



Taxonomic assessment of *Ololygon* gr. *argyreornata* (Anura, Hylidae) from northern Atlantic Forest reveals a new species from the Pernambuco Endemism Centre, northeastern Brazil

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Abstract. The Atlantic Forest in Brazil is a highly threatened biome, recognized as a biodiversity hotspot that hosts numerous endangered species with restricted distributions and facing intense human pressures. The Pernambuco Endemism Centre (PEC), located in Northeastern Brazil, represents one of the most concerning regions within the Atlantic Forest, as it comprises small, fragmented patches of pristine forest harboring several endangered species. Additionally, many species may be threatened but remain undocumented due to a lack of taxonomic studies, particularly within cryptic species complexes. In this study, we assessed the taxonomic status of populations assigned to the genus *Ololygon* from the state of Pernambuco. This genus presents several cryptic species with subtle morphological differences. Based on morphological, molecular, and acoustic data, we conclude that the populations from the state of Pernambuco constitute a previously undescribed species. Furthermore, we update the geographic distribution of the *Ololygon* gr. *argyreornata* species group, providing new morphological and acoustic data for *O. skuki* from the northern coast of Bahia state, and redescribe the advertisement call of *O. argyreornata* from Linhares, Espírito Santo.

Keywords: cryptic species, integrative taxonomy, *Ololygon skuki*, Scinaxinae, vocalization.

Introduction

The Pernambuco Endemism Centre (PEC) is a sub-biogeographical region of the Atlantic Forest domain in northeastern Brazil, located north of the São Francisco River (Silva and Tabarelli, 2001). Some authors consider PEC as a zoogeographical transition zone for vertebrates (Silva et al., 2024). It is regarded as the most threatened region within this domain (Ribeiro et al., 2009) and hosts a high diversity of amphibians,

including several threatened species (Roberto et al., 2017a; Dubeux et al., 2020).

Over the past 20 years, many new endemic frog species have been discovered in PEC (Carvalho-e-Silva et al., 2003; Carnaval and Peixoto, 2004; Cruz et al., 2011; Lima et al., 2011; Roberto et al., 2017b), including two species of hylid frogs from the genus *Ololygon*: *O. skuki* and *O. muriciensis*. Recently, the genus *Ololygon* was recognized as a well-supported

monophyletic group based on molecular analyses, characterized by 19 phenotypic synapomorphies and divided into seven species groups: *agilis*, *argyreornata*, *belloni*, *cardosoi*, *catharinae*, *feioi*, and *perpusilla* (Araujo-Vieira et al., 2023).

The *Ololygon argyreornata* species group was recently proposed by Araujo-Vieira et al. (2023). Previously, it was included within the *O. catharinae* clade (Faivovich, 2002; Faivovich et al., 2005). This group is strongly supported by molecular and total evidence analyses and is characterized by two phenotypic synapomorphies: a differentiated part of the *flexor accessorius* muscle inserting on the proximal end of the *tendo superficialis* of digit II, and fibers of the *abductor brevis plantaris* muscle of digit V reaching at most the proximal two-thirds of metatarsal V (Araujo-Vieira et al., 2023). The group includes two confirmed species: *O. argyreornata* and *O. skuki* and four unconfirmed candidate species (UCS) (*Ololygon* spp. 2, 4, 5 and 6, sensu Araujo-Vieira et al., 2023). These UCS were classified based on genetic distances above 3% in the mitochondrial 16S gene, a common threshold indicating potential new lineages in frogs (Vieites et al., 2009). However, no other diagnostic traits were found to distinguish these lineages from closely related species, so they could not be confirmed as distinct species without further taxonomic studies.

Ololygon argyreornata itself comprises two distinct lineages, with genetic distances in 16S ranging from 2.9 to 3.4%. *O. argyreornata* A occurs in the municipalities of Santa Teresa and Vitória, while *O. argyreornata* B is found in Linhares, in the state of Espírito Santo (Araujo-Vieira et al., 2023). The type locality of *O. argyreornata* was corrected by Bokermann (1966) to Rio Mutum, in Colatina, Espírito Santo, but there is no genetic data available for this population yet. The UCS *O. sp. 2* occurs in southern coast of the states of São Paulo, Rio de Janeiro and Paraná; *O. sp. 4* occurs in the municipality of Rio Preto, state of Minas

Gerais; and the *O. sp. 5* and *6* occurs in southern coast of the state of Bahia in northeastern Brazil (Araujo-Vieira et al., 2023).

Only one species of the *O. argyreornata* group occurs in the Pernambuco Endemism Centre: *O. skuki*, a small frog (14.6–17.1 mm in males and 20–24.2 mm in females), morphologically similar to *O. argyreornata* but distinguishable mainly by the presence of glandular acini on finger III and subtle differences in advertisement call (Nascimento et al., 2024).

Ololygon skuki was first described from the Environmental Protection Area of Catolé and Fernão Velho, in the municipality of Maceió, state of Alagoas, Northeastern Brazil, and was considered endemic to this locality (Lima et al., 2011; Dubeux et al., 2020). Nascimento et al. (2024) recovered *O. skuki* as the sister taxon of all the remaining species in the *O. argyreornata* group, provided a revised morphological diagnosis, described its advertisement call, and recorded the occurrence of the species along the northern coast of Bahia, in the municipality of São Sebastião do Passé. In the state of Pernambuco, there was only one record of *Ololygon skuki*, from an Atlantic Forest fragment in the municipality of Paudalho (IUCN SSC Amphibian Specialist Group, 2015), but no information was provided about its morphology, genetics, or vocalizations.

After a long period of monitoring anurans in the Atlantic Forest fragments of Pernambuco, we discovered new populations of *Ololygon* sp., morphologically similar to *O. skuki*. To verify its taxonomic status, we employed a series of species delimitation tests based on molecular methods, analyzed bioacoustic and morphological traits, and compared these to populations identified as *O. skuki* from northern Bahia and Alagoas. We conclude that the populations from Pernambuco have unique diagnostic features, different from *O. skuki*, and we describe them as a new species, placing it as a sister species to *O. skuki* within the *O. argyreornata* group.

Materials and methods

Tissue sampling and laboratory protocols

We collected individuals identified as *Ololygon* sp. in Parque Estadual Dois Irmãos (PEDI) (8°0'16.83"S, 34°56'58.85"W; 37.8 m a.s.l.), located in the municipality of Recife, and in Serra da Camaratuba (9°20'18.08"S, 35°50'46.41"W; 853 m a.s.l.), in the municipality of Bezerros, state of Pernambuco. At PEDI, we conducted bimestral field expeditions, each lasting 10 days (6 hours per person per day; 2-3 researchers), from 2015 to 2019 and again from 2020 to 2021 during the rainy season, between June and August. In total, we carried out 10 field trips, totaling 1,920 hours of active searching. After this effort, we recorded only 17 individuals of *Ololygon* sp., all found on the leaves of provisional ponds within the forest. Nine of these individuals were collected under authorization number 11218-1 SISBIO/Instituto Chico Mendes de Conservação da Biodiversidade.

At Serra da Camaratuba, we performed a single field trip from October 21 to 24, 2022, with a total sampling effort of 96 hours of visual search. During this expedition, we found nine juveniles on the leaves of the bromeliad *Hohenbergia ramageana*. Individuals were euthanized by applying 2% lidocaine to the skin, then fixed in 10% formalin and stored in 70% ethanol. Muscle tissue samples were taken and preserved in 99.5% ethanol for molecular analyses. Sex was determined based on the presence of vocal slits, nuptial pads, and vocal sacs in males, and the presence of egg masses in females.

We extracted DNA from muscle tissues using the DNeasy kit (Qiagen, Inc.), including: five individuals of *Ololygon* sp from the state of Pernambuco: two individuals from the municipality of Recife (CHUFC-A-9325, 9668) and three individuals from the municipality of Bezerros (CHUFC-A-12122, 12124 and 12128); six individuals of *O. argyreornata*, from the municipalities of Santa Teresa (CFBH 25444-45), Aracruz (CFBH 25452, 25455) and the Linhares (CFBH 38322-23), all in the state of Espírito Santo; three individuals of *O. sp. 2*, from the municipality of Maricá, state of Rio de Janeiro (CFBH 17885-86) and one individual from the municipality of Ubatuba, São Paulo (CFBH 17621); four individuals of southern Bahia, from the municipalities of Itabuna (CFBH 2888-89), Uruçua (TUESC 1070) and Porto Seguro (CFBH 32111). We amplified and sequenced a fragment of the 16S rRNA gene using the primers 16SAR and 16SBR developed by Palumbi et al. (1991). PCR mix and conditions were followed (Rojas et al., 2018), then the PCR products were purified with ExoSAP (ThermoFisher), and sent to ACTGene Molecular Analysis, Porto Alegre, Brazil. The chromatogram read was manually edited with Geneious 6 (Kearse et al., 2012). The new DNA sequences were submitted to GenBank (accession Nos SUB15698871).

Phylogenetic analysis and species discovery methods

To determine the phylogenetic position of *Ololygon* sp. from the state of Pernambuco, we performed a maximum parsimony phylogenetic analysis, following the same phylogenetic framework and using the same genetic dataset of

Araujo-Vieira et al. (2023: appendix S1), including portions of cytochrome b, cytochrome oxidase c subunit I, 12S-tRNA^{Val}-16S, NADH dehydrogenase subunit 1 and tRNA^{Ile} (ND1-tRNA^{Ile}) totaling 4,731 base pairs, the nuclear genes included portions of chemokine receptor type 4, proopiomelanocortin (POMC), seven in absentia homolog 1 (SIAH), recombination activating gene 1 (RAG1), rhodopsin exon 1 (RHOD), and tyrosinase (TYR), totaling 2,850 base. We expanded the dataset to include mitochondrial 16S rRNA gene sequences of *Ololygon skuki*, from northern coast of the state of Bahia, produced by Dória et al. (2018) and Nogueira et al. (2024) and topology sequences from Nascimento et al. (2024), and the new sequences of *Ololygon* sp from the state of Pernambuco mentioned above.

For the species delimitation methods, we used only the 16S rRNA, including the newly sequenced individuals mentioned in the previous section ($n = 18$), along with 192 sequences available in GenBank. These included all described species and unconfirmed candidate species (UCS) of *Ololygon* (sensu Araujo-Vieira et al., 2023) and *Julianus uruguayus* as the outgroup, totalling 209 sequences (supplementary table S1). Sequences were aligned using MAFFT version 7.273 (Katoh and Standley, 2013) with the G-INS-I algorithm and default parameters for gap opening and extension. We reduced the full dataset to unique haplotypes using the function hapCollapse in R environment (version 3.4.1; R Development Core Team, 2021). The final alignment contained 145 sequences and 576 base pairs, including gaps and missing data. Subsequently, we generated an ultrametric phylogenetic tree in BEAST 2.6.3 (Bouckaert et al., 2014) under the GTR+G+I nucleotide substitution model, selected as the best-fit model of molecular evolution using Jmodel Test 2.1.10 (Posada, 2008), based on the corrected Akaike Information Criterion (AICc). We applied an uncorrelated relaxed log-normal clock model and a coalescent constant population size prior. Two independent Markov Chain Monte Carlo (MCMC) runs were performed, each for 80 000 000 generations, with trees and parameters sampled every 8000 generations. We assessed convergence and effective sample size (ESS > 200) using Tracer v1.7 (Rambaut et al., 2018). The runs were combined using LogCombiner, and the maximum clade credibility tree (MCCT) was generated using TreeAnnotator (Bouckaert et al., 2014). All analyses were conducted via the CIPRES Science Gateway (Miller et al., 2010; <https://www.phylo.org>).

We performed three species delimitation analyses: the generalized mixed Yule-coalescent (GMYC) method (Pons et al., 2006; Fujisawa & Barraclough, 2013), the Poisson tree processes (PTP) method (Zhang et al., 2013), and the distance-based method Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al., 2021). Following the framework proposed by Machado et al. (2018), we performed the GMYC analysis using the splits package (version 1.0-19) in R. The ultrametric BEAST chronogram was transformed into a phylogram using the phangorn package (version 2.2.0; Schliep, 2011) to serve as input for the PTP analysis. All analyses were conducted in R version 3.4.1 (R Development Core Team, 2021). The optimized phylogram was submitted to the PTP web server (<http://species.h-its>).

org/). For the ASAP analysis, we used the aligned FASTA file of unique haplotypes and selected uncorrected distances as the substitution model on the ASAP web server (<https://bioinfo.mnhn.fr/abi/public/asap/>).

We estimated the minimum uncorrected pairwise distances between *Ololygon* sp and the described species of the *Ololygon argyreornata* group, including the UCS of Araújo-Vieira et al. (2023), using MEGA X (Kumar et al., 2018).

Morphological parameters and statistical analysis

We performed 11 measurements (in millimetres), seven following the standards of Duellman (1970): SVL (snout-vent length), HW (head width), HL (head length), ED (eye diameter), TD (tympanum diameter), TL (tibia length), and FL (foot length); the measurements IND (internarial distance), END (eye-nostril distance), IOD (interorbital distance), and THL (thigh length) followed Napoli (2005). Measurements were taken with a calliper to the nearest 0.1 mm (supplementary table S2).

To assess morphological differences between *Ololygon skuki* (from the type locality and the northern coast of the state of Bahia) and the populations of *Ololygon* sp. from the state of Pernambuco, we performed a multivariate ratio analysis (MRA). We first calculated the isometric size (isozize), defined as the geometric mean of all variables, and then conducted a principal component analysis (PCA) in the space of all ratios (PCA shape), which interprets both body shape and isozize.

We also performed a linear discriminant analysis (LDA) ratio extractor to identify the best measurement ratios that differentiate the groups and may be used in species diagnoses. In addition, we calculated the standard distance and the δ value (a measure of how well shape discriminates in comparison to size; see Baur and Leuenberger, 2011; Baur et al., 2014). These analyses were performed using the R script provided by Baur and Leuenberger (2020).

Subsequently, we performed analyses of variance (ANOVAs) using the first two components from the shape PCA and the isozize as response variables. Statistical significance was evaluated using Tukey's test, via the aov and TukeyHSD functions in the stats package (version 3.4.1; R Core Team, 2021) following Ferrão et al. (2022).

We analysed the webbing formula according to Savage and Heyer (1967), as modified by Myers and Duellman (1982). Digits were numbered II-V following Fabrezi and Alberch (1996). Snout shape terminology followed Heyer et al. (1990) and Lourenço et al. (2014).

For adult morphological comparisons with other species, we examined specimens housed in the following Brazilian collections: Célio F.B. Haddad Collection, Universidade Estadual Paulista Júlio de Mesquita Filho, Rio Claro, São Paulo (CFBH); Coleção Herpetológica da Universidade Federal do Ceará, Fortaleza, Ceará (CHUFC-A); Museu de História Natural da Universidade Federal de Alagoas, Maceió, Alagoas (MHNUFAL); Museu de Zoologia da Universidade Estadual de Santa Cruz, Ilhéus, Bahia (MZUESC); Museu de Biologia Mello Leitão, Santa Teresa, Espírito Santo (MBML); and Museu de História Natural

da Universidade Federal da Bahia, Salvador, Bahia (UFBA) (see Appendix 1).

We also examined photographs of type series and bibliographic data from species descriptions (e.g., Lutz and Lutz, 1939; Lutz, 1954; Barrio, 1962; Bokermann, 1967; Lutz, 1968, 1973; Cardoso and Haddad, 1982; Caramaschi and Kisteumacher, 1982; Haddad and Pombal, 1987; Carvalho-e-Silva and Peixoto, 1991; Pombal and Gordo, 1991; Cruz, Nunes, and Lima, 2001; Faivovich, 2005; Lourenço et al., 2009, 2014, 2016, 2019, 2020; Pombal et al., 2010; Lima et al., 2011; Andrade et al., 2018; Lacerda et al., 2021).

Bioacoustics

We analyzed 14 calls from two individuals of *Ololygon* sp. from the state of Pernambuco (CHUFC-A-9324 and CHUFC-A-9327). The audio files were deposited in the Sonographic Collection of the Universidade Federal do Ceará (CBUFC 542-43). Recordings were made in Parque Estadual Dois Irmãos, municipality of Recife, state of Pernambuco, on June 27 and 28, 2019, between 19:37 and 20:35, with air temperatures ranging from 24.1 to 24.3°C and relative humidity between 78 and 80%.

Calls were recorded using a TASCAM DR-40 digital recorder and a Sennheiser ME66/K6 unidirectional microphone, positioned approximately 50 cm from the calling male. The recorder was set to a 44.1 kHz sampling rate and 16-bit resolution. We also analyzed calls from one individual of *O. skuki* from the municipality of Mata de São João, on the northern coast of the state of Bahia (UFBA-S-127); one individual of *O. skuki* from the type locality (the same individual analysed by Nascimento et al., 2024); and one individual of *O. argyreornata* from the municipality of Linhares, state of Espírito Santo (FNJV_0031913).

Calls were analyzed using Raven Pro 1.6.1 (64-bit version; Cornell Lab of Ornithology, 2011) with the following settings: window type = Hanning, window size = 256 samples, 3 dB filter bandwidth = 248 Hz, overlap = 89.8% (locked), DFT size = 1,024 samples (locked), hop size = 0.590 ms, and spectral resolution (grid spacing) = 43.1 Hz. Figures were generated using the Seewave v.2.0.2 package (Sueur et al., 2008) in the R environment (version 3.4.1; R Development Core Team, 2021). Call terminology follows the note-centered approach proposed by Köhler et al. (2017) and Hepp et al. (2017).

To assess differences between the populations of *Ololygon* sp. from Pernambuco, *O. skuki* from the northern coast of Bahia, and *O. skuki* from the type locality, we performed a principal component analysis (PCA) using the following acoustic parameters: dominant frequency (DF; measured using the peak frequency function), note duration (ND), internote duration (IND), and number of pulses per note (PPN). Analyses were performed using the vegan package (Oksanen et al., 2018).

We then conducted a PERMANOVA using the adonis2 function from the vegan package to test whether the centroids of the morphospaces differed significantly among the groups based on PCA scores.

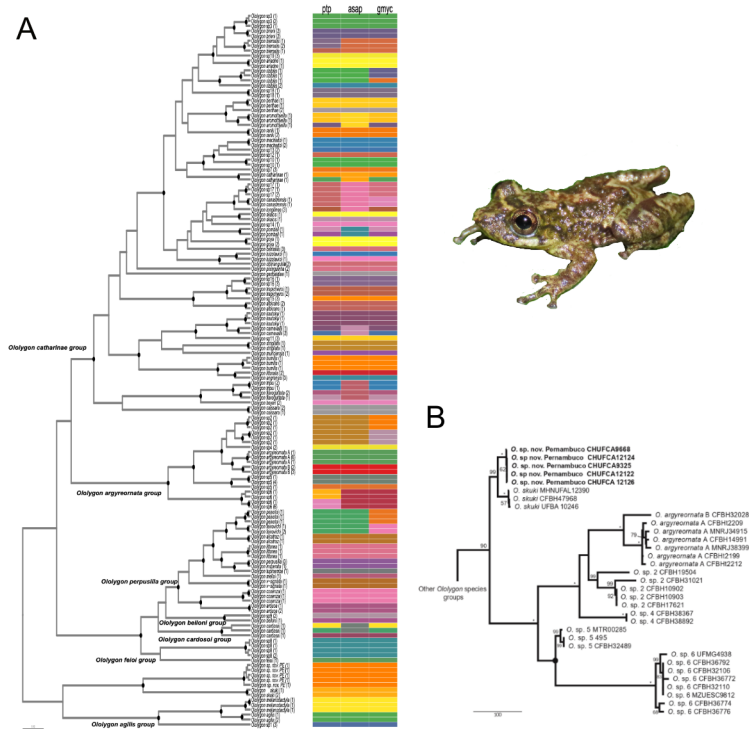


Figure 1. (A) Maximum clade credibility tree generated using BEAST 2.6.2 based on a dataset of 145 unique *Oloolygon* 16S rRNA haplotypes (576 aligned base pairs). Lineages identified through GMYC, ASAP, or PTP analyses (represented by colour groups) with posterior probabilities >0.95 are indicated by grey nodes. The number of unique haplotypes per lineage is shown in parentheses. (B) Phylogenetic relationships within the *Oloolygon argyreornata* group, as inferred from one of the 4,252 most parsimonious trees obtained using a molecular dataset with gaps treated as a fifth character state and equal transformation weights. Node values represent parsimony jackknife support; an asterisk (*) indicates 100% support. Nodes without values have <50% jackknife support. The black circle denotes a node that collapses in the strict consensus; all other displayed nodes are retained. For GenBank accession numbers and locality information, see Araujo-Vieira et al. (2023: Appendix S1) and Nascimento et al. (2024).

Conservation status

We performed a Geospatial conservation assessment tool using GeoCAT webserver (<https://geocat.iucnredlist.org/>). This software uses georeferenced distribution data to estimate the extent of occurrence (EOO) and area of occupancy (AOO), thereby classifying the species' conservation status according to the IUCN Red List criteria (Bachman et al., 2011).

Results

Phylogenetic relationships and species delimitation

The maximum parsimony analysis yielded 4252 most parsimonious trees (MPTs) with 91 693 steps. Our results indicate that the *O. argyreornata* species group forms a well-supported clade

(90% jackknife). *Oloolygon* sp. from the state of Pernambuco was recovered in a strongly supported clade (99% jackknife), as sister to *O. skuki* (fig. 1B). This clade, in turn, is sister to the remaining species within the *O. argyreornata* group.

Bayesian inference using only the 16S rRNA gene recovered a weakly supported clade (posterior probability (BI) < 0.95), composed of two well-supported species groups (BI > 0.95): *O. catharinae* and *O. argyreornata*. Within the *O. argyreornata* group, we recovered a well-supported clade composed of *O. sp. 2*, *O. sp. 4*, *O. argyreornata* (lineages A and B), and *O. sp. 5*, which is sister to *O. sp. 6*. *Oloolygon*

skuki was recovered outside the *O. argyreornata* species group, forming a well-supported clade with the *Ololygon* sp. from Pernambuco, as sister to the *O. agilis* species group; however, the relationship between these two groups lacked strong support. The sister clade to the *O. catharinae*+*O. argyreornata* groups is a well-supported clade comprising the *O. perpusilla*, *O. feioi*, *O. cardosoi*, and *O. belloni* species groups (fig. 1A).

The species delimitation methods recovered between 72 (ASAP; lowest score = 8.50, $p = 0.3740$, $w = 0.003$, distance threshold = 2.2%) and 82 (PTP) lineages, with GMYC recovering 79 lineages. Most currently recognized species were recovered by all methods, with some minor discrepancies (fig. 1). Within the *O. argyreornata* species group, both PTP and ASAP methods consistently recovered *O. sp. 2* and *O. sp. 4*. All methods recovered *O. argyreornata* lineages A and B as distinct. There was a consistent oversplitting of *O. sp. 5* into two lineages across all methods, and *O. sp. 6* was split into two lineages by PTP. The *Ololygon* sp. populations from the state of Pernambuco and *O. skuki* were recovered as distinct lineages in all species discovery methods (fig. 1A).

Our results also demonstrate that the UCS *O. sp. 5* from southern Bahia is more widespread than previously known, occurring in the municipalities of Uruçua, Itabuna, Ilhéus, and Canavieiras. In contrast, the *O. sp. 6* population remains restricted to Porto Seguro and Prado. The population from the coastal municipality of Maricá, state of Rio de Janeiro, belongs to *O. sp. 2*. *Ololygon argyreornata* lineage B was identified in the municipalities of Aracruz and Linhares, while lineage A was found in Santa Teresa, Vitória, and Ibirapu, all in the state of Espírito Santo.

Minimum genetic distances within the *O. argyreornata* species group ranged from 3% to 13%. The p-distances between *Ololygon* sp. from Pernambuco and the other members of the *O. gr. argyreornata* species group ranged

from 10% (*O. skuki*) to 13% (*O. sp. 4*) (see Supplementary Table S5).

Morphological analysis

We found a slight overlap in body shape along the first principal component (PCA shape) for males of *Ololygon skuki* and *O. sp.* from the state of Pernambuco (Supplementary fig. 1A, B). However, males showed statistically significant differences in body shape between the two groups (ANOVA: $S^2 = 0.43$, $F = 21.7$, $df = 1$, $p < 0.0001$). The variables that most contributed to group separation were interorbital distance (IOD), tympanum diameter (TD), and eye diameter (ED). A similar pattern was observed for females, which also showed significant differences in body shape (ANOVA: $S^2 = 0.17$, $F = 12.5$, $df = 1$, $p < 0.0001$; Supplementary fig. 1C, D).

In the second principal component of the shape PCA, the same pattern was observed, with significant differences between males (ANOVA: $S^2 = 0.43$, $F = 27.3$, $df = 1$, $p < 0.0001$), but not between females (ANOVA: $S^2 = 0.007$, $F = 0.50$, $df = 1$, $p = 0.49$). The isosize values also differed significantly between *O. skuki* and *O. sp.* from Pernambuco, with *O. skuki* being larger in both sexes (males: ANOVA: $S^2 = 0.14$, $F = 56.6$, $df = 1$, $p < 0.0001$; females: ANOVA: $S^2 = 0.10$, $F = 28.7$, $df = 1$, $p < 0.0001$; Supplementary fig. 3). The variation in PCA shape components and isosize values is summarized in the supplementary material (Supplementary table S3).

The LDA ratio extractor identified the best measurement ratios distinguishing males of *O. skuki* from *O. sp.* from Pernambuco as EY/IOD (standard distance = 3.26; $\delta = 0.39$) and HW/SVL (standard distance = 1.48; $\delta = 0.59$). For females, the most diagnostic ratios were IOD/THL (standard distance = 5.83; $\delta = 0.34$) and HL/SVL (standard distance = 5.05; $\delta = 0.37$) (Supplementary fig. 2).

Bioacoustics analysis

The PCA results revealed the presence of two distinct groups: *Ololygon skuki* (from the type locality and the northern coast of Bahia) and *Ololygon* sp. from the state of Pernambuco (Supplementary fig. 4). The number of pulses per note and internote interval, followed by note duration and dominant frequency, were the most important bioacoustic parameters contributing to PC1 and PC2 (Supplementary table S4). The PERMANOVA results also supported the distinction between the two species ($R^2 = 0.85$, $F = 521.7$, $df = 186$, $p < 0.0001$).

The call of one individual of *Ololygon skuki* from the municipality of Mata de São João, on the northern coast of Bahia, can be classified as a long call (sensu Hepp et al., 2017). The call has a mean duration of 3.41 seconds (range: 2.82–4.27 s; $n = 3$) and consists of a series of pulsed notes (mean = 38.3 ± 6.1 ; range: 33–45 notes per call; $n = 3$). Each note has a duration of 0.04 ± 0.01 s (range: 0.02–0.07 s; $n = 115$), with an internote interval of 0.04 ± 0.02 s (range: 0.02–0.16 s; $n = 112$), and is emitted at a rate of 11.3 ± 0.7 notes per second (range: 10.5–11.8; $n = 3$). The minimum frequency of the notes is 3383.6 ± 177.3 Hz (range: 2902.6–4043.4 Hz; $n = 115$), with a dominant frequency of 5218.9 ± 527.1 Hz (range: 3703.7–5684.8 Hz; $n = 115$) (Supplementary fig. 5A, B).

The call of one individual of *O. argyreornata* (lineage B) from the municipality of Linhares, state of Espírito Santo, is also classified as a long call, with a duration of 3.2 ± 0.9 s (range: 2.02–4.74 s; $n = 10$). It is composed of a series of pulsed notes (mean = 25.5 ± 7.8 notes per call; range: 13–37; $n = 10$), with note duration of 0.03 ± 0.004 s (range: 0.02–0.04 s; $n = 256$), and an internote interval of 0.10 ± 0.02 s (range: 0.07–0.16 s; $n = 246$), emitted at a rate of 7.8 ± 0.5 notes per second (range: 6.4–8.3; $n = 10$). The mean dominant frequency is 4432 ± 667.2 Hz (range: 3562.5–5520 Hz; $n = 113$) (Supplementary

fig. 5C, D). Additional call parameters were not analyzed due to the low quality of the recordings.

The call of *Ololygon* sp. from the state of Pernambuco, due to its distinctive pattern revealed in the PCA analysis, will be described in detail in the taxonomic section.

Taxonomic account

The populations of *Ololygon* sp. from the state of Pernambuco were recovered as a distinct evolutionary lineage in all molecular species delimitation methods. This lineage exhibits a high genetic divergence in the 16S rRNA mitochondrial gene fragment from its closest relative, *O. skuki* (10%), and has a distinct advertisement call. Based on these findings, and following the integrative taxonomy framework (e.g., Padial et al., 2010), we describe this lineage below as a new species.

The new species is assigned to the *Ololygon* gr. *argyreornata*, based on our Maximum Parsimony analysis, which recovered it as the sister species of *O. skuki*. Morphologically, the new species is similar to other members of the *O. argyreornata* species group, sharing the following traits: small body size in males (SVL 12.7–14.4 mm) and females (SVL 15.3–16.3 mm); absence of a pectoral fold and spicules on the nuptial pad, inner arm margin, and pectoral region; dorsal skin with scattered tubercles; postaxial webbing of toe I absent or basal; postaxial webbing of toe II extending to the midpoint of the penultimate phalanx; and a vocal repertoire composed of pulsed notes arranged in long multi-note calls (Araújo-Vieira et al., 2023).

Ololygon paulofreirei sp. nov.

urn:lsid:zoobank.org:act:13FA8089A90A-4C92-9CB2-2EE449F5FD7F *Scinax skuki*: IUCN SSC Amphibian Specialist Group, 2015

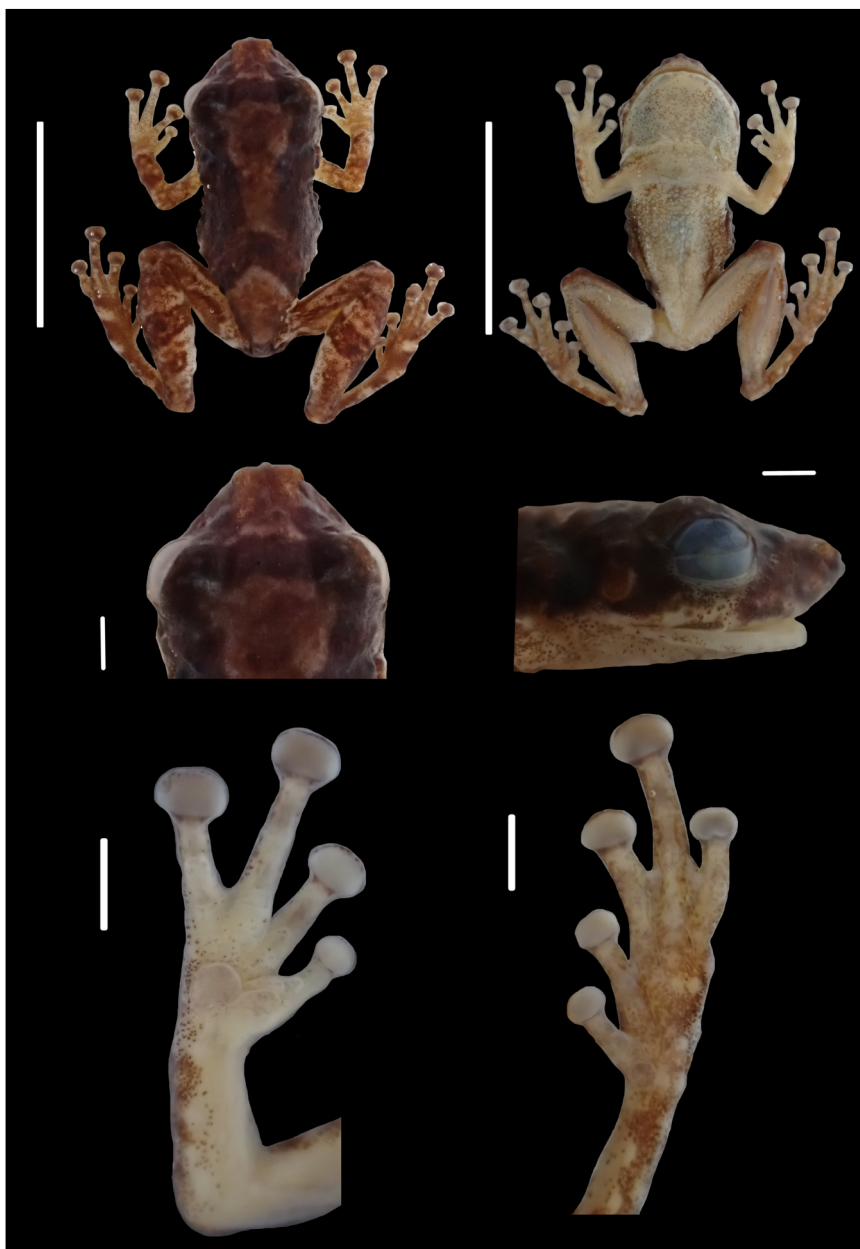


Figure 2. Holotype of *Ololygon paulofreirei* sp. nov. (UFC-A-9670): (Top) Dorsal and ventral views; (bottom) head in dorsal and lateral views; right hand and right foot in ventral views. Scale bars = 10 mm.

Holotype

CHUFC-A-9670, an adult male from the locality of Parque Estadual Dois Irmãos, municipality of Recife, state of Pernambuco, northeastern Brazil, 8°0'16.83"S, 34°56'58.85"W; 37.8 m a.s.l, collected on 20 July 2021 by I.J. Roberto,

E.G. Dias, E.M dos Santos, C.R. Oliveira, R.W. Ávila (fig. 2).

Paratopotypes

CHUFC-A-9327-9329, three adult males, collected on 29 June 2019 by E.M dos Santos e F.J.

Silva, CHUFC-A-9368-9369, two adult males, same collection date as for holotype.

Paratypes

MHNUFAL 10627, 10631-10640, 10642-10643, 13 adult males from locality of Reserva Ecológica Mata de Miritiba, Distrito de Chã de Cruz, municipality of Paudalho, state of Pernambuco, northeastern Brazil, 7°53'40.4"S, 35°3'36.2"W, 147 m a.s.l, collected on 5 March 2012 by M.G. Lima; MHNUFAL 10628-10630, MHNUFAL 10141, 10144, five females, same collection date as the male paratypes.

Additional material

Nine unsexed juvenile specimens (CHUFCA-A-12122-12130) from Serra da Camaratuba, municipality of Bezerros, state of Pernambuco, 9°20'18.08"S, 35°50'46.41"W, 853 m a.s.l.

Etymology

The specific epithet, a noun in apposition, is a tribute to the educator, philosopher, and writer Paulo Freire, who was born in the city of Recife in 1921. In 2021 he would be 100 years old. Paulo Reglu Neves Freire is recognized worldwide for his pedagogical work and is the patron of Brazilian education. His teaching method is used and referenced by educators from several countries who seek a transformative education.

Diagnosis

Ololygon sp. nov. is characterized by: (1) small size (SVL in males: 12.7-14.4 mm; females 15.3-16.3 mm); (2) absence of pectoral fold; (3) light colored and non-hypertrophied nuptial pad on finger III; (4) vocal sac single, subgular, well differentiated; (5) in life, inguinal region and the inner thigh and shank yellowish; (6) advertisement call with 0.22-2.24 s; (7) calls composed by 1-4 pulsed notes; (8) note rate of 1.4-3.2 notes/s; (9) notes composed by 3-6 pulses; (10) pulses with 0.16-0.30 s; (11) pulses

with amplitude modulation within pulses; (12) dominant frequency of 5857-6847 Hz.

Description of holotype

Body slender; head as wide as long; snout rounded with a mucronate tip in dorsal view and protruding in profile; *canthus rostralis* marked, rounded; loreal region slightly concave; nostrils prominent, elliptical opening dorsal-laterally after the tip of the snout; eyes large, protruding laterally; internarial distance 24% of head width; tympanum externally distinct, 33.5% of eye diameter; *annulus tympanicus* prominent, with a large tubercle posterior to the tympanum; supratympanic folder present, well-noticeable, covering the anterior *annulus tympanicus*, extending from the posterior corner of the eye to the shoulder; interorbital distance 37.1% of head width; large tongue, ovoid, free laterally and posteriorly; vocal slits present; elliptical choanae; two rows of vomerine teeth, each one with four teeth in each side, convex, posterior to the choana, in a central position; vocal sac single, median subgular, with partially loosened skin on the posterolateral portion of the gular region; forearm non hypertrophied, larger than upper arm; hand larger than upper arm; outer metacarpal tubercle rounded; inner metacarpal tubercle elliptical, smaller than the outer metacarpal tubercle; subarticular tubercles single and rounded, largely conic in finger V; few supernumerary tubercles present; nuptial pad light colored non-hypertrophied, without distinct epidermal projections; nuptial pad without epidermal projections on finger III; discs expanded broadened; webbing absent between fingers; finger size II > III > V > IV; hind limbs slender; tibia longer than the thigh, 54% of snout-vent length; inner metatarsal tubercle single rounded, larger than the small rounded inner metatarsal tubercle; subarticular tubercles rounded; supernumerary tubercles small, rounded, distributed across the plantar surface of the foot; relative toe length I > II > V > III > IV; webbing formula: I-1+II 3 IIII-3-IV 3^{1/2} 2^{1/2} V; toe discs elliptical, wider than long; pectoral

Table 1. Measurements (mm) of males and females in the type series of *Ololygon paulofreirei* sp. nov., including the holotype.

	Males (n = 22)	Females (n = 5)
SVL	13.5 ± 0.4 (12.7-14.4)	15.9 ± 0.4 (15.3-16.3)
HL	5.3 ± 0.4 (4.5-5.9)	6.3 ± 0.2 (6.0-6.7)
HW	5.2 ± 0.2 (4.8-5.6)	5.8 ± 0.05 (5.7-5.9)
IND	1.3 ± 0.2 (1.05-1.7)	1.6 ± 0.2 (1.3-1.9)
ED	1.5 ± 0.1 (1.1-1.7)	1.6 ± 0.2 (1.3-1.9)
END	1.8 ± 0.2 (1.5-2.1)	2.0 ± 0.2 (1.9-2.4)
IOD	2.3 ± 0.2 (1.9-2.8)	2.6 ± 0.1 (2.5-2.7)
TD	0.7 ± 0.2 (0.4-1.0)	1.0 ± 0.1 (0.8-1.1)
THL	6.4 ± 0.5 (5.4-7.2)	7.5 ± 0.3 (7.1-7.9)
TL	7.6 ± 0.3 (7.1-8.2)	9.0 ± 0.4 (8.5-9.5)
FL	5.3 ± 0.4 (4.6-6.3)	6.6 ± 0.5 (6.1-7.2)

Means±SD, ranges in parentheses.

fold absent; skin of dorsal surface and flanks covered with large tubercles of different sizes; ventral surface of gular, belly and limbs granular, covered with brown spots; cloacal opening at upper level of thighs.

Measurements of holotype (mm)

SVL = 13.7, HL = 5.06, HW = 5.09, IND = 1.2, ED = 1.5, TD = 0.5, END = 2.1, IOD = 1.9, THL = 6.5, TL = 7.5, FL = 5.1.

Colouration of holotype in preservative

Dorsal colouration is light brown, with a dark brown dorsolateral stripe and a dark brown chevron-shaped mark in the posterior region. Interocular region with a dark brown stripe. Dorsal surfaces of arms with dark brown blotches and dark brown transversal stripes in legs. Head heavily dark brown pigmented, with a light brown strip in the upper lip, extending towards the posterior margin of tympanum. Ventral region cream with scattered light brown dots.

Colouration of holotype in life

Same as in preservative, except for a perceptible black supratympanic fold, the head is less intense dark brown pigmented. Iris tan. Gular and inguinal region and hidden surfaces of thigh yellow. Belly with white dots in a creamy background.

Variation in type series

Measurement variation is presented in table 1. Females are larger than males, have more granules on the belly and upper limbs, and possess more slender upper arms compared to males. Some specimens exhibit a lower degree of tuberculation on the dorsum and a less protruding snout in lateral view (e.g., CHUFC-A-9325, 9326). Considerable polymorphism in coloration is observed (fig. 3), with most specimens displaying a lighter coloration than the holotype. For instance, male MHNUFAL 10627 shows a pronounced cream-colored dorsal vertebral stripe extending from the tip of the snout to the cloaca. Specimens MHNUFAL 10643, 10632, and 10634-10635 exhibit an inverted V-shaped marking at the sacral region. Variation is also evident in the distribution of brown dots on the ventral surfaces of the gular region, belly, limbs, fingers, and toes.

We observed variability in the presence of acini glands on finger III, which were prominent in CHUFC-A-9324, 9326, 9327, 9329, 9698, 9699, and MHNUFAL 10632 and 10644, but absent or not evident in CHUFC-A-9325, 9328, MHNUFAL 10627, 10631, 10633-10640, 10642, and 10643. The degree of looseness of the skin in the gular region also varied, ranging from partially loosened skin on the postero-lateral portion of the gular region (CHUFC-A-9324) to prominently distended skin externally



Figure 3. Adult specimens of *Ololygon paulofreirei* sp. nov. in life, Parque Dois Irmãos, municipality of Recife, state of Pernambuco, Brazil. (A-B) Holotype CHUFC-A-9670; (C) paratype CHUFC-A-9668; (D) paratype CHUFC-A-9669; (E-F) paratype CHUFC-A-9324.

evident in CHUFC-A-9325-9328 and 9698-9699.

Morphological comparison with other Ololygon species

Ololygon paulofreirei sp. nov. is distinguished from the species of the *O. agilis* species group (*O. agilis* and *O. melanodactyla*) by the absence of a pectoral fold (present) and an externally distended vocal sac (internal vocal sac). It can

also be distinguished from *O. melanodactyla* by its beige non-hypertrophied nuptial pad (epidermal black projections in the nuptial pad) (Lourenço, Luna and Pombal, 2014)

The smaller size of males (12.7-14.4 mm) and females (15.3-16.3 mm) distinguishes the new species from the species in the *O. belloni* species group (SVL in males 19.8-23.0 mm, females 26.3-29.2 mm); *O. cardosoi* species group (17.5-26.0 mm in males and 24.3-

29.1 mm in females), *O. catharinae* species group (SVL males 14.6–42.8 mm, SVL females 19.9–48.2 mm), *O. feioi* species group (in males 26.6–36.3 mm, females 41.5–45.5 mm) and *O. perpusilla* species group (1) SVL males 14.5–25.2 mm, females 18.6–31.6 mm) (see Araújo-Vieira et al., 2023).

The bright yellow background coloration of inguinal and hidden areas of the thigh and shank in *Ololygon paulofreirei* sp. nov. distinguishes from *O. ariadne* (violet or pink background; Bokermann, 1967), *O. muriciensis* (Cruz et al., 2001), *O. ranki* (Andrade and Cardoso, 1987), *O. skaios* (Pombal et al., 2010), *O. strigilata* (Pimenta et al., 2007), *O. tripui* (green; Lourenço et al., 2016), *O. brienii* (pale bluish; Lutz, 1973), *O. catharinae* (Pombal and Bastos, 1996), *O. humilis*, and *O. trapicheiroi* (light blue or white background; Lourenço et al., 2016), *S. hiemalis* (green; Haddad and Pombal, 1987), *O. obtriangulata* (dull grayish violet; Lutz, 1973), *O. agilis*, *O. albicans*, *O. angrensis*, *O. caissara*, *O. kautskyi*, *O. luizotavioi*, *O. melanodactyla*, *O. littoralis* (dark brown spots on light pale background; Lutz, 1973; Cruz and Peixoto, 1981; Carvalho-e-Silva and Peixoto, 1991; Caramaschi and Kisteumacher, 1989; Pombal and Gordo, 1991; Lourenço, Nascimento and Pires, 2009; Lourenço, Luna and Pombal, 2014), *O. carnevalli* (white with black blotches; Caramaschi and Kisteumacher, 1989) and *O. pixinguinha* (black blotches on a whitish-green background; Lacerda et al., 2021).

The well differentiated, externally expanded vocal sac in *Ololygon paulofreirei* sp. nov. promptly distinguishes from most species of *Ololygon*, with the exception *O. cardosoi* species group (Lourenço et al., 2020), *O. feioi* species group (Lourenço et al., 2020), *O. aromothyella*, *O. berthae*, *O. garibaldiae* and *O. rizibilis* (in the *O. catharinae* species group; Bokermann, 1964; Faivovich et al., 2005).

Ololygon paulofreirei sp. nov. is morphologically similar to *O. skuki* and *O. argyreornata* but can be distinguished from *O. skuki* by its smaller

body size (ANOVA: $S^2 = 0.17$, $F = 12.5$, $df = 1$, $p < 0.0001$). In life, *O. paulofreirei* has the inguinal region and hidden surfaces of the legs yellowish (orange in *O. skuki*). Compared to *O. argyreornata*, the new species is distinguished by its smaller size, with males measuring 12.7–14.4 mm and females 15.3–16.3 mm (compared to 15.5–17.2 mm in males and 18.4–23.7 mm in females of *O. argyreornata*; Araújo-Vieira et al., 2023; Nascimento et al., 2024).

Call description

The advertisement call of *Ololygon paulofreirei* sp. nov. consists of a series of long, multi-pulsed notes (1–4 notes per call), with a mean duration of 1.19 ± 0.65 s (range: 0.22–2.24 s; $n = 14$) (table 2, fig. 4). Each note contains three to six pulses, with amplitude modulation within pulses (pulse sub-units; Bang et al., 2017). The mean note duration is 0.23 ± 0.03 s (range: 0.16–0.30 s; $n = 36$), with an internote interval of 0.39 ± 0.16 s (range: 0.22–0.83 s; $n = 21$), resulting in a calling rate of 2.37 ± 0.56 notes per second (range: 1.41–3.16; $n = 13$). The dominant frequency of the notes ranges from 5857 to 6847.6 Hz.

Call comparison with other *Ololygon* species

The advertisement call of *O. paulofreirei* sp. nov. differs from all known species of *Ololygon* by its dominant frequency of 5857–6847.6 Hz, which is higher than: *O. albicans* (3300–4100 Hz), *O. angrensis* (2150–3700 Hz), *O. aromothyella* (4770–5410 Hz), *O. berthae* (4.47–5.28 Hz), *O. caissara* (3100–4400 Hz), *O. canastrensis* (2250–2340 Hz), *O. catharinae* (2250–3100 Hz), *O. centralis* (3490–4890 Hz), *O. goya* (2070–3100 Hz), *O. heyeri* (2840–3870 Hz), *O. hiemalis* (2250–3530 Hz), *O. humilis* (3090–3940 Hz), *O. littoralis* (1890–3530 Hz), *O. longilinea* (2070–2750 Hz), *O. luizotavioi* (2760–4130 Hz), *O. machadoi* (3500 Hz), *O. muriciensis* (2239.4–3445.3 Hz), *O. ranki* (2340–3420 Hz), *O. strigilatus* (2620–

Table 2. Acoustic parameters analysed on the advertisement call from *Ololygon* gr. *Argyreornata*.

	<i>Ololygon argyreornata</i> B	<i>Ololygon</i> sp 2 (São Paulo)	<i>Ololygon skuki</i> _(Bahia)	<i>Ololygon skuki</i> (topotype)	<i>Ololygon paulofreirei</i> sp. nov
Note duration (s)	0.03 ± 0.004 (0.02-0.04, <i>n</i> = 256)	0.02-0.04	0.04 ± 0.01(0.02-0.07, <i>n</i> = 115)	0.05 ± 0.01 (0.03-0.13, <i>n</i> = 93)	0.23 ± 0.03 (0.16-0.30, <i>n</i> = 36)
internote intervall (s)	0.10 ± 0.02 (0.07-0.16, <i>n</i> = 246)	-	0.04 ± 0.02(0.02-0.16, <i>n</i> = 112)	0.06 ± 0.01 (0.04-0.13)	0.39 ± 0.16 (0.22-0.83; <i>n</i> = 21)
Note rate	7.8 ± 0.5(6.4-8.3, <i>n</i> = 10)	-	11.3 ± 0.7 (10.5-11.8, <i>n</i> = 3)	9.2 ± 0.2 (9.1-9.4)	2.37 ± 0.56 (1.41-3.16; <i>n</i> = 13)
Notes per call	25.5 ± 7.8 (13-37, <i>n</i> = 10)	5	38.3 ± 6.1 (33-45, <i>n</i> = 3)	26-34	2.57 ± 0.85 (1-4; <i>n</i> = 14)
Call duration (s)	3.2 ± 0.9 (2.02-4.74, <i>n</i> = 10)	0.8	3.41 ± 0.8(2.82-4.27, <i>n</i> = 3)	3.4 ± 0.5 (2.8-3.7, <i>n</i> = 3)	1.19 ± 0.65 (0.22-2.24; <i>n</i> = 14)
Peak Frequency (Hz)	4432 ± 667.2 (3562.5-5520, <i>n</i> = 113)	5000-6300	5218.9 ± 527.1 (3703.7-5684.8, <i>n</i> = 115)	5125.3 ± 222.4(4565.0-5512.5)	6274.5 ± 216.1 (5857-6847.6; <i>n</i> = 36)
Pulses per note	-	2-25	-	36.1 ± 3.8 (25-42)	4.97 ± 0.7 (3-6; <i>n</i> = 36)
Minimum frequency (Hz)	-		3383.6 ± 177.3(2902.6-4043.4, <i>n</i> = 115)	4392.8 ± 327.3 (3273.0-4392.7)	2808.8 ± 564.6 (1740.8-3828.1, <i>n</i> = 36)

Two individuals of *Ololygon paulofreirei* sp. nov.: CHUFC-A 9324 (paratype) and CHUFC-A-9325 (paratype) from Parque Estadual Dois Irmãos, municipality of Recife, Pernambuco, Brazil; one individual of *O. skuki* (MHNUFAL-SOM 001; Nascimento et al., 2024) from Maceio, state of Alagoas; one individual of *O. skuki* (UFBA-S-127) from Mata de São João, state of Bahia; one individual of *O. argyreornata* B (FNJV_0031913) from Linhares, state of Espírito Santo; individuals of Ubatuba, state of São Paulo (Pombal Jr et al., 1995; Hepp et al., 2017), Brazil.

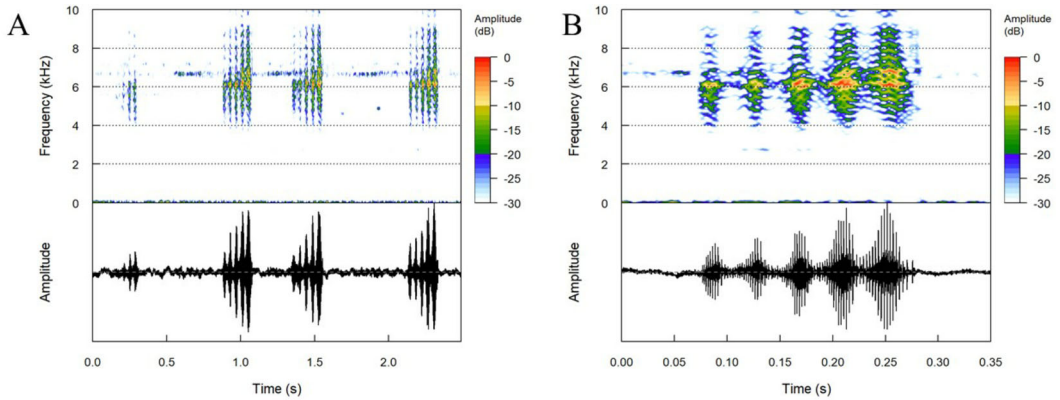


Figure 4. Advertisement call of *Ololygon paulofreirei* sp. nov. (A) Paratype (UFC-A-9324), recorded at Parque Estadual Dois Irmãos, municipality of Recife, state of Pernambuco, Brazil on June 27, 2019, showing a call composed by four distinct pulsed notes. (B) The last note of the same call, showing the five pulses, with amplitude modulation within pulses (subunits).

3380 Hz), *O. skaios* (2210-2240 Hz), *O. trapicheiroi* (2710-3320 Hz), and lower than *O. agilis* (7450-7920 Hz) (Bokermann and Sazima, 1973; Heyer, 1980; Cardoso and Haddad, 1982; Haddad and Pombal, 1987; Peixoto and Weygoldt, 1987; Andrade and Cardoso, 1987; Nunes et al., 2007; Lourenço et al., 2009; Pombal et al., 2010; Bastos et al., 2012; Garey et al., 2012; Pereyra et al., 2012; Mendes et al., 2013; Lourenço et al., 2016; Hepp et al., 2017; Dubeux et al., 2025).

The longer call duration (0.22-2.24 s) also differentiated *Ololygon paulofreirei* sp. nov from *O. muriciensis* (0.008-0.017 s) and *O. strigilata* (0.01-0.017 s) (Mendes et al., 2013). From *O. berthae* (3.20-52.04 s) (Pereyra et al., 2012) and *O. skaios* (4.42-7.90 s) (Pombal et al., 2010) it can be differentiated by its shorter calls. The lower number of notes per call (1-4) separates it from *O. canastrensis* (6-8), *O. heyeri* (6-9), *O. hiemalis* (6-17), *O. longilinea* (8-19), *O. ranki* (6-15), *O. machadoi* (6-7), *O. rizibilis* (7-23), *O. skaios* (42-73) (Bokermann and Sazima, 1973; Cardoso and Haddad, 1982; Andrade and Cardoso, 1987, 1991; Peixoto and Weygoldt, 1987; Pombal et al., 1995; Bevier et al., 2008; Hepp et al., 2017). The longer note duration 0.16-0.30 s distinguishes the new species from *O. albicans* (0.03 s), *O. angrensis*

(0.025 s), *O. caissara* (0.01-0.02 s), *O. canastrensis* (0.01-0.03 s), *O. catharinae* (0.04 s), *O. centralis* (0.02-0.03 s), *O. goya* (0.01-0.05 s), *S. heyeri* (0.002-0.01 s), *O. hiemalis* (0.02-0.09 s), *O. humilis* (0.002-0.006 s), *O. littoralis* (0.05 s), *O. longilinea* (0.02-0.07 s), *O. luizotavioi* (0.005-0.02 s), *O. machadoi* (0.05 s), *O. muriciensis* (0.008-0.017 s), *O. ranki* (0.01-0.05), *O. strigilata* (0.01-0.02 s), *O. skaios* (0.01-0.05 s). The note rate also separates the new species (1.4-3.2 notes/s), being higher than *O. angrensis* (0.07 notes/s) and lower than *O. canastrensis* (11.1-12.2 notes/s), *O. goya* (8.9-23.8 notes/s), *O. heyeri* (16.1-20.2 notes/s), *S. hiemalis* (5.7-10.5 notes/s), *O. humilis* (11.6-38.2 notes/s), *O. longilinea* (11.8-14.4 notes/s) and *O. ranki* (9-12.5 notes/s), *O. trapicheiroi* (3.6-8.2 notes/s) (Bokermann and Sazima, 1973; Heyer, 1980; Cardoso and Haddad, 1982; Haddad and Pombal, 1987; Peixoto and Weygoldt, 1987; Andrade and Cardoso, 1991; Nunes et al., 2007; Lourenço et al., 2009; Pombal et al., 2010; Bastos et al., 2011; Garey et al., 2012; Pereyra et al., 2012; Mendes et al., 2013; Lourenço et al., 2016; Hepp et al., 2017; Dubeux et al., 2025).

From the *O. argyreornata* species group the call of *O. paulofreirei* sp. nov. can be distinguished by its higher dominant frequency 5857-6847 Hz when compared to *O. skuki* (3703.7-5684.8 Hz), and *O. argyreornata* B (3562.5-5520 Hz); shorter call duration 0.2-2.2 s than *O. argyreornata* B (2.0-4.7 s) and *O. skuki* (2.8-4.3 s); longer note duration 0.16-0.30 s than *O. skuki* (0.02-0.13), *O. argyreornata* B (0.02-0.04), *O. sp. 2* (0.01-0.04); a longer internote interval 0.22-0.83 s than *O. skuki* (0.02-0.16 s); lower number of notes per call 1-4 and pulses per note 3-6 than *O. skuki* (25-45), *O. sp. 2* (8-12); lower note rate 1.4-2.2 notes/s than *O. skuki* (9.1-11.8 notes/s) and *O. argyreornata* B (6.4-8.3 notes/s) (table 2). The new species also have pulses with sub-units structure (within pulses amplitude modulation) (sensu Bang et al., 2017), absent in *O. skuki* and *O. sp. 2* and *O. argyreornata* B (Pombal et al., 1995; Hepp et al., 2017; Nascimento et al., 2024; this study).

Distribution

Known only from three Atlantic Forest fragments: Dois Irmãos State Park (*Parque Estadual Dois Irmãos*), a protected area and the largest fragment of Atlantic Forest in north-eastern Brazil, covering 1157.72 hectares and located within the urban zone of the municipality of Recife, state of Pernambuco, Brazil; the municipality of Paudalho, within the Environmental Protection Area of Aldeia Beberibe (*APA Aldeia de Beberibe*), located 18 km northwest of the type locality; and the Serra da Camaratuba mountain range, in the municipality of Bezerros, situated 104 km southwest of the type locality (Supplementary fig. 6).

Natural history

Ololygon paulofreirei sp. nov. was recorded at the type locality vocalizing on leaves and tree branches along the margins of a temporary pond formed after heavy rain, due to the terrain elevation. The pond measured approximately 4 m in length, 6 m in width, and had a depth of

50 cm. We observed only six males vocalizing synchronously, spaced 60 cm to 2 m apart, at heights ranging from about 40 cm to 2 m above the water surface, during June and July.

At the Paudalho locality, females collected in March had visible egg masses on their ventral surface. These observations suggest that the species' reproductive period may extend from March to July. During the dry season, in Serra da Camaratuba, juveniles were found on the leaves of an herbaceous tank bromeliad, *Hohenbergia ramageana*.

At the type locality, *Ololygon paulofreirei* occurs in syntopy with *Hylomantis granulosa*, *Dendropsophus branneri*, *D. gr. decipiens*, *Pristimantis ramagii*, *Phyllodytes edelmoi*, and *Leptodactylus natalensis*. For a complete list of anuran species recorded in Parque Dois Irmãos, see Pereira, Lira, and Santos (2016) and Melo et al. (2018).

Conservation status

The GeoCat analysis recovered an extent of occurrence (EOO) of 1507.813 km² and an area of occupancy (AOO) of 12 000 km² for *Ololygon paulofreirei* sp. nov, which classifies the species Endangered (EN) according to the IUCN Red List criteria.

Discussion

The three molecular species delimitation methods corroborate the hidden cryptic diversity found in the *Ololygon argyreornata* species group, delimiting all five unconfirmed candidate species and the two lineages in *O. argyreornata* (A and B) (see Araújo-Vieira et al., 2023). Our multilocus maximum parsimony analysis recovered *Ololygon paulofreirei* sp. nov. and *O. skuki* as the most basal species in the *O. argyreornata* group, sister to the remaining species and unconfirmed candidate species (UCS), similar to the results of Nascimento et al. (2024) that recovered *O. skuki* as the most basal species in

the *O. argyreornata* group. Our Bayesian phylogenetic analysis, using only a fragment of the 16S mitochondrial gene, failed to recover *O. skuki* and *O. paulofreirei* sp. nov. as part of the *O. argyreornata* species group. The low support of the relationships within the *Ololygon* species group in our molecular phylogenetic analysis may be due to the use of only part of the 16S fragment (see Almeida et al., 2021; Chan et al., 2022).

We found another population of *O. skuki* on the northern coast of Bahia. The specimens analysed from Mata de São João were larger and had an advertisement call similar to the calls of *O. skuki* from the type locality (Nascimento et al., 2024), with only differences regarding a higher note rate (10.5–11.4 notes/s) versus 9.1–9.4 notes/s, with the other parameters overlapping between both populations. The females analyzed from the municipality of Catu also fit the morphological diagnosis of *O. skuki* (Lima et al., 2011; Nascimento et al., 2020), with larger SVL than *O. paulofreirei* sp. nov. (Supplementary table 2).

Based on similarities in morphological and acoustic parameters and geographic location, *O. skuki* is more widespread on the northern coast of the state of Bahia, whereas *O. sp. 5* and *O. sp. 6* are restricted to the southern coast of Bahia. This biogeographic pattern of distinct genetic lineages and species on the northern and southern coasts of Bahia, and different lineages in the states of Pernambuco and Alagoas, similar to the distribution of *O. skuki* and *O. paulofreirei* sp. nov., has been previously found for *Pristimantis* spp. lineages, possibly as a result of climatic changes during the Quaternary (Carnaval et al., 2009; Silveira et al., 2019; Trevisan et al., 2020).

The conservative morphology of species within the *Ololygon argyreornata* group (Lima et al., 2011; Araujo-Vieira et al., 2023; Nascimento et al., 2024) has hindered clear taxonomic differentiation. However, bioacoustic parameters have proven to be valuable characters for distinguishing species within the group.

Bokermann (1966) provided only limited information on the call of *O. argyreornata* from Linhares, Espírito Santo, describing it as a long call composed of multi-noted pulses (sensu Hepp et al., 2017). We reanalyzed the call of *O. argyreornata* (lineage B) from Linhares, state of Espírito Santo, and observed a lower peak frequency, higher note rate, and greater number of notes per call compared to populations of *Ololygon* sp. 2 from Ubatuba, São Paulo (Pombal Jr. et al., 1995; Hepp et al., 2017). The advertisement call of *O. paulofreirei* sp. nov. is also notably distinct from most other species in the *O. argyreornata* group (Hepp et al., 2017; Andrade et al., 2018; Nascimento et al., 2024), resembling the sound of a katydid. Based on calling behaviour during recordings, we associate this call with “Type A” calls (Hepp et al., 2017), likely functioning as advertisement calls. These calls are long, composed of squawk-like notes with intermediate duration. Each note consists of distinct pulses exhibiting amplitude modulation. Spectral parameters are similar to the “short note” type of *O. aromothyella* and *O. berthae* (Pereyra et al., 2012), although both species show shorter note durations and lower peak frequencies than *O. paulofreirei* sp. nov.

Several *Ololygon* species have restricted geographic distributions, often limited to small forest fragments of the Atlantic Forest and Cerrado (Cruz et al., 2011; Lima et al., 2011; Lourenço et al., 2013, 2016, 2019, 2020; Lacerda et al., 2021). Some are already classified as endangered (*O. skuki*) or critically endangered (*O. muriciensis*) (IUCN SSC Amphibian Specialist Group, 2015a,b). Given its narrow distribution, small area of occupancy and extent of occupancy, habitat specificity, and small population size, *O. paulofreirei* sp. nov. can be classified as endangered. However, further data are needed to assess its conservation status accurately.

The presence of *O. paulofreirei* sp. nov. in a protected area, Parque Estadual Dois Irmãos, is encouraging. Nonetheless, only a small population was found in a limited area despite extensive surveys. Conservation measures are

urgently needed, especially considering that even protected areas face increasing threats due to political and economic pressures. In recent years, environmental policies and scientific research have suffered from budget cuts (Oliveira et al., 2020; Pereira et al., 2020), while deforestation and mining have intensified, including within conservation units and Indigenous lands (Siqueira-Gay et al., 2020; De Jesus & Catojo, 2021).

A significant threat is a proposed development project affecting the type locality of *O. paulofreirei* sp. nov., currently undergoing a concession process under Federal Law No. 10.147 (December 3, 2019), which includes Parque Estadual Dois Irmãos in the National Park Privatization Program (PND). Plans include constructing wider access roads and public visitation trails in the same area where the species was found. Proper zoning and a comprehensive environmental impact assessment are essential to mitigate threats to this rare species.

Additionally, two new developments are planned in the second known locality of *O. paulofreirei* sp. nov., within the Área de Proteção Ambiental (APA) Aldeia de Beberibe: the metropolitan ring road of Recife and a new military school. APA Aldeia de Beberibe harbors one of the largest remaining tracts of Atlantic Forest north of the São Francisco River. These projects pose significant risks to ecosystem services and regional biodiversity.

Supplementary materials. Data is available on <https://doi.org/10.1163/15685381-bja10241> under Supplementary Materials.

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Appendix: Examined specimens for comparison

Ololygon argyreornata

Brazil: Espírito Santo • municipality of Aracruz MBML 126, 661-662, CFBH 25452, 25454; municipality of Guarapari MBML 5618, 6322, 6657; municipality of Ibirapu MBML 7422-7423; municipality of Linhares 1994, 9402, 2199, CFBH 38322-38323; municipality of Santa Teresa MBML 9607-9608, 9670, CFBH 25444-25445; municipality of Sooretama MBML 9401, CFBH 14945.

Ololygon sp. 2

Brazil: Rio de Janeiro • municipality of Maricá CFBH 17885-17886; Paraná, municipality of Morretes UFBA 9151; São Paulo, municipality of Ilha do Cardoso CFBH 17763.

Ololygon sp. 5

Brazil: Bahia • municipality of Belmonte UFBA 14352-14353; municipality of Camacã, MZUESC 8473; municipality of Uruçuca, MZUESC 17905-17906, 18392-18393; municipality of Ilhéus, UFBA 10527-10529, MZUESC 14742, 19330, 19447.

Ololygon sp. 6

Brazil: Bahia • municipality of Itamaraju MZUESC 12938; municipality of Porto Seguro MZUESC 7872, 12942; municipality of Santa Cruz Cabralia, UFBA 14270.

Ololygon muriciensis

Brazil: Alagoas • Estação Ecológica de Murici, municipality of Murici; MHNUFAL 16171-16172; Fazenda Bananeiras, municipality of Murici, MHNUFAL 5856 (Paratype).

Ololygon skuki

Brazil: Alagoas • Área de Proteção Ambiental do Catolé e Fênão Velho, municipality of Maceió, MNRJ 7000 (Holotype), MNRJ 7001-7011 (Paratypes), MHNUFAL 2691-2692 (Paratypes), MHNUFAL 2565-2567, 2569, 4361-4365, 4370, 4505; Bahia: municipality of São Sebastião do Passé, UFBA 10242-10249; municipality of Catu, UFBA 10359-10360; municipality of Mata de São João, UFBA 4616, 4618-4622, 4971.

Ololygon machadoi

Brazil: Minas Gerais • Serra do Cipó, CHUFC-A-2171-2172

Ololygon ariadne

Brazil: São Paulo • Serra da Bocaina, municipality of São José do Barreiro, CHUFC-A-2096-2098

Ololygon hiemalis

Brazil: São Paulo • municipality of Botucatu, CHUFC-A-116

Ololygon trapicheiroi

Brazil: Rio de Janeiro • Floresta da Tijuca, municipality of Rio de Janeiro, CHUFC-A-2212-2213