Published in final edited form as:

J Physiol Paris. 2016 October ; 110(3 Pt B): 302–313. doi:10.1016/j.jphysparis.2016.10.002.

Evolution of electric communication signals in the South American ghost knifefishes (Gymnotiformes: Apterontidae): A phylogenetic comparative study using a sequence-based phylogeny

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Abstract

The electric communication signals of weakly electric ghost knifefishes (Gymnotiformes: Apterontidae) provide a valuable model system for understanding the evolution and physiology of behavior. Apterontids produce continuous wave-type electric organ discharges (EODs) that are used for electrolocation and communication. The frequency and waveform of EODs, as well as the structure of transient EOD modulations (chirps), vary substantially across species. Understanding how these signals have evolved, however, has been hampered by the lack of a well-supported phylogeny for this family. We constructed a molecular phylogeny for the Apterontidae by using sequence data from three genes (cytochrome c oxidase subunit 1, recombination activating gene 2, and cytochrome oxidase B) in 32 species representing 13 apteronotid genera. This phylogeny and an extensive database of apteronotid signals allowed us to examine signal evolution by using ancestral state reconstruction (ASR) and phylogenetic generalized least squares (PGLS) models. Our molecular phylogeny largely agrees with another recent sequence-based phylogeny and identified five robust apteronotid clades: (i) *Sternarchorhamphus* + *Orthosternarchus*, (ii) *Adontosternarchus*, (iii) *Apteronotus* + *Parapteronotus*, (iv) *Sternarchorhynchus*, and (v) a large clade including *Porotergus*, '*Apteronotus*', *Compsaraia*, *Sternarchogiton*, *Sternarchella*, and *Magosternarchus*. We analyzed novel chirp recordings from two apteronotid species

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jphysparis.2016.10.002>.

(*Orthosternarchus tamandua* and *Sternarchorhynchus mormyrus*), and combined data from these species with that from previously recorded species in our phylogenetic analyses. Some signal parameters in *O. tamandua* were plesiomorphic (e.g., low frequency EODs and chirps with little frequency modulation that nevertheless interrupt the EOD), suggesting that ultra-high frequency EODs and “big” chirps evolved after apteronotids diverged from other gymnotiforms. In contrast to previous studies, our PGLS analyses using the new phylogeny indicated the presence of phylogenetic signals in the relationships between some EOD and chirp parameters. The ASR demonstrated that most EOD and chirp parameters are evolutionarily labile and have often diversified even among closely related species.

Keywords

Apteronotidae; Phylogenetics; Animal communication; Electric organ discharge; Chirps; Signal evolution

1. Introduction

Communication signals transmit information about signalers and adaptively influence the behavior of receivers (Bradbury and Vehrencamp, 2011; Endler, 1993). Comparatively studying communication systems can elucidate processes that guide the evolution of animal behavior such as selective constraints on signal structure, evolution of sensory systems, and sexual selection (Endler, 1992; Endler et al., 2005; Ryan, 1998; Ryan and Rand, 1993). As with many behavioral traits, understanding the evolution of communication signals relies on both (i) an accurate and well-resolved phylogeny; and (ii) accurate quantitative data on signal parameters across a sufficient number of species within sampled clades. Phylogenetic comparative methods have been powerful tools for reconstructing the evolution of communication signals and sensory capacities in model systems where large datasets are available, such as visual displays in lizards (Ord and Martins, 2006), calls in frogs (Tobias et al., 2011; Wilczynski et al., 2001), and plumage coloration in birds (Hofmann et al., 2006; Odom et al., 2014).

The South American knifefishes (Order Gymnotiformes) are a diverse clade of weakly-electric teleost fishes distributed widely throughout Central and South America. These fish produce and detect weak electric fields, which are used for the identification of nearby objects, prey detection, and communication (Bullock et al., 2005; Hagedorn and Heiligenberg, 1985; Hopkins, 1972, 1974). The neural circuits that control the production and reception of these signals are well described (Berman and Maler, 1999; Carr and Maler, 1986; Heiligenberg et al., 1996; Metzner, 1999; Smith, 1999), and the signals are diverse both across and within species (Crampton, 1998; Crampton and Albert, 2006; Crampton et al., 2011; Hopkins, 1988; Kramer et al., 1981; Turner et al., 2007). Gymnotiform fishes have consequently become an established neuroethological model for comparative studies of the evolution and physiology of communication, sex differences, and sensory biology (Dulka, 1997; Dulka and Ebling, 1999; Hopkins, 1988; Krahe and Fortune, 2013; Krahe and Maler, 2014; Meyer et al., 1987; Smith, 1999; Turner et al., 2007; Zakon et al., 1999).

The ghost knifefishes (Apteronotidae) are the most speciose family of gymnotiform fishes (Crampton and Albert, 2006), with more than 90 species in 15 genera. They are only electric fishes whose electric organs are composed of nervous, rather than muscle, tissue (Bennett, 1971). They continuously produce high-frequency electric organ discharges (EODs) that act as communication signals conveying information about species identity, sex, and social rank. Both the frequency and the waveform of these fishes' EODs vary across apteronotid species, and in some species, the EOD is sexually dimorphic and may vary as a function of body size and/or dominance (Smith, 2013). These fish also transiently modulate EOD frequency (EODf) and/or amplitude to produce chirps that act as motivational signals during courtship or aggression. Like EODs, chirps also vary substantially across apteronotid species (Smith, 2013). EODs and chirps thus provide an ideal model for studying the evolution of communication. Most previous attempts to reconstruct the evolution of electric signals within the Apteronotidae, however, were hampered by relatively poorly resolved and conflicting phylogenies (Turner et al., 2007). Here, we present a new molecular phylogeny for Apteronotidae, which we compare to previous phylogenetic hypotheses, including a recent gymnotiform tree proposed by Tagliacollo et al. (2016).

The aims of this study were (1) to use concatenated molecular sequence data from two mitochondrial genes and one nuclear gene to further test hypothesized relationships among apteronotid species; and (2) to use the resulting phylogeny and a comprehensive and updated dataset of EOD and chirp parameters to examine the evolution and co-evolution of EODs and chirps in this family.

2. Materials and methods

2.1. Taxon sampling

We analyzed molecular sequence data from tissue samples of 84 individuals representing 32 apteronotid species in 13 genera. These samples also included members of *Apteronotus sensu stricto* and '*Apteronotus*', which represent two clades from the likely polyphyletic genus *Apteronotus* (Albert and Crampton, 2005; Crampton and Albert, 2006; de Santana, 2002; Triques, 2005). Species identifications, tissue sources, voucher accession numbers, and NCBI accession numbers for gene sequences are listed in Supplemental Table S1a. Outgroups included representatives of non-apteronotid gymnotiform families (*Electrophorus electricus*, *Eigenmannia virescens*, *Gymnotus carapo*, and *Sternopygus macrurus*) and two Siluriformes (*Ictalurus punctatus* and *Clarias batrachus*). Outgroup sequence data were obtained from public databases (sources and accession numbers listed in Table S1b).

2.2. DNA extraction, amplification, and sequencing

Genomic DNA was extracted from muscle or fin clips by using the standard tissue protocol for the Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, United States). PCR products were amplified by using weakly degenerate primers (sequences and references listed in Table S2) and a GoTaq polymerase kit (Promega North America, Madison, United States) and were purified with Qiagen Qiaquick Kits (Qiagen Inc., Valencia, United States). Cycle sequencing was performed by using the amplification primers (and two accessory internal primers for CytB; Table S2) and the BigDye Terminator v3.1 Cycle Sequencing Kit

(Life Technologies, Grand Island, United States). Samples were sequenced on an ABI3730 DNA Analyzer (Life Technologies, Grand Island, United States).

2.3. Sequence assembly and analysis

Sequence fragments were trimmed and assembled with Codon- Code Aligner v. 5.1.5 (CodonCode Corporation, Centerville, United States) or Geneious Pro 5.5.6 (Kearse et al., 2012). Consensus sequences for each individual were exported and aligned with the L-INS-I protocol in MAFFT (Kato et al., 2005). Alignments were visually confirmed with Mesquite (Maddison and Maddison, 2007). Sequence lengths of CytB were 900–1173 bp, while RAG2 sequence lengths were 729–1227 bp, based on variable read quality from internal sequencing primers across taxa. For COI, all samples were trimmed to a 490-bp fragment due to poor read quality at the ends of the gene for several samples. Overall mean genetic distances were calculated in MEGA 6.06 using the Tamura 3-parameter model (Tamura et al., 2013) with 500 bootstrap repetitions. To generate a concatenated matrix, a consensus sequence was created for each gene/species combination when multiple individual sequences were available. The sequences for all three genes were then combined into a single matrix. Concatenated sequence datasets produce robust and reliable phylogenetic topographies that are often more accurate than those produced by consensus trees generated from individual gene sequences (Gadagkar et al., 2005).

Maximum likelihood trees with 1000 bootstrap replicates were generated for all three individual genes and the concatenated sequences by using RAxML v. 7.4.2 (Stamatakis, 2006) on Indiana University's KARST computing cluster. The GTR + gamma substitution model was employed for all trees. All trees were rooted with *Ictalurus punctatus* (Siluriformes).

2.4. Phylogenetic comparative analysis of electrocommunication signals: recordings of EODs and chirps

We used the phylogeny generated in this study and phylogenetic comparative methods (Ancestral State Reconstruction (ASR) and Phylogenetic Generalized Least Squares (PGLS)) to examine the evolution and co-evolution of electric communication signal parameters across apteronotid species. Most of the signals used in these analyses were electric organ discharges (EODs) and playback-evoked chirps that were recorded in previously published studies that used similar methods (Kolodziejski et al., 2005; Turner et al., 2007; Zhou and Smith, 2006). These signals are available in a public database of electric fish signal recordings (<http://www.indiana.edu/~efishlab/catalog/>). The most commonly studied apteronotid communication signals are those of brown ghost knifefish. These fish are typically obtained through the commercial aquarium trade. Although they are frequently referred to as *Apteronotus leptorhynchus*, a recent morphological study (de Santana and Vari, 2013) differentiates nine similar species within a monophyletic *A. leptorhynchus* species group (*A. leptorhynchus*, *A. anu*, *A. galvisi*, *A. ferrarisi*, and *A. baniwa*, *A. rostratus*, *A. pemon*, *A. macrostomus*, and *A. spurrellii*). Because the EODs and chirps of species within this group have not yet been differentiated, we thus refer to the signals from these fish as *A. leptorhynchus* sp. to identify that they come from individuals from one of the species in this group. The phylogenetic analyses also included EODs and chirps that we recorded

from two species (*Sternarchorhynchus mormyrus* (n = 4) and *Orthosternarchus tamandua* (n = 1)) whose chirp signals had not been analyzed previously. Fish of these two species were collected from the Amazon River in Peru by commercial suppliers and were imported to Indiana University. Fish were housed individually in 38-l and 64-l tanks within an ~2000-l recirculating aquarium system. The tanks were kept on a 12 h:12 h light:dark cycle at 26.0–26.7 °C, pH 4.5–6.0, and conductivity of 100–300 $\mu\text{S cm}^{-1}$.

EODs and chirps of *S. mormyrus* were recorded in a “chirp chamber” and analyzed as in Turner et al. (2007). Because the *O. tamandua* individual in this study did not chirp in several sessions in a shelter tube within the chirp chamber, its chirps were recorded in the same chirp chamber, but while freely swimming instead of while being confined to a shelter tube. The chirp chamber was a 38-l tank containing water from the fish’s home tank and maintained at 25.8–27.0 °C. The fish were allowed to acclimate in the dark chirp chamber for 45 min. A pair of carbon electrodes was used to record the fish’s EOD, and an orthogonally-oriented pair of electrodes was used to present playback stimuli. The signal from the recording electrodes was bandpass filtered (0.1 Hz–10 kHz), amplified (100–1000 $\times$; model P-55 (Grass Instruments, West Warwick, RI, USA)) and digitized at 44.1 kHz on the left channel of a sound card in a computer running CoolEdit Pro (Syntrillium, Phoenix AZ, USA). Chirps were elicited with a series of playbacks of different frequencies meant to simulate the presence of a conspecific EOD in the tank with the subject. Playbacks were generated in CoolEdit Pro and were played via the sound card and a transformer to the carbon playback electrodes in the chirp chamber. Playbacks to *S. mormyrus* were sinusoidal voltage signals as used in previous studies. Playbacks to *O. tamandua* were previous EOD recordings of this fish that were temporally stretched or compressed to the appropriate frequency using Cool Edit Pro. Stimuli for *O. tamandua* were calibrated to a maximum amplitude of 1.5 mV/cm (measured parallel to the playback electrodes and midway between them), whereas stimuli for *S. mormyrus* were calibrated to 0.7 mV/cm. These stimulus amplitudes approximate the EOD of a conspecific in the tank with the subject. Fish received five playback stimuli with different frequencies relative to the subject’s own EOD frequency and that spanned the species-typical range of EOD frequencies. Both species received stimuli 20 Hz above and below (± 20 Hz) and 5 Hz below (-5 Hz) their own EOD frequency. *S. mormyrus* also received playback stimuli 150 Hz above and below (± 150 Hz) their own EOD frequency as in previous studies of other apteronotids (Turner et al., 2007). Because EOD frequency in *O. tamandua* is much lower than that in other apteronotids and spans a smaller range of frequencies, it received playbacks 100 Hz (rather than 150 Hz) above and below its own EOD frequency. In species with sexually dimorphic EODs, the -5 Hz and ± 20 Hz stimuli represent same-sex EODs, whereas the ± 100 or ± 150 Hz stimuli would simulate the presence of a conspecific opposite-sex EOD or a heterospecific EOD. Each recording consisted of a one-min. base line period with no playback stimulus, two min. with one of the five playback stimuli, and one min. of post-stimulus recording. Playback stimuli were presented in a random order and were separated by 10-min. intervals without stimulation to reduce habituation.

EOD frequency was measured from the baseline recording by using the frequency analysis function in CoolEdit Pro [fast Fourier transform (FFT) size = 65536]. EOD waveform complexity was quantified as the harmonic content as in Turner et al. (2007). Specifically,

the power (in dB) of the EOD signal at the fundamental frequency (F1) and at the second (F2) and third (F3) harmonics was measured from the FFT peaks. Waveform complexity was estimated as the power of F2 and F3 relative to that of F1 (i.e., F2-F1 and F3-F1). More positive values in F2-F1 and F3-F1 indicate more power in upper harmonics and thus a more complex waveform (Turner et al., 2007).

Chirps were identified and measured by using a custom-written procedure (efish, version 23e0, Brian Nelson; <http://bsnelson.org/eFish/efish.html>) running in Igor Pro (version 4.09, Wavemetrics) as described previously (Turner et al., 2007). Briefly, a phase-shifted and scaled copy of the playback signal was subtracted from the EOD recording to remove contamination created by the playback signal. An autocorrelation algorithm was used to calculate the fishes' EOD frequency during the recordings. EOD modulations (i.e., transient increases in EOD frequency, which include chirps and gradual frequency rises) were identified as any event in which EOD frequency departed from baseline EOD frequency by more than 3 Hz for between 10 ms and 60 s. Chirps, which are EOD modulations characterized by substantial (tens to hundreds of Hz), rapid, and transient EOD frequency increases, were distinguished from other EOD modulations (e.g., gradual frequency rises) by using criteria outlined previously (Turner et al., 2007). We measured and analyzed the same chirp parameters as Turner et al. (2007): chirp duration, frequency modulation (FM) of the positive phase of the chirp, relative amplitude modulation (%AM), FM of undershoots at the end of the chirp, positive FM slope, and negative FM slope (see Fig. 1 for explanation of how chirp parameters were measured). Mean chirp parameters for each individual were used as data points in the phylogenetic analyses.

2.5. Relationships between signal parameters

Turner et al. (2007) used phylogenetic generalized least squares (PGLS) models and morphology-based apteronotid phylogenies (Crampton and Albert, 2006; de Santana, unpublished) to test five *a priori* hypotheses about evolutionary relationships between EOD and chirp parameters. To determine whether these findings were robust with a phylogeny based on sequence data, we replicated these analyses using the phylogeny we generated from concatenated CytB, Rag2, and COI sequences. The phylogeny was reduced to include only relationship and branch length estimates for the 13 species for which we also had data on EOD and chirp parameters.

We used phylogenetic generalized least squared (PGLS) analyses with the consensus phylogeny to test the same five hypothesized relationships between EOD and chirp parameters tested previously with morphology-based phylogenies (Turner et al., 2007). Namely, we asked the following: (1) whether EOD frequency was associated with two measures of EOD waveform complexity (F2-F1 and F3-F1) based on the hypothesis that more complex waveforms might be more difficult to produce at high frequencies because the multiple reversals of electric organ current flow required to produce complex waveforms might be more difficult to accomplish at extremely high frequencies (see Turner et al., 2007); (2) whether the negative FM slope of chirps was associated with chirp undershoot FM based on the hypothesis that chirp undershoots result when fast decays from the chirp peak frequency do not allow sodium channels in the electric organ to recover fully from

depolarization-induced inactivation; (3) whether EOD frequency was associated with chirp FM based on the hypothesis that fish with higher EODf may be limited in how much more they can increase their EODf during a chirp; and (4–5) whether chirp AM (%) was associated with either chirp FM or with the peak EODf during a chirp based on the hypothesis that chirp AM results from sodium channel inactivation in the electric organ as its firing rate increases during a chirp.

We tested two additional hypothesized relationships based on recent findings that EOD waveform influences the conspicuousness of chirps (Petzold et al., 2016). Specifically, we asked whether EOD waveform complexity (F2-F1) was associated with chirp FM or chirp duration to test the hypothesis that EOD waveform complexity co-evolves with chirp parameters to maximize their conspicuousness.

PGLS correlations were run in the PGLS-RELATIONSHIPS module of COMPARE (version 4.6b; Martins, 2004). The PGLS model includes the parameter α that reflects the underlying evolutionary model for traits in the phylogeny. When $\alpha = 0$, phenotypic change and phylogenetic distance are related linearly (Turner et al., 2007), and PGLS closely approximates Felsenstein's independent contrasts model (FIC; Felsenstein, 1985; Martins et al., 2002). When $\alpha = 0$, evolutionary changes are assumed to happen through random genetic drift or Brownian motion, and phylogenetic relationships can strongly influence trait values (Turner et al., 2007). When $\alpha > 0$, the phenotypic change and phylogenetic distance are fit to an exponential model, and stabilizing selection around fixed optima is presumed (Turner et al., 2007). As α approaches its maximum, the phylogenetic influence on the trait is reduced, and the PGLS model approximates the TIPS model (i.e., non-phylogenetic regression using values at the tips of the tree) (Martins et al., 2002). The PGLS-relationships module provided separate results for the TIPS model ($\alpha = 15.5$), the FIC model ($\alpha = 0$) and a maximum likelihood (ML) estimate of α . Relationships between parameters were considered significant when the 95% confidence interval of the linear regression slope did not include 0. Comparing correlations across α values allowed us to determine the effect of different assumed evolutionary models on those relationships, and the ML α estimate provided an indication of the phylogenetic influence on trait evolution.

2.6. Ancestral state reconstruction

We also examined how chirp and EOD parameters evolved across apteronotid species by using the ancestral state reconstruction (ASR) module in COMPARE (Martins, 2004; Martins and Hansen, 1997). We used an exponential model within the ASR module that assumes evolution with a restraining force (i.e., stabilizing selection around fixed optima). The strength of the restraining force is parameterized as a factor α within the model. Large values of α model a stronger restraining force (stabilizing selection) whereas as α approaches 0, the model assumes linear (Brownian motion) evolution. Because we were uncertain about the strength of the restraining force acting on these traits, we followed the procedure used in Martins and Lamont (1998) by using a three different α values (0.1, 1, and 5) to evaluate the robustness of the ancestral states to different models of trait evolution (Martins and Lamont, 1998). The ASR algorithm uses measured variation in traits in extant species to estimate means and standard errors (SEMs) of trait values at ancestral nodes.

Significant evolution of traits along particular branches in the phylogeny were identified when the amount of evolutionary change along a branch exceeded the confidence intervals ($1.96 * SEM$) for evolutionary change estimated from the means and errors of the ancestral and descendent nodes.

3. Results

3.1. Apteronotid phylogeny based on concatenated sequences

Statistics for total alignment lengths, variable site number, and overall mean genetic distances for each gene are shown in Table S3. The overall genetic distances for the mitochondrial genes CytB and COI are similar to each other. RAG2 has a much lower overall mean genetic distance among sequences than CytB and COI, which is consistent with the slower rate of evolution of nuclear vs. mitochondrial genes (Brown et al., 1979).

The topologies of individual gene trees were largely consistent, but they were unable to provide well-resolved bootstrap values at nodes of varying depths (Figs. S1–S3). Specifically, the mitochondrial genes provided good resolution at recent nodes, with CytB providing the most robust support due to a larger number of informative characters (Fig. S1). In contrast, the nuclear RAG2 gene provided relatively strong support for intermediate nodes, but weak support within recently diverged clades (Fig. S3). As such, no individual gene trees provided robust bootstrap values across the range of divergence times within Apteronotidae.

The phylogeny based on the concatenated sequences of these three genes, however, provided relatively strong support for most of the nodes within the family (Fig. 2). The monospecific genera *Orthosternarchus* and *Sternarchorhamphus* form a clade that is the sister group to all other apteronotid lineages. The genus *Adontosternarchus* forms a monophyletic sister lineage to all apteronotids aside from *Sternarchorhamphus* + *Orthosternarchus*. Three other robust clades include (i) *Apteronotus* + *Parapteronotus*; (ii) *Sternarchorhynchus*; and (iii) a large clade including *Porotergus*, ‘*Apteronotus*’, *Compsaraia*, *Sternarchogiton*, *Sternarchella*, and *Magosternarchus*. This large clade contains four primary monophyletic groups: (i) the genus *Compsaraia*; (ii) the species *Sternarchogiton porcinum* and *S. nattereri*; (iii) the genera *Sternarchella* and *Magosternarchus*; and (iv) an interleaved clade of the genus *Porotergus* and ‘*Apteronotus*’ species. *Sternarchogiton preto* is within this large clade, but is not monophyletic with the other sampled *Sternarchogiton* species. The position of the genus *Platyurosternarchus* is uncertain, with a bootstrap value <50 for the corresponding node. Thus, the analysis was unable to conclusively place *Platyurosternarchus* more closely to *Apteronotus* + *Parapteronotus* versus *Sternarchorhynchus*.

3.2. Electric organ discharges and chirps of *Orthosternarchus tamandua* and *Sternarchorhynchus mormyrus*

EOD frequency ($Q_{10}^{\circ C}$ -corrected to 25 °C) in the *O. tamandua* recorded in this study was 458.5 Hz, and the waveform of its EOD was a relatively simple, monophasic head-negative wave (Figs. 3A and 4). The chirps of this fish occurred in pairs (Fig. 3A), and the first chirp of the pair caused the EOD to interrupt for approximately 7 cycles at its peak EODf. The

increase in EODf during both the first and second chirps in the pair was modest (60–70 Hz). The first chirp of the pair was slightly longer (~35 ms) than the second chirp (~25 ms).

Sternarchorhynchus mormyrus had a higher EOD frequency than any other species in this study (1429.2 ± 41.1 Hz), and the waveform of their EOD was less complex (more sinusoidal) than other *Sternarchorhynchus* species ($F2-F1 = -5.1 \pm 0.5$ dB; Fig. 4). *S. mormyrus* produced chirps that had short durations (26.3 ± 1.0 ms) and modest increases in EODf ($FM = 98.4 \pm 11.9$ Hz; Fig. 3B).

3.3. Relationships between signal parameters

The PGLS correlation coefficients to test hypothesized relationships between EOD and chirp parameters under three evolutionary models (TIPS/no phylogenetic signal ($\alpha = 15.5$), FIC/strong phylogenetic signal ($\alpha = 0$), and the maximum likelihood α model) are summarized in Table 1.

Chirps with greater FM had reduced amplitude during the chirp. Chirp AM and FM were strongly positively correlated with each other in all three models (Table 1). In addition, the rate at which EOD frequency returned to baseline from the peak of the chirp (i.e., chirp negative FM slope) tended to influence the extent to which EOD frequency went below baseline at the end of a chirp (i.e., the chirp undershoot). The negative FM slope and the undershoot FM were significantly correlated in the FIC model ($r = 0.57$, $p < 0.05$), but this relationship did not reach significance in the TIPS or ML α models. None of the other hypothesized relationships between EOD and chirp parameters were significant in any of the phylogenetic models.

The strength of the phylogenetic signal varied across the tested parameter relationships. The maximum likelihood estimates for α were maximal (indicating little phylogenetic signal) for relationships between chirp AM and FM and between chirp negative FM slope and undershoot FM (Table 1). Estimated ML α was intermediate for relationships between EOD waveform parameters ($F2-F1$ and $F3-F1$) and chirp parameters. ML α was low, indicating a stronger phylogenetic signal, for relationships between EOD frequency and either EOD waveform or chirp parameters.

3.4. Ancestral state reconstructions

Although we estimated ancestral states by using three different values for alpha (0.1, 1, 5) to simulate evolution under different strengths of stabilizing selection, we focus here on the ASRs for EOD and chirp parameters based on $\alpha = 1$ (i.e., intermediate stabilizing selection, Figs. 4–6). ASRs based on weaker ($\alpha = 0.1$) or stronger ($\alpha = 5$) restraining forces are shown in Supplemental Figs. S4–S6 to illustrate the robustness of the ASR to the assumed evolutionary models. With few exceptions, the evolutionary changes in EOD parameters were similar with the different values of α . Thus, the ASR findings presented here are relatively robust to evolutionary model (i.e., strength of stabilizing selection). For both EOD and chirp parameters all of the significant evolutionary changes were identified in the terminal branches of the phylogeny. Below we summarize the broad trends in the evolution of EODs and chirps.

3.4.1. EOD frequency and waveform complexity—EOD frequency (EODf) and waveform complexity are evolutionarily labile (Fig. 4). *Orthosternarchus*, which along with *Sternarchorhamphus* forms the sister group to all other apteronotids, has the lowest EOD frequency of any apteronotid (Crampton, 2007; Crampton and Albert, 2006). This suggests that ancestral apteronotids may have had lower EODf than most extant species in the family, and is consistent with the fact that most non-apteronotid gymnotiforms have lower EOD frequencies than apteronotids (Kramer et al., 1981). EODf in the other apteronotid species in this study ranged from ~700 to 1400 Hz. EODf has diverged significantly between closely-related species or genera in several clades (i.e., *Apteronotus leptorhynchus* sp./*albifrons*, *Sternarchorhynchus roseni*/*mormyrus*, and *Sternarchogiton*/*Sternarchella*). EOD waveform ranges from nearly sinusoidal (*A. albifrons*) to multiphasic and complex (*S. terminalis* and *S. roseni*). EOD waveform has also diversified recently in several clades (*Sternarchorhynchus*, *Porotergus*/“*Apteronotus*”, and *Sternarchogiton*/*Sternarchella*).

3.4.2. Chirp parameters—Like EOD parameters, chirp parameters also vary substantially across apteronotid species and have diverged across closely-related species (Figs. 5 and 6). Mean chirp FM within species ranged from ~60 Hz (*Orthosternarchus*, *S. roseni*, *A. leptorhynchus* sp.) to over 400 Hz (*P. hasemani*), and there were several lineages in which chirp FM increased significantly relative to ancestral states (*Adontosternarchus*, *P. hasemani*, *Porotergus*/“*Apteronotus*”, and *S. terminalis*; Fig. 5). Chirp AM is strongly linked across species with chirp FM (see Section 3.3 above), and some of the lineages with increased chirp FM also evolved significantly more chirp AM (e.g. *P. hasemani* and *Porotergus*/“*Apteronotus*”). However, this pattern was not universal. For example, *Orthosternarchus* chirps have high amounts of AM (i.e., complete interruptions) with very little chirp FM, and chirp AM in *S. mormyrus* decreased with no statistical change in chirp FM. Chirp duration was also evolutionarily labile, with chirps becoming longer relative to their most recent ancestral node in three species (*P. hasemani*, *A. albifrons*, *S. roseni*). The “shape” of chirps, as measured by the slopes of the rising or falling phase of chirp FM or the presence of chirp FM undershoots, also varied substantially across species (Fig. 6). One or more of these parameters changed significantly in terminal branches leading to most of the extant species studied, with the exception of *Orthosternarchus* and the two *Sternarchogiton* species. Changes in these parameters were often associated with changes in chirp FM, duration, or both. For example, substantial increases in chirp FM in *A. devenanzii*, *A. balaenops*, *P. hasemani*, *P. gimbeli*, and *S. terminalis* are accompanied by significant increases in the rising FM slope of the chirp. Similarly, the increase in chirp duration in *A. albifrons* was accompanied by a decrease in the falling FM slope of chirps.

4. Discussion

4.1. Comparison of phylogenies

The concatenated phylogeny generated in this study (Fig. 2) agrees with the sequence- and morphology-based phylogeny proposed by Tagliacollo et al. (2016) in all major respects. In particular, we confirm their conclusions that (i) the genus *Adontosternarchus* is a sister clade to all apteronotids besides *Sternarchorhamphus* + *Orthosternarchus*, (ii) “*Apteronotus*” species form a clade with the genus *Porotergus*, and (iii) *Sternarchorhynchus* is a sister

group to a large clade encompassing *Porotergus* + ‘*Apteronotus*’ + *Compsaraia* + *Sternarchogiton* + *Sternarchella* + *Magosternarchus*. The phylogeny generated in this study did not identify *Sternarchogiton* as a monophyletic genus, which is consistent with the molecular phylogeny of Tagliacollo et al. (2016). However, *Sternarchogiton* was monophyletic when both morphological traits and sequence data were included (compare Figs. 2A and 6 of Tagliacollo et al., 2016). Also, our results regarding *Porotergus* + ‘*Apteronotus*’ differ slightly from those of Tagliacollo et al. (2016). Our phylogeny contains two *Porotergus* species and two ‘*Apteronotus*’ species, which form an interleaved clade. Tagliacollo et al. (2016) found that *Porotergus gimbeli* was a sister species to a monophyletic ‘*Apteronotus*’ clade. However, because Tagliacollo sampled only a single *Porotergus* species, their results can neither contradict nor confirm our hypothesis that *Porotergus* and ‘*Apteronotus*’ form an interleaved clade. Testing this hypothesis will require further phyletic sampling within these genera.

Our phylogeny, like the recent molecular-morphological phylogeny (Tagliacollo et al., 2016), supports several monophyletic clades within Apterodontidae (Fig. 2). Most of these clades are consistent with earlier phylogenetic hypotheses based on morphological or more limited sequence data (Albert, 2001; Albert and Campos-da-Paz, 1998; Albert and Crampton, 2005; Albert et al., 1998; Alves-Gomes et al., 1995; Crampton and Albert, 2006; de Santana, 2002; Triques, 2005). However, some findings based on more recent molecular phylogenies contradict earlier apteronotid phylogenetic hypotheses. The position of *Adontosternarchus* is particularly interesting. *Adontosternarchus* emerges as the sister clade to all apteronotid lineages aside from *Sternarchorhamphus* + *Orthosternarchus*. This is inconsistent with most previous morphological hypotheses, which placed *Adontosternarchus* as a sister genus to *Sternarchogiton* (Albert and Campos-da-Paz, 1998; Albert and Crampton, 2005; Crampton and Albert, 2006). Similarly, earlier phylogenetic hypotheses placed *Sternarchorhynchus* + *Platyurosternarchus* as the sister clade to *Sternarchorhamphus* + *Orthosternarchus* (Albert and Crampton, 2005; Albert et al., 1998; Crampton and Albert, 2006). In contrast, both our study and Tagliacollo et al. (2016) place this clade within a large, multi-genus clade (including *Sternarchorhynchus*, *Sternarchogiton*, *Porotergus*, *Compsaraia*, *Magosternarchus*, and *Sternarchella*) that is the sister group to *Apteronotus s.s* + *Parapteronotus*. Finally, we failed to recover monophyly of the genus *Sternarchogiton*, with our analysis including three representatives from the five currently described *Sternarchogiton* species (de Santana and Vari, 2010). This lack of monophyly is caused by the position of *Sternarchogiton preto*, and this discrepancy will likely require full sampling of the genus to resolve. Tagliacollo et al. (2016) observed a similar phenomenon when only sequence data was used to build a phylogeny, although *S. porcinum* was the species that caused non-monophyly in their tree. The inclusion of morphological characters recovered *Sternarchogiton* as monophyletic in their analysis.

4.2. Electrocommunication signals of *O. tamandua* and *S. mormyrus*

We described EODs and chirps in two species whose chirps had not been previously recorded: *Orthosternarchus tamandua* and *Sternarchorhynchus mormyrus*. The EODs and chirps of *S. mormyrus* are mostly similar to those of other *Sternarchorhynchus* species, although there is some diversity of these signals within the genus. *S. mormyrus*’ EOD

frequency was somewhat higher and its EOD waveform less complex than those of some other recorded species (*S. curvirostris* and *S. roseni*; Fig. 4; Turner et al., 2007). This is consistent with a previous report that *S. mormyrus* had higher EOD frequency and lower “harmonic content” than most other *Sternarchorhynchus* species (Kramer et al., 1981). In addition, chirps in *S. mormyrus* tended to be shorter in duration and have less AM than previously recorded chirps of *S. roseni* or *S. curvirostris* (Turner et al., 2007). Thus, as in other apteronotid genera, the structure of these electric communication signals has diversified and has the capacity to convey species-identifying information among *Sternarchorhynchus* species.

The structure of signals in *O. tamandua* is particularly interesting because this species diverged from other apteronotids early in the family’s history (Fig. 2). *Orthosternarchus*’ position within the Apteronotidae is reflected in the divergence of *Orthosternarchus*’ EODs and chirps from those of other apteronotids. As has been noted previously (Crampton and Albert, 2006; Hilton et al., 2007), *Orthosternarchus* has the lowest EOD frequency of any apteronotid, and its EOD frequency is in the range of some non-apteronotid gymnotiforms (e.g., *Eigenmannia* spp. EODs are typically 200–600 Hz (Crampton, 1998; Crampton and Albert, 2006; Kramer et al., 1981; Hopkins, 1974). This suggests that evolution of higher EOD frequencies in other apteronotids likely occurred after *Orthosternarchus* and *Sternarchorhamphus* diverged from the rest of the family. EOD waveform is also distinct in the *Orthosternarchus* lineage. *Orthosternarchus* (and its sister genus *Sternarchorhamphus*) share a monophasic, head-negative EOD that is not found in other apteronotids or in non-apteronotid gymnotiforms (Bennett, 1971; Crampton and Albert, 2006).

Orthosternarchus chirps are distinct in two ways. First, despite the fact that their EOD frequency increases only modestly during their chirps, this frequency increase is often associated with a massive amplitude decrease that causes an interruption of the ongoing EOD. Although EOD interruptions occur in the chirps of other apteronotid species (e.g. *P. hasemani* and *P. gimbeli*) the extreme AM that comprises these EOD interruptions is typically associated with extreme frequency modulation during the chirp (Turner et al., 2007). The EOD interruption during a chirp with modest FM in *O. tamandua* is similar to chirps in some non-apteronotid gymnotiforms. For example, *Eigenmannia* spp. produce interruptions in which sudden, but relatively small, increases in EOD frequency cause the abrupt cessation of the EOD (Hagedorn and Heiligenberg, 1985). The production of relatively plesiomorphic chirps in *O. tamandua* may suggest that the constraint limiting the amount of chirp FM without interrupting the EOD has been relaxed in apteronotids after they diverged from *Orthosternarchus*. This would have allowed apteronotids to produce “big” chirps with larger amounts of FM. Another unusual feature of *O. tamandua* chirps was that they occurred in doublets. This non-random timing of chirps is reminiscent of the chirp bursts produced in *S. terminalis* (Turner et al., 2007), or of multi-peaked chirps that occur in “*A.*” *bonapartii* and *A. devenanzii* (Zhou and Smith, 2006; Ho et al., 2010).

4.3. Evolutionary relationships between signal parameters

Turner et al. (2007) used a PGLS approach and apteronotid phylogenies based on morphological characters to test several *a priori* hypothesized evolutionary relationships

between EOD and chirp parameters. The present analysis, using a more robust molecular phylogeny and signal data from additional species, largely replicated these previous findings. Specifically, like Turner et al. (2007), we confirmed a lack of covariation between most signal parameters, which suggests that many EOD and chirp parameters are evolving independently of each other. Like Turner et al. (2007), we found a significant relationship between chirp FM and AM (Table 1). This relationship likely reflects a tradeoff that results from inactivation of sodium channels (and resultant decreases in EOD amplitude) when the neurons that control the EOD fire faster to produce large increases in EOD frequency.

We also found a significant relationship between the falling FM slope of a chirp and the undershoot frequency in one evolutionary model (FIC), whereas Turner et al. (2007) found the same relationship in the TIPS model but not the FIC model. This supports a possible relationship between these chirp parameters, but suggests that the robustness of the relationship depends on the underlying evolutionary model. The relationship between chirp undershoots and chirp falling slope likely results from a physiological constraint. Specifically, if the excitatory input that causes EOD frequency to rise during a chirp is removed abruptly (leading to a steep falling FM slope), sodium channels that are inactivated during the chirp might not have time to fully recover. The pacemaker neurons that control EOD frequency might then be somewhat hyperpolarized and fire at lower rates, resulting in the EOD frequency undershoot.

We tested two additional hypothesized relationships between signal parameters. Petzold et al. (2016), found that the conspicuousness of chirps of different species was affected by parameters of both EODs and chirps. We hypothesized that EOD waveform and chirp parameters (FM and duration) might have co-evolved with each other to optimize the detection and/or discrimination of conspecific chirps. If so, this co-evolution might be revealed in phylogenetic correlations between these parameters. We did not find a significant relationship between EOD waveform complexity and either chirp duration or chirp FM. This could suggest that EOD waveform has not coevolved with chirp parameters or alternatively that these traits are related nonlinearly.

One important difference between the present analysis and that of Turner et al. (2007) is in the strength of the phylogenetic signal. For almost all of the PGLS models analyzed in the previous study, the maximum likelihood α was quite large, indicating little or no phylogenetic signal in the data (i.e., analyzing the data with a phylogenetic model provided no better fit than analyses ignoring phylogenetic relationships). In this study, the maximum likelihood α was low for several relationships between signal parameters (e.g., relationships between EODf and EOD waveform or chirp FM).. These low α values indicate phylogenetic influences on the signals. Thus, the present sequence-based phylogeny revealed phylogenetic relationships in signaling traits that were not apparent using previous phylogenies based solely on morphology. For other relationships (e.g., Chirp AM vs. FM and Chirp negative FM slope vs. undershoot FM), α was high in both the present study and in Turner et al. (2007). This indicates that evolution of relationships between these signal parameters may have occurred relatively rapidly, resulting in diversity in signals in closely related extant species and a lack of strong phylogenetic inertia on signals.

4.4. Ancestral state reconstructions and evolution of EODs and chirps

Our ASR analyses revealed widespread diversification of communication signals in Apterontidae. Numerous evolutionary changes were observed in every parameter of EODs and chirps, and changes in one or more signal parameter were present in lineages leading to almost every extant species.

All of the statistically significant evolutionary changes in signal parameters within the ASRs occurred at the tips of phylogeny. One explanation of this pattern of evolution at the tips rather than deep branches is methodological. The error in estimating ancestral states increases with phylogenetic distance, such that deep nodes have substantially higher errors (i.e., less precise estimates of mean trait values) than those for extant taxa (Martins, 1999). This accumulating error substantially reduces statistical power to detect more ancient evolutionary changes in traits, and ASR thus tends to bias estimates of evolutionary change towards terminal branches. A likely example of this is suggested qualitatively by the pattern of changes in EOD frequency across the phylogeny (Fig. 4). EOD frequency is substantially lower in *Orthosternarchus* than in all other apteronotids, which might suggest an evolutionary increase in EOD frequency occurred in one of branches diverging from the common ancestor of *Orthosternarchus* and the other apteronotids. Instead, the ASR identified evolutionary changes in EOD frequency only in some of the terminal branches leading to extant species. The inability of the ASR to identify significant early changes in EOD frequency is likely due, at least in part, to the large errors in the estimate of EOD frequency at the deep nodes of the phylogeny.

On the other hand, there are also clear examples within the ASRs of recent signal diversification. For example, chirp structure differs substantially across closely-related species in the *Parapteronotus* + *Apteronotus* clade, resulting in distinctive, species-specific chirps (Figs. 5 and 6). *P. hasemani* and *A. albifrons* evolved significantly longer chirps, *P. hasemani* evolved chirps with the most extreme FM and AM of all apteronotids, and *A. leptorhynchus* sp. evolved chirps with pronounced FM undershoots that are not present in the other two species. Thus, although the ASR likely does a poor job of estimating trait values at deep ancestral nodes and of identifying ancient evolutionary changes, it is a potentially useful tool for identifying patterns of diversification in signals, particularly within more recently diverged clades. It is important to note that although we were able to record and phylogenetically analyze the structure of EODs and chirps from 14 apteronotid species representing 10 genera, this is an incomplete sample of the more than 90 described species in this family. Additional phyletic sampling of signal structure in other species clarify how these signals evolved. In particular, analyzing signal structure in more species in clades with marked species variation in the structure of EODs (e.g., *Sternarchorhynchus* or *Sternarchella* + *Sternarchogiton*) or chirps (e.g., *Apteronotus* + *Parapteronotus*) could clarify how and why signals diversified in these clades.

Although this study comparatively examined many parameters of EODs and chirps, other EOD and chirp properties may also be relevant for communication. For example, we characterized EOD waveform based on recordings of the head-tail voltage of the discharging fish. These head-tail EOD measurements capture species-specific temporal patterns in the EOD's electric field, and fish are responsive to dipole playbacks of such signals (Engler and

Zupanc, 2001; Hupe, 2012; Kolodziejski et al., 2007; Tallarovic and Zakon, 2005; Triefenbach and Zakon, 2003). Nevertheless, the electric field of the EOD also has a complex spatial geometry that may depend on the relative position and orientation of the signaling and receiving fish as well as the geometry and temporal pattern of current flow within the electric organ (Assad et al., 1999; Kelly et al., 2008). Using electrode arrays to record species differences in the spatiotemporal geometry of the EOD's electric field may reveal evolution of additional behaviorally relevant signal components.

The rapid diversification of EOD and chirp structure highlighted in this study raises the question of what physiological mechanisms underlie signal diversity. Because the neural circuits that regulate EODs and chirps are well characterized and relatively simple, the diversity of these signals in apteronotid species provides an outstanding and largely unexploited opportunity to investigate how physiological mechanisms evolve to produce species diversity in behavior. Most recent studies of electromotor circuits in apteronotids have focused on *A. leptorhynchus* sp. The relatively few studies of electromotor circuits in other apteronotids suggest variation in these circuits that could contribute to species differences in EODs and chirps.

For example, the trajectory of electromotor neuron axons in the electric organ is associated with species differences in EOD waveform. In *A. albifrons*, which has a biphasic EOD waveform, the electromotor axons run rostrally within the electric organ for several segments before forming a 'hairpin' turn and then running caudally for several segments. The reversal in action potentials running tail-head and then head-tail within these axons was hypothesized to underlie the biphasic EOD in this species (Bennett, 1971). In *Sternarchorhamphus*, which has a head-negative monophasic EOD, electromotor neuron axons only run caudally once they enter the electric organ, and this simpler geometry of electromotor axons is hypothesized to underlie the simpler EOD waveform. These findings suggest that species diversity in EOD waveform in apteronotids may arise in part through the evolution of different patterns of axon guidance of electromotor axons within the electric organ during development. The trajectory of axons in the electric organs of apteronotid species with particularly complex EOD waveforms (e.g., *S. terminalis*, which has a triphasic EOD) has not been studied. It would be interesting to determine whether the association between electric organ morphology and EOD waveform is consistent across other apteronotid species, and in particular whether highly complex EOD waveforms are linked to additional complexity in the morphology of electromotor axons in the electric organ (e.g. multiple 'hairpin turns').

Another example of diversity in the electromotor circuit that might be linked to diversity in signal structure occurs in the synaptic inputs to the brainstem pacemaker nucleus, which controls EOD frequency. In *A. leptorhynchus*, relay cells in the pacemaker nucleus receive chemical synaptic inputs on dendrites and axon initial segments that are not present on the relay cells of *A. albifrons* (Elekes and Szabo, 1985). Although the functional significance of this species difference in synaptic inputs to relay cells is unknown, one possibility is that the additional synaptic inputs in *A. leptorhynchus* are related to species differences in chirping. In *A. leptorhynchus*, chirps are controlled by a glutamatergic input to relay cells from the diencephalic prepacemaker nucleus (Heiligenberg et al., 1996). Further studies of the

function of electromotor circuits and how chirps are produced in *A. albifrons* and other apteronotid species are needed to test this hypothesis.

4.5. Conclusions

In this work, we used concatenated sequence data from multiple genes to reconstruct the phylogeny of the family Apterontidae. The substantial agreement of this phylogeny with another recent comprehensive phylogeny (Tagliacollo et al., 2016) suggests that our phylogenetic hypothesis is robust. We used our phylogeny to complete the most detailed analysis of the evolution of electric signal structure in apteronotids to date. Our analyses demonstrate that some of the characteristic properties of apteronotid EODs and chirps, like extremely high EOD frequencies and chirps with greater than 100 Hz of frequency modulation, may have evolved after the divergence of *Orthosternarchus* + *Sterarchorhamphus* from other apteronotids. We also found extensive evidence that different properties of electric signals are highly evolutionarily labile, often evolving independently of each other and diversifying substantially between closely-related species. Multi-gene apteronotid phylogenies, such as the one presented here and by Tagliacollo et al. (2016) will allow further phylogenetic comparative studies that examine the evolution of other aspects of the behavior and physiology, such as species variation in sexual dimorphism of signals, in sociality, and in electrosensory mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

JAMO is grateful to TWAS-CNPq Postgraduate Fellowships program for support during this project. Special thanks to Mark Sabaj-Perez at the Academy of Natural Sciences in Philadelphia (ANSP) for providing sample tissues and species identifications. Support for processing tissue and DNA samples for sequencing was provided by the core laboratory of the Center for the Integrative Study of Animal Behavior (CISAB) at Indiana University.

Funding

This work was supported by NSF IOS 0950721 to GTS, INPA's internal funding Grant #12307 to JAG, and an NSERC Discovery Grant to NRL. Support for ARS and WWH was provided by the Common Themes in Reproductive Diversity training program at Indiana University (NIH-NIHCD 5T32HD049336-10). This research was supported in part by Lilly Endowment, Inc., through its support for the Indiana University Pervasive Technology Institute, and in part by the Indiana METACyt Initiative. The Indiana METACyt Initiative at IU is also supported in part by Lilly Endowment, Inc.

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Fig. 1.

Measurements of chirp parameters. Frequency (top), head-tail voltage (middle) and RMS amplitude (bottom) of the EOD of an *A. leptorhynchus* sp. individual producing a “large” chirp. Parameters measured in this study are illustrated on the traces. Frequency modulation (FM) was defined as the frequency difference between the positive start frequency and positive peak frequency during the chirp. Chirp duration was calculated by using the difference between positive chirp start time, and positive chirp end time. Note that this definition follows that of Turner et al. (2007) and includes only the ‘positive’ phase of the chirp. Rising slope, (the rate at which EOD frequency increases during the rising phase of

the chirp), was calculated as follows: Rising Slope = FM/(Positive Peak Time – Positive Start Time). Falling slope (the rate at which EOD frequency decreases during the falling phase of the chirp) was calculated as follows: Falling Slope = FM/(Positive Stop Time – Positive Peak Time). Some species produce chirps with undershoots, i.e., decreases of EOD frequency below the fishes' baseline EOD frequency at the end of the chirp. The FM of the undershoot was included as a parameter in the analyses, but because undershoots were not produced by most species, undershoots were not included in calculations of chirp durations or slopes. Chirp% Amplitude Modulation (AM) was calculated from the RMS EOD amplitude during the chirp as follows: $AM = (Amplitude\ Max - Amplitude\ Min) / Amplitude\ Max$.

Fig. 2.

Apterontid phylogeny based on maximum likelihood analysis of concatenated COI, CytB, and RAG2 sequences. Numbers at nodes represent bootstrap support.

Fig. 3.

Chirps of *Orthosternarchus tamandua* (A) and *Sternarchorhynchus mormyrus* (B) EOD frequency (top traces, red) and head to tail EOD voltage (bottom traces, blue) during representative chirps produced by *O. tamandua* and (A) *S. mormyrus* (B) in response to playbacks simulating conspecific EODs. The dashed line in (A) indicates a transient cessation (interruption) of the EOD during the chirp. Note that the *O. tamandua* chirps occurred in pairs.

Fig. 4.

Ancestral state reconstruction ($\alpha = 1$) for EOD frequency (blue) and waveform complexity (red, power of second harmonic vs. fundamental frequency (F2-F1)) for 13 apteronotid species. *A. leptorhynchus* sp. refers to the *Apteronotus leptorhynchus* species complex (de Santana and Vari, 2013). Estimated mean and <standard error> values are indicated at each node. Blue branches and asterisks indicate a statistically significant evolutionary change in EOD frequency along the branch (i.e., evolutionary change exceeds the confidence interval ($1.96 * SE$); Martins and Hansen, 1997). Red branches and asterisks indicate a statistically

significant change in EOD waveform. Representative head-tail EOD voltage traces are shown on the same time scale for each species.

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Fig. 5.

Ancestral state reconstruction of chirp amplitude modulation, frequency modulation, and duration ($\alpha = 1$) for 13 apteronotid species. Color coding of branches and asterisks indicate statistically significant evolutionary changes in chirp FM (red), chirp AM (blue), and/or chirp duration (orange). Representative traces of EOD frequency (red) and EOD head-tail voltage (blue) are shown on the right for representative chirps of each species. Time and frequency scales are the same on all traces. Dashed lines in the red traces indicate interruptions of the EOD during the chirp.

Fig. 6.

Ancestral state reconstruction ($\alpha = 1$), of chirp rising slope (Hz/ms, blue), chirp falling slope (Hz/ms, orange), and undershoot frequency modulation (Hz, red) for 13 apteronotid species. Color coding of branches and asterisks indicate statistically significant evolutionary changes in chirp rising slope (blue), chirp falling slope (orange), and/ or chirp undershoot FM (red). Representative traces of chirps are shown on the right as in Fig. 5.

Table 1

Phylogenetic correlations between signal parameters.

	Correlation coefficient		
	ML α^a	TIPS	FIC
Signal parameter relationship		$\alpha = 15.5$	$\alpha = 0$
EODf vs. WC (F2-F1)	0.23 (1.38)	0.26	0.19
EODf vs. WC (F3-F1)	0.03 (1.23)	0.02	-0.01
Chirp negative FM slope vs. undershoot FM	0.43 (15.5)	0.43	0.57*
EODf vs. chirp FM	-0.02 (1.2)	0.02	-0.03
Chirp% AM vs. chirp FM	0.78 (15.5)*	0.78*	0.81*
Chirp% AM vs. chirp positive peak frequency	-0.05 (6.7)	-0.04	0.05
Chirp FM vs. WC (F2-F1)	0.00 (6.43)	-0.01	0.11
Chirp FM vs. WC (F3-F1)	-0.21 (9.15)	-0.21	-0.08
Chirp duration vs. WC (F2-F1)	-0.18 (5.86)	-0.18	-0.08
Chirp duration vs. WC (F3-F1)	-0.18 (5.22)	-0.17	-0.11

^aMaximum likelihood estimate for α shown in parentheses.

*Indicates statistical significance (95% confidence interval of regression slope did not include zero).