Molecular systematic investigation of Philippine puddle frogs (Anura: Dicroglossidae: *Occidozyga* Kuhl and Van Hasselt, 1822) reveals new candidate species and a novel pattern of species dyads

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Abstract

Focusing on the phylogenetic relationships of puddle frog populations spanning the biogeographic interface between Sundaland (Borneo) and the Philippines, we demonstrate, for the first time, a widespread geographic pattern involving the existence of multiple divergent and co-distributed (sympatric) evolutionary lineages, most of which are not each other's closest relatives, and all of which we interpret as probable distinct species. This pattern of co-occurrence in the form of pairs of ecologically distinct puddle frog forms (dyads), prevails throughout northern Borneo, Palawan, Tawi-Tawi, the Sulu Archipelago, and western Mindanao (Zamboanga). Previously obscured by outdated taxonomy and logistical, legal, and security obstacles to field-based natural history studies, this pattern has remained hidden from biogeographers and amphibian biologists by an uncontested proposal that Philippine *Occidozyga laevis* is a single, "widespread," and "highly variable" species. In this paper we use an integrative synthesis of new genetic data, organismal phenotypic data, historical literature reports, and ecological observations to elucidate an interesting and potentially widespread pattern of puddle frog species coexistence at the Sundaland–Philippine biogeographic interface. Calling attention to this pattern opens promising possibilities for future research aimed at understanding the scope of this dyads pattern, and whether it extends to the more northern reaches of the Philippines. On either side of Huxley's and Wallace's lines, data suggest that the majority of puddle frog dyads at a given locality are not each other's closest relatives (are more distantly related, or non-monophyletic) and, thus, assembled ecologically, likely coexisting now as a result of their ecological tendencies toward distinct microhabitats (warmer stagnant pools in open areas, versus cool, flowing streams enclosed in forest). If these pairs of species types are determined to be the geographic norm among the more isolated, central, and northern, Philippine faunas, an obvious question will be whether they have evolved into dual ecological forms, possibly in response to ecological opportunity and/or reduced competition.

Keywords: biogeography, taxonomy, microhabitat, cryptic species

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Introduction

Puddle frogs of the genus *Occidozyga* Kuhl and Van Hasselt, 1822 are currently characterized with 12 species that are distributed throughout south and southeast Asia, ranging from eastern India through Bangladesh and southern China (as far east as eastern Fujian), and southwards throughout Indochina, the Thai-Malay peninsula, Indonesia (as far east as Sulawesi), Borneo, and the Philippines (Frost, 2020). Some species (referred to herein as "pond forms") occur in muddy puddles or stagnant water bodies that are associated with disturbed habitats such as rice fields, road-side ditches, irrigation canals, and rubber plantations (Brown et al., 2013b; Devan-Song and Brown, 2012; Inger, 1954; Sanguila et al.,

2016; Siler et al., 2011; Taylor, 1920), while others occur in swampy areas within secondary forests, ephemeral pools in primary forest, or stagnant, non-turbulent side pools disconnected from rivers (Brown et al., 2012; Inger, 1956, 1954; Iskandar et al., 2011; Sanguila et al., 2016). However, some species such as *O. baluensis* (Borneo; Inger et al. 2017), *O. semipalmata* and *O. tompotika* (Sulawesi; Iskandar et al., 2011) occur in shallow creeks with continuously running water. In the Philippines, one such species, *O. diminutiva* (Taylor, 1922) has been reported exclusively from these forest-associated riparian microhabitats (Alcala and Brown, 1998; Brown and Alcala, 1970; Inger, 1954; Taylor, 1922). Most *Occidozyga* are morphometrically similar yet exhibit substantial intra- and interspecific variation in color-pattern among certain wide-ranging species complexes. To compound matters further, numerous species have overlapping ranges, resulting in taxonomic disarray stemming from the inconsistent and interchangeable application of different species names by different authors (AmphibiaWeb, 2019; Frost, 2020; Iskandar et al., 2011).

Presently, two species of *Occidozyga* are known to occur in the Philippines: *O. laevis* (Günther, 1858) and *O. diminutiva* (Taylor, 1922). *Occidozyga laevis* is known throughout the archipelago (Brown and Alcala, 1970; Inger, 1954; Taylor, 1920), but the species' type locality has only been reported as "Philippinen (=The Philippines)" without more specific island, or locality information, and this species also has been reported from Borneo (Inger et al., 2017). However, due to morphological similarities, *O. laevis* has long been confused with *O. sumatrana* on Borneo and other parts of Sundaland (Berry 1975; Boulenger 1882; Chan-ard 2003; Chan et al. 2010a, b; Grismer et al. 2006; Inger 1966; Malkmus et al. 2002; Manthey & Grossmann 1997) and even now, their distributions are not satisfactorily characterized (Nutphund, 2001; Ohler, 2003). As such, their validity as distinct species have not been adequately confirmed and *O. laevis* continues to be uncritically included in taxonomic and geographic summaries of Borneo's amphibian fauna (AmphibiaWeb, 2019; Frost, 2020; IUCN, 2019) without accompanying data.

In contrast, *O. diminutiva* is only known from three localities within the Philippines: the Zamboanga Peninsula of Mindanao Island, Basilan Island, and Jolo Island of the Sulu Archipelago (Brown and Alcala, 1970; Frost, 2020; Inger, 1954; Taylor, 1922) and since its description in 1922, no studies have critically assessed its validity other than Inger (1954) who evaluated its generic placement and transferred the species to *Occidozyga* from *Micrixalus*. Since that study, no additional comparisons or consideration of the species' taxonomic status and geographical distribution has been undertaken (Diesmos et

al., 2015).

This study provides an initial molecular systematic investigation of *Occidozyga* in the southern Philippines with emphasis on populations that have had the potential of interacting with those from landmasses on the edge of the Sunda Shelf (Borneo Island). Given the biogeographical significance of this region (Brown et al., 2013a; Brown and Guttman, 2002; Brown and Alcala, 1970; Esselstyn et al., 2010; Lohman et al., 2011), namely its role in contributing to the conceptualization of the discipline and the implications of Huxley's modification of Wallace's Line, which traditionally extended the edge of the Sundaic biogeographical faunal region to include Palawan (Inger, 1954; Mayr, 1944), focal studies specifically targeting fine scale differentiation and terrestrial faunal distribution patterns across this faunal zone interface have been surprisingly few (Brown, 2016; Brown and Guttman, 2002; Esselstyn et al., 2010; Siler et al., 2012).

Occidozyga constitutes an ideal study group by virtue of its distribution that spans Sundaland and the Philippines. We focus on northern Bornean and southern Philippine *Occidozyga* to examine the diversity and taxonomic status of these ecologically-variable, seldom studied, and potentially taxonomically confused amphibians. We ask three central questions: First (1), given that *Occidozyga* taxonomy and species names have been indiscriminately applied by various authors working exclusively from one side of the northern Malaysian–southern Philippine political boundary (e.g. *O. sumatrana* and *O. laevis*), can a survey of genetic data resolve taxonomic ambiguities and elucidate species diversity and distribution across this faunal zone interface at the edge of the Sunda Shelf? Second (2), given the traditional view of Palawan Island as a faunal zone extension of Sundaland, are current taxonomic arrangements tenable or reflective of evolutionary relationships? In other words, could Palawan support populations more closely-related to Sundaland species such as *O. baluensis* or *O. sumatrana*, especially given the latter's notorious morphological similarity, which may render it indistinguishable from *O. laevis*? Finally (3) is it possible that the sympatric occurrence of species dyads (e.g. *O. laevis* and *O. diminutiva*, reported from the same areas but different microhabitats of the Sulu Archipelago and the Zamboanga Peninsula) could be more widespread?

Materials and methods

Morphological data and ecological observations

Given the scope of this study and our goals of providing an initial survey of populations immediately on either side of the Sundaland-Philippine biogeographic interface (Fig. 1), we examined the same general organismal attributes that distinguish
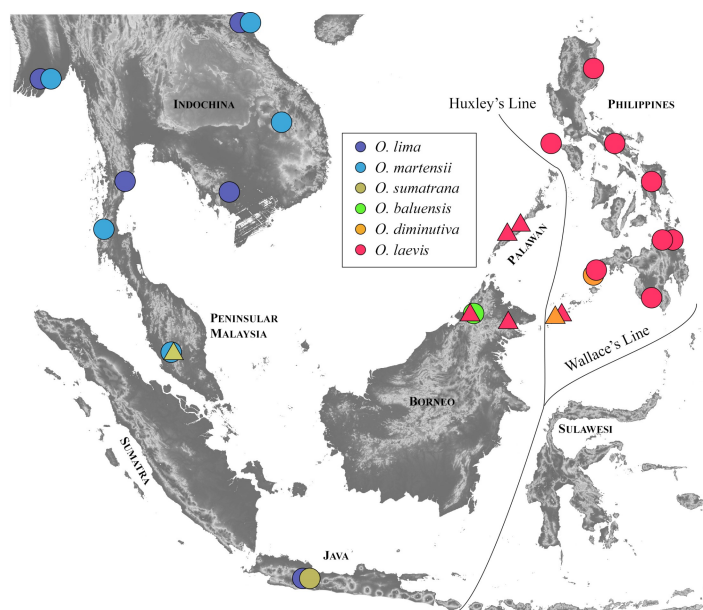
Figure 1. Distribution of samples used in this study. Circles denote nominal taxa, whereas triangles represent candidate species. Phylogenetically unrelated (Fig. 2) and sympatric Puddle Frog species dyads are identified at three sites across the Sundaland-Philippine biogeographic interface, indicated by differently colored symbols (Borneo, Tawi-Tawi, Zamboanga); whereas genetically divergent but phylogenetically related (sister lineages) constitute the proposed dyad, identified on Palawan.

the unambiguously distinct (valid) and sympatric species *O. diminutiva* and *O. laevis*: body size of males and females, general dorsal color pattern, ventral coloration, and microhabitat. Given that these categories of variation reliably allowed the last century of amphibian taxonomists to recognize these species (Alcala and Brown, 1998; Inger, 1954; Taylor, 1922), we assessed the same variables to determine whether they could differentiate taxa on Palawan (a single species, *O. laevis*, most closely-related to nominal *O. laevis* from the oceanic islands of the Philippines), the Sulu Archipelago (represented in our data set by Tawi-Tawi, the closest Philippine island to Borneo, and where *O. laevis* has been reported), and western Mindanao (where the two species, *O. diminutiva* and *O. laevis*, have been reported but unconfirmed with genetic data or confirmations of the phenotypes or ecological attributes discussed above). For some populations (both forms/localities on Palawan, *O. diminutiva* and *O. laevis* in Zamboanga) we (RMB only, for precision) were able to collect body size data (snout–vent lengths in mm, of males with nuptial pads indicating sexual maturity) for 10–15 specimens using digital calipers; for Bornean *O.* cf. *laevis* and *O. baluensis* we augmented literature reports (Inger 1966; Inger et al. 2017) with confirmation of ranges, using additional measurements by

RMB from additional Bornean specimens (Table 2), to confirm comparability and repeatability of methodology between Inger's (1966) and RMB's data collection. At one locality (Tawi-Tawi), size data are unavailable (voucher specimens corresponding to our tissues destroyed; no other modern collections exist) and so the biologist who did the field work and observed these animals in life (ELBR) simply characterized these animals as "large" or "small," based on memory, (such characterizations are included in quotes to indicate their subjectivity at this time).

Molecular sampling and phylogenetic estimation

For this study, we sequenced 38 samples from the holdings of the University of Kansas Biodiversity Institute (KU), and the Field Museum of Natural History, Chicago (FMNH). These include two outgroup samples (*Hoplobatrachus rugulosus* and *Limnonectes woodworthii;* Pyron and Wiens, 2011) and 36 ingroup samples including *Occidozyga sumatrana* from Peninsular Malaysia and Indonesia; *O. diminutiva* from Zamboanga (type locality) and the Sulu Archipelago, Philippines; and *O. laevis* from Borneo, Palawan, and numerous other populations throughout the Philippines (Fig. 1). DNA was extracted with the Promega Maxwell© RSC Instrument using the Maxwell© RSC Tissue DNA Kit. We used the primers 16Sc-L (5′-GTRGGCCTAAAAGCAGCCAC-3′), and 16Sd-H (5′-CTCCGGTCTGAACTCAGATGACGTAG- 3′) to amplify the 16S gene rRNA mitochondrial gene (Evans et al., 2003). Amplification was done using the following PCR thermal profile: 95 °C for 4min, followed by 35 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 70 s, and a final extension phase at 72 °C for 7min (McLeod, 2010). Amplified DNA products were subsequently visualized on 1.0% agarose gels and sequenced at Genewiz, Frederick, MD. Sequences were assembled, aligned (MUSCLE algorithm), and concatenated in Geneious Pro 5.3 (Kearse et al., 2012) prior to phylogenetic estimation. To provide a more comprehensive and accurate perspective of evolutionary relationships, we also supplemented our data with 20 additional 16S sequences of *O. lima* and *O. martensii* from GenBank. All molecular samples used in this study and their associated GenBank accession numbers are listed in Table 1.

We inferred phylogenies using maximum likelihood (ML) and Bayesian inference. The ML analysis was performed with IQ-TREE v1.6 (Nguyen et al., 2015) using the best-fit substitution model that was inferred by ModelFinder (Kalyaanamoorthy et al., 2017). Branch support was calculated based on 1,000 bootstrap replicates using the ultrafast bootstrapping method (Hoang et al., 2017). A Bayesian phylogeny was inferred using BEAST v2.6 (Bouckaert et al., 2019). The substitution model was estimated using bModelTest (Bouckaert and Drummond, 2017) and a relaxed log-normal

Table 1. Specimen identification, voucher information, localities, and Genbank accession numbers for sequences used in this study.

Taxa	Taxonomic identity inferred in this study	GB #	Museum Catalog #	Locality
From GenBank				
O. lima Java Island AB530619	*O. lima*	AB530619		Indonesia: Java
O. lima Vietnam AF206497	*O. lima*	AF206497		Vietnam
O. lima Thailand KR827958	*O. lima*	KR827958		Thailand
O. lima Cambodia KR827959	*O. lima*	KR827959		Cambodia
O. lima Laos KR827960	*O. lima*	KR827960		Laos
O. lima Myanmar MG935924	*O. lima*	MG935924		Myanmar
O. lima Myanmar MG935926	*O. lima*	MG935926		Myanmar
O. lima Myanmar MG935928	*O. lima*	MG935928		Myanmar
O. lima Kuala Lumpur, Malaysia AB488903	*O. martensii*	AB488903		Malaysia: Kuala Lumpur
O. martensii AF285214	*O. martensii*	AF285214		Unknown
O. martensii Vietnam DQ283357	*O. martensii*	DQ283357		Vietnam
O. martensii DQ458254	*O. martensii*	DQ458254		Unknown
O. martensii DQ458255	*O. martensii*	DQ458255		Unknown
O. martensii DQ458256	*O. martensii*	DQ458256		Unknown
O. martensii Ranong, Thailand AB530610	*O. martensii*	AB530610		Thailand: Ranong
O. martensii Thailand KP318725	*O. martensii*	KP318725		Thailand
O. martensii Myanmar MG935932	*O. martensii*	MG935932		Myanmar
O. martensii Myanmar MG935935	*O. martensii*	MG935935		Myanmar
O. martensii Myanmar MG935941	*O. martensii*	MG935941		Myanmar
O. baluensis Borneo, Malaysia DQ283143	*O. baluensis*	DQ283143	FMNH 242747	Malaysia: Sabah, Borneo
This study				
Hoplobatrachus rugulosus	*Hoplobatrachus rugulosus*	MT820164	UPLB(unknown)	Philippines: Los Baños, Laguna, Luzon
Limnonectes woodworthi	*Limnonectes woodworthi*	MT820165	KU 302234	Philippines: Catanduanes
O. laevis Quezon, Luzon	*O. laevis*	MT820166	PNM (unknown)	Philippines: Quezon, Luzon
O. laevis Isabela, Luzon	*O. laevis*	MT820167	PNM (unknown)	Philippines: Isabela, Luzon
O. laevis Zamboanga KU 314470	*O. laevis*	MT820168	KU 314470	Philippines: Pasonanca, Zamboanga, Mindanao
O. laevis Zamboanga KU 314471	*O. laevis*	MT820169	KU 314471	Philippines: Pasonanca, Zamboanga, Mindanao
O. laevis Misamis Oriental KU 319796	*O. laevis*	MT820170	KU 319796	Philippines: Misamis Oriental, Mindanao
O. laevis South Cotabato	*O. laevis*	MT820171	PNM (unknown)	Philippines: South Cotabato, Mindanao
O. laevis Oriental Mindoro KU 302322	*O. laevis*	MT820172	KU 302322	Philippines: Oriental Mindoro
O. laevis Western Samar KU 306301	*O. laevis*	MT820173	KU 306301	Philippines: Western Samar
O. cf. *diminutiva* Tawi-Tawi ELR 161	*O.* cf. *diminutiva*	MT820174	No voucher	Philippines: Tawi-tawi

Table 1. Specimen identification, voucher information, localities, and Genbank accession numbers for sequences used in this study.

Taxa	Taxonomic identity inferred in this study	GB #	Museum Catalog #	Locality
O. cf. *diminutiva* Tawi-Tawi ELR 170	*O. cf. diminutiva*	MT820175	No voucher	Philippines: Tawi-tawi
O. cf. *diminutiva* Tawi-Tawi ELR 202	*O. cf. diminutiva*	MT820176	No voucher	Philippines: Tawi-tawi
O. laevis Tawi-Tawi ELR 204	*O. cf. laevis*	MT820177	No voucher	Philippines: Tawi-tawi
O. laevis Agusan Del Norte	*O. laevis*	MT820178	No voucher	Philippines: Agusan Del Norte
O. laevis Borneo FMNH 230140	*O. cf. laevis*	MT820179	FMNH 230140	Malaysia: Sabah, Borneo
O. laevis Borneo FMNH 230732	*O. cf. laevis*	MT820180	FMNH 230732	Malaysia: Sabah, Borneo
O. sumatrana Peninsular Malaysia FRIM 1132	*O. sumatrana*	MT820181	FRIM 1132	Malaysia: Selangor
O. sumatrana Peninsular Malaysia FRIM 1133	*O. sumatrana*	MT820182	FRIM 1133	Malaysia: Selangor
O. sumatrana Peninsular Malaysia FRIM 1936	*O. sumatrana*	MT820183	FRIM 1936	Malaysia: Pahang
O. sumatrana Java Island RMB 2132	*O. sumatrana*	MT820184	MZB (unknown)	Indonesia: Java Island
O. sumatrana Java Island RMB 2133	*O. sumatrana*	MT820185	MZB (unknown)	Indonesia: Java Island
O. sumatrana Java Island RMB 2134	*O. sumatrana*	MT820186	MZB (unknown)	Indonesia: Java Island
O. cf. *laevis* 1 Irawan KU 326482	*O. cf. laevis 1*	MT820187	KU 326482	Philippines: Irawan, Palawan
O. cf. *laevis* 1 Irawan KU 326483	*O. cf. laevis 1*	MT820188	KU 326483	Philippines: Irawan, Palawan
O. cf. *laevis* 2 Brookes Point KU 326484	*O. cf. laevis 2*	MT820189	KU 326484	Philippines: Brookes Point, Palawan
O. cf. *laevis* 2 Brookes Point KU 326485	*O. cf. laevis 2*	MT820190	KU 326485	Philippines: Brookes Point, Palawan
O. cf. *laevis* 2 Brookes Point KU 326486	*O. cf. laevis 2*	MT820191	KU 326486	Philippines: Brookes Point, Palawan
O. cf. *laevis* 2 Brookes Point KU 326487	*O. cf. laevis 2*	MT820192	KU 326487	Philippines: Brookes Point, Palawan
O. cf. *laevis* 1 Irawan KU 308966	*O. cf. laevis 1*	MT820193	KU 308966	Philippines: Irawan, Palawan
O. cf. *laevis* 2 Brookes Point KU 309476	*O. cf. laevis 2*	MT820194	KU 309476	Philippines: Brookes Point, Palawan
O. cf. *laevis* 2 Brookes Point KU 309477	*O. cf. laevis 2*	MT820195	KU 309477	Philippines: Brookes Point, Palawan
O. cf. *laevis* 2 Brookes Point KU 309478	*O. cf. laevis 2*	MT820196	KU 309478	Philippines: Brookes Point, Palawan
O. cf. *laevis* 2 Brookes Point KU 309479	*O. cf. laevis 2*	MT820197	KU 309479	Philippines: Brookes Point, Palawan
O. cf. *laevis* 1 Brookes Point KU 309480	*O. cf. laevis 1*	MT820198	KU 309480	Philippines: Brookes Point, Palawan
O. diminutiva Zamboanga KU 321225	*O. diminutiva*	MT820199	KU 321225	Philippines: Pasonanca, Zamboanga, Mindanao
O. diminutiva Zamboanga KU 321226	*O. diminutiva*	MT820200	KU 321226	Philippines: Pasonanca, Zamboanga, Mindanao
O. diminutiva Zamboanga KU 321227	*O. diminutiva*	MT820201	KU 321227	Philippines: Pasonanca, Zamboanga, Mindanao

model was used as the clock prior. Two independent MCMC chains were implemented (20 million generations each) and convergence was assessed using Tracer v 1.6 (Rambaut et al., 2014). Converged trees were combined and a maximum clade credibility tree was inferred after the first 10% of sampled trees were discarded as burn-in.

Candidate species delimitation

We calculated uncorrected *p*-distances within and between nominal taxa using the complete-deletion function in MEGA-X (Kumar et al., 2018). To characterize and formally propose hypothesized (or "candidate") species boundaries, we performed species delimitation analyses using mPTP (Kapli et al., 2017) and bGMYC (Reid and Carstens, 2012), both of which have been shown to be effective for single-locus datasets (Blair and Bryson, 2017; Dellicour and Flot, 2018; Tang et al., 2014). For the mPTP analysis, we used the ML phylogeny as the input tree, and confidence of delimitation schemes were assessed using two independent MCMC chains at 5,000,000 generations each. Support values represent the fraction of sampled delimitations in which a node was part of the speciation process. To account for potential errors in phylogenetic estimation and uncertainty in model parameters, we implemented the bGMYC method that integrates over uncertainties in tree topology and branch lengths via MCMC (Reid and Carstens, 2012). As input, we used 100 randomly selected trees from the post burn-in combined runs of the BEAST analysis. For each tree, we ran the MCMC sampler for 50,000 generations with a burn-in of 40,000, retaining 10,000 post-burn-in generations with a thinning interval of 100. We then assessed species delimitation schemes from two conservative conspecificity probability thresholds (0.01 and 0.05).

Results

Phylogenetic relationships

Both ML and Bayesian phylogenies were concordant at all major nodes and all nominal species were monophyletic (Fig. 2). *Occidozyga lima* and *O. martensii* were inferred as the first two branching lineages, respectively. *Occidozyga baluensis* was reciprocally monophyletic with *O. diminutiva* and this clade was the sister lineage to *O. sumatrana* (Fig. 2). *Occidozyga laevis* from the Philippine islands of Luzon, Mindoro, Samar, and northern Mindanao formed a highly structured clade, but populations from Palawan formed a clade that was reciprocally monophyletic with populations from Tawi-Tawi and Borneo. Within Palawan, two divergent and highly supported reciprocally monophyletic clades were inferred [Palawan form 1

(PA1) and Palawan form 2 (PA2); Fig. 2]. Branch support for *O. laevis* clades was high, while support for other species was mixed (Fig. 2).

Candidate species delimitation

Multiple highly divergent populations were detected within *Occidozyga sumatrana, O. diminutiva,* and *O. laevis*, indicating that distinct, undescribed species may potentially be present within each of these nominal species. *Occidozyga sumatrana* from Peninsular Malaysia and Indonesia (Java) were 8–9% divergent from each other, while *O. diminutiva* from Tawi-Tawi versus Zamboanga were 6–7% divergent (Fig. 3). Divergences within *Occidozyga laevis* were the widest, ranging up to 15% (Tawi-Tawi vs. Samar). Populations from Palawan were 6–9% divergent compared to populations from Borneo + Tawi-Tawi, whereas Palawan form 1 (river edges, puddles) and Palawan form 2 (small rapidly-cascading streams) were 5% divergent from each other. *Occidozyga baluensis* was 9–12% divergent compared to *O. diminutiva.*
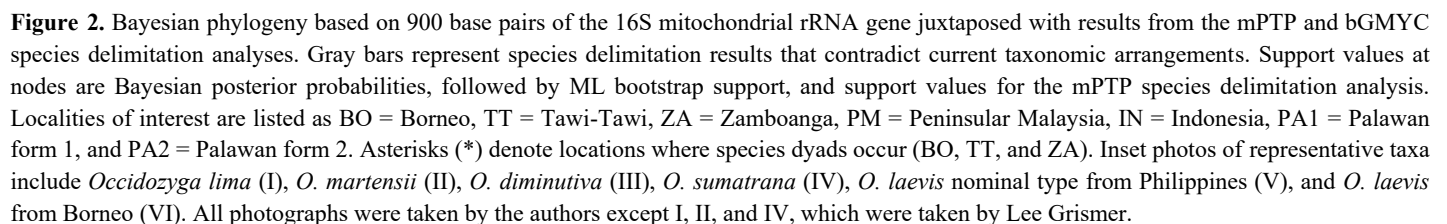
Both mPTP and bGMYC analyses supported the recognition of additional, undescribed species within *Occidozyga diminutiva, O. sumatrana,* and *O. laevis*. The mPTP analysis inferred *O. diminutiva* from Tawi-Tawi to be distinct from the Zamboanga population (Fig. 2). *Occidozyga sumatrana* was split into two species, represented by populations from Malaysia and Indonesia, while *Occidozyga laevis* was split into four species. At a conservative conspecificity probability threshold of 0.01, the bGMYC analysis lumped *O. baluensis* and *O. diminutiva* as a single species, but they were split at a threshold of 0.05. Similar to the mPTP analysis, *Occidozyga sumatrana* was also split into two species across both thresholds. At a threshold of 0.01, *O. laevis* was split into four species, while eight species were inferred at a threshold of 0.05 (Fig. 2).

Morphological attributes and microhabitat preferences

Based on a combination of (1) existing taxonomy (Taylor 1922; Inger 1954, 1966; Inger et al. 2017), (2) our assessment of traditional taxonomic character state differences from new localities and populations previously unreported in the literature (included herein), (3) available specimen-associated natural history museum data, field notes, new island records, and other occurrence records (summarized here), (4) our own field observations (microhabitat data, summarized together in this paper) and (5) limited body size information available from specimen data (Table 2) and the literature (Taylor 1922; Inger 1954, 1966; Inger et al. 2017), we found a general pattern of two apparent forms (Table 2) of puddle frogs at each of the landmasses spanning the Sundaland-Philippine faunal zone

Table 2. Species identification for four proposed puddle frog species dyads, at four sites (Fig. 1), general subjective (putative) classification as "pond" versus "stream" ecological types (see text for discussion), microhabitats, forest cover, general size, coloration, and phylogenetic relationships (polyphyletic vs monophyletic; see Fig. 2) inferred for each dyad. Data sources and voucher specimen information are included for reference. Table entries in quotes indicate qualitative impressions of field biologists in cases where no data are available and questions marks (?) indicate unknown. Data for Bornean species from Inger (1966) and Inger et al. (2017); Philippine populations data from Taylor (1920, 1922), Inger (1954) Alcala and Brown (1998), Brown et al. (2012, 2013b), Devan-Song and Brown (2012), Sanguila et al. (2016), Siler et al. (2011) with additional size ranges augmented and these earlier reports confirmed with data collected from voucher specimens (by RMB), corresponding to the same individuals and populations from which genetic data presented here (Figs. 2, 3) were derived; all other size data from specimens deposited at KU (Table 2). Data sources: T22 (Taylor 1922); AB98 (Alcala and Brown 1998) I54 (Inger 1954); I66 (Inger 1966); I17 (Inger et al. 2017); T (This study). All specimens correspond to museum-deposited voucher specimens and genetic material (KU: https://collections.biodiversity.ku.edu/KUHerps/; FMNH: https://collectionszoology.fieldmuseum.org/), unless one or the other has been lost/destroyed (voucher specimens from Tawi-Tawi Island [for which genetic material is available]). See Table 1 for correspondence between individual specimens from which genetic sequence data were derived.

Site/Taxa	Simplified ecological type	Forest cover	Male & female size (SVL)	Ventral coloration	Habitat substrate	Dyad relationship	Source	Voucher
Zamboanga & Basilan								
O. cf. laevis	pond	open areas & forest	27–38; 31–55	yellow	muddy water	polyphyletic	T22, AB98, I54,T	KU334623–29, 334630–35, 334636–37, 334638–41;
O. diminutiva	stream	forest	14–24; 22–29	Blotched white + brown	stream bed		T22, I54, T	KU314464–69, 321225–53; AS 62559, CAS20069–71, 0156–60.
Tawi-Tawi & Jolo								
O. cf laevis	pond	open areas & forest	"large"	yellow	muddy water	polyphyletic	T	ELR204 (genetic sample)
O. cf diminutiva	pond + stream?	disturbed forest	smaller"	Blotched white + brown	stream bed		T	ELR161, 170, 202; CAS60681–83, 62528–34.
Borneo								
O. cf laevis	pond	forest	21–31; 35–48	yellow	muddy water	polyphyletic	I66, I17, T	FMNH29081, 129091–111, 129113, 129120; KU155621–22, 155623–24.
O. baluensis	stream	forest	15–25; 25–35	blotched white + brown	stream bed		I66 I17, T	KU155617–20, 339545, 339547; FMNH36030–31, 137444, 138075–84, 138086–89, 138096–101.
Palawan								
O. cf laevis 1	pond	open areas & forest	22–33; 28–44	yellow	muddy water	monophyletic	I54, T	FMNH123585, KU79025–32, 91305, 309484–85, 326482–83, 308966.
O. cf laevis 2	stream	forest	22–29.7; 25.5–33.4	gray with dark flecks and blotches	stream bed		T	KU 79033, 308966, 309164, 309476–80, 326482, 326484–87.

Figure 2. Bayesian phylogeny based on 900 base pairs of the 16S mitochondrial rRNA gene juxtaposed with results from the mPTP and bGMYC species delimitation analyses. Gray bars represent species delimitation results that contradict current taxonomic arrangements. Support values at nodes are Bayesian posterior probabilities, followed by ML bootstrap support, and support values for the mPTP species delimitation analysis. Localities of interest are listed as BO = Borneo, TT = Tawi-Tawi, ZA = Zamboanga, PM = Peninsular Malaysia, IN = Indonesia, PA1 = Palawan form 1, and PA2 = Palawan form 2. Asterisks (*) denote locations where species dyads occur (BO, TT, and ZA). Inset photos of representative taxa include *Occidozyga lima* (I), *O. martensii* (II), *O. diminutiva* (III), *O. sumatrana* (IV), *O. laevis* nominal type from Philippines (V), and *O. laevis* from Borneo (VI). All photographs were taken by the authors except I, II, and IV, which were taken by Lee Grismer.

interface [Palawan, Tawi-Tawi, southwestern Mindanao (Zamboanga); each with two apparent morphological types], and Borneo (three or more forms). We referred loosely to these individual forms, which illustrate the duality of natural history observed at any single locality, as the "pond" form and "stream" form with the caveat that strong statements regarding adherence to this distinction and degree to which these forms actually exhibit truly more, or less specialization each will require verification (with substantially more data from detailed ecological studies and more sophisticated morphometric analyses based on large sample sizes of properly preserved specimens), but based on the following general set of observations. Typically, at a given site or general area, with sufficient survey and re-survey work, field biologists have recorded two forms of puddle frogs: one form ("pond") constitutes a larger-bodied type, usually seen/observed/collected in more open and/or disturbed habitats, and which is encountered most often submerged in muddy puddles, stagnant small ponds, water buffalo wallows, flooded rice fields and irrigation conduits, or unconnected side-pools of slow-flowing, low-elevation, silted rivers near coastal areas. Another form (the "stream specialist") has been detected by biologists in latter

stages of sampling, or has been detected only upon follow-up re-surveys in a particular area (Tawi-Tawi, Zamboanga) or following years of studies in an area (Palawan), perhaps suggesting differences in detectability which might reflect behavior and/or other ecological attributes and preferences—or just may reflect taxonomic or search-image bias on the part of field biologists. The "stream" form is usually smaller, it may be distinguishable with darker ventral coloration, also somewhat camouflaged dorsally, with spots and cross bars on the head, body, and limbs. In addition to its small body, darker, more cryptic coloration, the "stream" form appears associated with more enclosed, forested habitats, in some instances at higher elevation, and usually is not encountered in stagnant pools but, rather, in cooler forested areas, and situated in flowing or cascading streams.

Available evidence suggests that the loosely-applied characterization (above) is not perfect and that some exceptions should be enumerated. Thus, on Languyan Island, Tawi-Tawi, a "pond" form was found in rice fields and buffalo wallows and a "stream" form (*O.* cf. *diminutiva*) was found in ephemeral puddles on the floor of a selectively-logged, secondary forest; other specimens were taken from a small stream (ELBR, *pers. obs.*). At Pasonanca, Zamboanga Peninsula of western Mindanao, a "pond" form was found primarily in muddy pools on the edge of an agricultural area, and submerged in water of unconnected side-pools of a large low-elevation river with, and a "stream" form (*O. diminutiva*) was encountered in flowing tributaries and small seeps, both flowing into the same river, but set back in the forest at steeper terrains where water was cascading over rocks and cliff faces (RMB, *pers. obs.*). On Palawan, the situation is less clear. In some southern areas in the vicinity of Brookes Point, Quezon, and Narra, only one microhabitat specialist, the "pond" form, has yet been recorded (both in stagnant pools in open areas and forest rivers and streams (RMB *pers. obs.*), whereas at others (Montible, Roxas, Taytay, San Vicente; SS and RMB, *pers. obs.*), a second form is suggested by our genetic data, smaller specimens in collections which were only collected in forested streams (RMB, *pers. obs*), and a tendency for specimens encountered in small forested streams to be more colorfully- and cryptically-blotched (SS, *pers obs.*).

The puddle frog dyads pattern

Connecting our disparate lines of evidence, even while acknowledging each's associated caveats (e.g., the fact that our observations are from notes, records of field biologists, and specimen-associated data assembled by different investigators over a span of ~20 years), nevertheless led to the apparent conclusion of a dyads pattern of occurrence in puddle frogs

forms. The point at which our data intersected is the site-by-site (Fig. 1) accounting of our combined sources of information. Whether species dyads involved uncontroversial, named (Taylor 1922; Inger 1954, 1966; Alcala and Brown 1998; Inger et al. 2017), phylogenetically unrelated pairs of species (Borneo, southwestern Mindanao), a named species paired with a currently unrecognized candidate lineage (Tawi-Tawi), or a pair of haplotype clades which may correspond to disparate ecological observations and color pattern tendencies (Palawan: where the two forms may be sister species), an over-arching pattern of the apparently repeated duality of ecological and morphological types of puddle frogs exists at multiple localities immediately spanning Wallace's and Huxley's lines (Fig. 1). This is particularly evident when we considered that at most sites (1) our molecular phylogenetic analysis found strong statistical support for the placement of two separate forms (unrelated in Borneo, western Mindanao, and Tawi-Tawi, but possibly sister lineages on Palawan); that in all known dyads, both forms are (2) genetically divergent lineages; and that (3) our DNA-facilitated identification of formerly recognized species (previously named species, now newly confirmed with genetic material from each named species' type localities—and which enabled us to identify related but genetically distinct new allopatric candidate species such as *O.* cf. *diminutiva* on Tawi-Tawi, and *O.* cf. *laevis* in Borneo) suggests that other, putatively new, possibly undescribed, candidate species (with statistical support from various quantitative species delimitation analyses; Fig. 2) exhibit equivalent levels of genetic distinctiveness, at or above a level of genetic divergence estimated among formerly recognized, names species (Figs. 2, 3).

Discussion

Commenting on puddle frogs of northern Borneo, Inger et al. (2017) characterized the natural history, ecological tendencies, and morphological differences between *O.* cf. *laevis* and *O. baluensis*. Describing *O.* cf. *laevis* as effectively camouflaged against the drab color of the muddy, stagnant water in which the species is always found (puddles, small ponds, and wallows), the authors described the second species of the Borneo dyad (*O. baluensis*) as matching the variably-colored gravel, and red clay soil making up the substate of seeps and small flowing streams in forested areas (Table 2). Similarly, our survey of genetic, phenotypic, and ecological (microhabitat preference) variation in *Occidozyga* puddle frog populations on either side of the Sundaland–Philippine faunal zone interface revealed a surprising, widespread pattern of species occurrence in dyads, or repeated pairs of distinct evolutionary lineages, usually also ecologically constituting somewhat separate "pond"
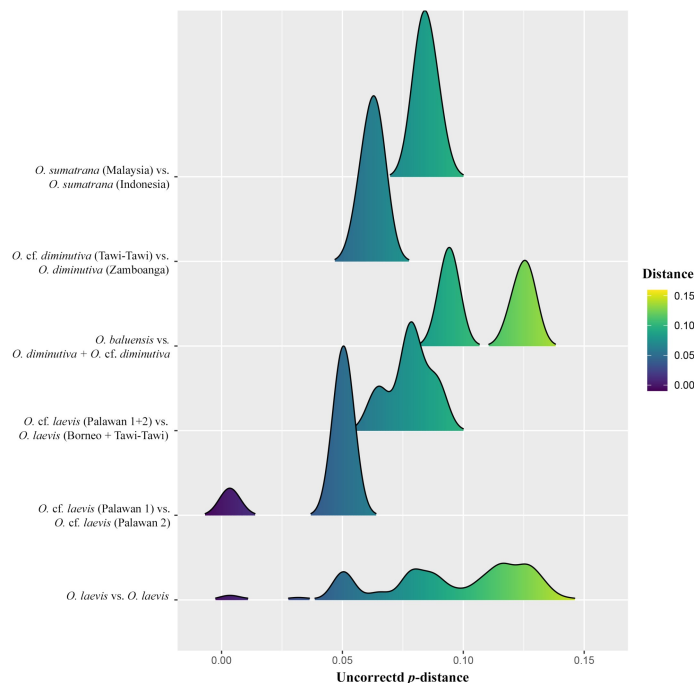
Figure 3. Density plots showing the distribution of uncorrected p-distances between reciprocally monophyletic focal clades.

versus "stream" forms; these involved previously recognized, named species, candidate species supported by our statistical species delimitation analyses (Fig. 2), and divergent forms with phenotypic variation (Table 2), which have diverged from congeners on the same scale as divergence found between taxonomically uncontroversial named species (Fig. 3). Aside from the question of how widespread this pattern may be, and whether it prevails on landmasses farther north (the oceanic portions of the Philippines) or south (southern Sundaland, and other portions of the Indo-Australian Archipelago; Fig. 1), what can we infer about evolutionary processes associated with, or possibly giving rise to, this newly-elucidated pattern of species dyads in geographic distributions of puddle frogs?

The question articulated above takes a more general form if we ask how have recently diverged species pairs (which we expect to be phenotypically and ecologically similar, at least at the onset of their divergence) empirically been shown to coexist? Or, how have seemingly similar species managed to coexist in time or space and avoid direct competition? We know from numerous comparative phylogenetic analyses of ecological niche differentiation, that phenotypically and/or ecologically similar land vertebrates appear to coexist due to divergence along key axes of organismal variation, often linked functionally to fitness via a phenotype–environment correlation (Schluter, 2000). In some heavily-cited cases of adaptive radiation, primary axes of variation involve evolutionary change, in a nearly deterministic fashion, in feeding niche and

related specializations (Lamichhaney et al., 2015), specialized utilization of structural microhabitats (Mahler et al., 2010), body size divergence and existence of optimal size categories (Setiadi et al., 2011) or other, critical, form-function relationship features (Alfaro et al., 2009; Cooney et al., 2017). In amphibians, additional conspicuous potential axes of variation involve larval biology (and the entire suite of associated variables, including dietary specializations, foraging guilds, life history trait variation, developmental timing, etc.; Alcala 1962; Duellman & Trueb 1986), reproductive mode, temporal segregation of the timing of reproduction, modality of mate-recognition signals and acoustic niche partitioning (Duellman and Pyles, 1983; Wells, 1977), and the overall fine-scale partitioning of spatial differentiation in response to ecological opportunity (Myers and Burbrink, 2012; Yoder et al., 2010), which may be tightly coupled to fine-scale microhabitat preferences (Blackburn et al., 2013; Setiadi et al., 2011). Which of these patterns (and inferred processes) might explain the species dyad distribution pattern revealed here in Philippine puddle frogs? At this point, data are quite limited, and these limitations prevent firm conclusions. The availability of only a single gene mitochondrial gene locus (preventing species tree inferences and characterizations of gene flow) and our lack of dense population-level sampling, prevent us from confidently distinguishing between geographic structure of genetic variation and possible species boundaries (Sukumaran and Knowles, 2017); these shortcomings will have to be ameliorated with future studies.

At this point, three primary sources of information hold the potential to substantially clarify the questions raised here. First, (1) genomic data (Chan *et al.* 2017; Chan *et al*. 2020; Hutter *et al.* 2019) collected from throughout the range of our sampling, and including key populations on either side of the Sundaland–Philippine faunal interface would greatly empower tests of species boundaries; second, (2) as the primary anuran mate-recognition signal (Wells, 1977), acoustic advertisement calls of frogs have the potential to reveal species boundaries, even in cases where morphological differentiation has not accompanied speciation (Brown et al., 2017, 2015); and third (3) there can be no substitute for the kind of natural history information, detailed microhabitat data, and behavioral clues that accompany long term, comprehensive, survey and re-survey field studies that focus on organisms in their natural environment (Brown et al., 2013b; Sanguila et al., 2016). With their exquisitely derived tadpoles (Haas et al., 2014), *Occidozyga* are known obligate carnivores, a fact which opens the possibility of fine-scale larval specialization on different prey items in slightly different microhabitats (e.g., stagnant pools and puddles, versus flowing or cascading, cooler, oxygenated water in forest streams). Thus, we strongly encourage students, protected area wardens, wildlife

managers, and conservation specialists to join forces in collaboration and focus on multilocus genetic datasets (Chan *et al.* 2020), large sample sizes of properly recorded (and vouchered) frog vocalizations (Köhler et al., 2017), multivariate analyses of adult phenotypic variation (Brown et al., 2017) and, most importantly, detailed field-based life history studies of *Occidozyga* microhabitats, larval biology, and adult behavior associated with the reproductive effort. Are puddle frog populations with apparent preferences for cool, running, enclosed forest stream environments truly "specialized" as a result of replicated evolutionary shifts in ecological preferences, tendencies, or physiological limits—and can we demonstrate (with ecological and physiological data and/or diet) that the hypothesized, contrasting "pond" forms found in open habitats, stagnant pools and puddles, in disturbed areas at low elevations have wider water pH tolerances, statistically broader temperature range limits, occur in more numerous kinds of bodies of water, and/or quantitatively exhibit a more variable diet? These and other questions will need to be the subject of focal studies, in different areas where puddle frog dyads occur, involving populations and species with distinct evolutionary histories, and occurring as part of communities of variable complexity; real data, collected properly in the field, and analyzed with appropriate inferential statistics will be required, before vindication or refutation of the pattern hinted at here, will be possible. Only then will a taxonomic resolution of this complex, archipelago-wide frog group be possible.

Opening our view through the window of amphibians natural history starts with modest, local-scale, observational studies requiring patience, persistence, and a desire to communicate science (Alcala 1955, 1956, 1957, 1958, 1962; Alcala and Brown 1955a,b, 1956, 1982, 1987). Here we have shown that rather than representing just a single common, widespread, human commensal species, the Philippine *Occidozyga* radiation encapsulates a series of unresolved, ongoing, general-interest conceptual questions in ecology and evolutionary biology—all of which hold tremendous potential for future studies.

Acknowledgements

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