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Unexpected species diversity in electric eels with a description of the strongest living bioelectricity generator

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Is there only one electric eel species? For two and a half centuries since its description by Linnaeus, *Electrophorus electricus* has captivated humankind by its capacity to generate strong electric discharges. Despite the importance of *Electrophorus* in multiple fields of science, the possibility of additional species-level diversity in the genus, which could also reveal a hidden variety of substances and bioelectrogenic functions, has hitherto not been explored. Here, based on overwhelming patterns of genetic, morphological, and ecological data, we reject the hypothesis of a single species broadly distributed throughout Greater Amazonia. Our analyses readily identify three major lineages that diverged during the Miocene and Pliocene—two of which warrant recognition as new species. For one of the new species, we recorded a discharge of 860 V, well above 650 V previously cited for *Electrophorus*, making it the strongest living bioelectricity generator.

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Is there only one electric eel species? Since Linnaeus’s description of *Electrophorus electricus* 250 years ago, electric eels have fascinated scientists and layperson alike by their capacity to generate strong (~650 V) electric organ discharges (EODs)\(^1\).\(^2\). Strong EODs facilitate hunting, prey capture, and defense, while weaker (~10 V) EODs allow electrolocation and communication\(^3\). Electric eels inspired the design of Volta’s first electric battery to provide constant current, provide a source of acetylcholinesterase for treating neurodegenerative diseases\(^4\), and recently encouraged the development of synthetic protocells with natural nanoconductors and capacitators\(^5\),\(^6\),\(^7\), and a stacked hydrogel battery that could be used to power medical implants\(^8\). Electric eels are also an emerging model for genomic studies of animal electrogenesis\(^9\). Due in part to their large size [up to 2.5 m\(^10\)], and specialized electrogenic morphology, electric eels have long been assumed to comprise a single species broadly distributed through Greater Amazonia—the superbasin comprising the Amazon, Orinoco, and coastal drainages of the Guianas e.g., refs.\(^11\),\(^12\).

To test the hypothesis of a single species of *Electrophorus*, we examine 107 specimens from across Greater Amazonia—including the type locality of *E. electricus* in Suriname\(^13\) (Supplementary Data 1). To explore species-level divergences, we adopt the General Lineage Concept (GLC)\(^14\), which recognizes species as separately evolving metapopulation lineages. The GLC unifies several pre-existing species concepts, which vary in their criteria for identifying the point of lineage divergence during speciation\(^14\). Practical applications of the GLC seek multiple, congruent lines of evidence for delimiting species, and to this end we subject a large dataset (comprising mitochondrial and nuclear DNA, morphology, and geographical and ecological distributions) to a range of empirical and model-based procedures. Our analyses lead us to conclude that there are three common species of *Electrophorus*, which occupy predominantly allopatric ranges (i.e., occupy different regions) in the Guiana Shield (*E. electricus*), Brazilian Shield (*E. voltai* sp. nov.) and in the lowland Amazon basin (*E. varii* sp. nov.). Here we describe these three species, and discuss their morphology, evolutionary history, and ecology.

### Results and discussion

**Genetic analysis.** Phylogenetic analyses based on the mitochondrial COI gene resolved three divergent and highly supported lineages corresponding to *E. electricus*, and the two proposed new species *E. voltai*, and *E. varii*—both with Bayesian Inference [posterior probability (PP) >0.95], and Maximum-Likelihood (ML) analysis (bootstrap >0.95; Fig. 1). Estimated evolutionary divergences of COI, using Kimura 2-parameter distances, are: 6.6% between *E. electricus* and *E. voltai*; 9.8% between *E. electricus* and *E. varii*; and 9.3% between *E. voltai* and *E. varii*. Intra-specific divergences range from 0.02% in *E. electricus* to 0.31 and 0.32% in *E. voltai*, and *E. varii*, respectively. Interspecific COI divergences are also well above the accepted threshold (~2%) used to recognize animal species, including fishes\(^15\). Finally, sequences were analyzed by pairwise distances to assess intra- and interspecific variation, without a priori species hypotheses, using Automatic Barcoding Gap Discovery (ABGD)\(^16\). ABGD clustered the sequences into the same three lineages.

Concatenated mitochondrial DNA (COI, ND4, ATPase6/8, 12S rDNA, and 16S rDNA) was analyzed with three General Mixed Yule Coalescent (GMYC) models: the Bayesian Poisson tree process (bPTP), single- (SML) and multi-threshold (MML) maximum-likelihood methods, and the Genealogical Sorting Index (GSI). The results for GMYC (bPTP, *E. electricus* 0.999; *E. voltai* 0.826; *E. varii* 0.973); SML (3 clusters, \(p = 5.7e\)-14), MML (3 clusters, \(p = 5.6e\)-14), and GSI (\(gsi = 1, p < 0.001\) for all

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**Fig. 1** Sampling localities and gene trees for the three species of *Electrophorus*. a Map of northern South America showing distributions of sampled records and type localities (indicated by numbers) for three electric eel species: *Electrophorus electricus* (red dots, 1 = Suriname River, Suriname); *E. voltai* (blue dots, 2 = Rio Jpitings, Brazil); and *E. varii* (yellow dots, 3 = Rio Goiap, Brazil). Bicolor dots (blue/yellow) indicate sympatric co-occurrence of *E. voltai* and *E. varii*. The map was created in ArcGIS (https://www.arcgis.com) with images available at Shuttle Radar Topography Mission, Global Multi-resolution Terrain Elevation Data, and HydroSHEDS database. b BEAST2.4 species tree (top cladogram; 94 specimens: 15 *E. electricus*, 41 *E. voltai*, 38 *E. varii* based on 5 mitochondrial (trees 1-5; 107 specimens: 19 *E. electricus*, 43 *E. voltai*, 45 *E. varii*) and 5 nuclear genes (6-10; 94 specimens). Higher shading densities represent areas where the majority of trees agree in topology and branch lengths (posterior probabilities >0.99), while lower densities represent areas of uncertainty (Supplementary Data 1)
three species) strongly support the same three lineages recovered from COI. Concatenated nuclear DNA (S7i-1, SH3PXX, 36298E1, 4174E20, and 55378E1) was analyzed under coalescent-based methods for species delimitation in the Bayesian Phylogenetics and Phylogeography program BP&P v3.217, and by Bayesian posterior probabilities (PP). Results derived from these analyses (Fig. 1) strongly support the same three lineages (PP = 1.0) recovered from COI and concatenated mitochondrial DNA. The full set of ten nuclear and mtDNA markers was analyzed using species delimitation in BP&P and Bayesian Evolutionary Analysis by Sampling Trees [*BEAST2.4]18 (Fig. 1). The same three lineages recovered by previous analyses were, again, overwhelmingly supported (PP = 1.0).

Morphological analysis. Due to uniform body shape and coloration, neither morphometric analyses of 19 linear body measurements (Supplementary Data 2) nor pigmentation characters unambiguously distinguish the three species of Electrophorus. However, a species-level assessment based on characters from the lower jaw, neurocranium, and cleithrum separate specimens of Electrophorus into two groups (Fig. 2): those possessing a dorsoventrally depressed skull (*E. electricus and *E. voltai), and those with a deepened skull (*E. varii). The cleithrum lies between vertebrae 5 and 6 in *E. electricus and *E. voltai and between 1 and 2 in *E. varii. We found additional diagnostic differences in head shape (Fig. 2), and non-overlapping ranges in the number of pectoral-fin rays (e.g., 32–38 in *E. electricus versus 20–28 in *E. varii) and lateral-line pores (e.g., 88–101 in *E. electricus versus 124–186 in *E. varii and 112–146 in *E. voltai; for more details, see Diagnoses). These historically overlooked characters unambiguously assign all individuals of Electrophorus to the same three species delimited by our genetic analyses.

Electrophorus interrelationships. The interrelationships among Electrophorus and outgroup genera are beyond the scope of this paper; however, some of our findings, based on a limited number of outgroup taxa, deserve comment. Our analyses recovered Gymnotus as part of an unresolved polytomy, with the genera Hypopomus and Sternopygus both constituting sister taxa to the polytypic Electrophorus (both in ML and in the trimmed terminals (n = 3) Bayesian analysis). In the full 113 terminal dataset Hypopomus is recovered as sister to Electrophorus (see Supplementary Fig. 1). In each of our analyses very long branches subtend all clades. The sampling schema undertaken herein, wherein many terminals within the genus Electrophorus are analyzed alongside the proposed sister lineage, i.e., Gymnotus, as well as a single species each of Hypopomus and Sternopygus the resultant topologies are not to be taken as proposal of new intergeneric relationships. Instead the sampling was chosen a priori based on previous work to calibrate divergence estimates, and provide a diversity of outgroup taxa with which to evaluate the local taxon Electrophorus. Based on previous research it has been proposed that Electrophorus is either a member of a monotypic Electrophoridae or part of the Gymnotidae. Regardless of familial placement and interrelationships the single branch that leads to Electrophorus, heretofore a single widespread species (and now comprising the nominal species and two new species) is representative of a unique lineage that is unlike other gymnotiforms.

Temporal diversification. We used estimates for the origin of the Isthmus of Panama with outgroup taxon sampling in additional gymnotids, i.e., Gymnotus carapo (South America) and G. cylin- dricus (Central America) (see ref. 21) as a calibration point for the multilocus species tree generated by *BEAST2.4, based on Maximum Clade Credibility, with a relaxed clock assumption for the mtDNA loci22 and a strict clock for the nDNA23. A normal
distribution was set to 10.5 Ma (see Time divergence estimates in Methods) and standard deviation of ±1.5 for the outgroup taxa spanning the Isthmus20. The resulting time-calibrated genealogies (Fig. 3) estimate the divergence between E. variii and E. electricus + E. voltai to have occurred by the late Miocene (7.1 Ma; 95% highest posterior density: HPD 8.9–5.2 Ma), with subsequent divergence between E. electricus and E. voltai in the Pliocene (3.6 Ma; 95% HPD 4.7–2.5 Ma).

**Ecological distributions, biogeography, and divergence events.** Electrophorus electricus is restricted to the Guiana Shield, and E. voltai occurs in generally north-flowing rivers of the Brazilian shield and south-flowing rivers of the Guyana shield. In contrast, E. variii occurs in lowland floodplain and terra-firme systems of the intercratonic Amazon Basin (Fig. 1; Supplementary Data 1). Electrophorus variii and E. voltai co-occur in some streams in the Guiana Shield (Fig. 1). The Miocene divergence of E. electricus + E. voltai and E. variii may reflect ecological specialization to shield versus lowland habitats. Shield streams and rivers are: (1) permanently normoxic (>3 mg/l dissolved oxygen); (2) uniformly low in conductivity (<30 µS/cm); and, (3) include rocky substrates, rapids, and waterfalls12. In contrast, the lowland Amazon: (1) include low conductivity blackwaters (<30 µS/cm) and high-conductivity whitewater (60–350 µS/cm); (2) include permanently normoxic terra-firme streams (>3 mg/l), and seasonally hypoxic floodplains (<0.5 mg/l); and, (3) are slow flowing—with non-rocky substrates and without rapids or falls12. Some morphological specializations may have attended divergence into shield versus lowland systems. For instance, the depressed skull of E. electricus and E. voltai may represent an adaptation for foraging in rocky substrates or withstanding higher flow—mirroring specializations in other rheophilic (fast-flow-adapted) fishes24.

We hypothesize that the divergence of E. voltai (Brazilian Shield) and E. electricus (Guiana Shield)—both restricted to low conductivity systems (Fig. 1a)—may have arisen from dispersal barriers imposed by the emergence of the Amazon’s modern (high-conductivity) river-floodplain course in eastern Amazonia (ref. 23 describes similar disjunct distributions in other taxa). The separation of the Guiana and Brazilian Shields by a major river-floodplain resulted from the reversal of a paleo west-flowing Amazon to the contemporary east-flowing Amazon during the Miocene-Pliocene. The Amazon River was initiated as a transcontinental river 9.4–9 Ma (late Miocene) by recent estimates26, began entrenchment about 6.8 Ma and developed its modern shape from about 2.4 Ma onwards27. Notwithstanding debate over the timing of these events26,28, our estimated 3.6 Ma (95% HPD 4.7–2.5 Ma) divergence of E. voltai and E. electricus (Fig. 3) is coincident with the later stages of the origins of the Amazon’s eastern course.

Do the geographical and ecological distributions of Electrophorus reflect predictive models of niche occupation? We used Ecological Niche Models (ENMs) based on climatic and geomorphological variables to test the premise of divergent habitat requirements for each species of Electrophorus. ENMs, based on MaxEnt presence-only algorithms, predicted the potential niche distributions of Electrophorus with strong confidence (Area Under the Curve, AUC ≥0.90, Fig. 4a–c). Likewise, observed geographic ranges are significantly influenced by the abiotic environmental factors included in our analyses: seasonality of air temperature (ST) and annual mean temperature (AMT)—strong predictors of flood pulse; altitude (AL), annual mean precipitation (AMP), and flow accumulation (FLA)—strong predictors of aquatic habitat structure; and; soil types (SOT 0, 3, 6, 11)—predictors of water chemistry. The
predicted niche area for *E. electricus* (Fig. 4a; AUC = 0.98) designates AL (44.7%) and AMT (25.3%) as the strongest contributors to the models. For *E. voltai* (Fig. 4b; AUC = 0.96), SOT (35.4%) and FLA (25.4%) contributed most. For *E. varii* (Fig. 4c; AUC = 0.90), AL (72.3%) and FLA (13.1%) contributed most. Despite strong performance, ENMs nonetheless generated some over-predictions of ranges (Fig. 4a–c). For instance, they inaccurately predicted *E. electricus* to occur in portions of the lowland Amazon basin between the Guiana and Brazilian Shields (Fig. 4a). Likewise, ENMs incorrectly predicted the occurrence of *E. varii* in the northern portion of the Guiana Shield (Fig. 4b).

A hypothesis of niche divergence among *Electrophorus* species was corroborated by multivariate analyses of variance (MANOVA) of climatic and geomorphological data. MANOVA confirmed significant differences among the niches modeled for each species (Pillai’s lambda = 1.1092, F = 10.414, P < 0.001).

In summary, while best regarded as approximations, our ENMs for *Electrophorus* support a hypothesis of divergent niche requirements and geographical ranges corresponding to distinct ecological conditions.

All the three species of *Electrophorus* have a low-voltage ( Sachs’ organ/posterior Hunter’s organ) electric organ discharges (EODs) and high-voltage (main/anterior Hunter’s organ) with a head-positive monophasic waveform. The low-voltage EOD varies in duration across the species as follows (Fig. 4d): *E. electricus* (2.03–2.19 ms, n = 2), *E. varii* (1.24–1.78 ms, n = 4), and *E. voltai* (1.72 ms, n = 1). The high-voltage EOD (Fig. 4d) ranges from 480 V at 760 mm TL, n = 1, in *E. electricus*; 151 V (200 mm TL) to 572 V (609 mm TL), n = 4, in *E. varii*; and 860 V at 1219 mm TL, n = 1, in *E. voltai*.

To explore similarity in EOD waveform structure between the three species of *Electrophorus* (Fig. 4d) we extracted prominent time-frequency features from all available low-voltage Sach’s organ EOD waveform recordings using the discrete wavelet transform (DWT) and subjected the resulting matrix of DWT coefficients to dimension reduction by pairwise ANOVA; see refs. 29,30. Finally, we subjected this reduced matrix to a nearest-neighbor (single linkage) multivariate hierarchical clustering procedure.

Nearest-neighbor clustering analysis (Fig. 4e) demonstrated that the Sach’s organ EOD waveform structures of *E. electricus* and *E. varii* cluster together, while the (single recorded) EOD of *E. voltai* is dissimilar to those of *E. electricus* + *E. varii*—primarily due to its shorter duration. The results of this clustering analysis were also congruent with measurements of the multivariate Mahalanobis distance (D^2) between the centroids of each species: D^2 for *E. electricus* to *E. varii* = 25, D^2 for *E. electricus* to *E. voltai* = 375; D^2 for *E. varii* to *E. voltai* = 540. The hierarchical classification of EOD waveform structure in Fig. 4e is not congruent with the phylogeny of *Electrophorus* (Fig. 3), suggesting that distances in EOD signal-space are not correlated to phylogenetic distance as would be expected if EOD structure evolves via non-adaptive drift. Instead, because the low-voltage Sach’s organ EOD may facilitate species-recognition (as documented in weakly-electric gymnotiforms 31), and because *E. voltai* and *E. varii* co-occur in geographical sympathy in parts of the lower Amazon, we hypothesize that the EODs of *E. voltai* may have diverged from that of *E. varii* as an adaptive response to costs associated with heterospecific mismating events (i.e., reproductive character displacement [RCD]; see ref. 32. Nonetheless, we stress that these analyses are based on small sample sizes (*E. electricus*, n = 2; *E. varii*, n = 4; *E. voltai*, n = 1). A thorough test of the RCD hypothesis will require a much larger dataset of signals with an expanded geographical coverage.
Systematic biology.

Electrophorus Gill, 1864

Electrophorus ref. 33: 152. Type species: Gymnotus electricus Linnaeus, 1766. Type by monotypy. Gender: masculine.

Electrophorus electricus (Linnaeus, 1766)

Gymnotus electricus1; Gymnotus tremuli ref. 34: 27, pl. 3; Gymnotus tremulus ref. 35: 111; Gymnotus electricus ref. 1: 427; Gymnotus regius ref. 36: 273.

Diagnosis: Ten nucleotides in COI (BOL-COI fish F1/R1; 569-bp fragment): G(8), A(50), T(76), T(77), T(107), C(119), C(182), G(272), G(494), A(560). Ventral outline of head U-shaped, widest at terminus of branchial opening (Fig. 2a) and lateral-line pores 88–101 (versus ovoid, widest anterior to branchial opening, Fig. 2b; 112–146 in E. voltai). Distinguished by skull depressed, cleithrum lies between vertebrae 5 and 6 (Fig. 2a), pectoral-fin rays 32–38, and lateral-line pores 88–101 (versus skull deep, cleithrum lies between vertebrae 1 and 2, Fig. 2c, 20–28, and 124–186 in E. variii, respectively).

Description: Species illustrated in Figs. 2, 3, and 5. Maximum size examined specimens 1000 mm TL. Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examined specimens 1000 mm TL (total length). Morphometric and meristic data in Supplementary Data 2. Body elongate; size examines...
Recently, genomic and proteomic tools have been used to greatly enhance our knowledge of the convergent origins of strong electric discharges. The results shown here suggest that sequencing and comparing the genomes of these three electric eel species will yield further advances towards the origins of, and underlying structures responsible for generation and output of high-voltage electric discharges. Assessment of further population and/or species diversity in *Electrophorus* will follow this study, based on the incorporation of additional specimens from targeted areas (including the upper Negro and Orinoco drainages). A comprehensive understanding of *Electrophorus* could also reveal a hidden variety of enzymatic or bioelectrogenic functions of interest to the broader scientific community.

**Methods**

**Taxon sampling and specimen collection.** To test the hypothesis of a single species of *Electrophorus* we examined 107 specimens (all sequenced for mitochondrial DNA, mtDNA, and 94 specimens for nuclear DNA, nDNA) from across Greater Amazonia including the type locality of *E. electricus* in Suriname (Supplementary Data 1). Outgroup species were *Gymnotus carapo*, *G. choco*, *G. cylindricus*, *G. panherinus*, *Hypopomus artemi*, and *Sternoptyx macrurus* (Supplementary Data 2). Specimens were collected and sampled in the field according to the Animal Care and Use standards of the depository institutions and the countries of origin of the tissue samples used in the DNA analyses. In addition, tissues and/or specimens were received from multiple institutions in North and South America and Europe following pertinent Material Transfer Agreements and the national and international guidelines for the shipment of museum specimens. Specimens were euthanized and muscle or fins removed and stored in 95% ethanol. All voucher specimens are deposited in the institutions listed in the abbreviation section.

**DNA sequencing.** Genomic DNA was isolated from muscle or fin using phenol-chloroform in the Autogen platform or DNeasy Tissue Extraction Kits (QIAGEN) following manufacturer’s instructions. The polymerase chain reaction (PCR) was used to amplify fragments of the mtDNA and nDNA and amplified using the primers compiled in Supplementary Data 4. PCRs for COI, 12S, 16S, *Atpase* 8/6, 361298E21, 417E420, 5537E20, and S7-I1 were carried out for 10 µl volumes as follows: 1 µl of 10x buffer, 0.5 µl of 10 µM dNTPs, 0.4 µl of 50 µM MgCl2, 0.3 µl of 10µM of each primer, 5 U of Taq DNA polymerase, 6.4 µl of denatured water, and 1µl of DNA extract. Thermal cycling conditions for genes were: 35 cycles, 95 °C for 30 s, 95 °C for 30 s, 72 °C for 45 s, and 72 °C for 300 s. In the case of ND4, PCR was carried out for 20 µl volumes and 1 µl of DNA extract. Nested PCRs for *SH3PX3* were carried out for 25 µl volumes and 2 µl of DNA extracts. Thermal cycling conditions for *SH3PX3* were: 35 cycles, 95 °C for 60 s, 94 °C for 30 s, 72 °C for 80 s, and 84 °C for 150 s. The annealing temperatures and times are provided in Supplementary Data 4. PCR products were purified using EXOSAP. DNA sequencing followed standard protocols employed in molecular systematics laboratories and were completed through a capillary sequencing technique on the LAB MAHVNN550 sequencer. All obtained sequences were deposited in GenBank (Supplementary Data 3).

**Sequence alignment.** Sequences were edited in the CodonCode Aligner (www.codoncode.com) and preliminarily aligned using ClustalW in MEGA 6.0.6. Alignments were checked by eye and manually adjusted when necessary. Kimura Two Parameter (K2P) pairwise distances were calculated using MEGA 6.0.6. For all analyses *Hypopomus artemi* and four species of *Gymnotus* were also included. 107 individuals of *Electrophorus* from throughout their range were included as the ingroup. The best model of nucleotide evolution for each locus was estimated using jModelTest, though codon-level estimates were not inferred or enforced. For introns sequences heterozygosity was noted with degenerate IUPAC codes. Insertion/Deletion mutations for the COI 36298E21 sequences were incorporated in the phylogenetic analyses e.g., ref. 32.

**Species delimitation.** Species delimitation was based on the subsequent evaluation of four molecular datasets. Dataset 1: Single locus (COI; 569 bp); Dataset 2: five mtDNA genes (COI, ND4, ATP8/6, 12S rDNA, and 16S rDNA; 2973 bp total); Dataset 3: five nDNA loci (one exon; *SH3PX3*: one intron; S7-I1; and three EPCs: 36298E20, 417E421, 5537E20; 2439 bp total); Dataset 4: concatenated mtDNA and nDNA genes (5432 bp). Dataset 1: Application of a simple barcoding approach for species delimitation, i.e., COI sequences, in combination with pairwise distance comparisons has resulted in highly successful species-level identifications in fishes, e.g., ref. 43. In spite of this, determination of the limits between inter- and intra-specific differences and the delimitation of the appropriate level of differences between species threshold has proven difficult, particularly in under-sampled phylogenies. For DNA taxonomy herein we utilize COI, which as noted above has previously demonstrated the ability to provide good resolution for species delimitation among fishes. We complement the traditional molecular taxonomic approach, i.e., a single gene or set of genes, by defining species clades and revealing the independent investigatory tool based on pairwise distances to automatically detect significant barcoding gaps without an a priori species hypothesis—the Automatic Barcoding Gap Discovery, ABGD.

Dataset 2: A computationally multi-faceted parametric inferential approach. Species validation can come in many forms; herein our approach begins with a GMYC model using the 5-gene concatenated mtDNA. Given that branching events, in this case the history of haplotypes along any phylogenetic tree, should be more recent within a species and more distant between species; implementation of the GMYC model seeks to distinguish between cladogenetic (species-level differences modeled by the Yule process) and tokogenetic (intra-specific differences modeled by the Coalescent process) events. A Bayesian, single-threshold ML method and multi-threshold ML methods are all available for the GMYC model and all three were used here. The single-threshold ML method is the most conservative approach, the multi-threshold method allows for variation in the depth of history at which tokogeny gives way to speciation and, there is a Bayesian implementation that takes into account error in reconstruction of phylogeny and model uncertainty. The full dataset (113 terminals) was reduced to unique haplotypes (63 terminals) prior to analyses. Beast 2.4.8 was run for 20 million generations sampling every 1000 generations, results were assessed using Tracer v1.5 (http://beast.bio.ed.ac.uk/Tracer) to ensure stationarity and to check that all parameters had acceptable effective samples sizes (~200) for use in generating the distribution of ultrametric trees. The derived maximum clade credibility (MCC) tree from the *BEAST2.4 run was used for the two GMYC analyses based on ML. These analyses were run on the GMYC web server (https://species.h-its.org/gmyc/) using the single and multi-threshold ML methods, as described above. The Poisson tree process (PTP) has been shown to better delineate species, particularly when divergences among lineages is low and, being Bayesian based implementation of this is available (http://species.h-its.org/ptp/). As two GMYC approaches were implemented we elected to use the BPTP method, to investigate a third approach to species number.

Dataset 3: Nuclear DNA used herein for *Electrophorus* and related species is a combination of three of EPIC loci (see Supplementary Data 3), a single nuclear ribosomal intron (S7-I1), and one exon (*SH3PX3*). Further estimates of species boundaries and validation of species were completed by analyzing the data in several different ways including analyzing each nuclear locus individually to determine the posterior probability support provided by each locus, analyzing the concatenated full nuclear DNA Dataset 3, incorporating coalescent-based methods for species delimitation of nDNA loci via Bayesian Phylogenetics and Phylogeography (BPPV3.3) and investigating the full ten locus dataset with the Genealogical Sorting Index, GSI and using 10000 permutations on the lattice server, as well as joint estimation of divergence times and the gene trees species tree using *BEAST2.4*. Details of these analyses are as follows: Each nuclear locus was analyzed individually and in a concatenated matrix using MrBayes v3.2.12. On the COI sequence data using *BEAST2.4.4*.

Dataset 4: Bayesian Phylogenetics and Phylogeography (BPPV3.3) was used to delimit species boundaries on the complete 10-locus dataset with all terminals included and with a reduced number of terminals (three individuals from each species). BPPV uses different Maximum Likelihood (ML) and Bayesian approaches, but infers phylogeny in a Bayesian framework. The program also accounts for population genetic uncertainties in incomplete lineage sorting associated with ancestral polymorphism conflicts in gene trees and species trees. In this program the Gamma prior G (α,β) is assigned to both population size (β) and age of the species root (α) and in our three analyses I used = 2 and = 1000; all other parameters of divergence time used the Dirichlet prior with the heredity scalar set to 0.25 for the mtDNA loci and to 1 for the five nuclear loci. The analyses were run twice to ensure consistency between the runs.

**Phylogenetic estimation (trees with outgroups).** We estimated the phylogeny of *Electrophorus* using the concatenated alignment of dataset 4 (5852 nucleotides total) in RAXML and MrBayes 3.2.612 both run on the CIPRES science gateway.

Time divergence estimates: We simultaneously estimated divergence time, based on an external calibration point, and the species trees from multilocus sequence data. We used *BEAST2.4* to fit a relaxed clock for the mtDNA loci and a strict clock for the nDNA23. A subject of much recent debate has been the chronological closing of the Central American Seaway via the Isthmus of Panama and its
consequences for biotic dispersal between North and South America and vicariance between Atlantic and Pacific Oceans9–11. On the younger side, 38 dated the formation of the Panama–Panamanian isthmus. Paleoenvironmental studies20,25 show a decrease in the transport of deep and intermediate Pacific waters into the Caribbean by 10 to 11 Ma, probably related to a closing Central American Seaway32. Based on uranium-lead geochronology in detrital zircons, 33 provided evidence that rivers originating on the Panama arc transported shallow marine deposits toward the landward margin of the Gulf of Mexico by the middle Miocene (13–15 Ma). Finally, 34 used both molecular and fossil data to argue for two significant waves of terrestrial dispersal at around 20 and 6 Ma. Based on these studies there is a wide time scale from which to select calibration points, each with support from the literature: 2.8 Ma35; and 5.1 Ma—75% of the fossil record. We used the latter time point for the DWT, using the Symmlet-4 wavelet base, to generate a matrix of 256 coefficients. The choice of the scaling base was based on the size of the dataset and the choices of similar studies22. Although of large size, the chosen wavelet base is the most appropriate for the size of the dataset. Finally, using the ROC (Receiver Operating Characteristic) curve72. Alternatives to AUC there choose randomly the presence locations in relation to randomly choosing the background locations, commonly used in SDM modelling1,2. This measure could be interpreted as average of true positives values (sensitivity) of all possible false positive values (specificity), producing a global measure of fit for the model. These variables were plotted (species-by-variable) and considered for nucleotide substitutions were performed using dismo and vegan packages for Maxent, and MANOVA in R software19.

**Molecular diagnosis.** Species of Electrophorus were diagnosed by unique nucleotide substitutions shared by all individuals of the distinct populations. Optimizations of the nucleotide substitutions among the species of Electrophorus were obtained from the MP topology using MEGA 6.0.6. Each numeric position was determined by the alignment between the species of Electrophorus with the outgroup. Screening for nucleotide substitutions performed manually post alignment using Mesquite (http://mesquiteproject.org).

**Phenotypic analysis.** Morphometric and meristic summaries do not include data from individuals smaller than 300 mm TL. Although of very large sizes compared to most species of Neotropical freshwater fishes, specimens of Electrophorus less than 300 mm are juveniles with pronounced differences in some meristic (e.g., number of anal/caudal-fin rays) and morphometric values (e.g., preanal-fin distance) relative to larger specimens. Internal anatomy was studied through radiographs.

**Meristics** follow11 with the addition of the number of lateral-line pores posterior of the gill opening. Anal/caudal-fin ray counts include the dorsal procurent rays, when present (made through radiographs). Merometrics are point-to-point distances taken with digital calipers with intra-specific ranges presented in tables. Measurements were taken from the left side of individuals, when possible, as follows: body width—the distance across the body at the pectoral-fin base; branchioporous—the distance from the dorsal to the ventral extremities of the opening; eye length—the horizontal distance between the anterior and posterior margins of the eye; eye-posterior naris distance—the distance from the anterior to the posterior margin of the nostril; eye maximum body depth—the greatest vertical extent of the body, usually at the origin of the anal fin along the posterior margin of the gill slit; head depth—the distance between the dorsal and ventral margins of the head at the vertical through the eye; head length—the distance from the tip of the lower jaw to the posterior margin of the opercle; head width—the horizontal distance between the dorsal limits of the branchial opening; internarial distance—the distance between the posterior margin of the anterior nares and the anterior margin of the posterior nares; interorbital distance—the distance between the medial margins of the eyes; mouth-eye distance—the distance from the posterior margin of the mouth to the ventral margin of the eye; mouth-maxilla length—the distance between the lateral corners of the mouth; preopercular-fin length—the distance from the base of the dorsal-most pectoral-fin ray to the distal most point on the fin margin; postorbital distance—the distance from the posterior margin of the eye to the posterior margin of the opercle; preanal-fin distance—the distance from the tip of the lower jaw to the anal-fin origin; preanal distance—the distance from the tip of lower jaw to the anterior margin of the anus; postanal distance—the distance from the anterior margin of the eye to the anterior margin of the lower jaw; snout-corner of mouth distance—the distance from the snout to the corner of the mouth; and total length—the distance from the tip of the lower jaw to the base of the central caudal-fin ray.

**Species distribution modelling and niche analysis.** According to77 the species distribution patterns are the consequences of three main factors: (1) dispersal ability; (2) the spatial distribution of environmental conditions that determine the survival of individuals and the persistence of populations; and (3) biotic interactions and dynamics of resources. The yields distributions are based on the set of climate variables in wide resolution scales (macroscale) that determine the distribution of organisms, i.e., Grinnell niche76.

To build the SDM models we used the MaxEnt algorithm, which works with presence data only78. Methods that use only presence data are common especially in studies of large gaps of information and high biodiversity such as the Amazon River basin, where there is no information about absence. MaxEnt estimates the probability of species distribution by fitting a function close to the uniform distribution under the environmental information associated to the occurrence points79. This method can discriminate between the environmental variables associated with the presence and occurrence points based on 10000 random points, i.e., the algorithm contrasts presence against the background location80. We used occurrence points of the three species described in this paper, to show the niche differences among them, Electrophorus electricus, E. voltai and E. voltai. We used 10000 points, i.e., one occurrence point per pixel, split into 20% test and 80% training.

The environmental variables were chosen according to their potential to represent the topographical and limnological characteristics in the Amazon freshwater ecosystem based on70 who showed that broad scale variables could be used as proxies for characteristics of the local aquatic environment for modelling fish species distributions in areas like the Amazon where large gaps exist in our understanding of distributions. The climatic macroscale variables were obtained from BioClim (www.worldclim.org); annual mean precipitation (AMP), annual mean temperature (AMT), seasonality of precipitation (SP) and seasonality of temperature (ST). We also used geomorphometric variables such as slope (SL), altitude (AL) and flow accumulation (FLA) obtained from Hydro1k (www.usgs.gov) database. Soil type characteristics (SOT) were gathered from FAO’s database (www.fao.org.br). All descriptor variables were obtained for pixels of 4 x 4 km of resolution.

Model evaluation was performed using the Area Under Curve (AUC), which is a threshold-independent measure based on ranking locations, i.e. the probability to choose randomly the presence locations in relation to randomly choosing the background locations, commonly used in SDM modelling1,2. This measure could be interpreted as average of true positives values (sensitivity) of all possible false positive values (specificity), producing a global measure of fit for the model. These values were plotted (species-by-variable) and considered for nucleotide substitutions were performed using dismo and vegan packages for Maxent, and MANOVA in R software44.

**Electric organ discharge analysis.** Low-voltage EOD waveform recordings. We measured the low-voltage electrolocation pulses generated irregularly (rates of ca. 0.1–10 Hz) by the Sachs’ electric organ1. Head-to-tail EOD waveforms sensu ref. 75 were recorded within 12 h of capture in inflatable swimming pools (2.5–3.0 m diameter, or rectangular ca. 3.0 × 2.5 m, or rectangular ca. 4.0 m × 3.0 m). The pools were placed at least 40 cm away from the collecting site. Temperature was standardized to 27 ± 0.2 °C. Submerged NiCr electrodes were placed at least 40 cm away from the head and tail of the fish, along the head–tail axis, and a train of low-voltage pulses acquired directly by an audio-digitizer (96 kHz sampling rate) or a National Instruments digital acquisition device (40 MHz, sampling rate 100–200 kHz). To choose randomly the presence locations in relation to randomly choosing the background locations, commonly used in SDM modelling1,2. This measure could be interpreted as average of true positives values (sensitivity) of all possible false positive values (specificity), producing a global measure of fit for the model. These values were plotted (species-by-variable) and considered for nucleotide substitutions were performed using dismo and vegan packages for Maxent, and MANOVA in R software44.

To test the significance of the niche differentiation among lineages, we performed the multivariate analyses of variance (MANOVA). All the analyses were performed using dismo and vegan packages for Maxent, and MANOVA in R software44.

High-voltage EOD amplitudes. We measured the high-voltage pulses generated in rapid volleys by the main electric organ and Hunter’s electric organ for predation and defense.6 We used a Fluor 190–202 storage oscilloscope to measure the peak voltage in the volley of high-voltage EODs. Soon after capture the subject specimen was stretched out on a dry heavy-duty (non-conductive) plastic sheet to isolate it from the load of water. In this position a DC-coupled voltage reading from snout to the distal end of the tail was taken by gently prodding the tip of the snout to elicit a volley of high-voltage discharges. The entire procedure was accomplished in less than one minute.

Low-voltage EOD quantitative analysis. All seven EODs were conditioned to a common sampling rate, energy-normalized to root mean squared (rms) amplitude and centered to the peak of the single EOD phase. Following the procedure described in refs. 44, 59 and using a custom MATLAB (The Mathworks, Natick, MA) program, we subjected the conditioned waveforms to the discrete wavelet transform (DWT), using the Symmlet-4 wavelet base, to generate a matrix of 256 DWT coefficients (256 unique coefficients at 8 wavelet scales [2^(8) – 1], and one scaling coefficient). The DWT is a popular time-frequency based procedure to decompose signals into a smaller number of features informative of temporal (waveform shape) and spectral (frequency) differences among groups of signals76. Following ref. 59 we then subjected the matrix of 256 DWT coefficients × 7 individuals to dimension reduction by pairwise ANOVA to extract those waveform features (DWT coefficients), which permit the most effective discrimination among the seven fish species. The distribution morphospace of the first two DWT coefficients for each individual. Finally, using the ‘cluster’ package in Statistica 13.3 (Tibco/Statsoft, Palo Alto, CA) we performed nearest-neighbor (single linkage) hierarchical
clustering of all individual EODs in the matrix of reduced DWT coefficients, with the Euclidean distance as a metric of distance in multivariate space.

**Nomenclatural acts.** This published work and the nomenclatural acts it contains have been registered in ZooBank, the proposed online registration system for the International Code of Zoological Nomenclature (ICZN). The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix "http://zoobank.org/". The LSIDs for this publication are: 75985E4C-EDC-43CE-A57B-CD71A9C99526, 7FA17DC2-5F58-43E6-8908-9E66E92E2458, and 142863F0-11F6-4789-A058-ECEC3CC022F.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Sequences for all molecular markers are available from the GenBank database (accession numbers are listed in Supplementary Data 3). Specimens from which DNA samples were analyzed were deposited along with tissue samples at the biodiversity collections listed in Supplementary Data 1. All data are available upon reasonable request.

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Author contributions
The experiments were conceived and designed by C.D.S., C.B.D., and W.G.R.C. C.D.S., C.B.D., R.G.F., and W.G.R.C. performed the experiments. Data were analyzed by C.D.S., C.B.D., R.G.F., and W.G.R.C. C.D.S., W.G.R.C., C.D.B., R.G.F., M.H.S., R.C., J.R., J.Z., R.R.O., R.N.M.J., D.A.B., T.F.T., J.M., W.O., N.C.C., L.A.P., C.N., L.S., L.F.A.M., F.R., J.C.W., N.M.P., R.P.V., and W.B.W. contributed reagents, materials, tools for analyses, and wrote the manuscript.

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