length 4.1; thigh length 5.2; crus length 5.2; Finger III length 2.8; Toe IV length 4.1; head length 10.2; head width 6.5; head height 3.7; eye–ear distance 2.4; eye–nostril distance 3.3; snout length 4.2; interorbital distance 1.4; internare distance 1.5; ear diameter 0.6; eye diameter 2.2; midbody dorsal scales 22; paravertebral scales 17; midbody ventral scales 14; ventral scales 16; supralabials 12; infralabials 11; circumorbital scales 32; circumnasals 4; snout scales 5; chin scales 9; Finger III total scansors 9; Finger III divided scansors 3; Toe IV total scansors 9; Toe IV divided scansors 3; precloacofemoral pores 25; cloacal spurs 1.

Variation.—Morphometric variation is summarized in Table 1. Among the two specimens examined, we observed variation in the number of midbody dorsal, paravertebral ventral, midbody ventral, supralabial, infralabial, and circumorbital scales, as well as Toe IV divided scansors (Supplemental Table 1).

Distribution.—*Lepidodactylus bakingibut* is known to occur on Mt. Cagua in Cagayan Province along the northernmost extent of the Sierra Madre mountain range, and likely also occurs in north-central and northwestern Luzon Island. As individuals of this lineage have been observed in Kalinga and Ilocos Norte Provinces, *L. bakingibut* may be more widespread across the northern extent of the island. Such a distribution across northern Luzon Island has been observed in other squamate reptiles in the Philippines (i.e. *Brachymeles ilocandia*; Siler et al. 2016b).

Ecology and natural history.—The type specimens for this species were both found in mixed primary and secondary-growth rainforest at mid- to high elevation on Mt. Cagua. Other

specimens for which only genetic data were available have been observed in lower elevation habitats converted for agricultural and residential purposes.

Etymology.—The name is based on terms in the dominant Ilocano dialect of northern Luzon Island, and chosen in recognition of the biogeographically and culturally distinct region in the northern Philippines. The specific epithet is derived from the Ilocano terms "bákir," meaning forest, and "alibut," meaning lizard or gecko, in reference to the observed habitat preferences of the new species. Suggested common name: Ilokano Scaly-toed Geckos.

Lepidodactylus ***nakahiwalay*** sp. nov.

(Fig. 6; Table 1)

Lepidodactylus sp. 3: Oliver et al. 2018:4.

Holotype.—PNM 9876 (formerly KU 320411; CDS Field No. 3931), adult male, collected 29 April 2009 at 98 m in Sitio Dangay, Barangay Vigo, Municipality of Lubang, Occidental Mindoro Province, Lubang Island, Philippines (13.78304°, 120.17246°), by CDS, J. B. Fernandez, and RMB.

Paratype (Paratopotype).—KU 320410 (CDS Field No. 3930), adult female, collected 29 April 2009 by CDS, J. B. Fernandez, and RMB.

Diagnosis.—*Lepidodactylus nakahiwalay* can be distinguished from congeners by the following combination of characters: (1) body size moderate to large (SVL 40.6–40.8 mm); (2) snout–forearm length moderate, 34.2–34.3% SVL; (3) total arm length short, 18.1–20.2% SVL; (4) crus length moderate, 11.8% SVL; (5) total leg length moderate, 23.5–26.1% SVL; (6) Finger III length long, 29.7–35.4% total arm length; (7) Toe IV length moderate, 33.0–36.5% total leg length; (8) head length moderate, 26.4–26.5% SVL; (9) head width moderate, 67.3–69.4% head

length; (10) snout length long, 43.5–44.9% head length; (11) eye diameter large, 22.4–25.0% head length; (12) midbody dorsal scale count within one eye diameter 22 or 23; (13) paravertebral scale count within one eye diameter 19 or 20; (14) midbody ventral scale count within one eye diameter 14–16; (15) circumnasal scales 4; (16) Finger III total scansors 7; (17) Toe IV total scansors 8 or 9; (18) precloacofemoral pores in males 23; and (19) rostral scale not in contact with nostril.

Comparisons.—Characters distinguishing *Lepidodactylus nakahiwalay* from all other Philippine species of *Lepidodactylus* are summarized in Table 1 and additional comprehensive comparisons are available in Supplemental Table 1. The new species most closely resembles *L. bisakol*; however, it differs in several characters, including having fewer paravertebral scales (19–20 vs. 21–23), a smaller relative snout–forearm length (34.2–34.3% SVL vs. 35.1–39.1%), relative crus length (11.8% SVL vs. 12.3–16.5%), and relative total leg length (23.5–26.1% SVL vs. 26.3–31.9%), a larger Toe IV length relative to total leg length (33.0–36.5% leg length vs. 25.7–32.7%) and a larger head width relative to head height (178.6–180.0% head height vs. 144.2–176.3%).

Considering all other Philippine congeners, *L. nakahiwalay* can be distinguished readily from *L. aureolineatus*, *L. herrei herrei*, *L. herrei medianus*, *L. labialis*, *L. lugubris*, and *L. planicaudus* by having a rostral scale separated from the nostril (vs. contacting); from *L. aureolineatus*, *L. balioburius*, *L. h. herrei*, and *L. h. medianus* by having more midbody dorsal scales (22 or 23 vs. < 22); from *L. h. herrei*, *L. h. medianus*, and *L. bakingibut* by having more paravertebral scales (19 or 20 vs. < 19); from *L. babuyanensis*, *L. h. herrei*, *L. h. medianus*, and *L. labialis* by having more midbody ventral scales (14–16 vs. < 14); from *L. bakingibut* by fewer ventral scales (12–15 vs. 16); from *L. aureolineatus*, *L. christiani*, and *L. h. herrei* by having more circumorbital scales

(31–34 vs. < 31); from *L. aureolineatus*, *L. h. herrei*, *L. labialis*, *L. lugubris*, and *L. planicaudus* by having more circumnasal scales (4 vs. 3); from *L. aureolineatus*, *L. balioburius*, *L. h. herrei*, *L. h. medianus*, *L. labialis*, *L. lugubris*, and *L. bakingibut* by having fewer total scansors on Finger III (7 vs. > 7); from *L. aureolineatus*, *L. h. herrei*, *L. h. medianus*, *L. labialis*, and *L. lugubris* by having fewer total scansors on Toe IV (8 or 9 vs. > 9); from *L. aureolineatus*, *L. h. herrei*, *L. h. medianus*, *L. labialis*, *L. lugubris*, and *L. bakingibut* by having a moderate number of precloacofemoral pores in males (23 vs. < 14 [*L. labialis*], > 24 [*L. aureolineatus*, *L. h. herrei*, *L. h. medianus*, *L. lugubris*, *L. bakingibut*]); from *L. labialis* and *L. lugubris* by having a linear pore series shape (vs. v-shaped); from *L. labialis* by having no cleft on the rostral scale (vs. cleft), more webbing between Toes II and III (1 or 2 vs. 0), and more cloacal spurs (2 or 3 vs. 0); from *L. aureolineatus*, *L. christiani*, *L. h. herrei*, *L. h. medianus*, *L. lugubris*, and *L. planicaudus* by having a larger axilla–groin distance relative to head length (193.5–198.1% head length vs. < 188.2%); from *L. aureolineatus*, *L. balioburius*, *L. christiani*, *L. h. medianus*, *L. bakingibut*, and *L. lugubris* by having a smaller relative snout–forearm length (34.2–34.3% SVL vs. > 34.4%); from *L. aureolineatus*, *L. balioburius*, *L. christiani*, *L. h. herrei*, *L. h. medianus*, *L. lugubris*, and *L. planicaudus* by having a larger relative head length (26.4–26.5% SVL vs. < 26.4%); from *L. aureolineatus*, *L. h. herrei*, *L. h. medianus*, *L. lugubris*, *L. bakingibut*, and *L. planicaudus* by having a larger snout length relative to head length (43.5–44.9% head length vs. < 43.3%).

Description of holotype.—Adult male in good condition; large incision in ventral surface from retrieval of genetic sample, hemipenes inverted from preservation. Body moderate, SVL 40.6 mm, axilla–groin distance 52.2% SVL; limbs well developed, moderately slender; tail original, detached; margins of limbs smooth, lacking cutaneous flaps or dermal folds; trunk lacking ventrolateral cutaneous fold.

Head moderate in size, largely undifferentiated from neck due to hypertrophied temporal musculature; snout rounded in dorsal and lateral aspects; head length 26.4% SVL, 267.5% head height; head width 67.3% head length, 180.0% head height; snout length 44.9% head length, 66.7% head width; dorsal surfaces of head homogeneous, with only slight prefrontal and interorbital concavities present; auricular opening large, elongated, angled anteroventrally and posterodorsally, positioned anterior to temporal swellings on either side of head; eye moderate; pupil vertical, margin straight; nostril contacting first supralabial, not contacting rostral; limbs and digits moderate in length, and moderately slender; legs longer than arms, thighs moderately thicker compared to brachium; thigh length 120.8% crus length; leg length 26.1% SVL, 129.3% arm length.

Rostral somewhat rectangular in anterior view, not cleft, bordered laterally by first supralabials, posterolaterally by anterior-most enlarged circumnasals, and posteriorly by three additional scales (= five snout scales); nostril surrounded by first labial and four equally sized enlarged circumnasals; supranasals separated by four heterogeneously sized median scales.

Total number of differentiated supralabials 14/13; total number of differentiated infralabials 12/12, bordered ventrally by slightly enlarged chin and undifferentiated gular scales; total number of chin scales between second and third infralabials 9; number of enlarged scale rows adjacent to chin scales one or two until fourth infralabials; patch of enlarged gular scales on anterior end of gular region continuing to a point in line with third infralabial on both sides.

Dorsal cephalic scales fairly homogeneous in size, shape, disposition, and distribution; cephalic scalation slightly convex, primarily round scales; prefrontal and interorbital depressions slight; undifferentiated posterior head scales slightly convex; gular and throat scales small, oval, rounded, and nonimbricate, making a moderately sharp transition in scalation towards posterior

end of neck on ventral surface, with enlarged rounded, hexagonal, non-overlapping scales; circumorbitals 31 (L). Dorsal body scales round, convex, juxtaposed, relatively homogeneous in size; dorsals sharply transition to flat, non-overlapping ventrals along lateral body surface; midbody dorsal scales within one eye diameter 22; paravertebral scales within one eye diameter 19; midbody ventral scales within one eye diameter 16; ventral scales within one eye diameter 15; scales on dorsal surfaces of limbs undifferentiated from dorsals; scales on dorsal surfaces of hands and feet similar to dorsal limb scales; ventral body scales flat, rounded, hexagonal, non-overlapping, much larger than dorsal body scales, relatively homogeneous in size. Enlarged precloacofemoral pore-bearing scales in a continuous, linear row 23; rectangular patch of moderately enlarged precloacal scales directly posterior to pore series and anterior to cloacal opening.

Digits moderately expanded and covered on palmar surface proximally with undivided bowed scansors and distally with divided scansors; total scansors on Finger III seven, first divided scansor on Finger III scansor three, last divided scansor on Finger III scansor seven; total scansors on Toe IV eight, first divided scansor on Toe IV scansor six, last divided scansor on Toe IV scansor eight; webbing between Toes II and III two, between Toes III and IV three.

Tail moderately round, detached, length moderate, 100.2% SVL; tail width 156.3% tail depth; caudals slightly convex, much more sub-rectangular than dorsals, subcaudals much more rectangular than ventrals; ventrolateral ridge with intermittent, enlarged, imbricate scales present; cloacal spurs at base of tail three.

Coloration of holotype in preservative.—Dorsal surface of body and limbs Grayish Horn Color (Color 268) with chevron patterning of Brownish Olive (Color 276) and Raw Umber (Color 280) running dorsolaterally on both sides of spine; dorsal surface of head Grayish Horn

Color (Color 268) with tear-drop shaped spot of Hair Brown (Color 277) present between eyes that extends to a point in line with back of the head; dorsal surface of tail Grayish Horn Color (Color 268) with moderate striping of Brownish Olive (Color 276); ventral surface of body and limbs Smoky White (Color 261) with light speckling of Smoke Gray (Color 266) present; ventral surface of tail has a base color of Smoky White (Color 261) but gradually transitions to Smoke Gray (Color 266) base color with Brownish Olive striping (Color 276) on posterior half of tail.

Measurements and scale counts of holotype (in mm).—Snout–vent length 40.6; axilla–groin distance 21.2; tail length 40.7; tail width 5.0; tail depth 3.2; snout–forearm length 13.9; upper arm length 4.0; forearm length 4.2; thigh length 5.8; crus length 4.8; Finger III length 2.9; Toe IV length 3.5; head length 10.7; head width 7.2; head height 4.0; eye–ear distance 3.5; eye–nostril distance 3.6; snout length 4.8; interorbital distance 1.7; internare distance 1.6; ear diameter 0.4; eye diameter 2.4; midbody dorsal scales 22; paravertebral scales 19; midbody ventral scales 16; ventral scales 15; supralabials 14; infralabials 12; circumorbital scales 31; circumnasals 4; snout scales 5; chin scales 9; Finger III total scansors 7; Finger III divided scansors 5; Toe IV total scansors 8; Toe IV divided scansors 3; precloacofemoral pores 23; cloacal spurs 3.

Variation.—Variation in mensural and meristic characters is summarized in Table 1.Among the two specimens examined, we observed variation in the number of midbody dorsal, paravertebral, midbody ventral, ventral, supralabial, infralabial, circumorbital, snout, and chin scales, Finger divided III scansors, Toe IV total scansors, and Toe IV divided scansors (Supplemental Table 1).

Distribution.—*Lepidodactylus nakahiwalay* occurs on Lubang Island in the Occidental Mindoro Province. This lineage may occur throughout the Lubang Island Group and may be

found on surrounding Ambil, Cabra, and Golo islands however current distribution is restricted to Lubang Island exclusively. It is unlikely that this lineage expands beyond the Lubang Island Group; examination of a *Lepidodactylus* specimen from Subic Bay on nearby Luzon Island suggests evolutionary distinction between localities.

Ecology and natural history.—*Lepidodactylus nakahiwalay* has only been observed in well-established secondary-growth rainforest habitats and has yet to be observed in more disturbed agricultural or residential areas along the coasts of the island. Like other members of the genus, this arboreal species was observed primarily on small branches or trunks of trees in the forest as well as along small stream systems.

Etymology.—The specific epithet is derived from the Tagalog term for isolated and is in reference to the biogeographically distinct and isolated island of Lubang which is believed to be surrounded by deep-ocean channels and never in historical contact with surrounding paleoisland landmasses. Suggested common name: Lubang Scaly-toed Geckos.

Lepidodactylus ***babuyanensis*** sp. nov.

(Figs. 3C, 7; Table 1)

Lepidodactylus sp. 4: Oliver et al. 2018:4.

Holotype.—PNM 9877 (formerly OMNH 46971; CDS Field No. 9198), adult male, collected 27 May 2018 at 72 m in Barangay Magsidel, Municipality of Calayan, Cagayan Province, Calayan Island, Philippines (19.27482°, 121.44701°), by CDS, K. Wang, J. Brown, E. D. Ellsworth, and S. N. Smith.

Paratypes (Paratopotypes).—OMNH 46977, 46978, 46979, 46982, 46989 adult males, OMNH 46980, 46981, 46983 46993, 47001 adult females collected 26 May 2018; OMNH

46970, adult male, OMNH 46972, 46973 adult females collected 27 May 2018; OMNH 47002 juvenile, OMNH 46974 subadult female, OMNH 46985 adult male, OMNH 46975, OMNH 46976, 46984, 46986, 46987, 46988, adult females collected 28 May 2018; OMNH 46992 adult male, OMNH 46990, 46991 adult females collected 29 May 2018; OMNH 47004 adult female collected 31 May 2018; OMNH 47005 adult female collected 31 May 2018; OMNH 47006 adult female collected 1 June 2018; OMNH 47007 subadult male collected 1 June 2018; OMNH 46994 subadult male, OMNH 47003 adult female collected 2 June 2018; OMNH 46996 subadult female, OMNH 46997, 46998, 46999, 47000, adult males, OMNH 46995 adult female collected 3 June 2018 by CDS, K. Wang, J. Brown, E. D. Ellsworth, and S. N. Smith.

Other paratypes.—KU 304603 (RMB Field No. 5723) subadult male, collected 6 March 2006, KU 304713 (RMB Filed No. 5834) subadult female, collected 9 March 2006 in Barangay Balatabat, Municipality of Calayan, Cagayan Province, Camiguin Island, Philippines. KU 306610 (RMB Field No. 6359), subadult male, collected 13 September 2006, KU 306755 (RMB Field No. 6388), juvenile, collected 15 September 2006, at Nipa Creek, Municipality of Calayan, Cagayan Province, Dalupiri Island, Philippines by J. B. Fernandez.

Diagnosis.—*Lepidodactylus babuyanensis* can be distinguished from congeners by the following combination of characters: (1) body size small (SVL 31.9–39.3 mm); (2) snout–forearm length short, 31.0–39.8% SVL; (3) total arm length short, 18.7–23.3% SVL; (4) total leg length short, 23.4–31.4% SVL; (5) head length short, 24.7–31.9% SVL; (6) Finger III divided scansors 3; (7) precloacofemoral pores in males 18–23; and (8) rostral scale not in contact with the nostril.

Comparisons.—Characters distinguishing *Lepidodactylus babuyanensis* from all other Philippine species of *Lepidodactylus* are summarized in Table 1 and additional comprehensive

comparisons are available in Supplemental Table 1. The new species appears to be quite cryptic in phenotype when compared to *L. balioburius*, with little distinction observed among morpholometric characters traditionally employed in systematic studies of the genus. However, examination of robust series of both *L. babuyanensis* (*n* = 40) and *L. balioburius* (*n* = 16) reveal that *L. babuyanensis* has tendencies towards being a larger species compared to *L. balioburius*, including a larger body size (maximum SVL 39.3 mm, mean SVL 35.1 mm vs. 35.0 mm, 32.4 mm), a larger relative axilla–groin distance (55.4% SVL, 50.9% SVL vs. 53.2%, 48.0%), a larger axilla–groin distance relative to snout–forearm length (174.0% snout–forearm length, 150.3% snout–forearm length vs. 144.3%, 129.8%), and a smaller eye diameter relative to head length (22% head length, 20.2% head length vs. 23.5%, 21.5%). Additionally, principal component analysis does recover some degree of separation between these lineages, primarily based on body size (Fig. 1).

Considering all other Philippine congeners, *L. babuyanensis* can be distinguished readily from *L. aureolineatus*, *L. herrei herrei*, *L. herrei medianus*, *L. labialis*, *L. lugubris*, and *L. planicaudus* by having a rostral scale separated from the nostril (vs. contacting); from *L. h. herrei* and *L. h. medianus* by having more midbody dorsal scales (15–22 vs. < 15); from *L. h. herrei* and *L. bisakol* by having a moderate number of paravertebral scales (15–20 vs. < 13 [*L. h. herrei*], > 20 [*L. bisakol*]); from *L. bisakol*, *L. nakahiwalay*, and *L. bakingibut* by having fewer midbody ventral scales (9–13 vs. > 13); from *L. bisakol* and *L. bakingibut* by having fewer ventral scales (8–12 vs. > 14); from *L. lugubris* by having fewer total scansors on Finger III (7–11 vs. > 11); from *L. h. herrei* and *L. planicaudus* by having fewer divided scansors on Finger III (3 vs. > 3); from *L. aureolineatus*, *L. h. herrei*, *L. h.medianus*, *L. lugubris*, *L. labialis*, and *L. bakingibut* by having a moderate number of precloacofemoral pores (18–23 vs. < 14 [*L. labialis*],

> 24 [*L. aureolineatus*, *L. h. herrei*, *L. h. medianus*, *L. lugubris*, *L. bakingibut*]); from *L. labialis* and *L. lugubris* by having a linear pore series shape (vs. v-shaped); from *L. labialis* by having no cleft on the rostral scale (vs. cleft) and more webbing between Toes III and IV (1–3 vs. 0); from *L. bakingibut* by having a larger thigh length relative to crus length (103.8–120.8% crus length vs. 100.0–102.0%); and from *L. christiani* by having a larger Finger III length relative to total arm length (21.7–35.5% arm length vs. < 21.4%).

Description of holotype.—Adult male in good condition; large incision in ventral surface from retrieval of genetic sample. Body small, SVL 37.3 mm, axilla–groin distance 50.1% SVL; limbs well developed, moderately slender; tail original; margins of limbs smooth, lacking cutaneous flaps or dermal folds; trunk lacking ventrolateral cutaneous fold.

Head moderate in size, largely undifferentiated from neck due to hypertrophied temporal musculature; snout rounded in dorsal and lateral aspects; head length 26.8% SVL, 285.7% head height; head width 70.0% head length, 200.0% head height; snout length 42.0% head length, 60.0% head width; dorsal surfaces of head homogeneous, prefrontal and interorbital concavities absent; auricular opening large, ovoid, angled slightly anteroventrally and posterodorsally, positioned anterior to temporal swellings on either side of head; eye small; pupil vertical, margin straight; nostril contacting first supralabial, not contacting rostral; limbs and digits moderate in length, and moderately slender; legs longer than arms, thighs moderately thicker compared to brachium; thigh length 104.1% crus length; leg length 26.8% SVL, 128.2% arm length.

Rostral pentagonal in anterior view, not cleft, bordered laterally by first supralabials, posterolaterally by anterior-most enlarged circumnasals, and posteriorly by four heterogeneously-sized additional scales (= six snout scales); nostril surrounded by first labial and

four equally sized enlarged circumnasals; supranasals separated by four homogeneously sized median scales.

Total number of differentiated supralabials 13/12; total number of differentiated infralabials 12/11, bordered ventrally by slightly enlarged chin and undifferentiated gular scales; total number of chin scales between second and third infralabials 10; number of enlarged scale rows adjacent to chin scales one until second or third infralabials.

Dorsal cephalic scales fairly homogeneous in size, shape, disposition, and distribution; cephalic scalation slightly convex, primarily round scales; prefrontal and interorbital depressions slight; undifferentiated posterior head scales slightly convex, slightly smaller than cephalic scales; gular and throat scales small, oval, rounded, and nonimbricate, making a gradual transition in scalation towards posterior of end of neck on ventral surface, with enlarged rounded, hexagonal, non-overlapping scales; circumorbitals 31 (L). Dorsal body scales round, slightly convex, juxtaposed, relatively homogeneous in size; dorsals sharply transition to flat, non-overlapping ventrals along lateral body surface; midbody dorsal scales within one eye diameter 18; paravertebral scales within one eye diameter 17; midbody ventral scales within one eye diameter 10; ventral scales within one eye diameter 10; scales on dorsal surfaces of limbs undifferentiated from dorsals; scales on dorsal surfaces of hands and feet similar to dorsal limb scales; ventral body scales flat, rounded, elongated, sub-imbricate to non-overlapping, much larger than dorsal body scales, relatively homogeneous in size. Enlarged precloacofemoral pore-bearing scales in a continuous, linear row 20; triangular patch of moderately enlarged precloacal scales directly posterior to pore series and anterior to cloacal opening.

Digits moderately expanded and covered on palmar surface proximally with undivided bowed scansors and distally with divided scansors; total scansors on Finger III nine, first divided

scansor on Finger III scansor seven, last divided scansor on Finger III scansor nine; total scansors on Toe IV seven, first divided scansor on Toe IV scansor five, last divided scansor on Toe IV scansor seven; webbing between Toes II and III two, between Toes III and IV three.

Tail moderately round, wide, length long, 119.3% SVL; tail width 143.3% tail depth; caudals slightly convex, sub-imbricate to overlapping, much more rectangular than dorsals, subcaudals much more rectangular than ventrals, anterior to posterior in direction; ventrolateral ridge with intermittent, enlarged, imbricate scales present; cloacal spurs at base of tail three.

Coloration of holotype in preservative.—Dorsal surface of body, limbs, and tail a mix of Smoke Gray (Color 266) and Grayish Horn (Color 268); dorsal surface of head similar in coloration to body except for large spot of Sepia (Color 279) extending a point in line with back of the eyes to a point in line with back of head; ventral surface of head, body, limbs, and tail all Smoky White (Color 261).

Coloration of paratype in life.—Based on photograph of OMNH 46977 in life (Fig. 3C). Dorsal surface of head, body, limbs, and tail mostly Drab (Color 19) to Smoke Gray (Color 266) with minimal speckling of Olive-Brown (Color 278) and Sepia (Color 279); very faint chevron patterning present along dorsal surface; faint post orbital stripe of Cream Color (Color 12) with minimal speckling of Sepia (Color 286) extends to a point in line with the posterior end of the head.

Measurements and scale counts of holotype (in mm).—Snout–vent length 37.3; axilla–groin distance 18.7; tail length 44.5; tail width 4.3; tail depth 3.0; snout–forearm length 11.8; upper arm length 3.8; forearm length 4.0; thigh length 5.1; crus length 4.9; Finger III length 2.4; Toe IV length 3.4; head length 10.0; head width 7.0; head height 3.5; eye–ear distance 2.6; eye–nostril distance 2.9; snout length 4.2; interorbital distance 1.4; internare distance 1.4; ear

diameter 0.7; eye diameter 2.0; midbody dorsal scales 18; paravertebral scales 17; midbody ventral scales 10; ventral scales 10; supralabials 13; infralabials 12; circumorbital scales 31; circumnasals 4; snout scales 6; chin scales 10; Finger III total scansors 9; Finger III divided scansors 3; Toe IV total scansors 7; Toe IV divided scansors 3; precloacofemoral pores 20; cloacal spurs 3.

Variation.—Morphometric variation within this series is summarized in Table 1. Among the 40 specimens examined, we observed variation in the number of midbody dorsal, paravertebral, midbody ventral, ventral, supralabial, infralabial, circumorbital, snout, and chin scales, Finger III total scansors, Toe IV total scansors, and precloacofemoral pores in males (Supplemental Table 1).

Distribution.—*Lepidodactylus babuyanensis* occurs throughout the Babuyan Island Group in Cagayan Province. Individuals have been collected from Calayan, Camiguin, and Dalupiri islands and we anticipate this lineage also inhabits Fuga and Babuyan islands as well.

Ecology and natural history.—*Lepidodactylus babuyanensis* has been found in patchwork secondary-growth rainforest habitat on multiple islands in the Babuyan Island Group. This species appears to be common in secondary-growth habitats, particularly on Calayan Island, where a large series of individuals were observed during our recent biodiversity surveys. *Lycodon alcalai* is a known predator of this species at least on Calayan Island (Griffing et al. 2019).

Etymology.—The specific epithet is chosen in reference to the biogeographically unique Babuyan Island Group of the northern Philippines, located in the Luzon Strait. The small archipelago is composed of five major islands (Babuyan Claro, Calayan, Camiguin Norte, Dalupiri, and Fuga), as well as associated small islets (Fig. 8). The Babuyan Island Group is

surrounded by deep-ocean channels and believed to have never been in historical contact with surrounding paleoisland landmasses. As such, the island group is home to a number of endemic vertebrate species. Suggested common name: Babuyan Scaly-toed Geckos.

DISCUSSION

The four species described here bring the total number of recognized Scaly-toed Geckos endemic to the Philippines to 11. Interestingly, *Lepidodactylus bisakol* and *L. bakingibut* represent the first endemic species described from Luzon Island proper, with *L. babuyanensis* and *L. nakahiwalay* described from small, peripheral, deep-water islands in close proximity to the Luzon PAIC (Calayan, Camiguin Norte, and Dalupiri islands, to the north in the Babuyan Island Complex or Lubang Island to the southwest). In addition to the Batan-Sabtang endemic (*L. balioburius*), the Orchid Island population (*L. yami*), plus probable species *L.* sp. 6 and *L.* sp. 7, this entire clade of as many as eight species has gone nearly unstudied for over the last half century (Brown and Alcala 1978; Ota 1987; Ota and Crombie 1989). These findings, of endemic Luzon PAIC taxa, stand in contrast to the earlier characterization of the Luzon fauna region as a biogeographic entity with no native *Lepidodactylus* fauna (Brown and Alcala 1978). In addition to the members of this novel clade characterized here we anticipate that, with targeted field work (Brown et al. 2012b, 2013; Devon-Song and Brown 2012) focused on *Lepidodactylus* microhabitats, and collection of genetic and phenotypic data, the large, geographically complex island of Luzon will eventually be recognized as home to greater—as of yet unsampled and unrecognized—species diversity (Siler et al. 2011; Brown et al. 2011b, 2013, 2020; Siler et al. 2014a).

Phylogenetic studies suggest that all four species described here are members of Brown and Parker's morphological Group III (1977), and two distinct phenotypic groups of *Lepidodactylus* do exist in the archipelago, as first recognized by Brown and Alcala (1978). However, phylogenetic evidence suggests that splitting Philippine *Lepidodactylus* into two sections, based on morphological characters alone, may lead to erroneous understandings of relationships. As opposed to retaining the sections "A" and "B" of Brown and Alcala (1978), we recognize instead the *L. lugubris* and *L. yami-balioburius* clades. Based on our phylogenetic results, the *L. lugubris* clade contains *L. aureolineatus, L. herrei, L. lugubris*, and *L. planicaudus*, whereas the *L. yami-balioburius* clade contains the two namesake species, *L. christiani*, and all four lineages described here. We abstain from placing of *L. labialis* into either clade, pending future availability of vouchered genetic material (Sanguila et al. 2016).

Although phylogenetic relationships within the *L. yami-balioburius* clade are clearer following this study, several uncertainties remain; these warrant further investigation. Oliver et al. (2018) provided genetic evidence for additional lineages in the *L. yami-balioburius* clade (*L.* sp. 6) and even from other nearby and distant insular nations (Fig. 2). *Lepidodactylus* sp. 6, for example, from Bulacan Province likely represents yet another distinct lineage from Luzon. However, at the time of this study, we were unable to obtain specimens of these putative lineages for examination of phenotypic variation and, therefore, cannot draw conclusions on their validity as potential species.

Additionally, one individual previously assigned to *L.* sp. 3 (KU 327768; Oliver et al. 2018) from Subic Bay on Luzon Island shows ~13% sequence divergence at the mitochondrial ND2 gene from the two *L. nakahiwalay* type specimens. Furthermore, phylogenetic analyses failed to show strong support for the Subic Bay and Lubang Island populations as a monophyletic group.

Unfortunately, due to the availability of a single specimen only from the Subic Bay population, we are not able to evaluate fully the distinctiveness of this lineage and reference it as *L.* sp. 7 pending the collection of additional vouchered material. Given this individual's genetic divergence and the recognized biogeographic distinctiveness of this region of Luzon Island from the deep-ocean island of Lubang (Brown and Diesmos, 2009; Devon-Song and Brown 2012), it would not be surprising if the population from Subic is a distinct species.

Finally, although *L. babuyanensis* is genetically distinct from both *L. yami* and *L. balioburius*, the latter two species show limited genetic differentiation from each other (< 2.7%). Further complicating this situation, two individuals from Sabtang Island in the Batanes Island chain, assigned to *L. balioburius* (KU 314011, 314012), exhibit less genetic distance from *L. yami* (~2.0–2.1%) than they do from other members of *L. balioburius* from Batan Island (~3.0–3.9%; Fig. 2). This is particularly puzzling because Sabtang is located less than 5 km away from Batan (the type locality of *L. balioburius*) and is over 170 km away from Lanyu (the type locality of *L. yami*; Fig. 7). Ota and Crombie (1989) struggled to find a single morphological trait to distinguish the two species and, instead, relied on a suite of traits for their diagnosis of *L. balioburius*. Now with phylogenetic evidence, the distinction between *L. yami* and *L. balioburius* is even less clear. In the description of *L. balioburius* (Ota and Crombie, 1989), type specimens were examined from Batan Island only; our expanded sampling and examinations of specimens from throughout the Batanes and Babuyan Islands may find the two to be conspecific. Due to our limited sample size for *L. yami*, we could not address the validity of *L. balioburius* and *L. yami* as distinct entities at this time.

A biogeographic link between Luzon and Lanyu herpetofauna has been documented previously in skinks (Ota and Huang, 2000) and even another gekkonid species in *Gekko kikuchii*

(Siler et al. 2014c). Interestingly, both examples are believed to be conspecific with individuals from Luzon Island and possible recent introductions (Ota and Huang, 2000; Siler et al. 2014c). The presence of *L. yami* on Lanyu Island, a potential conspecific in *L. balioburius* in the Batanes Islands, and a discreet species in *L. babuyanensis* in the Babuyan Island chain provides an intriguing opportunity to study the dispersal and speciation of herpetofauna across deep ocean barriers in this intervening biogeographic region (Fig. 7). Along with untangling taxonomic relationships between *L. yami* and *L. balioburius*, future studies could examine the directionality with which gekkonid species have moved historically between the Philippines and Taiwan and investigate modalities of faunal exchange between deep-oceanic geographic barriers (Siler et al. 2014c).

Despite this investigation, clarifying a small number of taxonomic uncertainties among Philippine *Lepidodactylus*, new systematic issues need further scrutiny to comprehend fully the relationships among Scaly-toed Geckos in the Philippines. Currently recognized taxa need further validation, and putative, novel lineages suggested by genetic data, should be explored. With over 20 gekkonid species having been described from the Philippines in the past decade alone (Uetz et al. 2020), our understanding of species diversity is improving rapidly, but still leaves many questions (Brown et al. 2020). Greater insight into Philippine gekkonid evolutionary lineage diversity will allow for novel, higher-level phylogenetic analyses (Wood et al. 2020) which must further explore relationships among the genera *Lepidodactylus*, *Luperosaurus*, and *Pseudogekko*. In depth, densely sampled future studies of these focal clades provide particularly promising opportunities for improving our understanding of reptile diversification patterns across the complex landscape of the Philippine archipelago.

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APPENDIX

Specimens Examined

Numbers in parentheses following species names indicate the number of specimens examined. Several sample sizes are greater than those observed in the description due to the examination of subadult specimens which were excluded from morphometric analyses.

Lepidodactylus aureolineatus (9): MINDANAO ISLAND, Agusan Province, Bunauan (MCZ R–26109–R–26117).

Lepidodactylus babuyanensis (42): CALAYAN ISLAND, Cagayan Province, Municipality of Calayan, Barangay Magsidel (Holotype PNM 9877, formerly OMNH 46971), (Paratopotypes OMNH 46970–47003); Sitio Longog (Paratypes OMNH 47004–47007); CAMIGUIN ISLAND, Cagayan Province, Municipality of Calayan, Barangay Balatabat (Paratypes KU 304603, 304713); DALUPIRI ISLAND, Cagayan Province, Municipality of Calayan, Nipa Creek (Paratypes KU 306610, 306755).

Lepidodactylus bakingibut (2): LUZON ISLAND, Cagayan Province, Municipality of Gonzaga, Barangay Magrafil, Mt. Cagua, (Holotype PNM 9875, formerly KU 330066), (Paratype KU 330065).

Lepidodactylus balioburius (22): BATAN ISLAND, Batanes Province, Municipality of Basco (KU 314000–314008), Municipality of Ivana (KU 314019, 314020), Municipality of Mahatao (KU 326207); SABTANG ISLAND, Batanes Province, Municipality of Sabtang (KU 314009–314018).

Lepidodactylus bisakol (8): LUZON ISLAND, Albay Province, Municipality of Tabaco, Barangay Mariroc, Sitio Nagsipit (Holotype PNM 9874, formerly OMNH 46002), (Paratopotype OMNH 46003), Municipality of Malinao, Barangay Tanawan (Paratype KU 331652); Sorsogon Province, Municipality of Irosin, Barangay San Rogue, Bulusan Lake, on Mt. Bulusan (Paratype TNHC 62481); Barangay Cawayan, Mt. Cawayan (Paratypes KU 347921, 348462); Barangay Cogon, Mt. Jormahan (Paratype KU 346536); Municipality of Bulusan, Barangay San Francisco, Bayugin Falls (Paratype KU 346537).

Lepidodactylus christiani (14): NEGROS ISLAND, Negros Oriental Province (CAS–SUR 24246–24250, CAS 129326, 129335, 129351, 129352, 133058, 133059), Municipality of Sibulan (CAS 128877–128879).

Lepidodactylus herrei herrei (18): NEGROS ISLAND, Negros Oriental Province (CAS 129297, 129298, 129353–129355, 129376, 129377, 132661–132667, 132675); Municipality of Valencia, Barangay Bongbong, Cuernos de Negros Mountain Range, Mt. Talinis (KU 327769, TNHC 62476, 62477).

Lepidodactylus herrei medianus (16): CEBU ISLAND, Cebu Province, Cebu City (CAS–SUR 27302, CAS 125239–125242, 140036, 140037), Municipality of Carmen (CAS–SUR 24813, CAS 131821), Municipality of Dalaguete (CAS 128434, 129047, 129063, 129064), Municipality of Minglanilla (CAS 185693); PORO ISLAND, Cebu Province, Municipality of Poro (CAS 125126, 125127).

Lepidodactylus labialis (15): MINDANAO ISLAND, Agusan del Norte Province, Municipality of Cabadbaran (CAS 133209, 133210, 133238, 133243, 133258, 133314–133317, 133329, 133338, 133339, 133353–133356, 133790).

Lepidodactylus lugubris (20): GREAT AND LITTLE GOVENEN ISLANDS, Basilan Province (MCZ R–26087, R–26088, R–26092, R–26093, R–85747–R–85750); BASILAN ISLAND, Basilan Province (CAS 60507, 60508, 60510, 60513–60518, 60520); LUZON ISLAND, Albay Province, Municipality of Malinao (KU 331651, 331653).

Lepidodactylus nakahiwalay (2): LUBANG ISLAND, Occidental Mindoro Province, Municipality of Lubang, Barangay Vigo, Sitio Dangay (Holotype PNM 9876, formerly KU 320411), (Paratopotype KU 320410).

Lepidodactylus planicaudus (15): MINDANAO ISLAND, Cotobato or Sulturan Kudarat Province, Tatayan to Saub, Cotobato coast (MCZ R–26094–R–26099, R–26101, R–26102, R–163938, R–163939, R–163941, R–163943–R–163945); Davao del Sur Province, Mt. Apo (KU 327715).

Lepidodactylus yami (2): *TAIWAN*, LANYU ISLAND, Imoro (USNM 267944), Lung Men (USNM 291811).

Lepidodactylus sp. 7 (1): LUZON ISLAND, Zambales Province, Municipality of Olongapo, Subic Bay (KU 327768).

1 TABLE 1.—Summary of morphological characters in Philippine species of *Lepidodactylus*. In parentheses, mean ± one standard deviation follows ranges.

	aureolineatus (4 m, 4 f)	*babuyanensis* (17 m, 23 f)	*balioburius* (7 m, 9 f)	*bakingibut* (1 m, 1 f)	*bisakol* (3 m, 2 f)	*christiani* (10 m, 2 f)
Snout–vent length (SVL)	32.7–37.8 (35.6 ± 1.9)	31.9–39.3 (35.1 ± 1.9)	28.1–35.0 (32.4 ± 1.8)	35.9, 37.7	34.5–39.2 (36.9 ± 1.7)	33.1–39.0 (36.2 ± 1.8)
Axilla–groin distance/SVL	45.0–53.1% (47.0 ± 3.2%)	43.5–55.4% (50.9 ± 2.7%)	39.8–53.2% (48.0 ± 3.7%)	49.3, 49.6%	47.3–54.2% (50.4 ± 3.3%)	41.6–52.5% (48.4 ± 2.7%)
Snout–forearm length/SVL	37.9–41.5% (39.8 ± 1.3%)	31.0–39.8% (34.0 ± 2.0%)	34.6–40.1% (37.0 ± 1.4%)	34.5, 35.4%	35.1–39.1% (36.6 ± 1.6%)	34.9–41.4% (37.9 ± 1.9%)
Total arm length/SVL	22.3–25.7% (24.3 ± 1.2%)	18.7–23.3% (20.7 ± 1.2%)	18.3–24.4% (20.9 ± 1.7%)	21.2%	18.1–29.6% (21.2 ± 4.7%)	20.9–28.1% (24.4 ± 1.9%)
Total leg length/SVL	28.7–32.4% (30.5 ± 1.1%)	23.4–31.4% (27.9 ± 1.6%)	25.4–28.7% (27.1 ± 1.2%)	27.6, 28.7%	26.3–31.9% (28.2 ± 2.2%)	26.2–32.0% (29.1 ± 1.8%)
Midbody dorsal scales	16–20 (17.9 ± 1.3)	16–21 (18.7 ± 1.8)	20–24 (21.3 ± 1.9)	19, 22	20–24 (21.8 ± 2.0)	18–22 (20.1 ± 1.3)
Midbody ventral scales	9–14 (12.6 ± 1.8)	9–13 (11.0 ± 1.1)	10–16 (12.1 ± 1.7)	14, 16 (7.4 ± 1.1)	15–17 (16.2 ± 0.8)	12–14 (12.8 ± 0.8)
Total pores (in males)	25–31 (27.5 ± 2.5)	18–23 (20.7 ± 1.5)	19–23 (21.1 ± 1.5)	25	23–27 (25.0 ± 2.8)	20–27 (24.3 ± 2.3)
Circumnasal scales	3	3 or 4	4	4	4	4
Rostral contacting nares	yes	no	no	no	no	no

TABLE 1.—(*continued*).

	herrei herrei (6 m, 2 f)	*herrei medianus* (8 m, 3 f)	*labialis* (8 m, 7 f)	*lugubris* (1 m, 14 f)	*nakahiwalay* (1 m, 1 f)	*planicaudus* (5 m, 17 f)
Snout–vent length (SVL)	41.6–50.8 (46.0 ± 2.6)	38.1–44.7 (41.4 ± 2.2)	42.5–52.8 (47.9 ± 3.2)	36.1–44.0 (39.7 ± 2.3)	40.6, 40.8	29.1–37.6 (32.2 ± 2.5)
Axilla–groin distance/SVL	45.2–50.8% (47.8 ± 2.0%)	42.3–54.7% (46.6 ± 3.4%)	45.9–56.4% (50.8 ± 2.7%)	39.6–51.8% (45.2 ± 3.7%)	51.2, 52.2%	33.9–50.5% (43.4 ± 4.7%)
Snout–forearm length/SVL	31.3–38.4% (36.8 ± 2.3%)	35.2–43.2% (38.8 ± 2.5%)	31.5–38.1% (34.8 ± 2.1%)	34.5–43.1% (38.9 ± 2.4%)	34.2, 34.3%	32.2–39.6% (37.3 ± 2.0%)
Total arm length/SVL	20.8–23.8% (22.3 ± 1.1%)	20.0–24.7% (22.6 ± 1.4%)	20.2–26.5% (23.9 ± 1.7%)	19.1–25.6% (22.9 ± 2.0%)	18.1, 20.2%	16.2–22.0% (20.0 ± 1.6%)
Total leg length/SVL	26.6–31.3% (28.7 ± 1.5%)	28.2–32.9% (30.3 ± 1.5%)	25.5–31.8% (27.9 ± 1.6%)	26.0–33.0% (29.5 ± 1.9%)	23.5, 26.1%	24.8–30.9% (27.1 ± 1.6%)
Midbody dorsal scales	9–11 (9.9 ± 0.8)	11–14 (12.5 ± 1)	18–24 (20.5 ± 1.9)	16–22 (19.1 ± 2.0)	22, 23	17–27 (20.8 ± 2.6)
Midbody ventral scales	9–11 (10 ± 0.8)	9–12 (10.7 ± 1.0)	9–12 (10.2 ± 0.7)	10–15 (12.5 ± 1.4)	14, 16	11–16 (13.8 ± 1.4)
Total pores (in males)	31–37 (34.2 ± 1.9)	33–39 (36.7 ± 2.1)	11–13 (12 ± 0.8)	32	23	18–23 (20.3 ± 2.2)
Circumnasal scales	3	3 or 4	3	3	4	3
Rostral contacting nares	yes	yes	yes	yes	no	yes

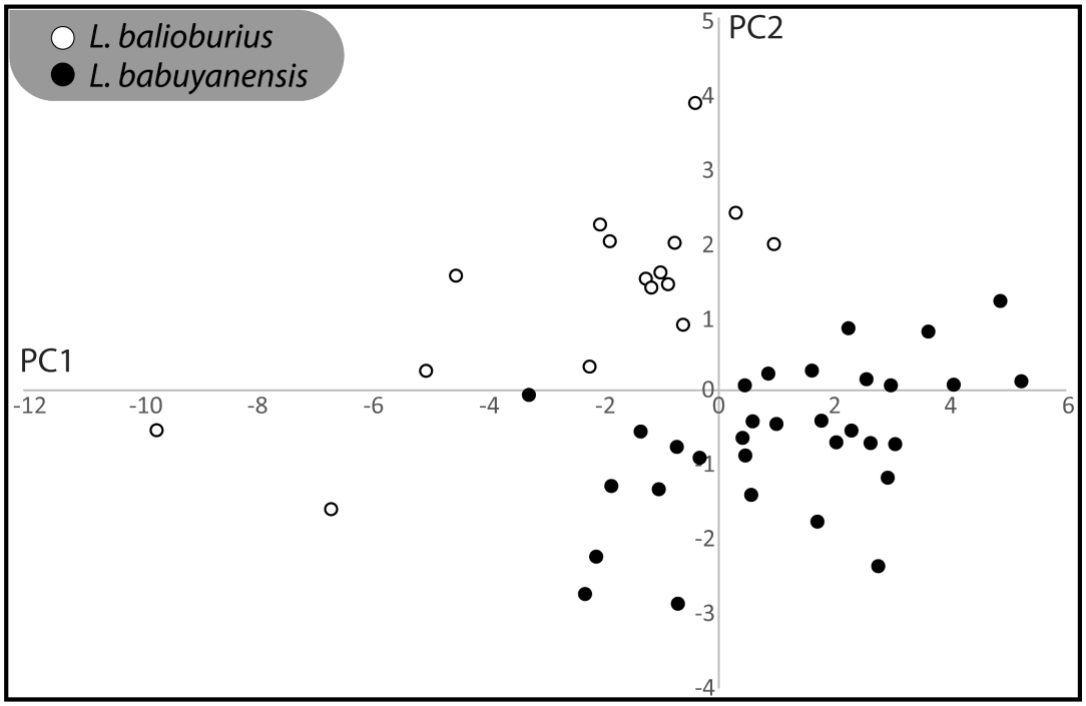

FIG. 1.—Two-dimensional principal component analysis comparing 21 meristic morphological features of adult *Lepidodactylus balioburius* and *L. babuyanensis* specimens.

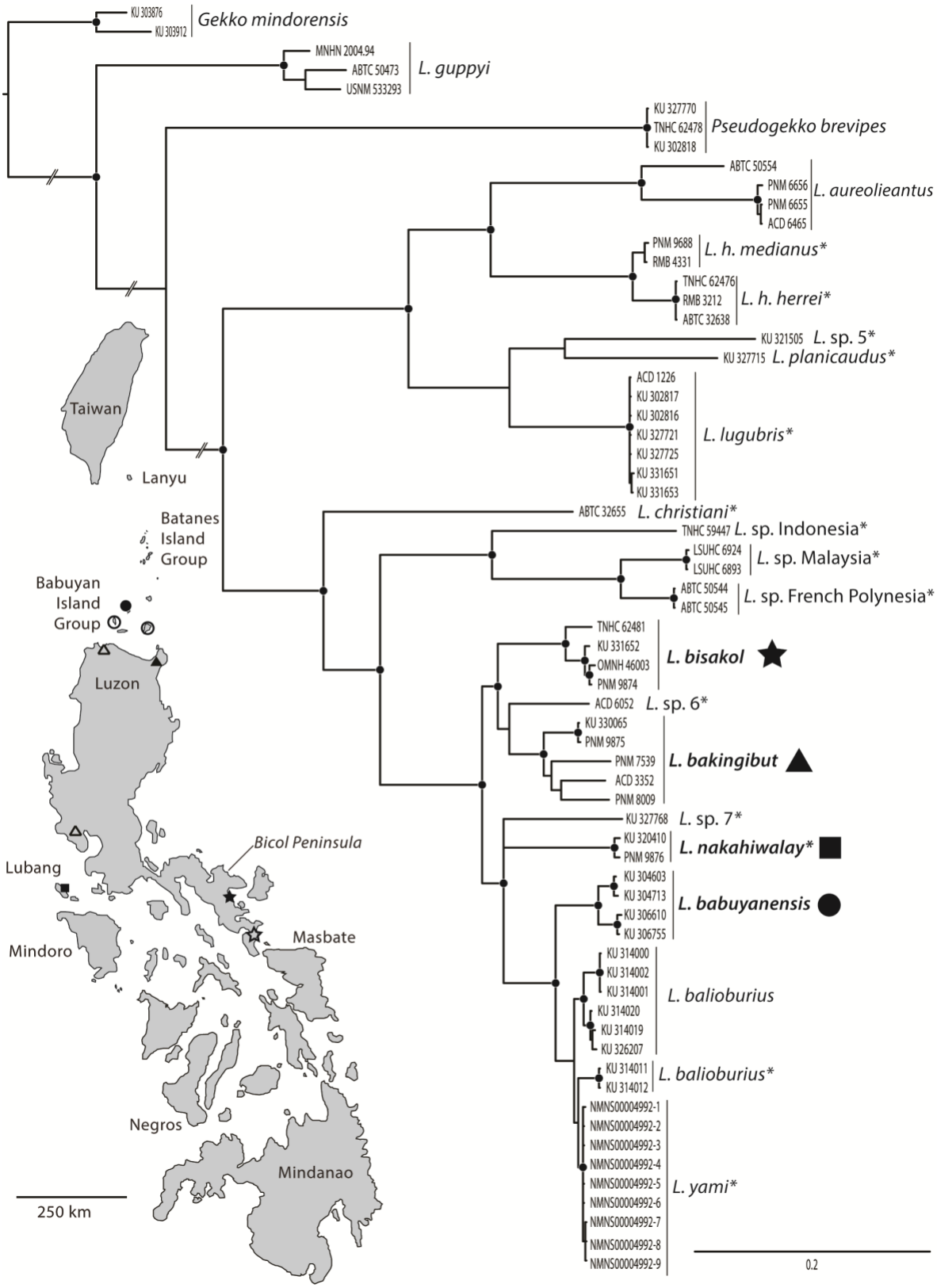

FIG. 2.—Maximum clade credibility topology resulting from Bayesian analysis of the

mitochondrial ND2 coding region for geckos of the genus *Lepidodactylus*. *Gekko mindorensis*, *Lepidodactylus guppyi*, and *Pseudogekko brevipes* samples were used as outgroups following higher level phylogenetic analyses (Oliver et al. 2018). Black circles at nodes indicate Bayesian posterior probabilities ≥ 0.95; nodes shown without circles were supported by posterior probabilities < 0.95. Asterisks following taxonomic names on the topology denote lineages delimited in Poisson Tree Processes (PTP) modeling analysis; taxa without asterisks were subdivided by PTP analysis. A reduced map of the Philippine islands is presented on the bottom left, showing the location of type localities of the four species described herein by shapes matching those denoted on the topology. Filled shapes denote type localities while open shapes represent paratypes and/or referred specimens.

FIG. 3.—Photographic plate of (A) *Lepidodactylus bisakol* (holotype PNM 9874), (B) *Lepidodactylus bakingibut* (paratype KU 330065), and (C) *Lepidodactylus babuyanensis* (paratype OMNH 46977) in life.

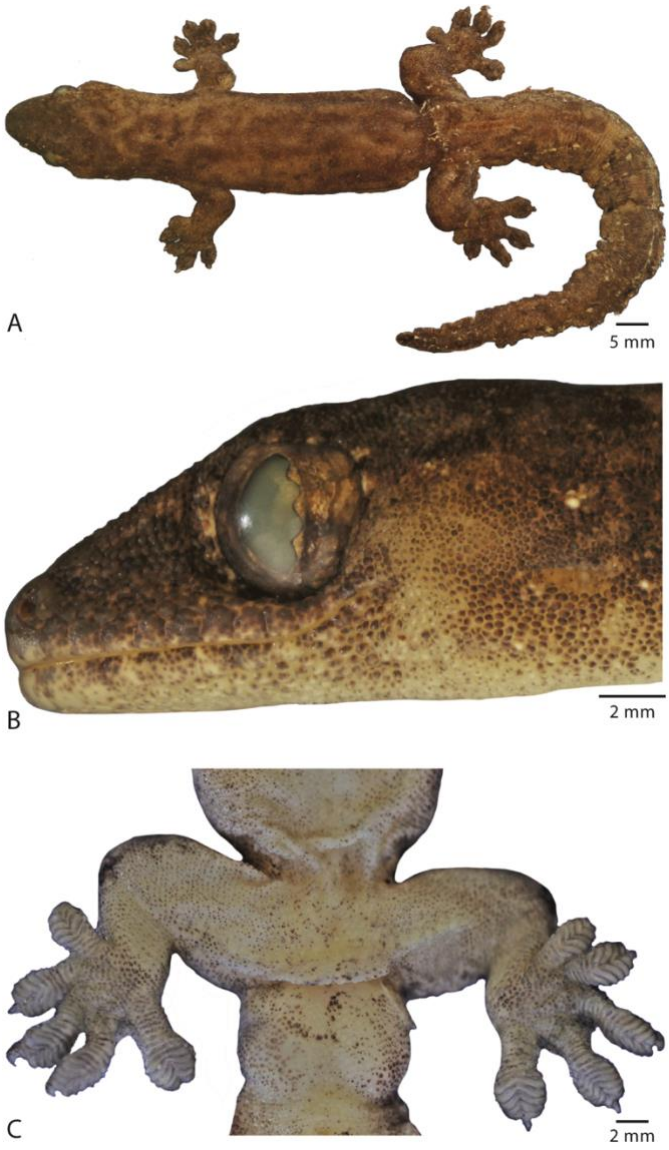

FIG. 4.—Dorsal body (A), head (B), and cloacal region (C) of the holotype of *Lepidodactylus bisakol*.

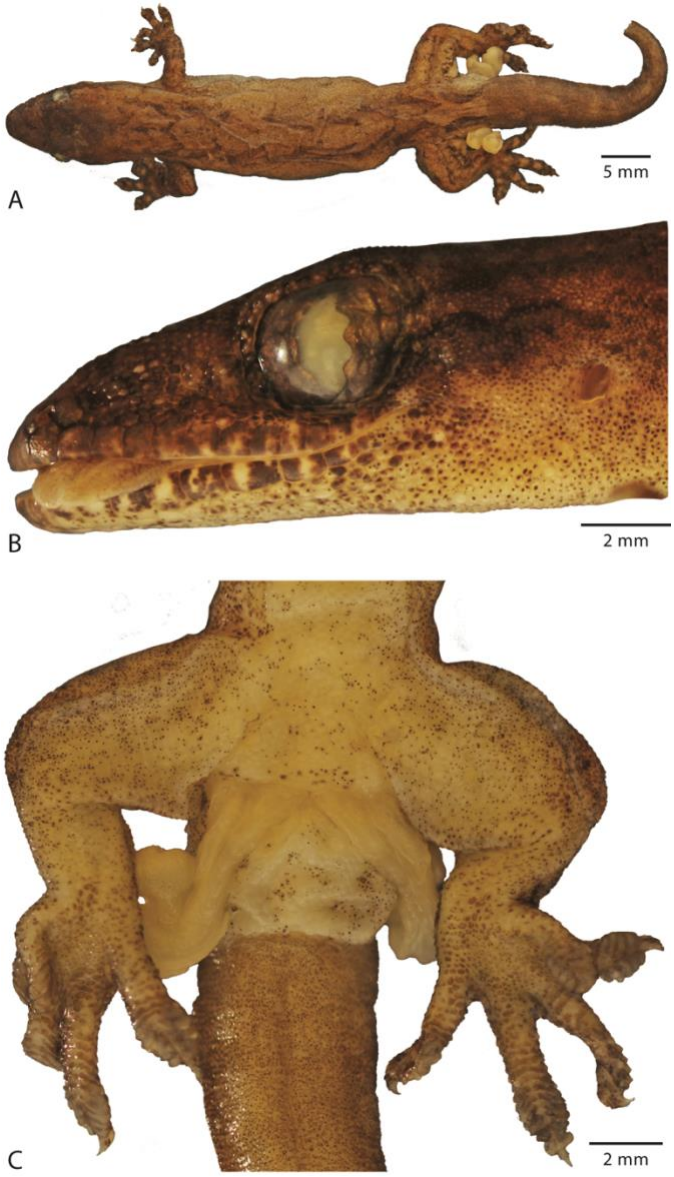

FIG. 5.—Dorsal body (A), head (B), and cloacal region (C) of the holotype of *Lepidodactylus bakingibut*.

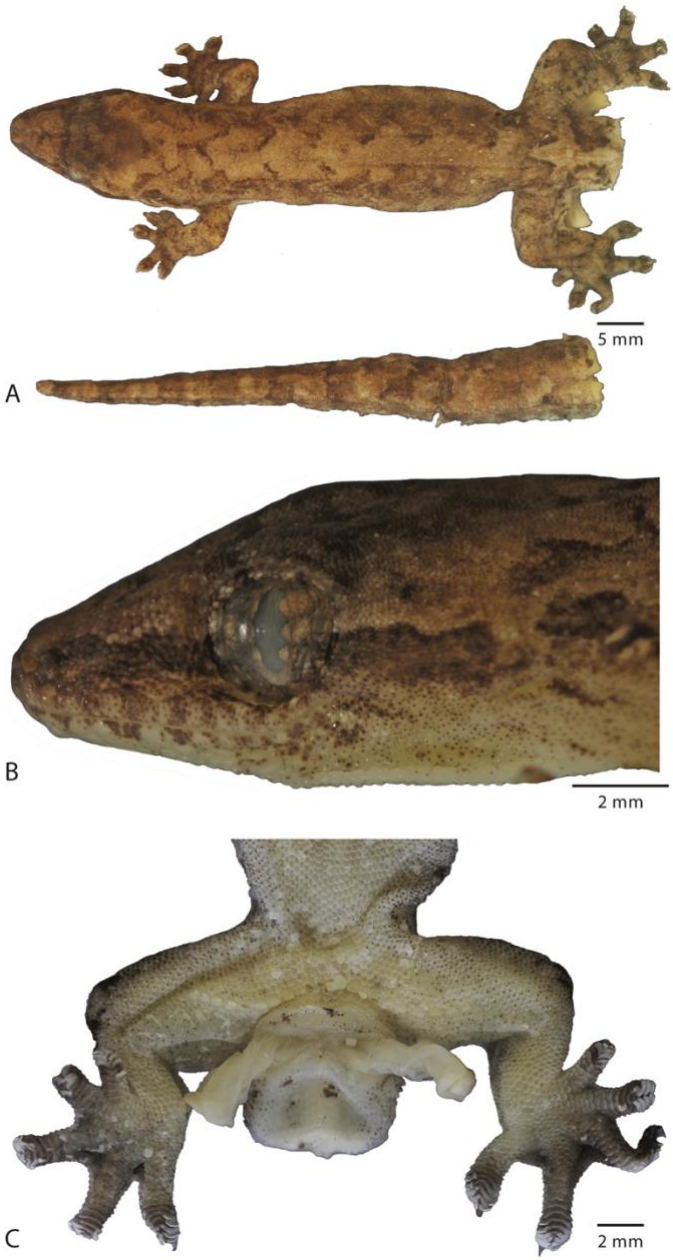

FIG. 6.—Dorsal body (A), head (B), and cloacal region (C) of the holotype of *Lepidodactylus nakahiwalay*.

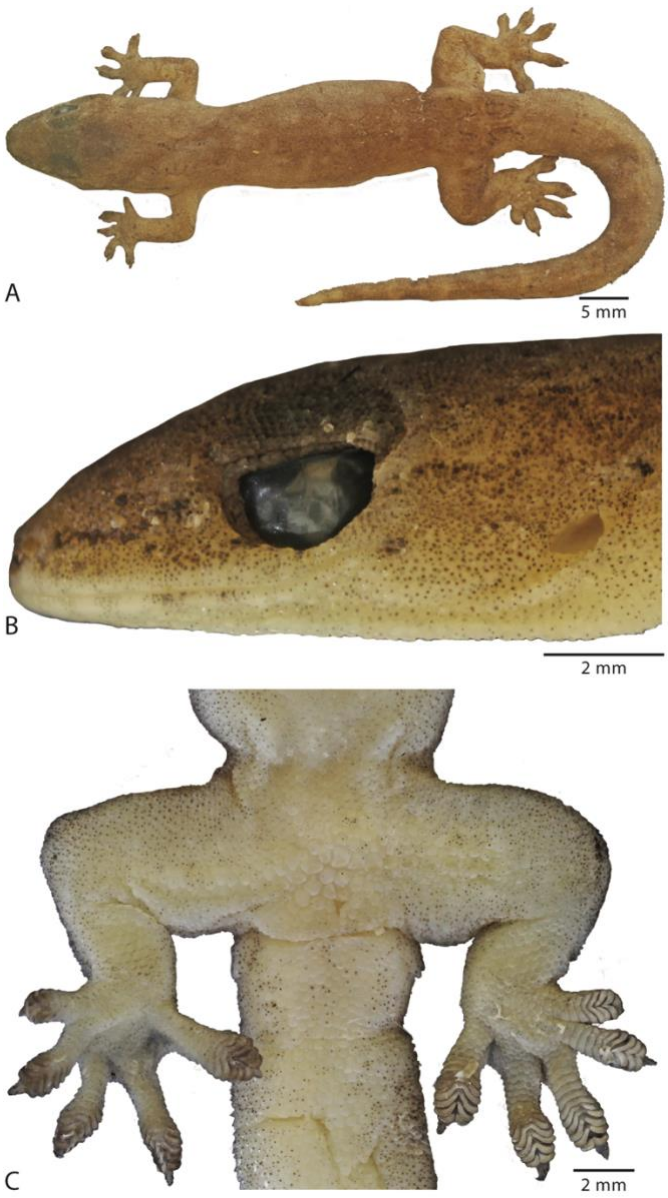

FIG. 7.—Dorsal body (A), head (B), and cloacal region (C) of the holotype of *Lepidodactylus babuyanensis*.

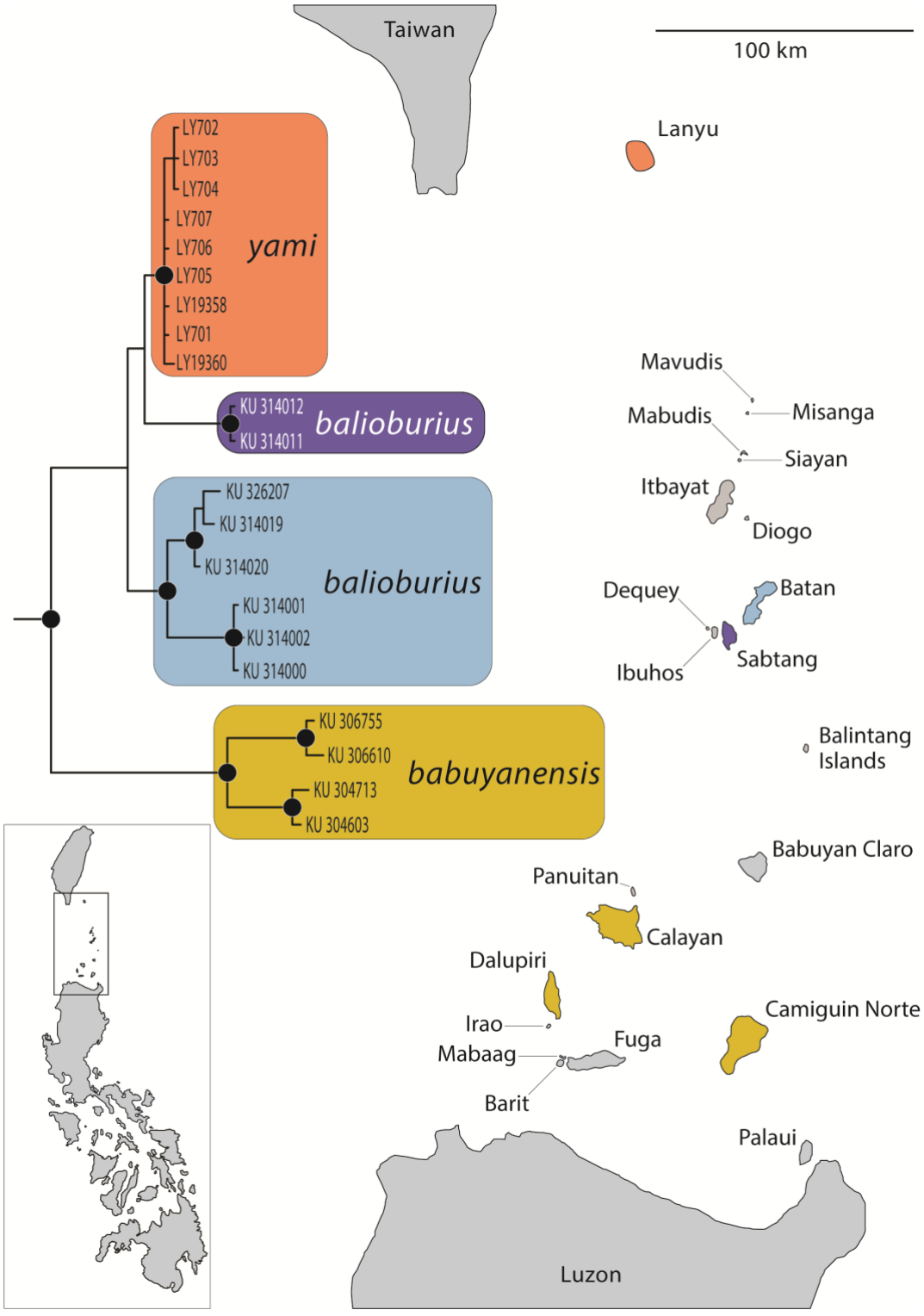

FIG. 8.—Island distributions of sampled individuals of *Lepidodactylus babuyanensis*, *L. balioburius*, and *L. yami* across the northern Philippines and southern Taiwan. Color-coded

clades correspond to the islands of specimen origination. Note the non-monophyly of *L. balioburius* populations from neighboring Batan and Sabtang islands. Asterisks following taxonomic names on the topology denote lineages delimited in PTP modeling analysis; taxa without asterisks were subdivided by PTP analysis.

Chapter 2: Gut microbial ecology of Philippine gekkonids: ecoevolutionary effects on microbiome compositions

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Abstract

Given the rapidly changing landscapes of habitats across the globe, a sound understanding of host-associated microbial communities and the ecoevolutionary forces that shape them is needed to assess general organismal adaptability. Knowledge of the symbiotic endogenous microbiomes of most reptilian species worldwide remains limited. We sampled gut microbiomes of geckos spanning nine species and four genera in the Philippines to (i) provide baseline data on gut microbiota in these host species, (ii) test for significant associations between host phylogenetic relationships and observed microbial assemblages, potentially indicative of phylosymbiosis, and (iii) identify correlations between multiple ecoevolutionary factors (*e.g.* species identity, habitat tendencies, range extents, and maximum body sizes) and gut microbiomes in Philippine gekkonids. We recovered no significant association between interspecific host genetic distances and observed gut microbiomes, providing limited evidence for phylosymbiosis in this group. Philippine gekkonid microbiomes were associated most heavily with host species identity, though marked variation among conspecifics at distinct sampling sites indicates that host locality influences gut microbiomes as well. Interestingly, individuals grouped as widespread and microendemic regardless of host species identity displayed significant differences in alpha and

beta diversity metrics examined, likely driven by differences in rare OTU presence between groups. These results provide much needed insight in host-associated microbiomes in wild reptiles and the ecoevolutionary forces that structure such communities.

Introduction

Endogenous microbial communities inhabiting vertebrate and invertebrate hosts are increasingly recognized as essential in maintaining organismal well-being, influencing a variety of traits from host development and behavior to immune response and metabolism (Fraune and Bosch 2010; Cho and Blaser 2012; Lee and Hase 2014). Furthermore, these gut microbiomes likely contribute to host phenotypic plasticity, allowing for rapid adaptation to changing environments (Alberdi *et al*. 2016; Bahrndorff *et al*. 2016; Littleford-Colquhoun *et al*. 2019). Given the dramatic alteration of habitats globally during the Anthropocene, a sound understanding of host-associated microbial communities and the forces that influence them is needed to predict general organismal adaptability to future conditions (Amato 2013; Alberdi *et al*. 2016; Stumpf *et al*. 2016; Trevelline *et al*. 2019; Zhu *et al*. 2021).

At a broad taxonomic scale (generally at the level of family or higher), gut microbial communities often mirror phylogenetic relationships among hosts; a phenomenon known as phylosymbiosis (Ley *et al*. 2008; Amato 2013; Sanders *et al*. 2014; Groussin *et al*. 2017; Youngblut *et al*. 2019; Lim & Bordenstein 2020). Identifying signs of phylosymbiosis is a requisite first step towards understanding the ecoevolutionary forces that drive observed assemblages (Lim & Bordenstein 2020). Evidence of this association, however, varies considerably at differing taxonomic levels. For example, phylosymbiosis is supported among turtle ants of the genus *Cephalotes*, where gut microbiota are strongly correlative with host

phylogenetic relationships (Sanders *et al.* 2014). Similarly, significant associations between host phylogenetic affinities and microbial communities have been noted among all seven sea turtle species (Scheelings *et al.* 2020) and among 51 species of passerine birds (Kropackova *et al.* 2017). Interestingly though, among passerine bird microbiomes examined, most microbial variation in assemblages remains unexplained after accounting for host phylogeny, and factors operating at the within-species level are suspected of contributing to most individual variance (Kopackova *et al.* 2017). Other studies find less conclusive evidence for the presence of phylosymbiosis. In lizards of the genus *Anolis* and among 31 species of Afrotropical bats, significant, yet weak, associations between host phylogenetic relationships and microbial compositions have been recovered (Ren *et al.* 2016; Lutz *et al.* 2019). In both such instances, host microbial assemblages in individuals are believed to be influenced more by contemporary ecological features rather than phylogenetic ones (Ren *et al.* 2016; Lutz *et al.* 2019). Additional information is needed to better disentangle the influences of various ecoevolutionary factors on observed host-associated microbial communities, particularly within taxonomic groups that have received limited attention from host-associated microbial studies to date.

Reptiles represent one of the most speciose vertebrate groups on the planet, with over 11 000 recognized lineages distributed across all continents except Antarctica (Uetz, Freed, and Hošek 2021). These species vary tremendously in body morphologies, habitat preferences, reproductive strategies and more (Vitt and Caldwell 2013). Despite a striking array of species diversity and a subcosmopolitan distribution, relatively little is known about the symbiotic gut microbiomes of most reptilian species worldwide (Colston and Jackson 2016; Kohl *et al.* 2017). A critical facet of reptile microbiome research in particular need of further investigation pertains to the ecological and evolutionary traits that structure these gut communities.

In the few studies that examine multiple reptile species to date, host taxon identity is a prominent indicator of microbial assemblages, with interspecific differences in microbiome compositions generally greater than intraspecific distinctions (Lankau *et al*. 2012; Ren *et al*. 2016; Kohl *et al*. 2017). Analyses of host ecomorphs recover mixed findings. Galapagos land and marine iguanas, which differ significantly in diet, show significantly distinct microbiomes (Lankau *et al*. 2012) though few features distinguish various Caribbean anole ecomorphs, which all tend to be generalist species (Ren *et al*. 2016). Within species, individual diet has clear influences on gut microbiota in reptiles (Lankau *et al*. 2012; Ren *et al*. 2016; Jiang *et al*. 2017; Kohl *et al*. 2017). Host locality shows strong correlations with microbial compositions in Puerto Rican anoles (Ren *et al*. 2016) and both Galapagos land and marine iguanas (Lankau *et al*. 2012) but no such significant correlations have been noted in gopher tortoises across the southeastern United States (Gaillard 2014). Host internal microbial community dynamics clearly can be influenced strongly across both ecological and evolutionary scales (Lankau *et al*. 2012).

In this study, we sought to better understand gut microbial community diversity and structure in reptiles using a unique study system, wild gekkonid lizards in the Philippines. The insular nation of the Philippines in Southeast Asia is home to a remarkable array of reptilian diversity and is considered a global hotspot for reptiles (Mittermeier *et al*. 1999; Roll *et al*. 2017). Over 350 species can be found across the ~7,500 islands in the Philippines (Uetz, Freed, and Hošek 2021). The Philippines archipelago is home to a spectacular assortment of reptile species diversity in part because of its complex geographic history. Seven Pleistocene Aggregate Island Complexes (PAICs; Brown *et al*. 2013a) are generally recognized though many of these PAICS can be divided further still into various endemic biogeographic and even sub-faunal regions of native flora and fauna (Heany 1993; Vallejo 2014). In this complex landscape, geckos represent

one of the most taxonomically diverse groups of all vertebrates with 49 species described across multiple genera (Uetz, Freed, and Hošek 2021). Precise dietary information for all gekkonid species in the Philippines is lacking, though most are thought to be insectivorous (Bauer 2013; Goldberg *et al*. 2016). Although sharing generalist dietary strategies, Philippine gekkonids display a wide variety of body sizes, distributions, and hypothesized habitat preferences to accompany their phylogenetic distinctiveness (Brown *et al*. 2008,2009,2010,2011,2013b; Welton *et al*. 2010). The marked array of evolutionarily distinct lineages of gekkonids coupled with microendemics and widespread species across the Philippines provide an exceptional study system to test for phylosymbiosis among confamilials and to complete ecoevolutionary comparisons of reptile hosts and their microbial assemblages.

We sampled gut microbial communities in 47 individual geckos from nine species and four genera in the Philippines to (i) provide baseline data on endogenous microbiota in these host species, (ii) test for evidence of phylosymbiosis; microbial community relationships that parallel phylogenetic relationships among gekkonid hosts at the family level, and (iii) test for correlations between broad ecoevolutionary factors and gut microbial community compositions in wild gekkonids, including host species: identity, range, habitat preferences, and maximum body size as well as individual sampling locality and sampling biogeographic region in the Philippines.

Materials and Methods

Host species examined

We analyzed gut microbial communities sampled via cloacal swabbing from 47 wild gekkonid lizards. These lizards represent the following nine species and four genera from the Philippines: *Cyrtodactylus philippinicus* (*n* = 12), *Gekko crombota* (*n* = 7), *G. gecko* (*n* = 4), *G. kikuchii* (*n* =

1), *G. mindorensis* (*n* = 4), *G. rossi* (*n* = 9), *Hemidactylus frenatus* (*n* = 3), *H. platyurus* (*n* = 3), and *Luperosaurus macgregori* (*n* = 4).

Sampling localities

To better address the possible influences of locality-specific factors on gut microbiota in wild gekkonids, we sampled hosts opportunistically at seven distinct localities in the central and northern Philippines. These sites were spread across four discrete biogeographic regions: the Babuyan Islands, northern Luzon, the Bicol Peninsula, and Negros Island. We conducted fieldwork on Calayan and Camiguin Norte (Babuyan Island Group), Luzon, and Negros islands in the Philippines during three field expeditions carried out between 2016 and 2018. Individual sampling localities included Magsidel and Tapao Falls in the Babuyan Island Group, Mariroc and Tulay na Lupa on the Bicol Peninsula of Luzon, Mt. Palali and Nasiping in northern Luzon, and Cagbang on Negros Island (Table 1, Fig. 1).

Animal and sample collection

Geckos were captured by hand between 1600 and 0200 h and for all locality records we used the WGS-84 datum. We collected cloacal swabs to inventory host-associated gut microbial communities, which have been shown to be effective proxies for endogenous microbiome sampling in reptiles (Colston *et al*. 2015; Eliades *et al*. 2021). To collect cloacal microbiome samples, we inserted sterile rayon-tipped swabs approximately 3 cm into the cloacal opening of each animal and rotated them 10 times (Smith *et al*. 2021). For efficient preservation of DNA, we then placed swabs into individual screw-top 1.5 mL cryovials with 750 ul Xpedition™ Lysis/Stabilization Solution (Zymo Research Products). Cloacal swabs were stored at ambient

temperature while in the field before transportation to the Sam Noble Oklahoma Museum of Natural History for curation and storage in a -20°C freezer until DNA extraction (Smith *et al.* 2021).

Microbial inventories

Sample processing, data curation, and analysis closely reflect processes from Eliades *et al.* (2021). All DNA extraction and library preparation steps were completed at the Sam Noble Museum's Shared Genetics Laboratories at the University of Oklahoma. We extracted total DNA from 56 gekkonid samples using Zymo Quick-DNA Fecal/Soil Microbe Kits. Cloacal swabs were incubated at 65° C for 15 minutes on a dry heating block and then vortexed for 15 minutes on an Eppendorf ThermoMixer® at 23°C and maximum speed (2000 rpm) immediately prior to beginning Zymo's recommended protocol. We amplified the V4 region of the 16S rRNA gene using published protocols index primers and PCR protocols (Kozich *et al.* 2013). PCR products were cleaned, normalized, and pooled using a Sequel Prep Normalization Plate Kit (Invitrogen). Pooled libraries were purified using Agencourt® AMPure® magnetic bead capture and sent to the University of Oklahoma's Consolidated Core Lab (CCL) for sequencing using 515F and 806R primers targeting 2x300bp reads on an Illumina MiSeq sequencing platform (Caporaso *et al.* 2012). Libraries were prepared and sequenced in two iterations with 24 samples sequenced in 2018 and 32 samples sequenced in 2019.

Raw sequences from both sequencing iterations were processed concurrently. Reads were first paired and trimmed using AdapterRemoval2 v2.2.2 with default parameters (Lindgreen 2012; Schubert, Lindgreen, and Orlando 2016). Cleaned sequences were clustered de novo into operational taxonomic units (OTUs) using UPARSE in USEARCH v11.0.667 at a minimum

sequence identity of 97% and a minimum abundance of four (Edgar 2013). Remaining sample curation and analysis was carried out in QIIME v1.9.1 (Caporaso *et al*. 2010). Taxonomies were assigned to OTUs using GreenGenes v13.8 (DeSantis *et al*. 2006). Archaea, chloroplast, mitochondria, PhiX, and other non-bacterial sequences were removed from processed OTU tables to ensure only bacterial sequences were included in downstream analyses. OTUs found in sample extraction negatives and PCR negatives were filtered and removed from all samples. These samples produced 1,063,934 reads with a minimum read depth of 111, maximum of 47,747, and a median of 15,504 reads per sample. Sequences were rarified to 1000 reads per sample (Good's coverage mean = 0.95 ± 0.03), and samples with insufficient sequencing depth (n = 9) were removed from further analyses, resulting in 47 samples examined (Table 1). All raw 16S rRNA sequences have been deposited in the Sequence Read Archive (SRA) under accession no. XXXX.

Assessments for phylosymbiosis

We used Mantel tests in QIIME with default parameters to test whether host phylogeny, as measured in cophenetic genetic distances, is correlated with observed variation in microbial communities of Philippine gekkonid hosts (Caporaso *et al*. 2010). To generate host genetic distances, we downloaded previously published sequence data available on GenBank for the coding region of the mitochondrial NADH dehydrogenase 2 (ND2) gene for all nine host species included in this study and 10 other extant gekkonid species to improve phylogenetic resolution and to serve as appropriate outgroups (Appendix 1; Siler *et al*. 2012). To estimate a time-calibrated phylogeny, we employed an available fossil calibration, *Yantarogekko* (Bauer *et al*. 2005), in divergence dating analyses (Appendix 1), which is estimated to date to the Paleogene

(33.9–55.8 Ma). Sequence data were aligned in MUSCLE (Edgar 2004) and trimmed to 1,041 base pairs of the coding region. We used JModelTest v2.1.10 to identify the substitution model GTR + I + Γ for further use with sequence data (Darriba *et al.* 2012).

We estimated an ultrametric, time-calibrated topology in BEAST v2.6.3 (Bouckaert *et al.* 2014), using the Fossilized Birth Death Model following protocols described in (Heath 2014), with an initial, minimum date of 33.9 Ma (Bauer *et al*. 2005) set for the fossil *Yantarogekko*. To calibrate our analyses, we used a uniform prior distribution, U(33.9, 55.8), Branch-specific rates of substitution were allowed to vary across the tree according to uncorrelated lognormal distributions (Drummond *et al*. 2006), with exponential prior distributions with a mean of 0.01 for the standard deviation. All remaining priors were left at default values. We ran four independent analyses of 10 million generations, logging parameter values every 1000 generations, and assessed stationarity of the analyses by plotting parameter values and likelihood scores of all four chains over generations to confirm congruence. Conservatively, we discarded the first 20% of samples from each run as burn-in and combined and summarized the remaining 8000 samples across all four independent MCMC chains in TreeAnnotator within BEAST.

The resulting consensus chronogram was used to generate a cophenetic distance matrix via ape v5.4-1 (Paradis & Schliep 2019) in R v3.6.2 (R Core Team 2013) for use in Mantel tests to assess phylosymbiosis in gekkonid microbiomes. We used vegan v2.5-6 (Oksanen *et al.* 2016) to compare cophenetic distances between the nine species sampled in this study and summarized interspecific beta diversity metrics including weighted UniFrac, unweighted UniFrac, and Jaccard distances.

Endogenous microbial community comparisons

We compared a variety of community membership metrics considering multiple ecoevolutionary lenses. For all comparisons, we first calculated alpha diversity measurements including numbers of observed OTUs, the Shannon index (Shannon 1948), and Faith's Phylogenetic Diversity (Faith's PD; Faith 1992). Alpha diversity measurements were compared using analysis of variance (ANOVA) tests in R v3.6.2 (R Core Team 2013) with the Tukey Test used for post-hoc analyses. ANOVA tests with Bonferroni corrections were used in QIIME to compare relative abundances of bacterial taxa between groups of interest.

Community diversity and structure were compared using principal coordinates analysis (PCoA) on beta diversity metrics including weighted and unweighted UniFrac distances (Lozupone & Knight 2005) and the binary Jaccard index (Jaccard 1901). Beta diversity matrices and PCoA plots were generated from the same rarefied datasets used to measure alpha diversity metrics. The adonis function in the vegan v2.3_4 package (Oksanen *et al*. 2016) of R v3.3.1 (R Core Team 2013) was used on beta diversity distance matrices with 999 permutations to compare community composition between groups statistically.

We analyzed bacterial composition among all 47 samples to document host-associated microbes in these species and to visualize patterns across microbial communities in Philippine gekkonids. Initial analyses grouped samples first by host species identity and then by a suite of ecoevolutionary categories to identify potential correlations with observed gut microbiomes. These schemes included grouping by host species general habitat tendencies, range extents, and host maximum body sizes. We compared species considered human commensal against those believed to be forest obligates, then widespread and microendemic species, and finally larger- (maximum SVL > 95 mm) versus smaller-bodied (maximum SVL < 95 mm) hosts (Table 1).

After such initial comparisons, we next analyzed samples as grouped by sampling locality and broader biogeographic region.

After analyzing all 47 samples included in this study concurrently, we examined microbial communities from specimens within the genus *Gekko* (*n* = 25) exclusively to narrow the taxonomic distinctiveness between hosts in analyses and reran ecoevolutionary tests. With this subset, we compared alpha and beta diversity metrics using the same analyses between the following four groups: species identity, habitat tendencies, range extents, and maximum body sizes.

Following these interspecific comparisons within the *Gekko* genus, we next analyzed microbiomes of only *Cyrtodactylus philippinicus* specimens (*n* = 12) to focus purely on intraspecific variability among distinct, allopatric populations. We compared both alpha and beta diversity metrics between sampling sites using the methods described above.

Finally, to lessen the influence of locality as a variable separating host species, we compared samples retrieved from three distinct collection sites: Cagbang (*n* = 8), Magsidel (*n* = 17), and Mt. Palali (*n* = 11). Here, multiple, sympatric gekkonid species were sampled. In each subset, we compared our alpha and beta diversity metrics by host species identity to ask whether sympatric species presence and overlapping interspecific ranges may mitigate the host species-specific microbial compositions generally observed in reptiles.

Results

General patterns in gekkonid microbiota

Individual phyla dominating each species' microbial communities varied by host species (Fig. 2), although three phyla, Proteobacteria (54.1%), Firmicutes (20.9%), and Bacteroidetes (16.6%),

were most abundant across all rarefied reads. Philippine gekkonid samples averaged 103 OTUs per 1000 rarified sequences, the Shannon index varied from 1.40–6.39 (mean = 4.19 ± 1.39), and Faith's PD varied from 3.62–21.78 (mean = 11.25 ± 4.20). The average Jaccard distance between all pairs of samples was 0.83. Six OTUs were found across rarefied sequences from ≥ 70% of all host cloacal samples, including: two *Acinetobacter* spp., *Serratia* sp., *Staphylococcus* sp., *Bacteroides* sp., and an unidentified taxon in the family Enterobacteriaceae.

Across Philippine samples from hosts in the family Gekkonidae, Mantel tests recovered no significant association between host species genetic distances and microbial assemblages as measured by any of the three beta diversity metrics examined (weighted UniFrac r = 0.207, P = 0.438; unweighted UniFrac r = 0.110, P = 0.607; Jaccard r = 0.119, P = 0.599).

In comparing alpha diversity metrics across Philippine gekkonid microbiome samples, we found no significant differences in the number of OTUs or the Shannon index among host species. There was, however, a significant difference between host species in Faith's PD (F = 2.636, P = 0.021; Sup. 1A). Tukey post-hoc analyses identified the *Gekko mindorensis*-*Luperosaurus macgregori* and *Cyrtodactylus philippinicus*-*L. macgregori* pairwise comparisons as likely driving such differences. Grouping samples by host habitat preferences (human commensal or forest obligate) and maximum body size (larger or smaller) failed to retrieve significant differences in any alpha diversity metrics. In comparing widespread and microendemic samples, significant differences were noted in observed OTUs (mean widespread = 120.11, microendemic = 80.95; F = 8.225, P = 0.006), Shannon index (F = 5.467, P = 0.024), and Faith's PD (F = 8.866, P = 0.005; Sup. 2B). Differentiating by individual host sampling locality and biogeographic region both showed significant distinction in number of observed

OTUs (locality $F = 3.672$, P = 0.005; region $F = 5.464$, P = 0.003) and in Faith's PD (locality $F = 4.301$, P = 0.020; region $F = 6.021$, P = 0.016) but not in the Shannon index (Sup. 1B, 2A).

We found strong, significant associations between host species and microbial compositions based on all three beta diversity metrics examined (weighted UniFrac $R^2 = 0.310$, P = 0.005; unweighted UniFrac $R^2 = 0.285$, P = 0.001; Jaccard $R^2 = 0.257$, P = 0.001; Fig. 3). Adonis comparisons of beta diversity metrics showed no differences in human commensal and forest obligate groupings. Significant, yet weak, differences in two beta diversity metrics were found by widespread and microendemic species distribution patterns (unweighted UniFrac $R^2 = 0.060$, P = 0.001; Jaccard $R^2 = 0.051$, P = 0.001) and in the Jaccard index between maximum body size conditions ($R^2 = 0.029$, P = 0.043). Correlations between host locality and microbial communities were found in all three metrics (weighted UniFrac $R^2 = 0.208$, P = 0.028; unweighted UniFrac $R^2 = 0.209$, P = 0.001; Jaccard $R^2 = 0.201$, P = 0.001). Slightly weaker results were recovered in grouping cloacal samples by source host biogeographic region as opposed to specific locality (weighted UniFrac $R^2 = 0.132$, P = 0.021; unweighted UniFrac $R^2 = 0.119$, P = 0.001; Jaccard $R^2 = 0.111$, P = 0.001).

Endogenous microbiota across geckos in the genus *Gekko*

We collected samples from five *Gekko* species at five distinct sites across the Philippines: Cagbang, Magsidel, Mt. Palali, Nasiping, and Tapao Falls (Table 1, Fig. 1). We only sampled one species of *Gekko* per site and, as such, host species and host locality are confounded in subsequent analyses here and only host species comparisons are included. Microbial compositions within samples from species of *Gekko* varied by host taxon with consistent, high prevalence of Proteobacteria, Firmicutes, and Bacteroidetes (Figs. 2, 4). No OTUs were found to

vary in relative abundance across all five host species in the genus *Gekko*. Only nine OTUs were found in ≥ 70% of all rarified samples from these hosts although within species, a greater number of shared OTUs was common (Appendix 2). For instance, 24 OTUs were found in all *G. mindorensis* samples while 13 OTUs were identified in all *G. gecko* samples, four in all G. *crombota* samples, and four in most (≥ 85%) *G. rossi* samples.

We found no significant difference in the number of OTUs between host species nor a difference in the Shannon index, though there was significant differentiation in Faith's PD between *Gekko* species ($F = 3.287$, P = 0.032). Post-hoc analyses indicated this significance was driven by the *G. rossi*-*G. mindorensis* pairwise comparison (P = 0.013; Sup. 3A). Grouping by host range extents and host body size classes respectively found significant differences in observed OTUs (widespread mean = 130.22, microendemic = 92.00; $F = 5.198$, P = 0.032; larger body mean = 96.10, smaller = 144.40; $F = 5.909$, P = 0.023; Sup. 4A) and Faith's PD ($F = 6.004$, P = 0.022; $F = 9.846$, P = 0.005; Sup. 4B). No significant differences were found when grouping by forest obligates and human commensals.

Microbial community composition varied significantly by host species in the unweighted UniFrac and Jaccard index metrics ($R^2 = 0.270$, P = 0.001; $R^2 = 0.256$, P = 0.001; Fig. 4) but not in the weighted UniFrac metric, suggesting rare OTUs rather than more relatively abundant taxa are driving observed differences in microbial assemblages between hosts in the genus *Gekko*. Aside from host species identity, multiple other ecoevolutionary factors showed statistically significant, yet weaker differences between grouping schemes in the unweighted UniFrac and Jaccard distance metrics. These included grouping by species distribution patterns ($R^2 = 0.076$, P = 0.014; Jaccard $R^2 = 0.073$, P = 0.003) and host maximum body size (unweighted UniFrac $R^2 = 0.104$, P = 0.001; Jaccard $R^2 = 0.080$, P = 0.001). Grouping by broad habitat associations only

recovered significant, yet weak, distinctions in the Jaccard metric ($R^2 = 0.064$, $P = 0.009$). The average Jaccard distance between any two species within the genus was 0.88 and pairwise comparisons between species varied from 0.84 on average between *G. crombota* and *G. mindorensis* and 0.94 between *G. gecko* and *G. kikuchii*.

Endogenous microbiomes at the species level in *Cyrtodactylus philippinicus*

We sampled gut microbial communities in 12 *Cyrtodactylus philippinicus* specimens at four distinct sites, three on Luzon Island and another on Negros Island, to assess intraspecific variability in host microbiomes at discreet sampling localities (Table 1, Fig. 1). Phyla dominating microbial compositions in *C. philippinicus* hosts differed by locality, with Proteobacteria always most common (Fig. 5). Firmicutes, Bacteroidetes, and Actinobacteria comprised most of the remaining reads though proportions in individual hosts varied by site (Fig. 5). Just three specific OTUs were shown to differ statistically between localities including *Ochrobactrum* sp., an unidentified taxon in the order Bacillales, and another in the family Bacteriovoracacea (Appendix 3). Three OTUs were found in all *C. philippinicus* specimens sampled, two *Acinetobacter* spp. and a *Serratia* sp., while 13 OTUs were found in ≥ 70% of *C. philippinicus* hosts.

The number of OTUs per 1000 rarefied sequences did not vary significantly between sites, neither did the Shannon index, nor Faith's PD. Principal coordinates analysis revealed a degree of clustering by locality in weighted and unweighted UniFrac measures and pronounced grouping in Jaccard distances. Adonis tests found significant differentiation by locality in all three beta diversity metrics (weighted UniFrac $R^2 = 0.513$, $P = 0.002$; unweighted UniFrac $R^2 = 0.350$, $P = 0.035$; Jaccard $R^2 = 0.362$, $P = 0.001$; Fig. 6), suggesting distinct microbial

compositions between sampling sites. Grouping host-associated microbiota by host biogeographic region rather than specific host locality produced similar, though weaker, results in weighted UniFrac (R^2 = 0.335, P = 0.014), unweighted UniFrac (R^2 = 0.241, P = 0.035), and Jaccard metrics (R^2 = 0.245, P = 0.008). Jaccard distances varied by locality, ranging from 0.68 within samples at Tulay na Lupa, to 0.74 at Mariroc, 0.84 at Cagbang, and finally 0.86 at Mt. Palali.

Locality-specific assessments of microbial inventories in gekkonids

Three sites yielded samples from multiple, sympatric gekkonid species: Cagbang, Magsidel, and Mt. Palali. At Cagbang, on Negros Island, we sampled *Cyrtodactylus philippinicus* (*n* = 2), *Gekko gecko* (*n* = 4), *Hemidactylus frenatus* (*n* = 1), and *H. platyurus* (*n* = 1) hosts (Table 1, Fig. 1). There was a high degree of intraspecific variation within host microbial compositions at this site (Sup. 5A). Most microbiomes were dominated by Proteobacteria, Firmicutes, and Bacteroidetes, except for our single *H. frenatus* sample at this site, with a high proportion of Tenericutes (Sup. 5A). We found no significant difference in microbial community structure between host species based on alpha diversity metrics measured. Despite limited sampling sizes, clustering was apparent in PCoA plots with strong, significant distinction in community composition between host species in weighted UniFrac (R^2 = 0.678, P = 0.0200), unweighted UniFrac (R^2 = 0. 566, P = 0.008), and Jaccard distances (R^2 = 0. 534, P = 0.004; Sup. 6A–C). At this site, Jaccard distances within *C. philippinicus* hosts were 0.84 and among *G. gecko* samples 0.81. Jaccard distances between these two species averaged 0.92, suggesting a greater proportion of shared diversity intraspecifically rather than interspecifically.

At Magsidel, in the Babuyan Island chain, both *G. rossi* (*n* = 9) and *Luperosaurus macgregori* (*n* = 4) were sampled with marked variability apparent among individual *G. rossi* compositions, where some samples were dominated by Bacteroidetes, others Firmicutes, and others still Proteobacteria (Sup. 5B). Cloacal samples from *L. macgregori* hosts were composed predominantly of Proteobacteria followed by Firmicutes (Sup. 5B). Significant differences were found in the number of OTUs observed per 1000 sequences between each host species (*G. rossi* mean = 86.78, *L. macgregori* mean = 36.75; $F = 5.308$, $P = 0.042$), Shannon index ($F = 6.787$, $P = 0.025$), and Faith's PD ($F = 5.732$, $P = 0.036$). Grouping in PCoA plots was unclear, with insignificant differentiation between species in weighted UniFrac measures and significant, yet weak distinctions in unweighted UniFrac ($R^2 = 0.179$, $P = 0.009$) and Jaccard distances ($R^2 = 0.145$, $P = 0.008$; Sup. 6D–F). Jaccard distances within *G. rossi* and *L. macgregori* samples were both 0.86, while interspecific comparisons averaged 0.91.

Finally, we sampled *C. philippinicus* (*n* = 5), *G. mindorensis* (*n* = 4), and a lone *H. frenatus* specimen at Mt. Palali on Luzon Island. Proteobacteria dominated gut microbial communities in geckos sampled on Mt. Palali followed in relative abundance by Firmicutes then Bacteroidetes across all host species (Sup. 5C). At this site, no significant differences in alpha diversity metrics were recorded between host species. Statistically significant community clusters between host taxa were most clear in weighted UniFrac composition plots ($R^2 = 0.481$, $P = 0.015$), unweighted UniFrac ($R^2 = 0.294$, $P = 0.023$) and Jaccard distance ($R^2 = 0.288$, $P = 0.007$) clusters were more ambiguous (Sup. 6G–I). Average Jaccard distances within *C. philippinicus* samples at this site were 0.86 and were 0.82 for *G. mindorensis*. Between *C. philippinicus* and *G. mindorensis* the average Jaccard distance was 0.87, suggesting slightly more shared OTUs within species rather than between species on average. Between *H. frenatus* and the other two sympatric confamilials,

the Jaccard distances between species averaged 0.89 and 0.94 compared to *G. mindorensis* and *C. philippinicus,* respectively.

Discussion

This study provides baseline information on symbiotic gut microbes in wild Philippine gekkonids and points toward several ecoevolutionary forces shaping such compositions. At the family level in Philippine gekkonids, we found limited evidence of phylosymbiosis or host evolutionary history strongly reflecting current microbial compositions. Although previous studies have noted that evolutionary history influences gut microbial communities at various taxonomic levels (Ley *et al*. 2008; Sanders *et al*. 2014; Groussin *et al*. 2017; Youngblut *et al*. 2019), such impacts may be unevenly distributed across taxonomic groups. For instance, Youngblut *et al*. (2019) found that evolutionary history had a stronger effect on intestinal microbiome diversity in mammals than in non-mammalian species. Ren *et al*. (2016) noted only weak associations between host genetic distances and microbial assemblages in congeneric reptiles of the genus *Anolis*. High degrees of intraspecific variation in host-associated microbiomes may explain the lack of evidence for phylogenetic past reflecting contemporary community compositions here and in other reptile hosts (Brooks *et al*. 2016; Ren *et al*. 2016). At the scale of host family, various ecoevolutionary factors outside of phylogenetic histories likely serve pivotal roles in shaping and maintaining gut microbial communities in gecko hosts from the Philippines (Kropackova *et al*. 2017; Lutz *et al*. 2019).

Of all ecoevolutionary factors examined, we found that observed host-associated microbial assemblages were most correlative with host species, as seen previously in other reptile groups (Lankau *et al*. 2012; Ren *et al*. 2016; Kohl *et al*. 2017). Sampling locality and broader

biogeographic zones irrespective of host species identity were also significantly associated with microbial assemblages, agreeing with previous findings (Lankau *et al*. 2012; Ren *et al*. 2016). Within our interspecific comparisons, we note that observed patterns may be at least partially confounded with host species due to uneven opportunistic sampling of wild gekkonid specimens (Table 1, Fig. 1). Although future sampling designs that can capture spatial variation within and between species would help resolve such confounding, the variation in host-associated assemblages in *Cyrtodactylus philippinicus* samples observed across multiple sites here (Fig. 6) suggests that individual host locality does influence gut microbiomes significantly even within species.

The site specificity seen in compositions from *C. philippinicus* specimens at discrete locations shows that locality can and does influence observed intraspecific variation in microbial compositions (Fig. 6). Site-specific factors that alter these compositions need further investigation in wild hosts (Ren *et al*. 2016). Preliminary evidence suggests that differences in individual diet at least in captive reptiles may be responsible for marked intraspecific variation in host-associated gut microbiomes (Jiang *et al*. 2017; Fong, Sung, and Ding 2020). Studies on specific microhabitat tendencies and improved ecological knowledge on host species are needed to better understand drivers of gut microbiome formation and maintenance.

All gecko species included in this study have only broad ecological data available (Brown *et al*. 2008,2009,2010,2011,2013b; Welton *et al*. 2010). The categorizations used in this investigation failed to recover much differentiation based on ecological traits; however, it is possible that more fine-scale ecological partitioning would prove intuitive (Lankau *et al*. 2012; Ren *et al*. 2016). Widespread and microendemic species comparisons offer promising avenues for more targeted testing, as alpha and beta diversity metrics were significantly distinct between

groups (Sup 2B). Widespread species sampled in this study showed greater OTU diversity, Shannon Index values, and Faith's PD as compared to microendemic counterparts. They also displayed distinct communities in the unweighted UniFrac and Jaccard metrics as compared to microendmic counterparts, suggesting differentiation in rare OTU presence. This significance could be preliminary evidence for a valid ecological phenomenon in which widespread and microendemic hosts exhibit distinct strategies in harboring endogenous microbiome compositions. Internal microbial communities are critical facets of organismal adaptability to novel environments (Amato 2013; Stumpf *et al*. 2016; Trevelline *et al*. 2019), and the increased diversity presence or transient microbe acquisition may play a role in widespread species' capacities to invade novel habitats (Alberdi *et al*. 2016). Additional insight into the functional capacity of reptile microbiomes and the way endogenous microbiota influence adaptive capacity of hosts is of critical importance for conservation considerations of reptile species in the future (Brooks *et al*. 2016; Colston and Jackson 2016; Littleford-Colquhoun *et al*. 2019; Trevelline *et al*. 2019).

Here we expand upon what is known on endogenous microbial communities in wild reptiles and identify a suite of contemporary and historical influences that structure such compositions using gekkonids from across the Philippine archipelago. We found no correlations between host genetic distances and observed microbial compositions, suggesting a muted influence of evolutionary history on present variation in Philippine geckos. Despite this, host species was consistently the greatest determinate in microbial assemblages with marked intraspecific variation observed based on sampling locality. While these results suggest that contemporary ecological traits may play a more central role than do evolutionary pasts in the maintaining of

enteric microbial diversity in gekkonid hosts, future research investigating these factors more precisely in wild specimens remains essential.

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Conflict of interest

The authors declare no conflict of interest.

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Table

Table 1. Sampling table of 47 gekkonid hosts examined across the Philippines. Specimens included nine species from four genera collected at seven localities in the archipelagic nation. Contemporary factors examined were habitat preferences (forest obligates or human commensals), host species range (widespread vs. microendemic), and host maximum body size (SVL > or < 95 mm).

Genus	**Species**	**Total Sampled**	**Biogeographic Region (# sampled)**	**Localities (# individuals sampled)**	**Habitat**	**Range**	**Body Size**
Cyrtodactylus	*philippinicus*	12	Luzon (5), Bicol (5), Negros (2)	Cagbang (2), Mt. Palali (5), Mariroc (2), Tulay na Lupa (3)	Forest	Wide	Large
Gekko	*crombota*	7	Babuyan (1)	Tapao Falls (7)	Forest	Micro	Large
Gekko	*gecko*	4	Negros (1)	Cagbang (4)	Human	Wide	Large
Gekko	*kikuchii*	1	Luzon (1)	Nasiping (1)	Human	Wide	Small
Gekko	*mindorensis*	4	Luzon (4)	Mt. Palali (4)	Forest	Wide	Small
Gekko	*rossi*	9	Babuyan (9)	Magsidel (9)	Forest	Micro	Large
Hemidactylus	*frenatus*	3	Luzon (2), Negros (1)	Cagbang (1), Mt. Palali (1), Nasiping (1)	Human	Wide	Small
Hemidactylus	*platyurus*	3	Luzon (2), Negros (1)	Cagbang (1), Nasiping (2)	Human	Wide	Small
Luperosaurus	*macgregori*	4	Babuyan (4)	Magsidel (4)	Forest	Micro	Small

Figures and Figure Legends

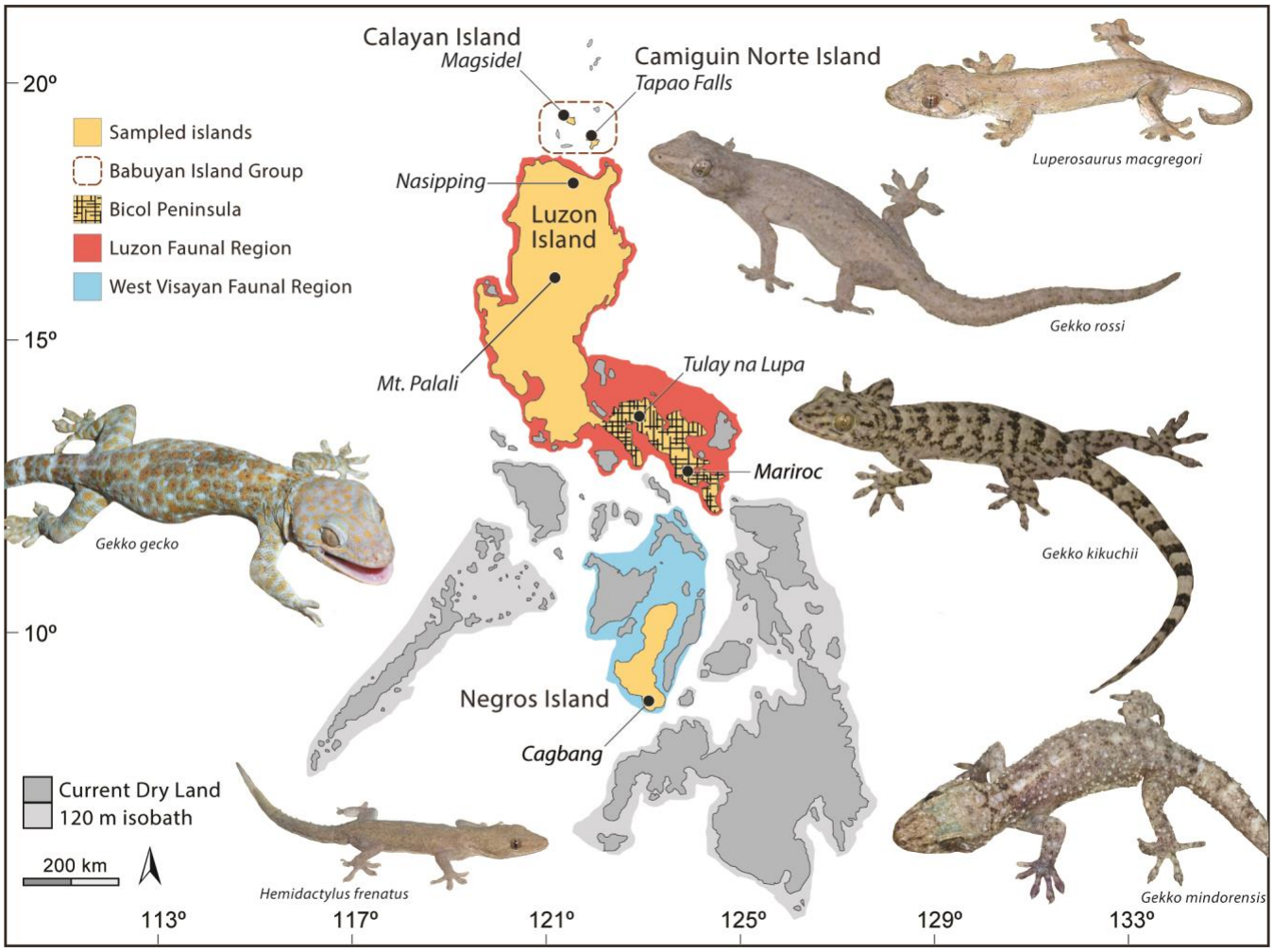

Fig. 1. Map of Philippine archipelago with a shaded 120 m isobath around major island groups. Major biogeographic regions of note in this study include the Babuyan Island Group, Luzon Island, the Bicol Peninsula of southern Luzon, and Negros Island. Specific localities sampled in this investigation are included in italics. (Photographs of *G. rossi*, *H. frenatus*, and *L. macgregori* courtesy of Kai Wang, *G. mindorensis*, *G. gecko*, and *G. kikuchii* by CDS).

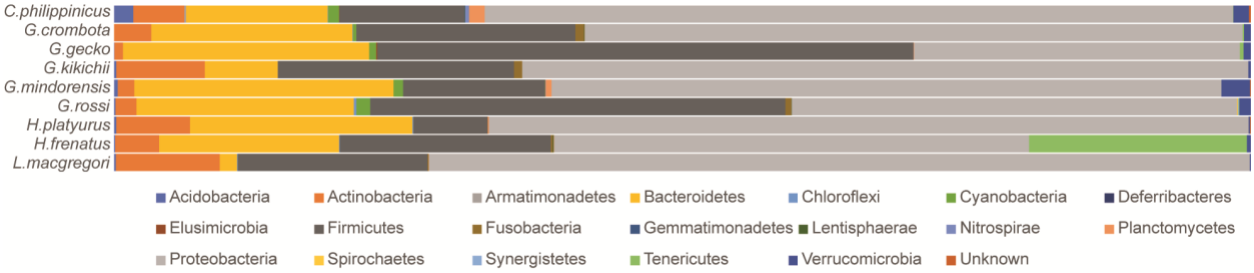

Fig. 2. Stacked barplot of average gut microbiome compositions by phyla across Philippine gekkonid hosts.

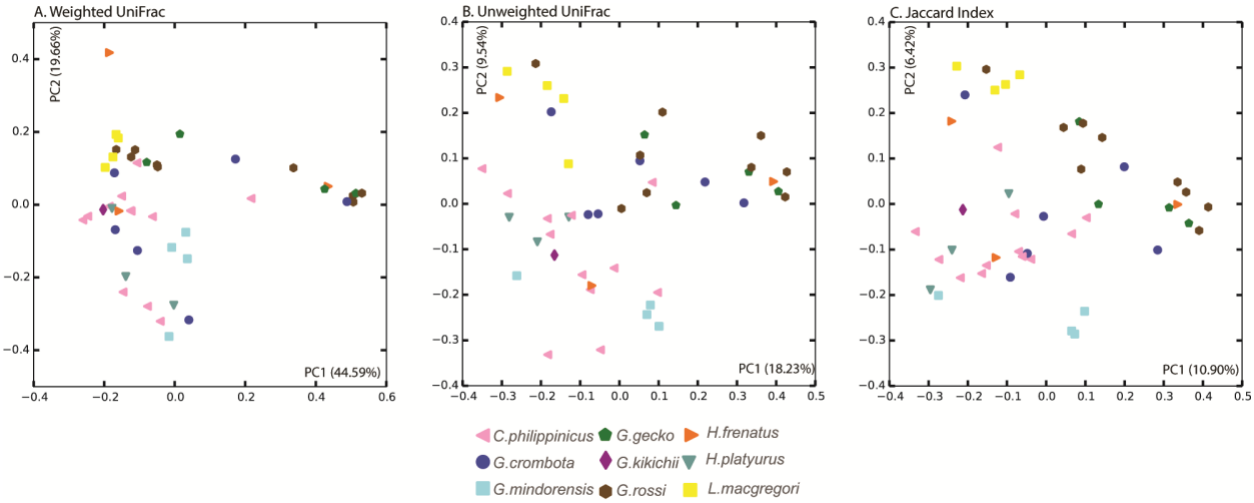

Fig. 3. Principal coordinates analysis plots of gut microbiomes from gekkonid hosts as measured by beta diversity metrics including A) Weighted UniFrac, B) Unweighted UniFrac Distances, and C) Jaccard Index.

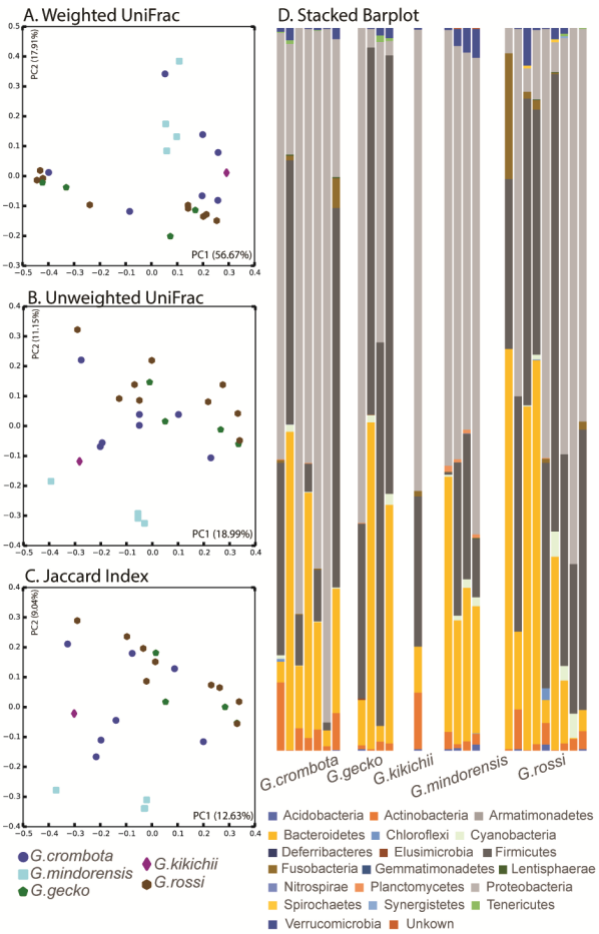

Fig. 4. Principal coordinates analysis plots of beta-diversity metrics from geckos in the genus *Gekko* (A–C). Stacked barplot (D) of individual microbial compositions of hosts, grouped by taxonomic identity.

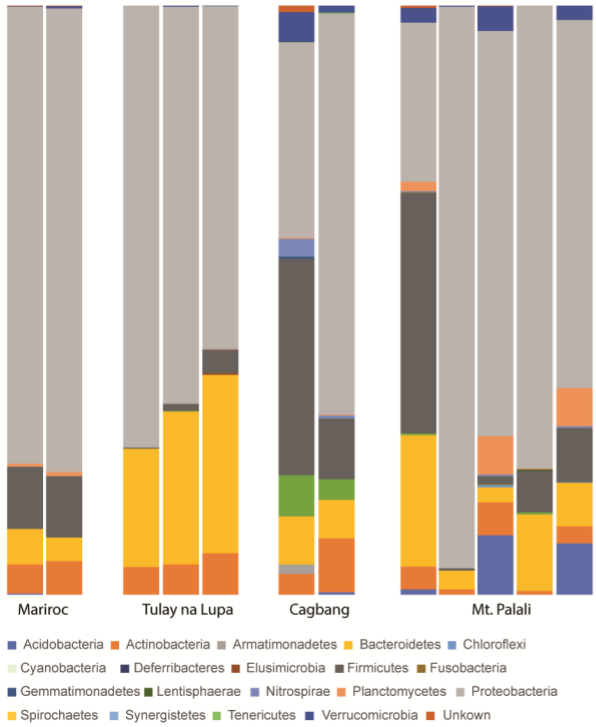

Fig. 5. Microbiome compositions at the phylum level from *Cyrtodactylus philippinicus* hosts grouped by sampling locality.

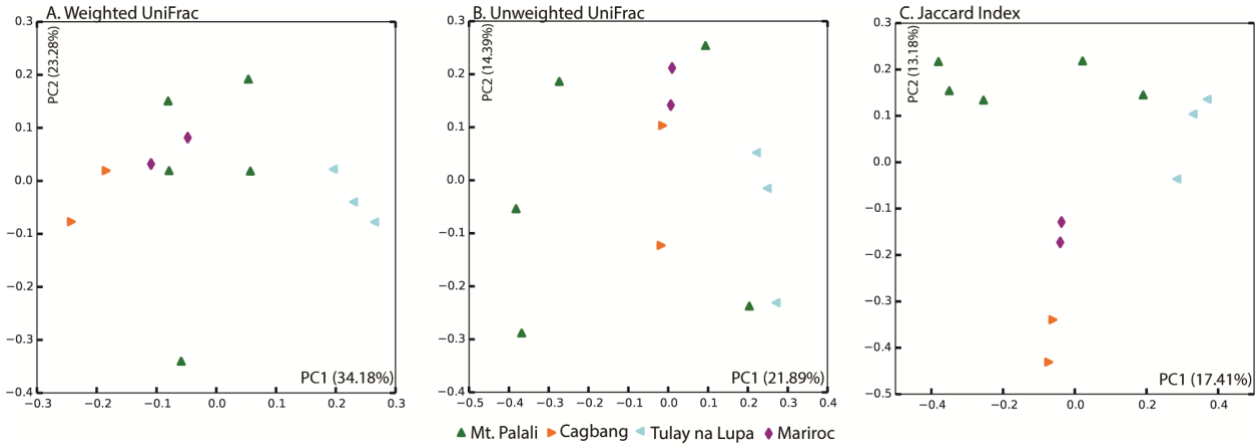

Fig. 6. Principal coordinates analysis plots of *Cyrtodactylus philippinicus* samples designated by sampling locality.

Chapter 3: Gut microbial ecology of the Critically Endangered Fijian crested iguana (*Brachylophus vitiensis*): effects of captivity status and host reintroduction on endogenous microbiomes

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ABSTRACT

Animals often exhibit distinct microbial communities when maintained in captivity as compared to when in the wild. Such differentiation may be significant in headstart and reintroduction programs where individuals spend some time in captivity before release into native habitats. Using 16S rRNA gene sequencing, we (i) assessed differences in gut microbial communities between captive and wild Fijian crested iguanas (*Brachylophus vitiensis*) and (ii) resampled gut microbiota in captive iguanas released onto a native island to monitor microbiome restructuring in the wild. We used both cloacal swabs and fecal samples to further increase our understanding of gut microbial ecology in this IUCN Critically Endangered species. We found significant differentiation in gut microbial community composition and structure between captive and wild iguanas in both sampling schemes. Approximately two months post-release, microbial communities in cloacal samples from formerly captive iguanas closely resembled wild counterparts. Interestingly, microbial communities in fecal samples from these individuals remained significantly distinct from wild conspecifics. Our results indicate that captive upbringings can lead to differences in microbial assemblages in headstart iguanas as compared to wild individuals even after host reintroduction into native conditions. This investigation

highlights the necessity of continuous monitoring of reintroduced animals in the wild to ensure successful acclimatization and release.

KEYWORDS

Conservation, headstart, husbandry, microbial restructuring, reptiles, wildlife management

1 | INTRODUCTION

Gastrointestinal microbial communities are critical to host health, contributing to an array of functions that impact host fitness and reproductive success such as nutrient acquisition based on digestive efficiency, hormone balance, and immune response (Cho & Blaser, 2012; Colston & Jackson, 2016; Fraune & Bosch, 2010; Ley et al., 2008). Given that gut microbiota serve essential roles in maintaining host well-being, the study of these communities is a novel tool for wildlife conservation initiatives, particularly in programs involving ex-situ animal care (Bahrndorff, Alemu, Alemneh, & Lund Nielsen, 2016; Jiménez & Sommer, 2017; Redford, Segre, Salafsky, del Rio, & McAloose, 2012; West et al., 2019). With few exceptions, a variety of species housed in captivity show disparate gut microbiomes compared to wild counterparts which may be caused by dietary differences, antibiotic treatments, exposure to other species in captivity, or various other potential drivers that alter microbial compositions (Alfano et al., 2015; Cheng et al., 2015; Clayton et al., 2016; Eigeland et al., 2012; McKenzie et al., 2017; West et al., 2019; Zhu, Wu, Dai, Zhang, & Wei, 2011). Such differences may be signs of dysbiosis, or perturbations of microbial communities that hinder system function and are often associated with negative health outcomes in hosts (Gilbert et al., 2016; West et al., 2019). For example, captivity has been linked to increases of potential pathogens within gastrointestinal microbial communities

in mammals (Amato et al., 2016; Cheng et al., 2015; Wan et al., 2015; Wasimuddin et al., 2017), birds (Xie et al., 2016), and reptiles (Jiang et al., 2017; Kohl et al., 2017). Distinct gut microbiota between captive and wild hosts is especially significant in headstart and reintroduction conservation programs, as altered microbial communities or introduced pathogens in captive animals slated for release could hinder reintroduction success and survivorship in the wild due to reduced dietary efficiency or compromised immune response affecting survivorship (Bahrndorff et al., 2016; Jiménez & Sommer, 2017; Redford et al., 2012; West et al., 2019).

Headstart programs have become increasingly common management strategies to supplement declining wildlife populations at risk of extinction (McGowan, Traylor-Holzer, & Leus, 2017; Redford et al., 2011; Tear, Scott, Hayward, & Griffith, 1993). In these programs, young animals are reared in captivity past their most vulnerable life stages before being released to reinforce wild populations (Alberts, 2007; Ferguson, Brown, & DeMarco, 1982). Historically, however, effective reintroduction of captive animals into the wild has been rare, with as few as 13% of such projects being deemed successful (Fischer & Lindenmayer, 2000; Mathews, Orros, McLaren, Gelling, & Foster, 2005). Multiple factors have been linked to animal headstart and reintroduction difficulties including individual animal behavior (Alberts, 2007; Mathews et al., 2005) and ill-suited release sites (Pérez-Buitrago et al., 2008). More recently, microbial incompatibilities also have been suggested as possible impediments to reintroduction success (Bahrndorff et al., 2016; Jiménez & Sommer, 2017; Redford et al., 2012; West et al., 2019). However, no studies to date have examined gut microbiota in reintroduced species both pre- and post-release to analyze microbial composition and acclimation of these communities to native habitats. Improved understanding of host natural microbiomes and microbial shifts associated

with captivity and headstart animal release could help management practitioners to better prepare animals for reintroduction and increase headstart success of imperiled species.

The Fijian crested iguana (*Brachylophus vitiensis*) is an herbivorous lizard species endemic to dry and littoral forests in western Fiji (Fisher et al., 2019; Harlow, Fisher, & Grant, 2012). Since the species' discovery in 1981, it has experienced sharp population declines throughout most of its limited range due to habitat loss and introduced predators (Fisher et al., 2019; Gibbons, 1981; Harlow et al., 2007). The Fijian crested iguana is listed on CITES Appendix 1 and as Critically Endangered by the IUCN Red List (Fisher et al., 2019; Harlow et al., 2012). To ensure the long-term viability of this species in Fiji, a captive breeding and headstart program was established in 2010 with a specific focus on animals from the uninhabited island of Monuriki (Chand et al., 2016; Fisher et al., 2019). Monuriki Island crested iguanas are genetically distinct from all other crested iguana populations (Keogh, Edwards, Fisher, & Harlow, 2008), and the 2008 Iguana Species Recovery Plan prioritized Monuriki as the single most important site for immediate conservation action for this taxon (Fisher et al., 2019; Harlow, Hudson, & Alberts, 2008). From 2010–2012, 20 adult iguanas were caught in the wild from Monuriki Island and transported to Kula Eco Park on the large island of Viti Levu to develop a captive breeding colony (Chand et al., 2016). Over the next six years, these 20 wild caught individuals were successfully bred in managed care at Kula Eco Park with the intention of headstarting and returning the offspring to their source island of Monuriki (Chand et al., 2016; Fisher et al., 2019). In mid-May 2015, 32 captive-bred crested iguanas were released onto Monruki Island, with an additional 32 captive-bred iguanas and 16 of the original adult wild founder iguanas released onto Monuriki in February 2017.

In 2017, we completed extensive sampling of gut microbial communities from Fijian crested iguanas in captivity at Kula Eco Park, wild iguanas on Monuriki Island, and previously captive iguanas released onto Monuriki to better understand how endogenous microbiomes are influenced by both human care and host reintroduction. In this study, we not only compare gut microbiomes in captive and wild lizards of a Critically Endangered species, but also assess the restructuring of microbiota in headstart animals reintroduced into native habitats. Additionally, by inventorying gut microbiota in Fijian crested iguanas using two sampling techniques, cloacal swabs and fecal samples, we address how sampling regime influences microbial data recovered and subsequent downstream analyses. While gut microbial diversity reported from cloacal and fecal sampling is often similar, significant discrepancies in relative abundances of microbial taxa between sampling types are well noted (Colston, Noonan, & Jackson, 2015; Kohl et al., 2017; Stanley, Geier, Chen, Hughes, & Moore, 2015). We used both techniques to maximize our understanding of gut microbial ecology in *B. vitiensis* and to mitigate potential shortcomings associated with employment of a single sampling technique (Colston et al., 2015; Ren, Kahrl, Wu, & Cox, 2016). The Fijian crested iguana headstart initiative represents a unique opportunity to address two important research questions: (i) How does captivity status effect the diversity and structure of gut microbiomes? (ii) How do such communities respond to host reintroduction into native habitats? The results of this study have direct implications for the management and conservation of this Critically Endangered reptile species and for headstart and reintroduction programs globally.

2 | MATERIALS AND METHODS

2.1 | Animal maintenance and sample collection

Located 45 km northwest of the main Fijian island of Vitu Levu, Monuriki Island (17° 37'S, 177° 02'E) is a small (45 ha, 216 m ele.), uninhabited island belonging to the Mamanuca island group in western Fiji (Fig. 1). From 2010–2012, 10 male and 10 female adult Monuriki Island crested iguanas were harvested from the wild and brought to Kula Eco Park on Viti Levu to initiate a captive breeding headstart program. These 20 wild caught crested iguanas were maintained at Kula Eco Park in a private facility specifically built for captive-breeding of Monuriki crested iguanas. Iguana cages were made from galvanized steel and mesh, measuring 92 cm tall and 92 cm wide, with wood branches for arboreal perching. Iguanas were maintained on a daily diet of fresh salad made from local mixed greens and fruits. Adult iguanas were housed in pairs, while all captive-bred offspring were kept in small groups of two to four individuals per cage. Nest boxes were placed in cages for gravid females. Once eggs were deposited by a female, they were immediately removed and placed in a separate incubator until hatching. Hatchlings were fed in the same manner as adults and juveniles, but salads were cut into smaller pieces. We implanted unique passive integrated transponder (PIT) tags subcutaneously into all iguanas for identification in the wild.

We collected samples from crested iguanas of four distinct life history groups: the original wild caught adult founder iguanas from Monuriki brought to Kula Eco Park for captive breeding (wild caught founders; WCF) from 2010–2012, captive-born individuals released onto Monuriki in 2015 (CB2015), captive-born individuals released onto Monuriki in 2017 (CB2017), and wild individuals on Monuriki (Wild). Further, we sampled microbiota in WCF and CB2017 individuals while in captivity and approximately two months after relocation onto Monuriki Island.

From 22–24 February 2017, we inventoried gut microbiota in WCF and CB2017 Fijian crested iguanas at Kula Eco Park using two sampling techniques, cloacal swabs and fecal samples. To collect cloacal samples, sterile, rayon-tipped swabs were inserted approximately 3 cm into the cloacal opening of each animal and rotated 10 times. For fecal sample collection, iguanas were placed in individual pre-washed pillowcases overnight and feces were retrieved opportunistically within 4–8 hours. Pillowcases were washed subsequent to each use. For efficient preservation of DNA in both sample types, swabs and fecal samples were placed into individual screw-cap 1.5mL cryovials with 750 ul Xpedition™ Lysis/Stabilization Solution. These vials were subsequently inserted into a custom 3D-printed plastic sleeve to hold the vials, bolted to a reciprocating saw attachment, inserted into a Milwaukee M12 Hackzall battery-operated reciprocating saw, and shaken vigorously for 5 minutes to act as a mechanical homogenization device. Samples were stored at ambient temperature while in the field before transportation to the Sam Noble Oklahoma Museum of Natural History for curation and storage.

On 24 February 2017, we transported 16 WCF and 32 CB2017 (aged 12–28 months) iguanas from Kula Eco Park to Monuriki Island for assimilation into their source population. From the time of release to mid-July 2017, we conducted standard night surveys for *Brachylophus* (Harlow et al., 2007) on Monuriki island to monitor iguanas and sample gut microbial communities in the wild. Once iguanas were captured, the presence of a PIT tag allowed us to determine if the individual was a WCF, CB2017, or CB2015 iguana, while all iguanas lacking PIT tags were classified as Wild individuals. Gut microbial samples were collected using the same methodologies as for iguanas in captivity at Kula Eco Park.

2.2 | Microbial inventories

We extracted total DNA from 94 samples (52 cloacal and 42 fecal) from 39 host lizards using Zymo Quick-DNA Fecal/Soil Microbe Kits. Both cloacal swabs and fecal samples were incubated at 65° C for 15 minutes on a dry heating block and then vortexed for 15 minutes on an Eppendorf ThermoMixer® at 23°C and maximum speed (2000 rpm) immediately prior to beginning Zymo's recommended protocol. We amplified the V4 region of the 16S rRNA gene using the index primers and PCR protocols of Kozich, Westcott, Baxter, Highlander, and Schloss (2013). PCR products were cleaned, normalized, and pooled using a Sequel Prep Normalization Plate Kit (Invitrogen). Pooled libraries were purified using Agencourt® AMPure® magnetic bead capture and sent to the University of Oklahoma's Consolidated Core Lab (CCL) for sequencing using 515F and 806R primers targeting 2x300bp reads on an Illumina MiSeq sequencing platform (Caporaso et al., 2012).

Raw sequences were first paired and trimmed using AdapterRemoval2 v2.2.2 with default parameters (Lindgreen, 2012; Schubert, Lindgreen, & Orlando, 2016). Cleaned sequences were clustered *de novo* into operational taxonomic units (OTUs) using UPARSE in USEARCH v11.0.667 at a minimum sequence identity of 97% and a minimum abundance of four (Edgar, 2013). Remaining sample curation and analysis was carried out in QIIME v1.9.1 (Caporaso et al., 2010). Taxonomies were assigned to OTUs using GreenGenes v13.8 (DeSantis et al., 2006). Archaea, chloroplast, mitochondria, PhiX, and other non-bacterial sequences were removed from processed OTU tables to ensure only bacterial sequences were included in downstream analyses. All 16S rRNA sequences have been deposited in the Sequence Read Archive (SRA) under accession no. PRJNA702127.

Among all samples (n = 94), a number were either duplicates (i.e. multiple subsamples of a single fecal deposit or cloacal swabs collected from the same host consecutively) or failed to

generate sufficient sequencing coverage to produce meaningful microbial assessments. In instances where duplicate samples existed (n = 9), we retained only the sample with the greater sequencing depth. Of the remaining samples, those with fewer than 500 sequences (n = 2) were also removed to maximize sample inclusion against OTU coverage. The finalized dataset used for all subsequent analyses consisted of 83 samples (46 cloacal and 37 fecal) from 38 Fijian crested iguanas (Appendix S1). Within these datasets, five Fijian crested iguana hosts had complete time-series sets (pre- and post-release sampling) via cloacal swabbing and five had them through fecal sampling. Three individuals occurred in both groups and had complete sampling sets from the two methodologies (Appendix S1).

Rarefaction depths varied by comparison based on Good's coverage estimates (Good, 1953) and rarefaction curves to maximize sample inclusion against OTU coverage (Fig. S1). For analyses inclusive of all samples and of cloacal samples exclusively we rarefied to 500 reads per sample (Good's estimate all samples = 0.92 ± 0.03, range: 0.86–0.99; cloacal samples = 0.94 ± 0.03, range: 0.87–0.98). In analyses involving fecal samples exclusively, we rarefied to 3,350 sequences per sample (Good's estimate fecal samples = 0.98 ± 0.005, range: 0.97–0.99).

We compared a variety of community membership metrics across samples from Fijian crested iguana hosts. For all comparisons, we first calculated alpha diversity measurements including number of observed OTUs, the Shannon index (Shannon, 1948), and Faith's Phylogenetic Diversity (Faith's PD; Faith, 1992). Alpha diversity measurements were compared using analysis of variance (ANOVA) tests in R v3.6.2 (R Core Team, 2013) with the Tukey Test used for post-hoc analyses. Kruskal-Wallis tests with Bonferroni corrections were used in QIIME to compare relative abundances of bacterial taxa between treatment groups. In examining specific OTUs, BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990) was used to compare

novel sequences against those available in the National Center for Biotechnology Information's (NCBI) Nucleotide database.

Community diversity and structure were compared using principal coordinates analysis (PCoA) on beta diversity metrics including weighted and unweighted UniFrac distances (Lozupone & Knight, 2005) and the binary Jaccard index (Jaccard, 1901). Beta diversity matrices and PCoA plots were generated from the same rarefied datasets used to measure alpha diversity metrics. The adonis function in the vegan v2.3_4 package (Oksanen et al., 2016) of R v3.3.1 (R Core Team, 2013) was used on beta diversity distance matrices with 999 permutations to compare community composition between groups statistically.

2.3 | Sample comparisons

We first analyzed bacterial composition across all 83 samples (Appendix S1) and then split the dataset into cloacal and fecal subsets to examine general patterns between sample types. Following broad overviews of the data, we tested the effects of captivity status on gut bacterial communities in crested iguana hosts and examined for microbial restructuring in reintroduced lizards post-release.

To determine the influences of captivity status on gut microbial communities we used snapshot analyses of cloacal and fecal samples taken from WCF, CB2017, CB2015, and Wild lizards. For cloacal comparisons, we included 35 samples collected between 22 February and 2 March 2017 (Appendix S1). This subset included 10 WCF, 13 CB2017, three CB2015, and nine Wild individuals. In our subsequent fecal analyses, we included 26 fecal samples collected between 22 February and 1 March 2017 (Appendix S1). This dataset encompassed fecal samples from nine WCF, nine CB2017, two CB2015, and six Wild iguanas. In addition to comparing

microbial communities across four treatments, we also ran all analyses between just two conditions, captive (WCF and CB2017 grouped) and non-captive (CB2015 and Wild grouped) (Ren et al. 2016).

We sought to assess the effects of release on lizard microbiota using both cloacal and fecal samples collected roughly two months after host reintroduction to Monuriki. We collected cloacal samples from five recently released lizards, one WCF and four CB2017, between 24 April and 11 May 2017 (Appendix S1). We compared microbial communities from these samples against those in the initial 23 captive animal cloacal samples (10 WCF, 13 CB2017) as well as the initial 12 non-captive samples (nine Wild, three CB2015). We also compared six novel fecal samples (one WCF, five CB2017) collected between 2 and 17 May (Appendix S1) against the 18 initial captive fecal samples (nine WCF, nine CB2017) and eight non-captive fecal samples (two CB2015, six Wild). In both instances, we sought to determine if gut microbiomes were more similar to captive communities or non-captive communities two months after host reintroduction.

3 | RESULTS

3.1 | General patterns in Fijian crested iguana microbiota

Our curated dataset of 83 samples generated 898,625 reads with a minimum read depth of 540, a maximum of 30,503, and a median of 9,883 reads per sample. Among the 46 cloacal samples only, 410,545 reads were recovered with a minimum read depth of 540 sequences per sample, maximum of 25,304, and median read depth of 8,521.5. The 37 fecal samples produced 488,080 reads with a minimum, maximum, and median read depth of 3,378, 30,503, and 12,558 reads per sample respectively.

Fijian crested iguana microbiome samples averaged 85 unique OTUs per 500 reads, the Shannon index varied from 0.93 to 6.32 (mean = 4.77 ± 1.28), and Faith's PD varied from 2.76 to 14.33 (mean = 9.34 ± 2.87). The average Jaccard distance between pairs of samples was 0.83 suggesting that any two samples shared ~17% of their OTUs on average. Across rarefied sequences, six OTUs were found in ≥70% of all samples, one *Oscillospira* sp., one *Phascolarctobacterium* sp., two unidentified taxa in the family Enterobacteriaceae, and two unidentified taxa in the families Clostridiaceae and Lachnospiraceae. At a rarefied depth of 500 reads per sample, most sequences (91.8%) belonged to four phyla: Firmicutes (48.3%), Proteobacteria (18.4%), Actinobacteria (13.9%). and Bacteroidetes (11.1%).

The average number of OTUs per cloacal sample was 68 (sequence depth = 500 rarified reads/sample), the Shannon index varied from 0.81–6.07 (mean = 4.09 ± 1.32), and Faith's PD varied from 2.8–12.66 (mean 7.88 ± 3.04). Jaccard distances averaged 0.86 across pairs of cloacal samples, a slight increase when compared to that among all samples. Just four OTUs were identified in ≥70% rarefied cloacal sequences, one *Corynebacterium* sp., an unidentified microbe in Clostridiaceae, and two unidentified taxa in Enterobacteriaceae. The majority of cloacal reads (95.1%) belonged to the same four dominant phyla as in all samples: Firmicutes (37.2%), Proteobacteria (27.7%), Actinobacteria (24.3%), and Bacteroidetes (5.9%).

Within fecal samples and at a sequencing depth of 3,350 quality-controlled reads, the average number of OTUs found was 224, the Shannon index varied from 5.14 to 6.63 (mean = 5.90 ± 0.37), and Faith's PD varied from 11.94 to 21.69 (mean = 17.13 ± 2.05). The average Jaccard distance between any pair of fecal samples was 0.65, suggesting more similarity among fecal samples compared to among cloacal samples. Across all fecal samples, 90 OTUs were found in ≥70% of samples and seven OTUs were found in 100% of fecal samples. These included three

Bacteroides spp., one *Parabacteroides* sp., an unidentified taxon in Lachnospiraceae, one in Enterobacteriaceae, and a third in Ruminococcaceae. Most rarefied reads (86.6%) belonged to just three phyla: Firmicutes (61.5%), Bacteroidetes (18.1%), and Proteobacteria (7.0%), while Actinobacteria comprised only 0.8% of rarified fecal reads.

3.2 | Comparison of microbiota in captive and non-captive iguanas via cloacal samples

Comparisons of cloacal samples from Fijian crested iguanas of treatment groups WCF, CB2017, CB2015, and Wild yielded no significant differences in measured alpha diversity metrics (Fig. S2). This lack of differentiation remained even when samples were grouped as captive (WCF and CB2017 grouped) and non-captive (CB2015 and Wild grouped) treatments (Fig. S2). PCoA plots of beta diversity metrics showed limited clustering when grouping both by four treatments and by captive and non-captive lizards (Fig. 2A). Among all four treatments, adonis tests determined significant differentiation in unweighted UniFrac distances (R^2 = 0.1412, P = 0.004), and Jaccard distances (R^2 = 0.1445, P = 0.001) while weighted UniFrac distances (R^2 = 0.1448, P = 0.075) were not significantly distinct. Grouping by captive and non-captive types produced similar, yet weaker, results in unweighted UniFrac distances (R^2 = 0.0641, P = 0.007), Jaccard distances (R^2 = 0.0646, P = 0.002), and weighted UniFrac distances (R^2 = 0.0460, P = 0.174). The average Jaccard distance between pairs of cloacal samples in this subset was 0.85 and remained similar within treatment groups (WCF = 0.81, CB2017 = 0.82, CB2015 = 0.75, Wild = 0.88; captive = 0.83, non-captive = 0.86).

Rarefied cloacal samples across all groups in this subset were dominated by Firmicutes (37.7%), Proteobacteria (26.2%), Actinobacteria (24.9%), and Bacteroidetes (6.5%) with some differentiation among treatments (Fig. S3). At 500 sequences per sample, Kruskal-Wallis tests

identified two OTUs that varied significantly in relative abundance between all four treatments following Bonferroni corrections. These included one *Cupriavidus* sp. (WCF mean reads = 0, CB2017 = 0, CB2015 = 0.7, Wild = 0), and an unidentified taxon in Coriobacteriaceae (WCF mean reads = 0, CB2017 = 0, CB2015 = 2.0, Wild = 0). Both of these differentiations are likely due to limited sampling in the CB2015 category (n = 3). When comparing captive and non-captive samples, one OTU, an unidentified taxon in Micrococcaceae, was found to differ between treatment groups (mean captive reads = 19.1, non-captive = 0). BLAST queries of this specific sequence returned a 99.6% match to *Nesterenkonia* sp. strain MadaFrogSkinBac.DB-.3605. While not significantly distinct between treatments, a number of OTUs were present in rarefied captive samples that were absent in non-captive ones (Appendix S2). Notably, these included another *Nesterenkonia* sp. (captive mean reads = 37.7), one *Brevibacterium* sp. (captive mean reads = 12.5), and one *Brachybacterium* sp. (captive mean reads = 11.2).

3.3 | Comparison of microbiota in captive and non-captive iguanas via fecal samples

We found significant differences in the number of OTUs ($P = 0.005$; WCF = 223, CB2017 = 233, CB2015 = 181.5, Wild = 190) and in Faith's PD ($P = 0.001$; WCF = 17.0, CB2017 = 17.8, CB2015 = 14.1, Wild = 15.1) but not in the Shannon index when comparing fecal samples across all four treatments (Fig. 3). Post-hoc analyses of observed OTUs found significant differentiation between CB2017 and Wild samples ($P = 0.012$) while remaining comparisons were insignificant. Post-hoc analyses of Faith's PD results revealed significant differentiation between CB2017 and CB2015 ($P = 0.011$) and CB2017 and Wild ($P = 0.005$) treatments. Remaining pairwise comparisons were insignificant. Grouping by captive and non-captive statuses again resulted in significant differences in the number observed OTUs ($P < 0.001$; captive mean = 229, non-

captive mean = 188) and in Faith's PD ($P < 0.001$, captive mean = 17.4, non-captive mean = 14.9) but not in the Shannon index (Fig. S4). PCoA plots showed evident clustering among all four treatments and when grouped as captive and non-captive samples (Fig. 2B). Adonis analyses showed significant differences between the four conditions in weighted UniFrac distances ($R^2 = 0.4297$, $P = 0.001$), unweighted UniFrac distances ($R^2 = 0.3302$, $P = 0.001$), and Jaccard distances ($R^2 = 0.3142$, $P = 0.001$). Captive and non-captive comparisons showed similarly significant yet slightly weaker results in weighted UniFrac ($R^2 = 0.3162$, $P = 0.001$), unweighted UniFrac ($R^2 = 0.2122$, $P = 0.001$), and Jaccard distances ($R^2 = 0.2036$, $P = 0.001$). Pairs of fecal samples averaged a Jaccard distance of 0.65 with some deviation within treatment groups (WCF = 0.57, CB2017 = 0.55, CB2015 = 0.57, Wild = 0.63; captive = 0.57, non-captive = 0.64).

The most prevalent phyla among rarefied fecal reads included Firmicutes (66.5%), Bacteroidetes (16.1%), and Proteobacteria (6.5%) contributing to 89.2% of sequences. Synergistetes (2.4%), Planctomycetes (2.3%), Tenericutes (2.0%), and Verrucomicrobia (1.9%) also contributed to general relative diversity present while Actinobacteria accounted for just 0.7% of rarefied reads (Fig. S5). Kruskal-Wallis tests identified one OTU that varied in abundance across all four groups, an unidentified Clostridiales (WCF mean reads = 0, CB2017 = 0, CB2015 = 1.5, Wild = 0) though significance of this difference is likely due to limited sampling of CB2015 individuals (n = 2) in this subset. Comparisons of captive and non-captive microbial communities from crested iguana fecal samples identified seven OTUs that varied significantly between treatments. Three of these OTUs, one *Coprococcus* sp. (captive mean = 0.3, non-captive = 26.5), an unidentified Coriobacteriaceae (captive mean = 0, non-captive = 3.9), and an unidentified Mogibacteriaceae (captive mean = 0, non-captive = 4.3), were more

prevalent in non-captive animals than in captive ones (Appendix S2). The remaining four OTUs were common in rarefied captive animal communities but absent from non-captive counterparts. These OTUs included one *Ruminococcus* sp. (captive mean = 95.3, non-captive = 0), an *Acetobacterium* sp. (captive mean = 82, non-captive = 0), an unidentified Christensenellaceae (captive mean = 53.4, non-captive = 0), and one *Bacteroides* sp. (captive mean = 25.3, non-captive = 0; Appendix S2). References of the unidentified Christensenellaceae sequence against published data in BLAST returned hits only to uncultured bacterial clones. A litany of additional OTUs were present in rarefied captive fecal samples that were not recovered in non-captive ones (Appendix S2). Among these included an unidentified taxon in Synergistaceae (captive mean = 116.9), two unidentified Christensenellaceae (captive means = 58.6, 38.9), one *Akkermansia* sp. (captive mean = 29.3), another *Ruminococcus* sp. (captive mean = 18.7), an unidentified Clostridiales (captive mean = 15.1), and one *Coprococcus* sp. (captive mean = 11.0). BLAST searches of the unidentified taxon in Synergistaceae returned a 100% match to *Cloacibacillus porcorum* strain CL-84, while the two unidentified Christensenellaceae and the Clostridiales paired only to uncultured bacterium.

3.4 | Temporal variation of cloacal microbiota in captive crested iguanas post-release

Comparisons of microbial communities from five cloacal samples taken shortly after host reintroduction against both captive and non-captive microbial communities revealed no significant variation in alpha diversity metrics (Fig. S6). Comparisons of reintroduced individuals with complete time-series sampling yielded no significant difference in alpha diversity metrics pre- and post-release. PCoA plots revealed limited clustering across all three conditions in weighted and unweighted UniFrac metrics though some grouping between

reintroduced and non-captive samples was apparent in Jaccard plots (Fig. 4A). Plots of only individuals with complete time-series sampling also showed inconsistent groupings (Fig. S7). Adonis tests between reintroduced, captive, and non-captive samples found significant differentiation in unweighted UniFrac (R^2 = 0.0883, P = 0.006) and Jaccard distances (R^2 = 0.0907, P = 0.001). Further pairwise comparisons between reintroduced samples and non-captive samples uncovered no distinction in any beta metrics. Reintroduced samples were, however, significantly distinct from captive ones in the Jaccard metric (R^2 = 0.0601, P = 0.006). The average Jaccard distance among pairs of samples from reintroduced lizards was 0.83.

Microbial communities sourced from cloacal swabs in this subset were largely dominated by three phyla: Firmicutes, Proteobacteria, and Actinobacteria in all treatment groups. However, proportions of these taxa shifted between reintroduced, captive, and non-captive conditions (Fig. S8). We identified a single OTU that varied statistically between all three groups based on Kruskal-Wallis tests, the unidentified taxon in Micrococcaceae matching *Nesterenkonia* sp. strain MadaFrogSkinBac.DB-.3605 (reintroduced mean reads = 0, captive = 19.1, non-captive = 0). Interestingly, a number of OTUs that were commonly found in cloacal samples from captive animals including the additional strain of *Nesterenkonia*, the *Brevibacterium* sp., and the *Brachybacterium* sp. were nearly or entirely absent in rarefied reads of samples from reintroduced hosts (reintroduced mean reads = 0, 0.2, 0.2 respectively; Appendix S2).

3.5 | Temporal variation of fecal microbiota in captive crested iguanas post-release

We compared microbial communities in six fecal samples from reintroduced iguanas against those from captive and non-captive samples and found significant differences in the number of observed OTUs (P < 0.001; reintroduced mean reads = 252, captive = 229, non-captive = 188)

and in Faith's PD ($P < 0.001$, reintroduced mean reads = 18.9, captive = 17.4, non-captive = 14.9; Fig. 5). Post hoc analyses of observed OTUs found significance only between reintroduced and non-captive communities ($P = 0.001$). Faith's PD post hoc tests found significance between reintroduced and non-captive communities ($P = 0.003$) as well as between captive and non-captive communities ($P = 0.003$). Comparisons of reintroduced individuals with complete time-series sampling yielded no significant difference in alpha diversity metrics pre- and post-release. Plotted beta diversity metrics showed some clustering between treatment groups with reintroduced animals associating most closely with captive samples (Fig. 4B). Significant differences in adonis tests were recorded in weighted UniFrac distances ($R^2 = 0.3417$, $P = 0.001$), unweighted UniFrac distances ($R^2 = 0.2291$, $P = 0.001$), and Jaccard distances ($R^2 = 0.2197$, $P = 0.001$) between reintroduced, captive, and non-captive samples. Pairwise comparisons between reintroduced and captive samples were significantly distinct for all three metrics: weighted UniFrac ($R^2 = 0.2493$, $P = 0.001$), weighted UniFrac ($R^2 = 0.0899$, $P = 0.001$), and Jaccard distances ($R^2 = 0.0915$, $P = 0.001$). Comparisons between reintroduced and non-captive samples also produced significant differentiation in weighted UniFrac distances ($R^2 = 0.3992$, $P = 0.006$), unweighted UniFrac distances ($R^2 = 0.3862$, $P = 0.002$), and Jaccard distances ($R^2 = 0.3747$, $P = 0.001$). PCoA plots of individuals with complete time-series sampling exclusively showed clustering with some overlap between groups (Fig. S9). The average Jaccard distance among pairs of samples after release was 0.61.

Microbial communities found in fecal samples from reintroduced, captive, and non-captive samples were primarily dominated by three phyla: Firmicutes, Bacteroidetes, and Proteobacteria. Relative abundances of these phyla varied between conditions (Fig. S10). In comparing OTU relative abundances, Kruskal-Wallis tests retrieved nine OTUs that differed between all three

treatments (Appendix S2). These included one *Acetobacterium* sp. (reintroduced = 3.2, captive = 82, non-captive = 0), one *Akkermansia* sp. (reintroduced = 164.8, captive = 29.3, non-captive = 0), one *Butyricimonas* sp. (reintroduced = 1.7, captive = 0, non-captive = 0.3), one *Coprococcus* sp. (reintroduced = 2.5, captive = 0.3, non-captive = 26.5), one *Ruminococcus* sp. (reintroduced = 7.7, captive = 95.3, non-captive = 0), an unidentified Christensenellaceae (reintroduced = 121.7, captive = 53.4, non-captive = 0), an unidentified Coriobacteriaceae (reintroduced mean reads = 0, captive = 0, non-captive = 3.9), an unidentified Lachnospiraceae (reintroduced = 0.3, captive = 7.7, non-captive = 0), and an unidentified Mogibacteriaceae (reintroduced = 0.5, captive = 0, non-captive = 4.3). Scrutiny of additional taxa found in captive lizards yet absent from non-captive ones yielded mixed results with some bacterial strains becoming more prevalent in reintroduced hosts and others becoming less prevalent (Appendix S2). The unknown Synergistaceae matching *C. porcorum* for example increased in mean relative abundance between conditions (reintroduced mean reads = 122.5, captive = 116.9) as did the noted *Bacteroides* sp. (reintroduced = 134.7, captive = 25.3; Table 4). Meanwhile the second *Ruminococcus* sp. (reintroduced = 0.8, captive mean = 18.7), *Coprococcus* sp. (reintroduced = 0.7, captive mean = 11.0), and unidentified Clostridiales (reintroduced = 0.2, captive mean = 15.1) all decreased in relative abundances in reintroduced hosts (Appendix S2).

4 | DISCUSSION

Our findings show that captive and non-captive Fijian crested iguanas harbor distinct microbial communities regardless of sampling regime (cloacal versus fecal). These results expand on a growing body of evidence that suggests animals housed in captivity have distinct microbiomes when compared to wild conspecifics (Alfano et al., 2015; Cheng et al., 2015; Clayton et al.,

2016; Eigeland et al., 2012; Jiang et al., 2017; Kohl et al., 2017; McKenzie et al., 2017; Ren et al., 2016; West et al., 2019; Zhu et al., 2011). In both cloacal and fecal sampling, captive (WCF and CB2017 grouped) and non-captive (CB2017 and Wild grouped) iguanas harbored significantly different microbial communities in at least two beta diversity metrics (Fig. 4). Further, Jaccard distances were consistently lower within captive treatments, suggesting a greater degree of shared OTU breadth and potentially homogenization among captive individuals. These findings are consistent with those seen in *Anolis sagrei* where alpha diversity measures were generally higher in captive animals compared to wild conspecific hosts, yet gut communities were more homogenous, and beta diversity metrics separated wild and captive hosts (Ren et al., 2016). In addition to harboring distinct microbial communities, a number of specific OTUs, particularly potential pathogens, were seen in greater abundances in captive over non-captive Critically Endangered Fijian crested iguanas.

The introduction of potentially pathogenic bacteria has been documented previously in wild reptiles brought into temporary captivity (Jiang et al., 2017; Kohl et al., 2017) but not in a conservation initiative specifically designed to release captive animals into the wild. In cloacal samples from captive Fijian crested iguanas, one *Brachybacterium* sp., one *Brevibacterium* sp., and two *Nesterenkonia* spp. were present in rarefied reads while absent from non-captive counterparts (Appendix S2). All three of these genera have species implicated as potential pathogens at least in humans (Gruner, Pfyffer, & von Graevenitz, 1993; Nakayama, Ohkusu, & Tateda, 2009; Tamai et al., 2018). Fecal samples produced similar results where strains from multiple genera, including *Bacteroides, Cloacibacillus*, and *Ruminococcus* were found commonly in captive samples but absent in rarefied, non-captive reads (Appendix S2). These three genera are also potentially pathogenic strains in humans (Domingo et al., 2015; Titécat,

Wallet, Vieillard, Courcol, & Lo, 2014; Wexler, 2007). Although determining the exact pathogenic capacities of particular microbes is outside the realm of this investigation, high abundances of potential pathogens in animals under human care supports the possibility that headstart animals can harbor disease-causing bacteria at significantly higher rates than animals living in the wild (Redford et al., 2012). Although microbial communities in hosts can shift rapidly on the scale of days to even hours in some cases (Costello, Gordon, Secor, & Knight, 2010; Ren et al., 2016), the impacts of releasing animals with elevated levels of what could be pathogenic microbiota have received little attention to date (Redford et al., 2012).

Reintroduction of captive Fijian crested iguanas into native habitats promoted restructuring of gut microbiomes towards non-captive communities. After two months on Monuriki Island, cloacal samples from reintroduced iguanas appeared to harbor gut microbial communities more similar to non-captive than to captive compositions (Figs. 4, S6). Additionally, noted potential pathogens in captive individuals were either absent or diminished in reintroduced hosts. Microbial assemblages generated from fecal samples, however, did not produce similar results. Instead, microbiota from fecal samples of reintroduced lizards seemingly resembled captive hosts more closely rather than non-captive hosts (Figs. 4, 5). Potential pathogens also displayed differing trends with *Ruminococcus* spp. becoming less abundant in host iguanas two months after release and *Bacteroides* sp. and *Cloacibacillus* sp. becoming more abundant in samples taken from individuals after reintroduction. Such findings support previously proposed hypotheses that pathogens associated with human care may continue to impact headstart or reintroduced animals even after release (Bahrndorff et al., 2016; Redford et al., 2012; West et al., 2019). Despite fecal samples from reintroduced iguanas being significantly distinct from non-captive samples, this differentiation does appear to be temporary. Released animals relocated

onto Monuriki Island in 2015 (CB2015) contained gut microbial assemblages more closely associated with true wild iguanas rather than captive ones in both cloacal and fecal samples, suggesting that re-acclimation of wild-type microbiomes can occur after prolonged survival in native habitats (i.e. two years; Fig. 2).

Although both cloacal and fecal sampling techniques recovered significant differentiation in gut microbial communities between captive and non-captive Fijian crested iguanas (Figs. 2,4), specific OTUs that varied between treatments were inconsistent. Further, differences were apparent in comparing assemblages from reintroduced lizards to those in captive and non-captive hosts based on sampling regime (Fig. 4). Cloacal samples from reptiles generally encapsulate the breadth of gut microbial diversity but vary significantly in abundances compared directly to hindgut samples while fecal samples tend to better represent gut diversity and abundances (Colston et al., 2015; Kohl et al., 2017). When assessing microbial communities in captive lizards for potential disease-causing microbes, or in evaluating the restructuring of host microbiomes post-release, multiple non-lethal gut microbial sampling techniques may be necessary to fully elucidate trends of interest.

Gut microbial communities in captive Fijian crested iguanas are distinct from those in non-captive iguanas and this differentiation prevails for some time post-release. However, the duration in which a host's microbial composition shifts to closely resemble true wild counterparts remains unclear. A continued need exists to monitor microbial communities in headstart animals post-release to track animal well-being (Bahrndorff et al., 2016; Jiménez & Sommer, 2017; Redford et al., 2012; West et al., 2019). Such studies could determine the influences of potential disease-causing bacteria associated with captive upbringings on host survival, growth, and reproduction in the wild. Further, wild conspecifics in populations with

introduced animals should be monitored closely for introduction of novel pathogens brought on from interaction with animals sourced from headstart programs (West et al., 2019). Such scenarios may justify the use of soft-releases or probiotics prior to animal release to acclimatize gut microbiota in headstart individuals to natural conditions and eliminate possible disease-causing agents before complete reintroduction to the wild (Redford et al., 2012; West et al., 2019). Along with increased monitoring of animal health, additional scrutiny of specific OTUs seen in differential abundances between headstart and wild animals that may be pathogenic is necessary to determine the virulence of such bacterial strains. Should these OTUs be minimally pathogenic then no additional action may be necessary to mitigate their increased abundances while animals are in captive settings. Ultimately, consistent monitoring of hosts post-release and further examination of possible pathogens are the next step towards improving our understanding of gut microbial ecology in endangered species with conservation significance.

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DATA ACCESSIBILITY STATEMENT

All 16S rRNA sequences have been deposited in the Sequence Read Archive (SRA) under accession no. PRJNA702127.

AUTHOR CONTRIBUTIONS

J.C.B. and R.N.F. coordinated fieldwork and collected samples; S.J.E, J.C.B, and C.D.S. completed sample processing and laboratory analyses; S.J.E. conducted molecular and phylogenetic analyses; and T.J.C assisted with study design and statistical analyses. S.J.E produced figures and wrote the manuscript. All authors provided critical review of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FIGURES AND FIGURE LEGENDS

Fig. 1. Adult Fijian crested iguana (*B. vitiensis*) perched in native habitat on Monuriki Island (Photo by J.C.B.).

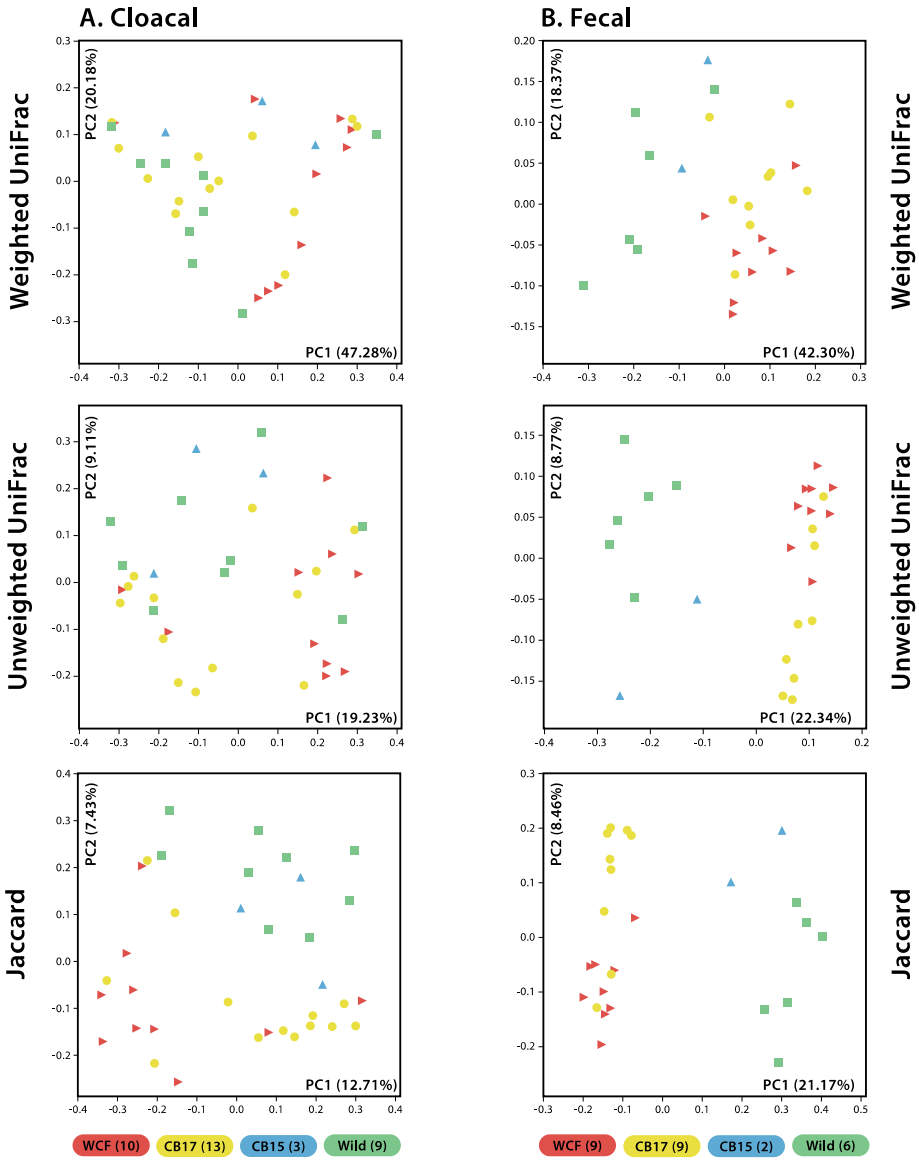

Fig. 2. Principal coordinates analysis plots of initial (2017) A) cloacal swabs and B) fecal samples across four Fijian crested iguana treatment groups. Treatment groups include wild caught founders (WCF) in captivity and captive-born headstart individuals (CB2017) in captivity at Kula Eco Park on Viti Levu, Fiji as well as captive-born individuals released onto Monuriki Island in 2015 (CB2015) and fully wild individuals on Monuriki Island (Wild). Number of individual samples per treatment group is indicated in parentheses.

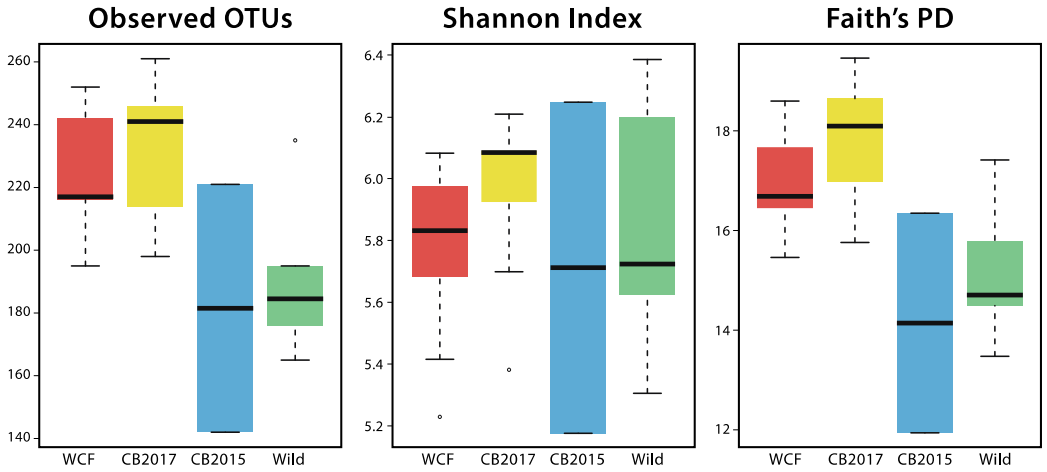

Fig. 3. Alpha diversity metrics of initial (2017) fecal samples across four treatment groups. Treatments included wild caught founder (WCF) iguanas in captivity, captive-born headstart individuals (CB2017) in captivity, captive-born individuals released onto Monuriki in 2015 (CB2015), and fully wild individuals on Monuriki Island (Wild). Paired symbols denote significantly distinct treatment groups.

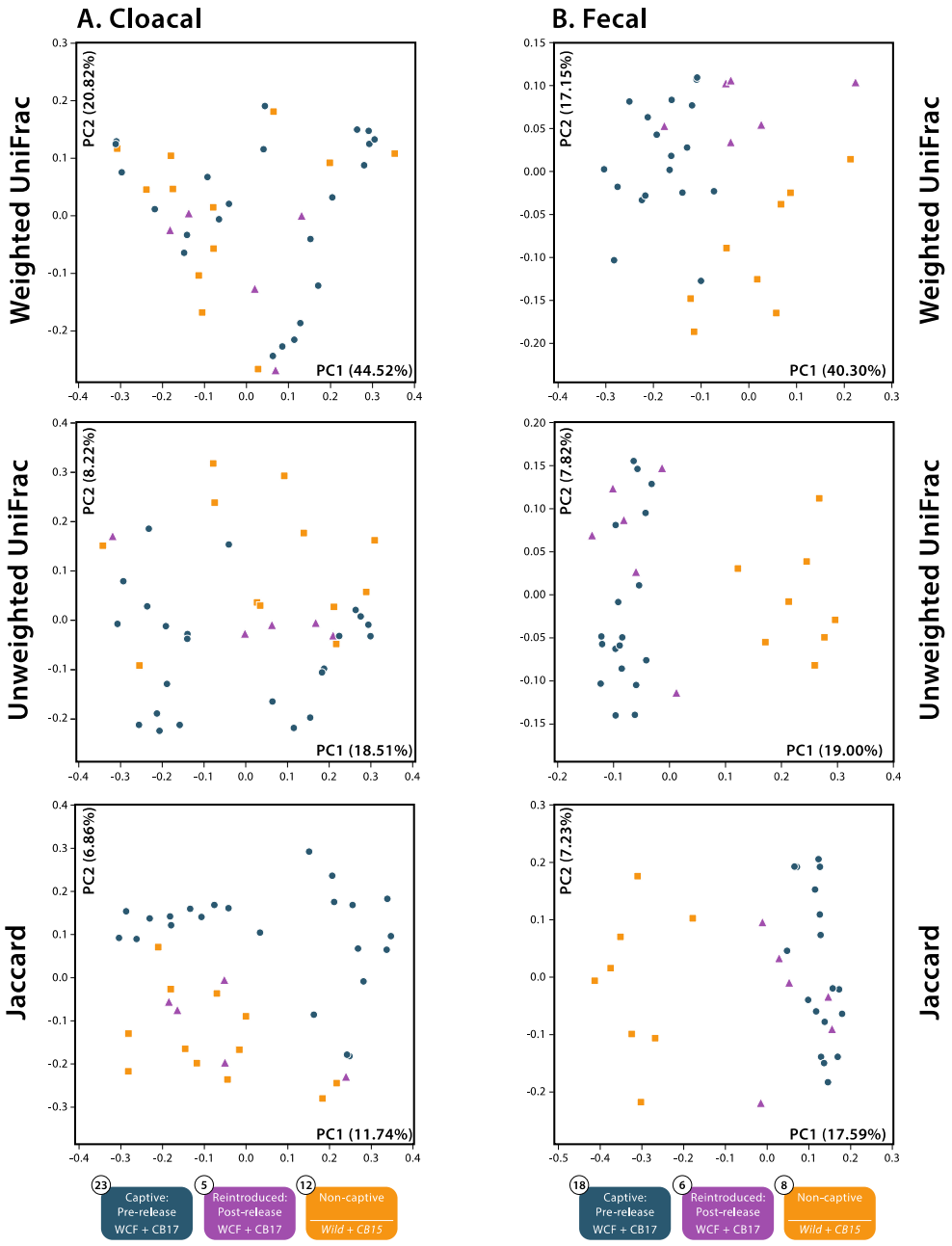

Fig. 4. Principal coordinates analysis of reintroduced and initial (2017) A) cloacal swabs and B) fecal samples across three Fijian crested iguana treatment groups. Captive pre-release samples include wild caught founders (WCF) in captivity and captive-born headstart individuals (CB2017) in captivity at Kula Eco Park collected February 2017. Non-captive individuals consist of captive-born individuals released onto Monuriki island in 2015 (CB2015) and fully wild

individuals on Monuriki island (Wild). Post-release treatments include formerly captive WCF and CB2017 individuals sampled in late April 2017, two months after release onto Monuriki Island.

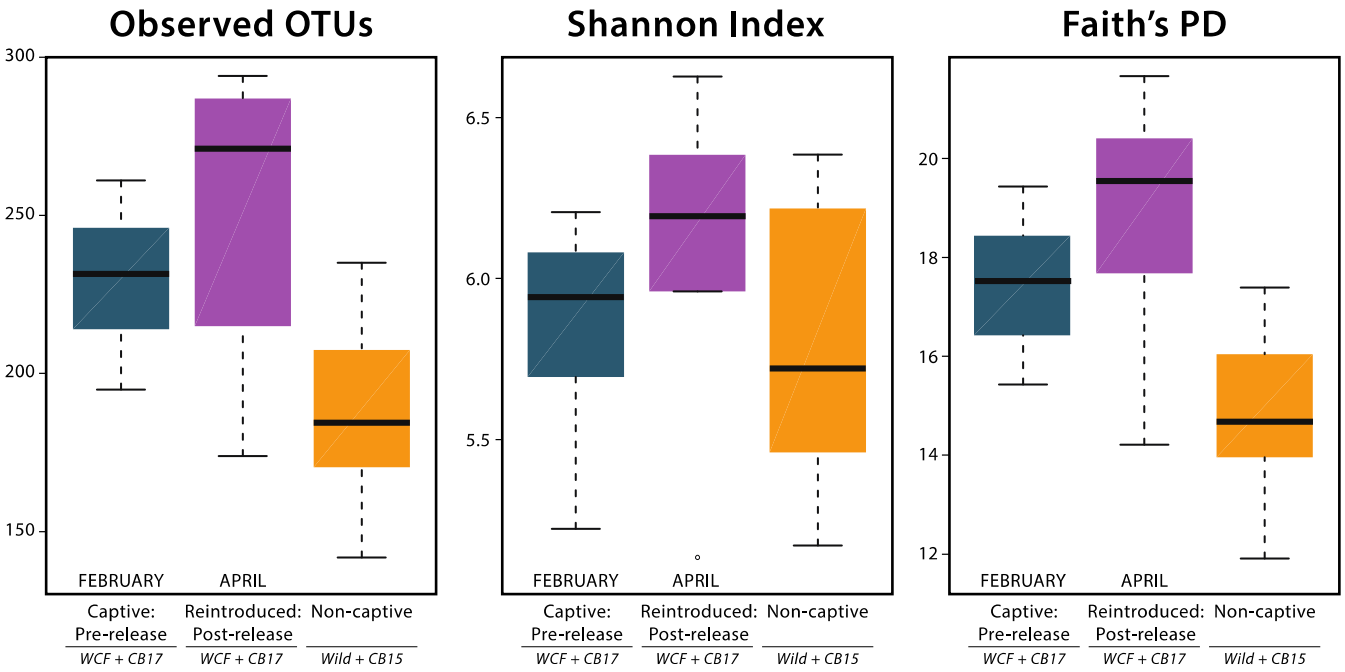

Fig. 5. Alpha diversity metrics of fecal samples from reintroduced Fijian crested iguana hosts compared against initial samples. Captive pre-release samples include wild caught founders (WCF) in captivity and captive-born headstart individuals (CB2017) in captivity at Kula Eco Park. Non-captive individuals consist of captive-born individuals released onto Monuriki island in 2015 (CB2015) and fully wild individuals on Monuriki island (Wild). Initial sample collection occurred in Febraury 2017. Post-release treatments include formerly captive WCF and CB2017 individuals sampled two months after release onto Monuriki Island in April 2017. Paired symbols denote significantly distinct treatment groups.

Chapter 4: Gut microbiota shifts through headstart and reintroduction of the locally threatened Texas horned lizard (*Phyrnosoma cornutum*)

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Formatted for Zoo Biology

ABSTRACT

Animals raised in captivity as part of reintroduction programs must adapt rapidly to novel conditions when released into native habitats. The host's microbiome may be critical in this acclimation process, as gut microbiomes often vary significantly between captive and wild conspecifics. We sought to better understand the interconnectedness of wildlife headstart and reintroduction programs and host-associated gut microbial communities in Texas horned lizards (*Phyrnosoma cornutum*) in Oklahoma, a Tier I Species of Greatest Conservation Need in the state. We raised hatchling *P. cornutum* at the Oklahoma City Zoo and Botanical Garden for either one or two years before reintroducing them to their source population on Tinker Air Force Base in Midwest City, Oklahoma. We did this to 1) document differences in gut microbial communities between captive-reared and wild lizards and 2) assess whether gut microbiota in reintroduced Texas horned lizards shifted to closely resemble wild counterparts following release. Within three months of reintroduction, headstart Texas horned lizard microbiomes were substantially more similar to wild counterparts than while housed in captivity. These results suggest that reintroduced animals have the capacity to rapidly alter gut microbiota to reflect microbial communities found in naturally occurring host populations. This study offers promising signs for the plasticity of the microbiota in reintroduced hosts and underscores the need for continuous monitoring efforts in headstart programs.

KEYWORDS

Captive management, conservation, headstart, microbiome, reintroduction, reptiles

INTRODUCTION

The Texas horned lizard (*Phrynosoma cornutum*) is one of the most iconic reptile species in North America. Well known for its distinctive horns, lateral spikes, and flattened body, *P. cornutum* is native to desert and grassland habitats in the south-central United States and Mexico with sandy or loose soils that facilitate burrowing for nesting and brumation (Price 1990; Burrow et al. 2001). Historical accounts of this species have been documented as far north as Kansas, east to Missouri, west to Colorado, and south into Mexico (Price 1990). Despite an expansive geographic range, local extirpations of Texas horned lizards have been recorded regularly across many of its known localities, particularly since the 1950s (Price 1990; Carpenter et al. 1993; Donaldson et al. 1994; Busby and Parmelee 1996). These declines have been linked to several factors including overexploitation for the pet trade (particularly in the first half of the 20th century), the spread of invasive red imported fire ants (*Solenopsis invicta*), and habitat disruption, including loss, fragmentation, increased road mortality, and declines of their primary food source, native ant species (Price 1990; Carpenter et al. 1993; Donaldson et al. 1994).

Though Texas horned lizard populations have become increasingly isolated within fragmented landscapes, subpopulations are able to persist so long as sufficient suitable habitat remains (Carpenter et al. 1993; Donaldson et al. 1994; Stark 2000; Endriss et al. 2007; Vesy et al. 2021). The species generally requires mosaic landscapes to thermoregulate between open and shaded areas throughout the day and between seasons (Bogosian et al. 2012; Wolf et al. 2015), with home ranges varying greatly in size from 100–8,400m^2 based on age and sex of lizards as well as seasonality (Fair and Henke 1999; Vesy et al. 2021). Despite this species' ability to

persist in small, isolated pockets, increased efforts are being undertaken to monitor population health and proactively address continued population declines as human encroachment continues.

One possible measure that can be taken to directly address declining wildlife population trends is conservation translocation, involving deliberate movement and release of organisms to meet defined conservation objectives (IUCN/SSC 2013). Headstart programs are a type of translocation initiative employed in wildlife management programs to reinforce populations of threatened species (Redford et al. 2011; Burke 2015; McGowan et al. 2016; Bennett et al. 2017). The goal of headstart programs is to increase hatchling or juvenile survival rates by rearing individuals in captivity during vulnerable life stages before reintroduction under the expectation that larger and healthier reintroduced individuals will have increased survivorship in the wild (Alberts 2007; Burke 2015; Bennet et al. 2017). Such tactics have been used to aid species from various taxonomic groups including mammals, birds, amphibians, and reptiles (Fischer and Lindenmayer 2000; Mathews et al. 2005; Germano and Bishop 2009), and these programs continue to serve an important role in population management and wildlife recovery plans (e.g., Bennet et al. 2017; Brown et al. 2021; Eliades et al. 2021). This includes recent efforts to combat declining population trends observed for Texas horned lizards.

In Oklahoma, the Texas horned lizard is designated a Tier I Species of Greatest Conservation Need, as population declines have been observed statewide (Oklahoma Department of Wildlife Conservation 2016). The best studied population of *P. cornutum* in the state occurs at Tinker Air Force Base (TAFB), in Midwest City, Oklahoma, where a long-term and ongoing wildlife monitoring program has been in place for more than 20 years, investigating population demographics, survival rates, habitat requirements, and general behavior of the small, isolated population (Endriss et al. 2007; Moody et al. 2007; Bogosian et al. 2012; Wolf et al. 2013, 2014,

2015; Mook et al. 2017; Vesy et al. 2021; Eliades et al. 2022). Since the start of horned lizard studies at TAFB in 2003, overall population size has fluctuated drastically, as have individual survival rates at all life stages, and small-scale habitat restoration studies have shown little effect on increasing suspected population size (Endriss et al. 2007; Wolf et al. 2013; Vesy et al. 2021). Demographic studies have found that increased hatchling and juvenile survivorship have the greatest effect on *P. cornutum* population growth on TAFB (Wolf et al. 2014). To bolster this small urban population, a headstart program for Texas horned lizards at the Oklahoma City Zoo and Botanical Garden (OKC Zoo) was initiated in 2019. This program began with the goal of establishing a viable methodology to increase hatchling survivorship during early development before releasing more robust lizards back into the source population on TAFB (Endriss et al. 2007; Wolf et al. 2014).

Of particular interest in the creation and implementation of this headstart program for Texas horned lizards in Oklahoma has been the study of gut microbiomes in lizards while in human care and after release into native habitat. Animals housed in captivity generally harbor distinct gut microbial assemblages as compared to wild counterparts (Bahrndorff et al. 2016; Jiménez and Sommer 2017; Trevelline et al. 2019; West et al. 2019; Zhu et al. 2021; Dallas and Warne 2022). Captive hosts tend to have lower alpha diversity and more homogenized microbial compositions than their wild counterparts (Eigeland et al. 2012; Amato et al. 2013; Cheng et al. 2015; Clayton et al. 2016; Kueneman et al. 2016; Stumpf et al. 2016; West et al. 2019). Given the integral role that microbiomes play in maintaining host health via nutrient intake, immune response, and even organismal behavior (Fraune and Bosch 2010; Cho and Blaser 2012; Lee and Hase 2014), well-suited microbiomes may be needed for organisms to persist when released from captive conditions. Translocated individuals without locally adapted gut bacterial

communities may be unprepared for native habitats, leading to reduced survivorship in the wild (Redford et al. 2012; Bahrndorff et al. 2016; Jiménez and Sommer 2017; Trevelline et al. 2019; West et al. 2019; Zhu et al. 2021). Initial reintroduction studies on Tasmanian Devils (Chong et al. 2019) and Fijian crested iguanas (Eliades et al. 2021) suggest that microbiomes in translocated hosts are sufficiently plastic to eventually mirror resident populations. However, additional examinations of reintroduction effects on microbiomes in different host species and the rates at which transitions in microbial communities may occur following host release are needed.

In 2021, we translocated 34 Texas horned lizard juveniles raised at the OKC Zoo to native prairie habitat on TAFB to reinforce the wild population. We collected fecal samples as proxies for endogenous microbial communities in wild lizards at TAFB and captive-reared hosts both before and after translocation to 1) identify differences in gut microbial communities between captive-reared and wild individuals and 2) assess whether gut microbiota in reintroduced lizards adjust to resemble wild counterparts more closely within one active season on TAFB from April through September. We anticipated to see initial disparities in gut microbiomes between captive and wild hosts and eventual shifts in endogenous microbial assemblages of captive-reared hosts to mirror wild individuals after translocation. These microbial datasets will be essential in adjusting future captive husbandry conditions to promote beneficial microbial assemblages that may improve reintroduced horned lizard survivorship in the wild (Redford et al. 2012; West et al. 2019; Yang et al. 2020).

MATERIALS AND METHODS

Animal maintenance and sample collection

Texas horned lizard eggs and hatchlings were collected from TAFB and transported to the OKC Zoo for rearing in captivity during the summers (June–August) of 2019 and 2020. In total, 34 Texas horned lizards were raised for a joint reintroduction onto TAFB in June 2021 (15 individuals from 2019 cohort, 19 individuals from 2020 cohort). Husbandry conditions for animals while at the OKC Zoo are outlined in detail by Barrett et al. (2022). All Texas horned lizards were housed individually in 20-gallon aquariums. These enclosures were kept at stable temperatures year-round with an ambient temperature of ~24°C and a basking temperature of 37°C available for eight hours daily (9:00–17:00). Light and watering cycles were also constant, with UVB fixtures on for 11 hours per day (8:00–19:00) and misting systems programed to turn on twice daily for ~30 seconds at 10:30h and 14:00h. All lizards were fed a stable diet of pinhead crickets and fruit flies dusted with Repashy Calcium Plus for their one- or two-year rearing periods.

To collect fecal samples from captive lizards at the OKC Zoo, animals were placed in individual, sterilized, 6 qt shoebox containers within their home aquaria and left for one hour (Fig. 1A). Occasionally, food was offered via broadcast feeding while in these containers to stimulate activity and metabolic function. Fecal samples deposited by lizards during this period were stored immediately in Zymo DNA/RNA Shield at ambient temperatures for a maximum of 30 days following manufacturer guidelines before being transported to the Sam Noble Museum for long-term storage at -20°C.

On 1 June 2021, 34 Texas horned lizards were translocated from the OKC Zoo to four soft-release pens on TAFB for a five-week soft-release acclimation period. Soft-release pens were ~ 10 ft x 10 ft and consisted of one piece of aluminum siding dug four inches into the ground with

mesh netting over top to avoid potential predation (Fig. 1C). Lizards were placed in groups of 7–10 individuals from the same age cohorts and split between the four pens. On 9 July 2021, we removed 21 headstart Texas horned lizards from soft-release pens (10 mortalities, 3 unknown fates during acclimation period), and lizards were given complete access to their release site on TAFB. All lizards were equipped with Cell Track Tech LifeTags (Cellular Tracking Technologies, Cape May, NJ) and/or Recco harmonic radar diodes (RECCO Rescue Systems, Lidingo, Sweden) to identify and relocate individuals following complete reintroduction.

We were not able to recover any fecal samples from headstart lizards during the five-week soft-release phase (1 June–9 July 2021), but we collected fecal samples after complete release on TAFB to analyze microbiome shifts associated with complete animal translocation. Lizards were tracked via LifeTags or harmonic radar diodes, caught by hand, and placed in sterilized 6 qt shoebox containers in the nearest shaded area to the site of encounter (Fig. 1B). No food was offered during sample collection on TAFB. Individuals were left in tubs for roughly one hour and then placed back at the exact site of encounter. We tracked and monitored lizards on TAFB from the time of translocation to the end of the active season in early October (Wolf et al. 2013; Vesy et al. 2021). Field sampling of wild and translocated Texas horned lizards occurred from 23 June–15 September and 15 July–28 September respectively (Table 1). We again stored fecal samples immediately in Zymo DNA/RNA Shield in the field and then moved samples to an air-conditioned research facility on TAFB for temporary storage up to 30 days before eventual transportation to the Sam Noble Museum for long-term storage at -20°C.

Microbial inventories and comparisons

We sent 95 fecal samples and one negative control of Zymo DNA/RNA Shield to Zymo Research for DNA extraction through their ZymoBIOMICS™ service. Our 96 DNA extracts were returned along with two positive controls and two negative controls to the Sam Noble Museum, where we amplified the V4 region of the 16S rRNA gene (Kozich et al. 2013). Remaining sample processing and analyses closely mirror protocols from Eliades et al. (2021). PCR products were cleaned, normalized, and pooled using a Sequel Prep Normalization Plate Kit. Pooled libraries were purified using Agencourt® AMPure® magnetic bead capture and brought to the University of Oklahoma's Consolidated Core Lab for sequencing using 515F and 806R primers targeting 2x250bp reads on an Illumina MiSeq sequencing platform (Caporaso et al. 2012).

We paired and trimmed raw sequences using AdapterRemoval2 v2.2.2 with default parameters (Lindgreen 2012; Schubert, Lindgreen, and Orlando 2016). Cleaned sequences were clustered *de novo* into operational taxonomic units (OTUs) using UPARSE in USEARCH v11.0.667 at a minimum sequence identity of 97% and a minimum abundance of four (Edgar 2013). Remaining sample curation and analysis was carried out in QIIME v1.9.1 (Caporaso et al. 2010). Taxonomies were assigned to OTUs using GreenGenes v13.8 (DeSantis et al. 2006). Archaea, chloroplast, mitochondria, PhiX, and other non-bacterial sequences were removed from processed OTU tables to ensure only bacterial sequences were included in downstream analyses. All 16S rRNA sequences have been deposited in the Sequence Read Archive (SRA) under accession no. XXXX.

From our 95 fecal samples sequenced, we removed duplicates that were subsamples from the same fecal deposit and retained only the sample with greater sequencing depth (n = 7). Samples with fewer than 850 reads (n = 6) were also removed to ensure sufficient sequencing depth was

present for each sample. Finally, we removed all samples collected outside of March–September 2021 (n = 14) to ensure all sampling was done within one active season for Texas horned lizards on TAFB (Vesy et al. 2021) and minimize the potential influences of time and seasonality as confounding variables in microbiome comparisons. Our finalized dataset consisted of 68 total samples taken from 14 wild Texas horned lizards and nine captive-reared lizards translocated from the OKC Zoo to TAFB (Table 1). Sampling was uneven across treatment groups (Table 1). We retrieved 23 fecal samples from the 14 wild Texas horned lizards encountered on TAFB, 22 samples from eight headstart lizards pre-release at the OKC Zoo, and 23 samples from all nine captive-reared individuals after reintroduction onto TAFB; Table 1). One translocated lizard (ID 991-1) lost its only pre-release sample during filtering due to insufficient sequencing depth (Table 1). In the pre-release group, the maximum number of samples taken from a single lizard at different time points was seven and two lizards were represented by a single sample. In the post-release treatment, the maximum number of samples included from an individual was six and four lizards were represented by a single sample. Finally in the wild group, one individual was sampled four times while nine other animals were only sampled once (Table 1). This curated dataset of 68 samples returned 6,570,445 reads with a minimum read depth of 857 reads and a maximum of 600,876 per sample. We rarefied all samples from this finalized pool to 850 reads per sample (Good's estimate = 0.96 ± 0.01, range: 0.93–0.99) before continuing with analyses.

To better understand the effects of reintroduction on microbiota in Texas horned lizards, we compared rarefied sequence data using a variety of community membership metrics across samples. Treatment groups included captive lizards pre-release, captive lizards post-release, and wild lizards on TAFB (Table 1). Because sample sizes were uneven between individuals, we repeated analyses individually for the eight lizards sampled before and after translocation to

support conclusions drawn from preliminary treatment groupings (Table 1). We first calculated alpha diversity measurements including number of observed OTUs, the Shannon index (Shannon 1948), and Faith's Phylogenetic Diversity (Faith's PD; Faith 1992). Alpha diversity measurements between treatments were compared using analysis of variance (ANOVA) tests in R v3.6.2 (R Core Team 2013) with the Tukey Test used for post-hoc analyses. We used Kruskal-Wallis tests with Bonferroni corrections to compare relative abundances of bacterial taxa between treatment groups. To more accurately identify specific taxa that differed between treatments, we used BLAST (Altschul et al. 1990) and compared our novel sequences against those available in the National Center for Biotechnology Information's (NCBI) Nucleotide database.

To compare community diversity and structure between treatment groups, we used principal coordinates analyses (PCoA) on beta diversity metrics including weighted and unweighted UniFrac distances and the binary Jaccard index (Jaccard 1901; Lozupone and Knight 2005). The adonis() function in the *vegan* v2.3_4 package (Oksanen et al. 2016) of R v3.3.1 (R Core Team 2013) was used through QIIME on beta diversity distance matrices to run PERMANOVA tests with 999 permutations comparing community composition statistically between our three groups.

RESULTS

General patterns in Texas horned lizard gut microbiomes

Across all 68 Texas horned lizard fecal samples from the OKC Zoo and TAFB, samples averaged 90.37 ± 22.48 unique OTUs per 850 reads, with a maximum of 139 and minimum of 12. The Shannon index ranged from 2.63 to 5.97 (mean = 4.65 ± 0.73) and Faith's PD ranged from 2.02 to 11.67 (mean = 8.74 ± 1.53). Interestingly, 88.79% of rarified reads were assigned to

just two phyla, Bacteroidetes (47.42%) and Firmicutes (41.37%) (Fig. 2). We found 10 OTUs assigned to the order Clostridiales in 80% of all Texas horned lizard samples collected (Appendix S1), indicating a core set of microbes in both captive and wild lizards.

Comparison of microbiota between pre-release, post-release, and wild lizards

We found no significant differences in alpha diversity measures (number of OTUs [$F = 1.86$ $P = 0.164$], Shannon index [$F = 0.655$, $P = 0.523$], or Faith's PD [$F = 0.645$, $P = 0.528$]) when comparing samples from our three treatment conditions: headstart lizards pre-release, post-release, and wild hosts (Fig. 3). However, PERMANOVA results revealed significant differences among treatment groups in weighted UniFrac ($R^2 = 0.15018$, $P = 0.001$), unweighted UniFrac ($R^2 = 0.16277$, $P = 0.001$), and Jaccard distances ($R^2 = 0.16814$, $P = 0.001$). PCoA plots of the three treatments indicate differentiation between groups in beta diversity metrics (Fig. 3). Within-group Jaccard distances were 0.71 for captive individuals pre-release, 0.68 post-release, and 0.70 for wild hosts. Between groups, captive and wild Jaccard distances averaged 0.84, pre-release and post-release 0.77, and post-release and wild 0.74, suggesting greatest compositional differences in the fecal microbiota between captive and wild hosts, followed by pre-release and post-release lizards, and greatest compositional similarity between translocated and wild hosts based on the Jaccard index.

We identified 38 specific OTUs that varied significantly in rarified abundances between treatment groups from Kruskal-Wallis tests (Appendix S2). BLAST searches of these OTUs returned at least two strains of clinical interest. One OTU matched to *Bacteroides thetaiotaomicron* (mean reads per sample = 35.59 pre-release, 0.09 post-release, 0.09 wild) and the other *Providencia rettgeri* (mean reads per sample = 61.09 pre-release, 25.17 post-release,

0.30 wild). In both instances, bacterial strains with possible clinical significance in humans (Murphy et al. 2011; Ye et al. 2020) were found to be significantly more common in captive Texas horned lizards before reintroduction compared to after release or to wild conspecifics.

To directly identify if captive Texas horned lizards harbored distinct microbiomes compared to wild conspecifics before release, we ran analyses looking at the pre-release and wild treatment groups exclusively. We found no significant differences in alpha diversity metrics examined including number of OTUs ($F = 0.453$, $P = 0.505$), Shannon index ($F = 0.047$, $P = 0.830$), and Faith's PD ($F = 0.003$, $P = 0.960$). Gut microbial compositions were significantly distinct in PERMANOVA findings for weighted UniFrac ($R^2 = 0.13633$, $P = 0.001$), unweighted UniFrac ($R^2 = 0.16901$, $P = 0.001$), and Jaccard ($R^2 = 0.18165$, $P = 0.001$) metrics. Visualization of differences is apparent in PCoA plots (Fig. 4A).

We conducted additional analyses looking at headstart Texas horned lizards pre- and post-release to better understand gut microbial shifts associated with host reintroduction to native habitat on TAFB. Although there were still no differences in the Shannon index ($F = 0.805$, $P = 0.374$) or Faith's PD ($F = 0.805$, $P = 0.374$), we did find a significant increase in the number of observed OTUs after lizards were released from captivity ($F = 4.517$, $P = 0.039$; mean reads pre-release = 84, post-release = 97). Beta diversity metrics were again statistically distinct in PERMANOVA tests of weighted UniFrac ($R^2 = 0.11267$, $P = 0.002$), unweighted UniFrac ($R^2 = 0.12541$, $P = 0.001$), and Jaccard distances ($R^2 = 0.11673$, $P = 0.001$) and clustering in PCoA plots was somewhat apparent in all three indices used (Fig. 4B).

Finally, we sought to determine if gut microbial compositions in headstart Texas horned lizards had shifted after release to align with wild counterparts more closely within three months of reintroduction by comparing post-release samples with wild ones. We found no significant

differences in observed OTUs (F = 1.402, P = 0.243), Shannon index (F = 1.020, P = 0.318), and Faith's PD (F = 0.851, P = 0.361). Significant statistical distinctions were recovered via PERMANOVA though weaker compared to previous pair-wise comparisons in weighted UniFrac (R^2 = 0.09679, P = 0.004), unweighted UniFrac (R^2 = 0.08134, P = 0.001), and Jaccard indices (R^2 = 0.09054, P = 0.001). Interestingly, clusters were not readily distinguishable in PCoA plots (Fig. 4C).

Comparison of microbiota individually in headstart lizards pre- and post-release

We found that most individuals (5/8) sampled both before and after translocation displayed a consistent pattern of increased average observed OTUs after reintroduction to native habitat on TAFB (Fig. 5). In comparing beta diversity metrics within individuals pre- and post-release, results were less consistent. Due to limited sample sizes within individuals, PERMANOVA analyses were not always possible. However, most lizards displayed patterns of distinct clustering in microbiome composition pre-release and less apparent separation between post-release and wild conspecific samples in PCoA plots of beta diversity metrics (Fig. S1). This tendency was especially clear in individual ID 849-11, the lizard with the most complete sampling time series before and after release (seven samples from captivity, four following release; Fig. 6). We found significant statistical distinction in this individual pre-release and post-release and wild conspecifics in weighted UniFrac (R^2 = 0.22854, P = 0.002), unweighted UniFrac (R^2 = 0.22365, P = 0.001), and Jaccard indices (R^2 = 0.25157, P = 0.001).

DISCUSSION

In this study, we show that captive-reared Texas horned lizards at the OKC Zoo harbored microbial communities that were similarly diverse yet compositionally distinct from wild counterparts. Interestingly, this distinction largely faded within three months of host reintroductions to native habitat on TAFB (Figs. 3, 4). By the end of one active season, headstarted Texas horned lizards had microbial compositions that closely reflected wild conspecifics as indicated by lessened Jaccard distinctions and weakened PERMANOVA differentiation in all three beta diversity metrics. Our findings on gut microbiota from a headstart program thus have major implications for the study of gut microbiomes in future conservation initiatives (Redford et al. 2012; Bahrndorff et al. 2016; Jiménez and Sommer 2017; West et al. 2019; Dallas and Warne 2022).

A well-established body of evidence supports that captivity alters gut microbiomes in species across a broad taxonomic spectrum including reptiles (Alfano et al. 2015; Cheng et al. 2015; Clayton et al. 2016; Kueneman et al. 2016; Jiang et al. 2017; Kohl et al. 2017; McKenzie et al. 2017; Fong et al. 2020; Eliades et al. 2021). Furthering this trend, we found that fecal samples from captive Texas horned lizards showed distinct communities in beta diversity metrics compared to wild counterparts and even samples collected from hosts after reintroduction to TAFB. We show that Firmicutes and Bacteroidetes dominate the gut microbiomes of captive and wild Texas horned lizards alike, and Jaccard distances suggest that rare OTUs were most different between pre-release and wild treatment groups, while post-release and wild lizards were most similar in microbial compositions. Limited differences in alpha diversity and apparent differences in beta metrics have been found in other captive reptile species, including those of conservation significance (Kohl et al. 2017; Fong et al. 2020; Eliades et al. 2021).

We identified several OTUs including potential pathogens that differed significantly in abundances between pre-release, post-release, and wild Texas horned lizard treatment groups (Appendix S2). In two cases, possible pathogenic bacteria identified as *Bacteroides thetaiotaomicron* and *Providencia rettgeri* were found readily in captive lizards at the OKC Zoo but rarely in individuals after translocation or in wild individuals on TAFB. Previous studies of microbiota in captive reptiles suggest that the introduction of potential pathogens to animals in human care may not be uncommon (Jiang et al. 2017; Kohl et al. 2017; Fong et al. 2020; Eliades et al. 2021). Further, a study on reintroduced Fijian iguanas in the genus *Brachylophus* found that potential pathogens associated with host captivity may become less abundant after host reintroduction (Eliades et al. 2021). Serious consideration should be taken before reintroducing animals in sympatry with conspecifics as horizontal transmission of pathogens could be highly dangerous for imperiled populations (Bahrndorff et al. 2016; West et al. 2019; Eliades et al. 2021; Dallas and Warne 2022).

Captive-reared Texas horned lizards experienced rapid restructuring of microbiota following release onto native prairie habitat at TAFB. However, it should be noted that we only reintroduced lizards at one and two years old and not adult, reproductive individuals. Ontogenetic shifts in microbiome composition between juvenile and adult life stages have been reported previously in multiple reptile taxa (Troyer 1984; Yuan et al. 2015; Price et al. 2017). It is possible that by releasing juveniles, we introduced animals that still had a degree of flexibility in microbiome composition, but that raising and releasing adults could have different outcomes (Dallas and Warne 2022). Additional studies on ontogenetic development of microbiomes in reptiles is needed to better understand possible implications for reintroduction efforts.

Nevertheless, the adaptability of microbiota in reintroduced Texas horned lizards offers significant promise for the viability of captive-rearing programs to bolster declining wildlife populations. Reintroduction programs historically have suffered low success (e.g., low survivorship post-reintroduction) and microbial incompatibilities have been hypothesized as partially responsible for these setbacks though studying such impacts remains challenging (Redford et al. 2012; Bahrndorff et al. 2016; Jiménez and Sommer 2017; West et al. 2019; Dallas and Warne 2022). For instance, we were able to collect fecal samples before and after translocation from only eight of 34 individual lizards reintroduced onto TAFB in 2021. Fate tracking remains a costly and time-intensive endeavor that obfuscates our capacity to truly understand the success of reintroduction programs (Chong et al. 2019). Our findings of lizards with microbiomes distinct from wild types in captivity and then comparable to wild types after reintroduction could be indicative of several possibilities. First, it may indicate that in general, most lizards shift from a captive-type microbiome to a wild-type after release to native habitat. Alternatively, it could be that the few lizards we recaptured and sampled had survived due in part to their capacity to shift microbiota, providing an adaptive advantage. Those translocated individuals unable to acclimate microbial assemblages rapidly enough may have died. Future release efforts with improved fate-tracking techniques will help to better understand the role of gut microbiomes in reintroduction success (Chong et al. 2019; Yang et al. 2020).

We found that captive-reared juvenile Texas horned lizards clearly have distinct microbiomes from wild conspecifics on TAFB and that these communities shift rapidly to reflect native types after host reintroductions. Additional reintroduction efforts focused on adults could provide insight into the plastic nature and adaptability of gut microbiomes in hosts at various life stages (Dallas and Warne 2022). Further, animal husbandry manipulations such as the use of

probiotics or diet augmentation in captive lizards slated for release would be invaluable in assessing how modified microbial assemblages impact success and survivorship following release (Loudon et al. 2014; Kueneman et al. 2016; Stumpf et al. 2016; West et al. 2019; Yang et al. 2020). This study provides crucial baseline data on microbiomes in a threatened, iconic North American reptile species. Future works expanding on these conclusions will be able to more concretely untangle the role that microbiomes play in headstart and reintroduction program success in translocated species.

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AUTHOR CONTRIBUTIONS

S.J.E. oversaw animal husbandry and fecal sample collection at the OKC Zoo and in the field. K.M.S. and S.J.E tracked lizards on TAFB and collected fecal samples in the wild. R.W.M coordinated field activities on TAFB. B. L., L.P.B, and R.J.S., assisted in headstart program establishment and operations at the OKC Zoo. C.D.S. and J.L.W. led supply procurement, organized fieldwork operations, and headed funding acquisition. S.J.E. completed laboratory procedures, molecular analyses, and wrote the manuscript. All authors provided critical review of this manuscript.

Table 1. Background data of 68 Texas horned lizard fecal samples included in this study. Treatment conditions included samples pooled as pre-release, post-release, and wild.

THL ID	Treatment	Cohort	Pre-release Sample Dates (total)	Post-release Sample Dates (total)
849-1	Headstart	2019	4/16, 4/21, 5/14 **(3)**	7/19, 7/21, 9/8 **(3)**
849-11	Headstart	2019	3/5, 4/7, 4/14, 4/16, 5/5, 5/14, 5/17 **(7)**	8/25, 9/8, 9/21, 9/28 **(4)**
849-2	Headstart	2019	3/5, 5/20 **(2)**	7/15 **(1)**
849-4	Headstart	2019	4/16, 4/19, 5/14 **(3)**	8/31, 9/21, 9/28 **(3)**
991-1	Headstart	2020	**(0)**	8/10 **(1)**
991-14	Headstart	2020	4/14, 4/21 **(2)**	7/21, 7/22, 8/26 **(3)**
2-3	Headstart	2020	5/14 **(1)**	7/15 **(1)**
2-5	Headstart	2020	4/28 **(1)**	7/15, 7/21, 8/10, 8/21, 8/31, 9/21 **(6)**
4-1031	Headstart	2020	4/19, 4/23, 5/20 **(3)**	7/15 **(1)**

THL ID	Treatment	Age Class	Wild Sample Dates (total)
1032	Wild	Adult	6/23, 7/26, 8/11, 9/14 **(4)**
1067	Wild	Adult	7/20 **(1)**
1072	Wild	Adult	7/13, 7/20, 7/27 **(3)**
1079	Wild	Adult	7/21 **(1)**
1038	Wild	Subadult	8/10, 8/11 **(2)**
1052	Wild	Subadult	8/18 **(1)**
1053	Wild	Subadult	8/11 **(1)**
1064	Wild	Subadult	8/11 **(1)**
1081	Wild	Subadult	8/11, 9/9, 9/14 **(3)**
1043	Wild	Juvenile	6/25 **(1)**
1054	Wild	Juvenile	7/13, 7/14 **(2)**
1087	Wild	Hatchling	8/18 **(1)**
1109	Wild	Hatchling	9/15 **(1)**
1071	Wild	Unknown	6/23 **(1)**

FIGURES AND FIGURE LEGENDS

Fig. 1. A) Headstart Texas horned lizard at the OKC Zoo placed in 6 qt plastic tub and offered food for fecal sample collection. B) Translocated lizard caught on TAFB and placed in plastic tub with fecal sample visible. C) One of four reintroduction pens used on TAFB to house and acclimate translocated Texas horned lizards from the OKC Zoo.

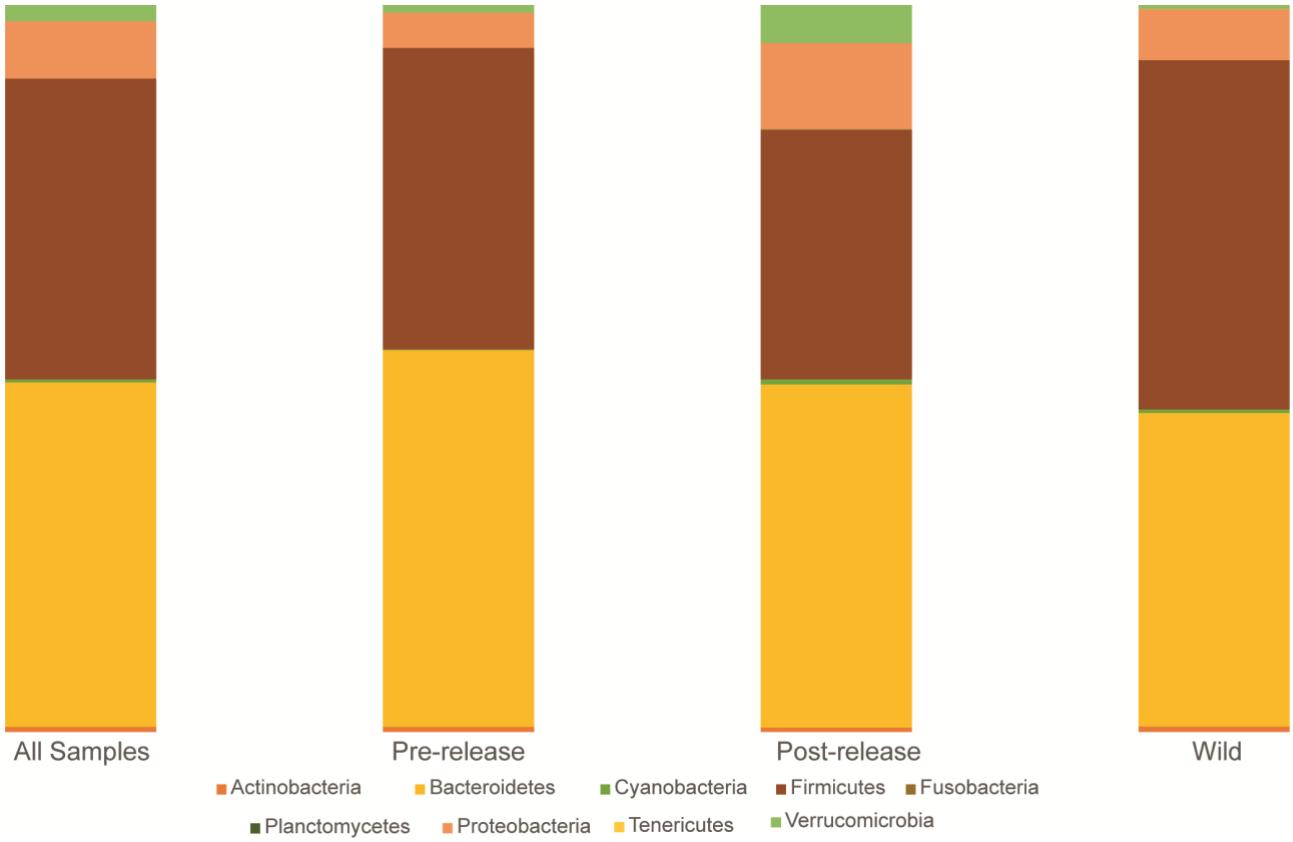

Fig. 2. Stacked barplots of average microbial composition in Texas horned lizard treatment conditions at the phylum level. Among all conditions, Bacteroidetes and Firmicutes are the most dominant phyla observed.

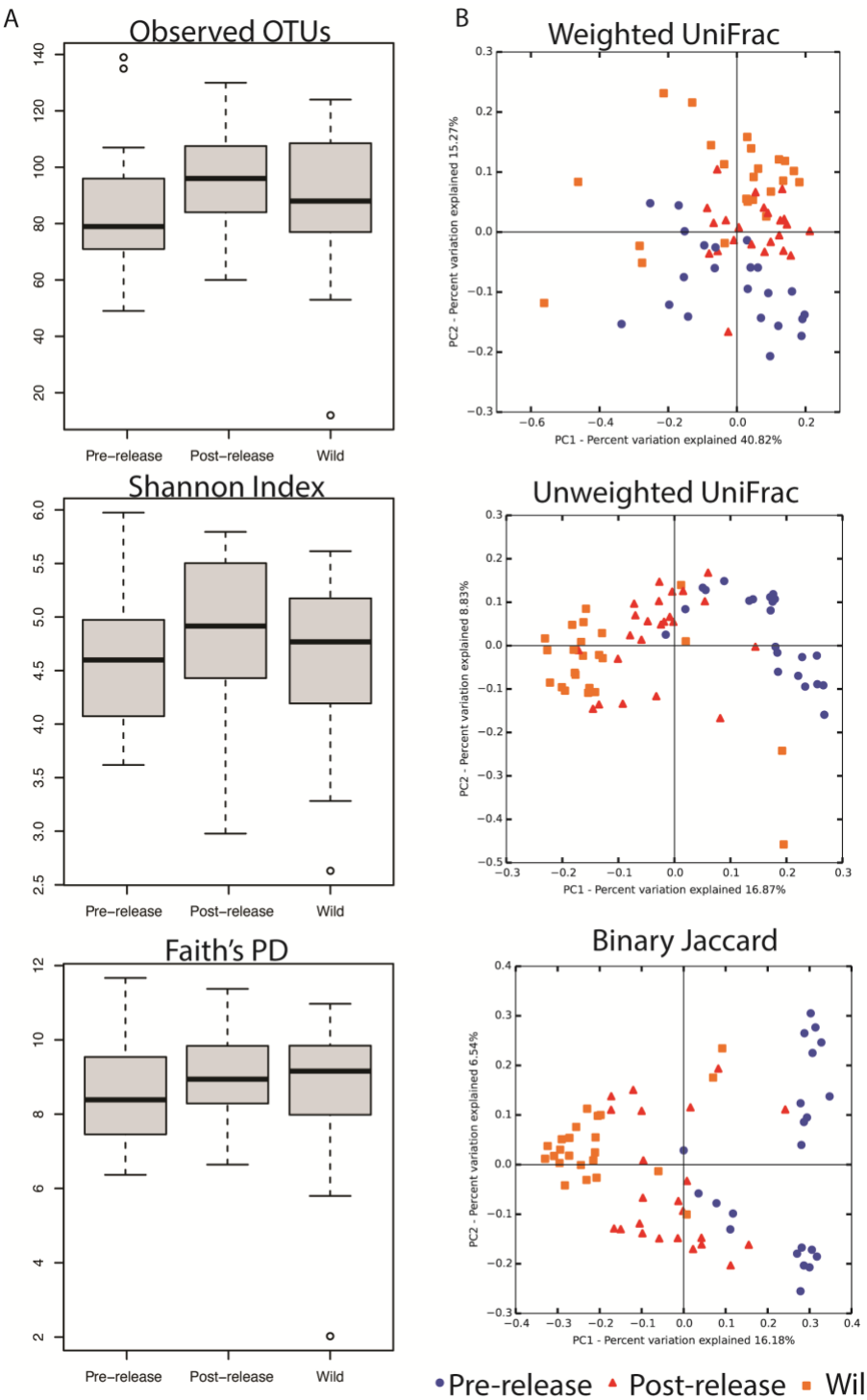

Fig. 3. A) Alpha diversity metrics of observed OTUs, Shannon index, and Faith's PD from fecal samples across three Texas horned lizard groups: pre-release, post-release, and wild. B) Principal coordinates analysis (PCoA) plots of fecal sample beta diversity metrics including weighted UniFrac, unweighted UniFrac, and Jaccard distances between all three treatment groups.

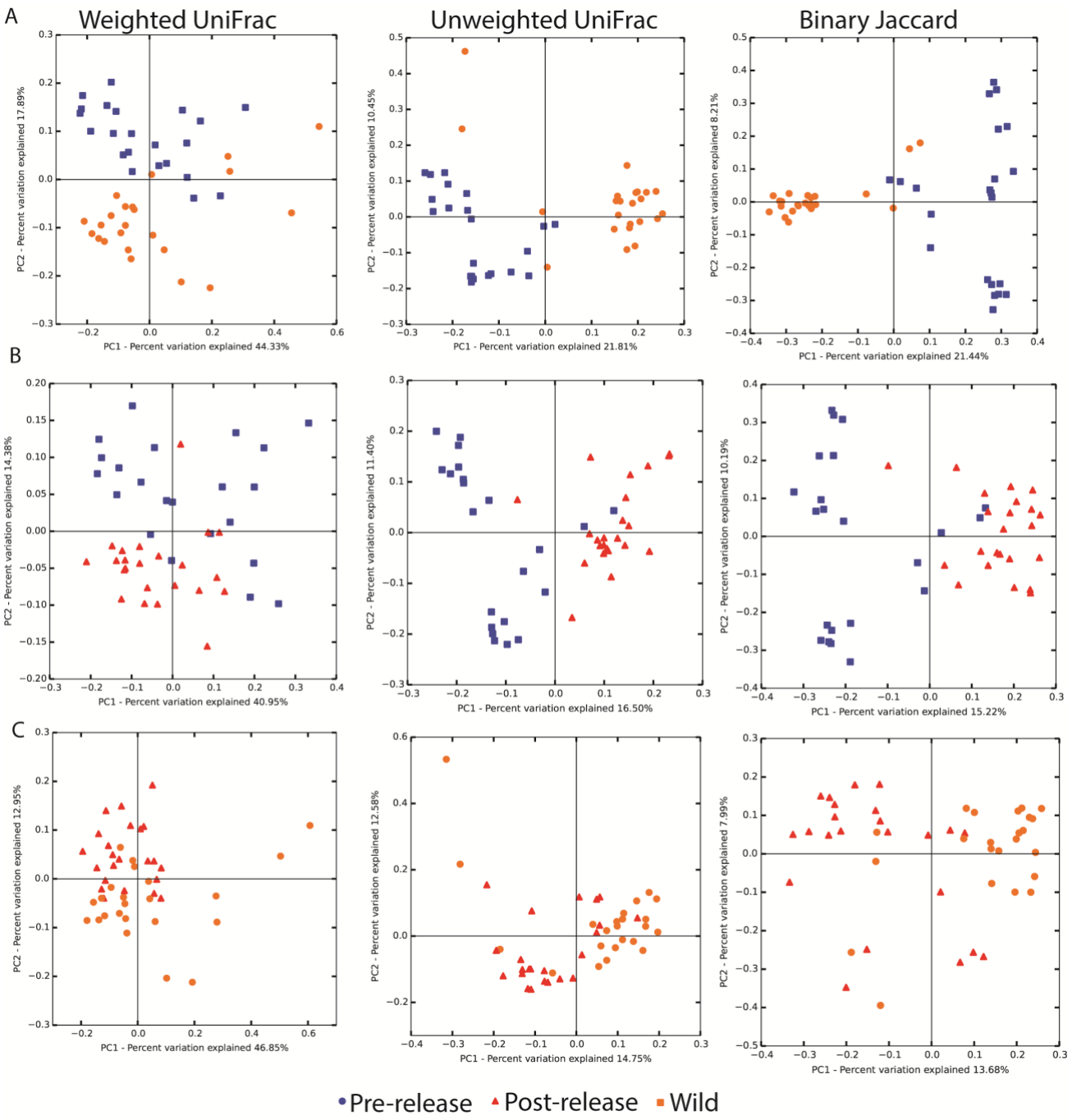

Fig. 4. Principal coordinates analysis (PCoA) plots of Texas horned lizard fecal samples comparing A) captive and wild, B) pre- and post-release, and C) post-release and wild conditions directly in pair-wise comparisons.

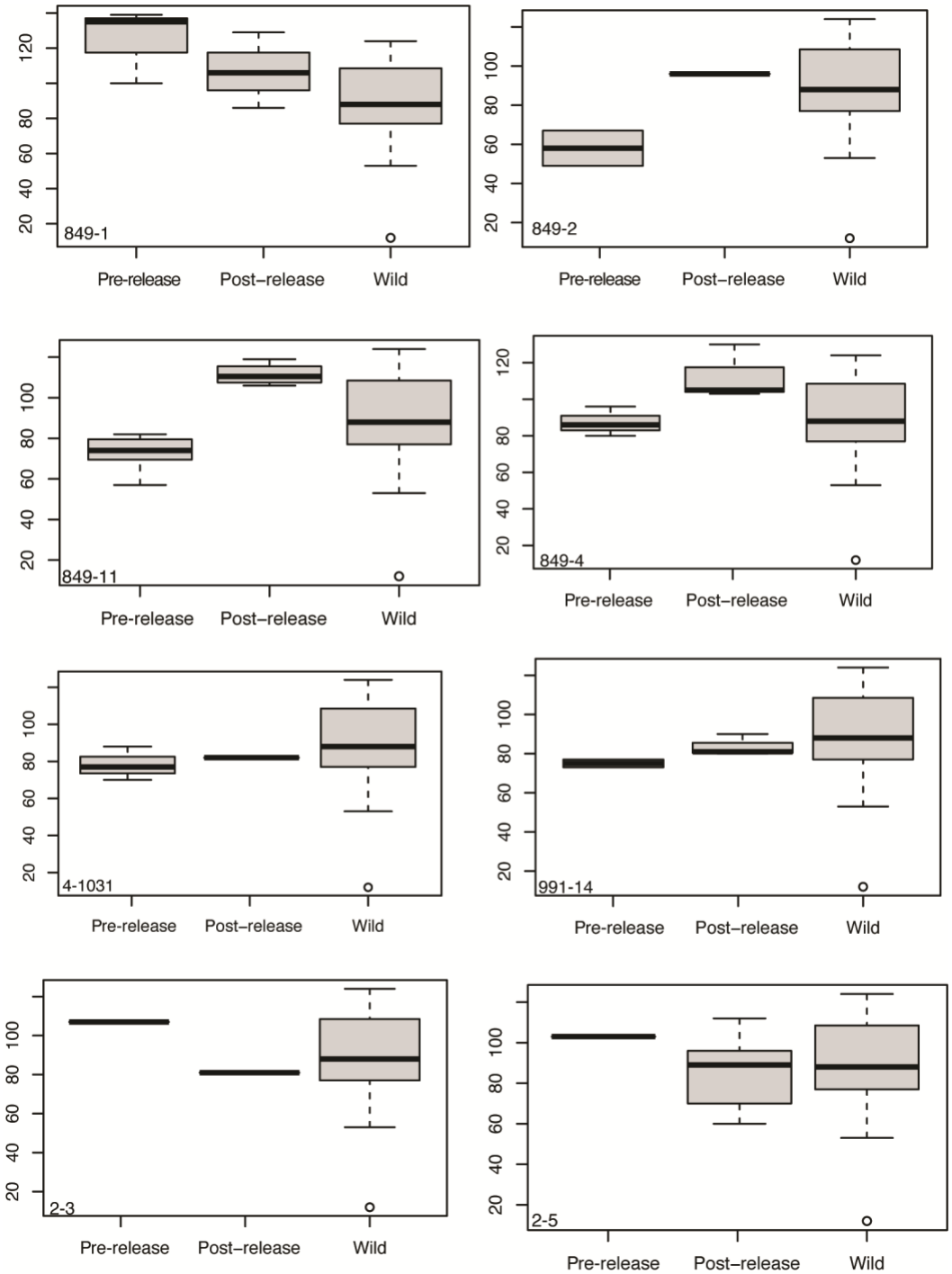

Fig. 5. Observed OTUs in eight (8) Texas horned lizard hosts sampled before and after reintroduction to TAFB compared to wild averages. Of hosts sampled, five of the eight show increased OTU diversity following translocation.

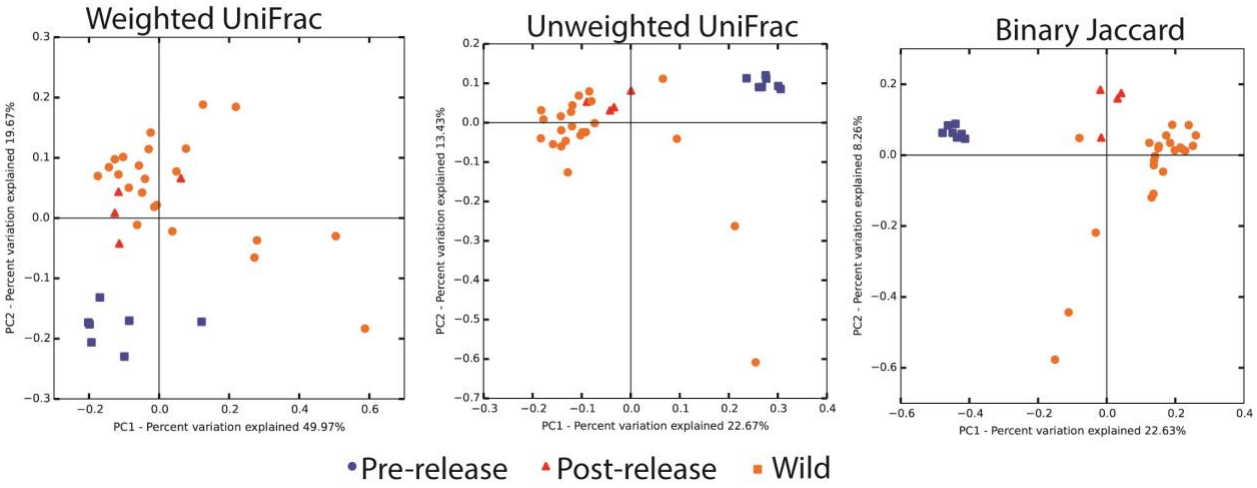

Fig. 6. Principal coordinates analysis (PCoA) plots of fecal samples from headstart Texas horned lizard ID 849-11.