



# Drift-driven evolution of electric signals in a Neotropical knifefish

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Communication signals are highly diverse traits. This diversity is usually assumed to be shaped by selective forces, whereas the null hypothesis of divergence through drift is often not considered. In Panama, the weakly electric fish *Brachyhypopomus occidentalis* is widely distributed in multiple independent drainage systems, which provide a natural evolutionary laboratory for the study of genetic and signal divergence in separate populations. We quantified geographic variation in the electric signals of 109 fish from five populations, and compared it to the neutral genetic variation estimated from cytochrome oxidase *I* (COI) sequences of the same individuals, to test whether drift may be driving divergence of their signals. Signal distances were highly correlated with genetic distances, even after controlling for geographic distances, suggesting that drift alone is sufficient to explain geographic variation in electric signals. Significant differences at smaller geographic scales (within drainages) showed, however, that electric signals may evolve at a faster rate than expected under drift, raising the possibility that additional adaptive forces may be contributing to their evolution. Overall, our data point to stochastic forces as main drivers of signal evolution in this species and extend the role of drift in the evolution of communication systems to fish and electrocommunication.

**KEY WORDS:** Animal communication, *Brachyhypopomus occidentalis*, electric organ discharge, genetic drift, signaling/courtship, weakly electric fish.

Communication signals are highly diverse phenotypes (Endler 1992; Bradbury and Vehrencamp 2011) that can serve many functions, including attracting and courting mates, maintaining territories, indicating fitness, and avoiding heterospecific matings. Identifying evolutionary processes leading to this intraspecific diversity is therefore important to understand how biodiversity, and ultimately species, arise and are maintained. This can be challenging as a multitude of different drivers can act on communication signals (Bradbury and Vehrencamp 2011). Thus, how signals evolve, including the relative contribution of selective and stochastic processes, is not only a long-standing debate (Endler

1992; Foster and Endler 1999) but also remains poorly understood.

One fruitful approach to discriminate the roles of selection and stochasticity in signal evolution is studying patterns of geographic variation of intraspecific signals (Wilczynski and Ryan 1999). Such geographic patterns have been well-documented in the acoustic signals of passerine birds (Nicholls et al. 2006; Ruegg et al. 2006; Dingle et al. 2008; Irwin et al. 2008), frogs (Heyer and Reid 2003; Loughheed et al. 2006; Pröhl et al. 2006, 2007; Ohmer et al. 2009), and mammals (Helweg et al. 1998; Campbell et al. 2010; Jiang et al. 2010). Three major hypotheses have

been put forward to explain signal divergence patterns. First, signal divergence results from adaptation that enhances transmission efficiency across different signaling conditions, such as specific microclimate or vegetation structures, as has been demonstrated by extensive research on birds (Nicholls et al. 2006; Ruegg et al. 2006; Dingle et al. 2008). Second, signal evolution is driven by sexual selection (Panhuis et al. 2001; Ritchie 2007), for example, through differences in mate preference (Moriarty Lemmon 2009). Third, in the absence of selection, signal divergence is simply the outcome of neutral evolution acting through genetic drift (Lande 1976; Lynch and Hill 1986). Although this third hypothesis is the fundamental alternative to adaptive forces, it is not consistently addressed in studies of signal divergence.

Testing drift-driven signal divergence requires direct comparisons of signal distances and neutral genetic distances, for which strong correlations are expected if signal divergence is largely stochastic (Wilkins et al. 2012). In theory, strong signal and genetic correlations could also be consistent with mutation-order (M-O) processes (Wilkins et al. 2012), whereby signal divergence arises due to the chance occurrence and fixation of different beneficial mutations in different populations adapting to the same selection pressures (Mani and Clarke 1990). In practice, though, it remains unknown what the actual contribution of M-O processes to signal divergence is, as we still do not know what selective factors facilitate M-O speciation (Schluter 2009), and, to our knowledge, no direct evidence has been found for it (but see Martin and Mendelson 2012). On the other hand, poor or no correlations are expected if divergent selection, either ecological or sexual, is driving most of the signal divergence (Wilkins et al. 2012). In case of drift-driven signal divergence, covariation of signals with other ecological or sexual traits or environmental variables should be small (Wilkins et al. 2012).

Weakly electric fishes are prime candidates to study the evolutionary drivers of signal divergence (Arnegard et al. 2010; Leal and Losos 2010; Tiedemann et al. 2010). The African Mormyri-formes and South American Gymnotiformes have independently evolved an active electric sense that they rely on for communication and localization of objects in their environment. They possess an electrogenerative organ that produces a weak electric field (referred to as electric organ discharge [EOD]) in the surrounding water (Bennett 1971). Using an array of electroreceptors distributed over their skin, they can sense perturbations of their self-generated electric field caused by objects in their environment or by the EODs of other electric fish (Moller 1995; Zupanc and Bullock 2005). Similar to the diversity of anuran calls or passerine bird songs, the EODs of electric fishes vary greatly across families, genera, and species (Sullivan et al. 2000; Crampton and Albert 2006). Of great advantage is that EODs are readily quantifiable (Turner et al. 2007; Crampton et al. 2008), thus offering an excellent model for the study of signal evolution.

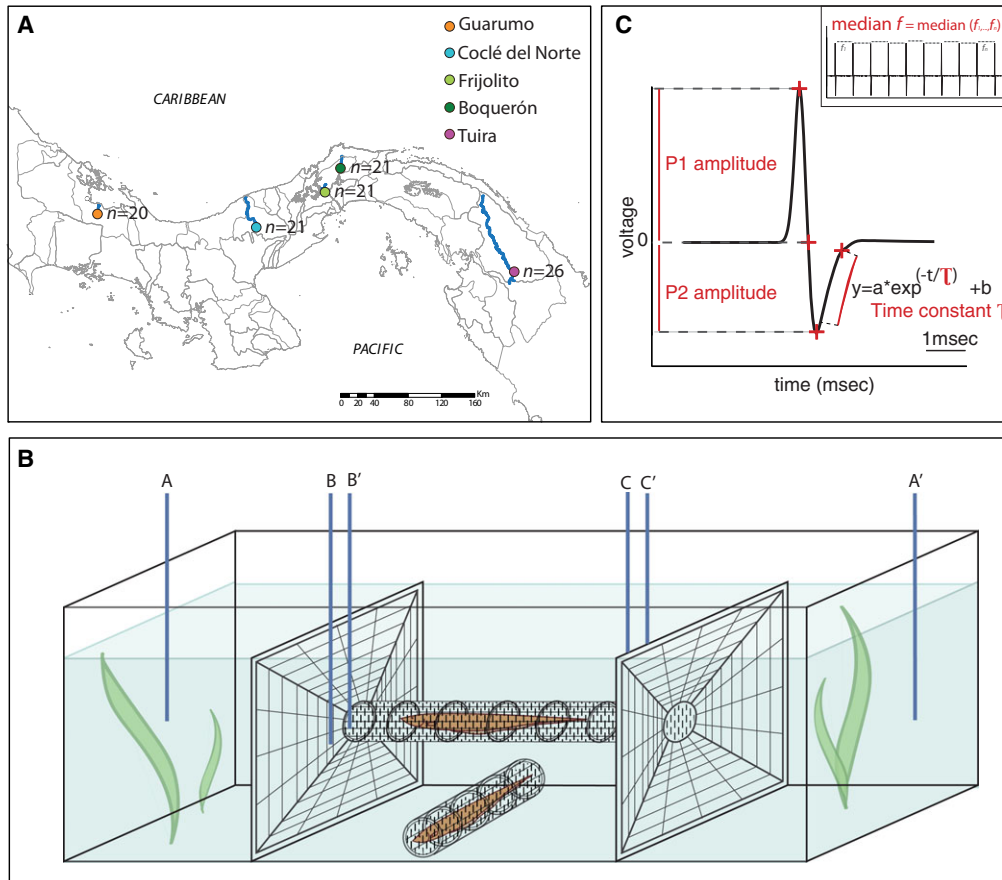
It has been suspected that divergence of signals involved in sexual selection and species recognition plays a critical role in the evolution of prezygotic reproductive isolation in weakly electric fishes (Arnegard et al. 2006; Markowski et al. 2008; Feulner et al. 2009; Fugère and Krahe 2010; Crampton 2011). Multiple, non-exclusive hypotheses have been proposed for the factors shaping signal divergence in electric fishes (Crampton et al. 2013), including genetic drift (Gallant et al. 2011), sexual selection (Feulner et al. 2009; Arnegard et al. 2010), and adaptation to environments with different electroreceptive predator pressures (Stoddard 1999). Here, we assess evidence for neutral divergence of EOD properties in allopatric populations of the weakly electric fish *B. occidentalis*. A previous phylogeographic analysis of this species has shown a complex history of multiple colonizations of Central America and subsequent drainage isolation, leading to highly divergent lineages, some of which later came into secondary contact (Picq et al. 2014). Whether genetic differentiation among these lineages is accompanied by proportional levels of signal variation is still unknown. Thus, the main question of our study is to what extent signal divergence in allopatric populations of *B. occidentalis* is stochastic. By focusing on the relative role of drift, our study remains non-exclusive of other potential forces, such as ecological or sexual selection, which could also be acting on signals (e.g., Irwin et al. 2008). In fact, drift may not only promote signal divergence but may also be an important promoter of speciation, even in concord of sexual selection (Uyeda et al. 2009).

Here, we test the hypothesis that signal divergence in *B. occidentalis* is largely stochastic, for which we expect signal divergence to correlate with neutral genetic divergence. For that purpose, we quantified the correlation between the variation of recorded electric signals and mitochondrial DNA sequences among five populations of *B. occidentalis*.

## Materials and Methods

### STUDY SYSTEM

The distribution of *B. occidentalis* extends from Venezuela to the Sixaola River in Costa Rica (Albert and Crampton 2003a). It is the only electric fish species widely distributed in Panama in nearly all Caribbean and Pacific drainages (Meek and Hildebrand 1916; Bussing 2002; Albert 2003a,b; Albert and Crampton 2003b; Campos-da-Paz 2003; Eschmeyer 2015). Two evolutionary lineages have been identified: the Bocas lineage, formed exclusively by populations in the westernmost Caribbean drainages of Bocas del Toro, and the Panama-Colombia lineage, which holds all the remaining populations from Panama and northwestern Colombia. The two lineages represent independent colonizations from South America, one in the Miocene and one in the Early Pliocene (Picq et al. 2014).



**Figure 1.** (A) Map indicating the five recording and sampling sites for *B. occidentalis* in Panama. Colored circles represent independent drainages with the respective rivers indicated in blue: Guarumo (orange), Coclé del Norte (blue), Tuirá (purple), and Chagres (green). Dark and light green circles indicate two rivers within the Chagres drainage: Frijolito (light green) and Boquerón (dark green). Sample size for signal and genetic data is indicated for each recording site. Gray lines indicate all the independent drainages in Panama. (B) Portable setup for automated recordings of free-swimming fish in the field (adapted from Stoddard et al. (2003)). Side compartments of the tank were accessible to the recorded fish and joined by a mesh tube accessible via two mesh funnels. Tube electrodes (BB' and CC') detect the presence of the fish in the tube and trigger automated EOD recordings from tank-end electrodes AA'. The second fish was placed in a closed mesh tube at a 90° angle relative to the AA' electrodes. (C) Parameters measured from each EOD waveform: peak P1 amplitude, peak P2 amplitude, and time constant of the repolarization of the second phase of the EOD pulse ( $\tau$ -P2) were measured individually for each chosen waveform. Median frequency was calculated as the median of all instantaneous frequencies between adjacent pulses of the chosen 1-sec recording. The frequency of the peak in the power spectrum was determined from a fast Fourier transform of the EOD pulses (not shown in figure).

## SAMPLING

Five study sites were selected belonging to four independent drainages representing four reciprocally monophyletic clades of *B. occidentalis* in Panama (Picq et al. 2014), one locality representative of the highly divergent Bocas lineage (Guarumo drainage), and four localities from three drainages representing three clades within the Panama-Colombia lineage (Coclé del Norte, Chagres, and Tuirá drainages). Within the Chagres drainage, two rivers (Frijolito and Boquerón) were selected to explore intradrainage signal variation (Fig. 1A).

Field work was conducted during the dry season of Panama (January–May 2012), when *B. occidentalis* breed and exhibit sex

differences in their EODs (Hagedorn 1988). Fish were localized using an EOD detector consisting of two wire electrodes placed on a plastic rod, which, when submerged in the water, allows acoustic localization of electric fish via a mini amplifier-speaker (RadioShack, Fort Worth, TX). Specimens were then collected using dipnets.

## ELECTRIC SIGNAL RECORDINGS

*Brachyhypopomus occidentalis* generates a sexually dimorphic, biphasic pulse-type EOD, which is highly plastic across environmental and social conditions, and displays circadian variations, with higher amplitudes and longer durations of the EOD pulses

at night (Hagedorn 1995). Therefore, only mature males without tail damage were selected for recordings. Fish ( $n = 109$ , 20–26 per site) were transferred in situ to individual 114L plastic coolers (95 cm  $\times$  38 cm  $\times$  40 cm) filled with river water from the collection site and were given 1 h to acclimate. Recordings were performed overnight, starting before nightfall (between 4 and 6 p.m.) and ending after sunrise (between 7 and 9 a.m.). Our goal was to extract for each fish one EOD pulse at the peak of their night time EOD modulation and then compare these pulses across individuals and populations.

The portable setups were designed for automated EOD recordings of free-swimming fish, because production of communication signals in weakly electric fish can differ between restrained and free-swimming situations (Dunlap and Larkins-Ford 2003). Each cooler was divided into three compartments by funnels made from plastic mesh (Fig. 1B). A horizontal tube of 5 cm diameter made from dark plastic mesh permitted the fish to move freely between the two-end compartments and also served as a hiding spot for the fish. A second fish confined to another mesh tube was added to the central compartment to avoid social isolation of the recorded fish, which may cause a decline of EOD duration and amplitude (Hagedorn and Zelick 1989; Franchina et al. 2001). Water temperature was measured throughout the night every 5 min with a temperature data logger (UA-002-08; HoboWare).

To achieve standardized recordings of the focal fish's EOD, its signal was recorded between electrodes mounted at the ends of the tank (A-A' in Fig. 1B), aligned with the horizontal mesh tube. Two additional pairs of electrodes (B-B' and C-C') were mounted directly at the ends of the mesh tube to detect the fish's presence inside the tube, which triggered automated 1-sec recordings from the A-A' electrodes. Four identical tanks permitted parallel recording of up to four fish per night. Signals were amplified by a splash-proofed EXM-04D 4-channel extracellular differential amplifier (npi electronic GmbH, Tamm, Germany; DC, 0–20 kHz bandwidth) and digitized with a USB-6211 data-acquisition device (National Instruments, Austin, TX) at a sampling rate of 40 kHz per channel. Custom-written software (Matlab; The Mathworks, Natick, MA) controlled the acquisition and the automated switching between the four tanks via a custom-built switch (see Appendix S1 for recording details). The design of this setup was modified from Stoddard et al. (2003) and adapted for use in the field. After overnight EOD recordings, all fish were euthanized according to the animal care protocol approved by the McGill University Animal Care Committee, with an overdose of MS-222. Sex was confirmed by gonadal inspection.

#### ANALYSIS OF ELECTRIC SIGNAL VARIATION

A single EOD pulse was extracted from each fish's overnight recordings. To do this, we first selected the 10 waveforms with

the highest peak amplitude of the first head-positive EOD phase (P1) from each 1-sec recording. Peak P1 amplitude, ratio of P1/P2 amplitude, time constant of the return to baseline from P2 ( $\tau$ -P2), median frequency, and frequency of peak power (Fig. 1C) were extracted for each waveform and mean values were plotted as representatives of each 1-sec recording against time. Then, we chose for each fish the 1-sec recording showing the highest values of these parameters (except peak frequency, for which the lowest value was targeted), and within this recording, we picked a single waveform that showed no contamination from the EOD of the second fish.

To compare waveform similarity between and within *B. occidentalis* populations, we performed cross-correlations between all possible pairs of the 109 representative EOD waveforms in the dataset, as described by Carlson et al. (2011). We used the maximum of the absolute values of the cross-correlation function as a measure of pairwise waveform similarity, resulting in a matrix of pairwise similarities ranging from 0 (dissimilar waveforms) to 1 (identical waveforms). Multidimensional scaling (MDS) was applied to this cross-correlation matrix using the "mdscale" function in Matlab with Kruskal's normalized stress 1 criterion (Kruskal and Wish 1978). In our MDS analysis, the number of dimensions was set to  $N = 2$ , which resulted in a stress of 0.0555, considered to give a good ordination representation with low probability of misinterpretation (Clarke 1993). Pairwise Euclidean distances in this MDS space were computed between all individuals using "vegan" (Oksanen et al. 2012) in R version 2.15.1 (R Development Core Team 2012).

To test the null hypothesis of no differentiation between the signal waveforms from different populations, we performed a permutational MANOVA (PERMANOVA) directly on the Euclidean pairwise signal distance matrix using 999 permutations to determine probability values. We also performed pairwise PERMANOVA comparisons to determine which populations differed from each other based solely on locality, using Bonferroni-adjusted alpha values ( $\alpha' = 0.05/10 = 0.005$ ), correcting for 10 pairwise population comparisons.

To assess which EOD features showed differences, we performed multivariate analysis (MANOVA) on the five extracted signal variables. To identify which variables contributed most to differences among localities, we used univariate ANOVA tests with Bonferroni-adjusted alpha values to correct for the five comparisons between the five EOD variables ( $\alpha' = 0.05/5 = 0.01$ ). Pairwise comparisons between populations were conducted to identify which ones differed most for a given variable, using Bonferroni-adjusted alpha values accounting for 10 pairwise population comparisons ( $\alpha'' = \alpha'/10 = 0.01/10 = 0.001$ ).

We considered four factors that have been proposed to affect electric signal variation: (1) Total body length may affect EOD amplitude, as longer fish can accommodate more electrocytes in

their electric organ (Bennett 1971; Hopkins et al. 1990). (2) Tail width has been associated with longer P2 durations in *B. occidentalis* males compared to immature males and females. Also, tail width varies depending on the level of sexual maturity (Hagedorn and Carr 1985). (3) Sex of the second fish can affect the EOD, as demonstrated in isolated *B. pinnicaudatus* males, which showed a faster recovery in their P2 duration and amplitude when interacting with a conspecific male than with a female (Franchina et al. 2001). (4) Low water temperature may also increase P2 duration, although not all gymnotiforms appear to be temperature sensitive in that respect (Caputi et al. 1998; Silva et al. 1999). We conducted multivariate analyses of covariance (MANCOVA) to determine if signal variables were significantly different among localities after controlling for the effect of these four factors by including them as covariates. All statistical analyses were performed using “vegan” in R.

Additionally, because lineages are part of a hierarchically structured phylogeny and cannot be considered as drawn from independent distributions (Felsenstein 1965), we performed a phylogenetic MANOVA (Garland et al. 1993) to evaluate whether signal variables differed among localities when accounting for the lack of independence among populations, using “geiger” (Harmon et al. 2014) in R. When identified via these phylogenetic comparative methods, significant differences in traits provide strong evidence for evolution due to selection as the effects of evolutionary history are ruled out. This was done using the phylogenetic tree based on mitochondrial data (see below).

### GENETIC DATA COLLECTION AND ANALYSIS

Gill arches with filaments were excised from all specimens and preserved at ambient temperature in saturated 20% DMSO and 0.5 MEDTA solution at pH 8. Specimens were individually tagged and deposited in the Neotropical Fish Collection (NFC-STRI) at the Smithsonian Tropical Research Institute, Panama.

Genomic DNA was isolated using DNeasy Tissue Kits (QIAGEN, Valencia, CA). Primers, PCR amplification, and sequencing of the 5' portion of the COI gene were performed as in Picq et al. (2014). Sequences were edited and aligned using Geneious version 5.6.3 (Drummond et al. 2011) and deposited in GenBank under accession numbers KX060632-KX 060740.

Phylogenetic relationships among unique haplotypes were inferred by Bayesian and maximum likelihood methods (Guindon and Gascuel 2003; Ronquist and Huelsenbeck 2003) using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003) and Garli version 2.0 (Zwickl 2006), respectively (see Appendix S2 for analysis details). The evolutionary model that best fit our data (TIM1 + I) was determined in jModelTest (Posada 2008) using the Akaike information criterion. We generated a median-joining haplotype network (Bandelt et al. 1999) using Network version 4.610 (fluxus-engineering.com).

Pairwise genetic  $p$ -distances between all individuals, as well as mean  $p$ -distances between localities were calculated using MEGA 5 (Tamura et al. 2011). Standard errors were estimated by 1000 bootstrap replicates.

We tested deviations from a model of neutral evolution in each population, which could be caused by selection or demographic changes (Ramírez-Soriano et al. 2008), using Tajima's  $D$  (Tajima 1989), Fu's  $F_s$  (Fu 1997), and  $R_2$  (Ramos-Onsins and Rozas 2002) in DnaSP version 5.10.1 (Librado and Rozas 2009). Statistical significance was assessed by 1000 coalescence simulations.

### COMPARISON OF GENETIC AND SIGNAL DIVERGENCE

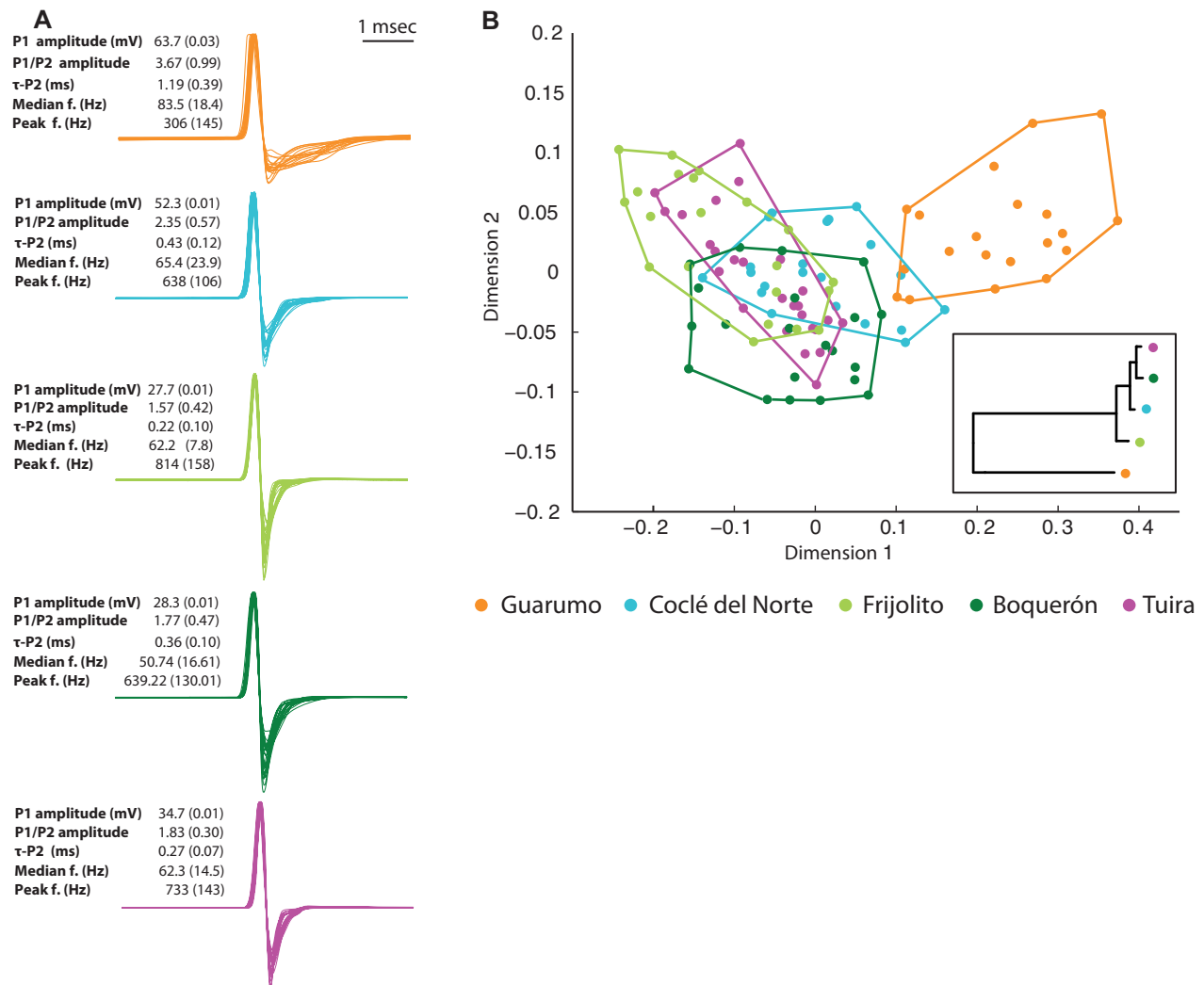
To investigate whether electric signals evolve at a rate consistent with neutral evolution, we tested the correlation between signal and genetic divergence between populations with a Mantel test using pairwise COI sequence divergence as a metric of neutral evolution and pairwise Euclidean distances between coordinate data in the MDS plot as signal distances. Mantel tests between signal and geographic distances, and between genetic and geographic distances were also performed. Geographic distances were estimated as linear distances between recording sites. All tests were performed using “vegan” in R and significance of association between matrices was assessed with 1000 permutations.

## Results

### GEOGRAPHIC VARIATION IN EOD WAVEFORMS

We found substantial within-site variability in EOD waveform (Fig. 2A), as well as significant variation among recording localities (PERMANOVA on Euclidean signal distances,  $F_{4,108} = 46.591$ ,  $P < 0.001$ ). In the MDS plot, the minimum polygon enclosing the signals of the Guarumo males formed a clearly differentiated group, whereas all other population polygons overlapped to some degree (Fig. 2B). The high divergence of Guarumo was most evident in the signal distance dendrogram, which also showed the highest signal similarity between Tuirá and Boquerón (Fig. 2B, inset). Pairwise PERMANOVA comparisons of signal distances indicated significant EOD waveform differences between Guarumo and all other populations, as well as between Frijolito and Coclé, and interestingly between Frijolito and Boquerón, the only two rivers within the same drainage (Table 1).

Our MANOVA identified significant EOD variation among localities ( $F_{4,412} = 10.66$ ,  $P < 0.001$ ), even when considering each variable independently (P1 amplitude:  $F_{4,104} = 20.94$ ,  $P < 0.001$ ; ratio of P1/P2 amplitude:  $F_{4,104} = 42.88$ ,  $P < 0.001$ ; time-constant  $\tau$ -P2:  $F_{4,104} = 79.90$ ,  $P < 0.001$ ; median frequency  $F_{4,104} = 8.52$ ,  $P < 0.001$ ; peak power frequency  $F_{4,104} = 40.54$ ,



**Figure 2.** (A) Individual EOD waveforms from each recorded male in the five recording localities (Guarumo;  $n = 20$ ; Frijolito;  $n = 21$ ; Coclé del Norte;  $n = 21$ ; Boquerón;  $n = 21$ ; Tuira;  $n = 26$ ). EOD waveforms were normalized in amplitude with respect to the first positive (P1) peak and aligned at the zero-crossing position between P1 and P2 and overlaid for each locality. Means and standard deviations in parentheses of the five extracted signal variables shown. (B) Minimum polygons enclosing EOD waveforms from each recording locality in a two-dimensional representation of signal space (obtained through multidimensional scaling of signal cross-correlations). Each point represents the EOD of a recorded male ( $n = 109$ ). The inset represents a signal dendrogram built from the within-group and between-group Euclidean signal distances.

$P < 0.001$ ). Population means for these variables are shown in Figure 2A. All pairwise MANOVA tests showed at least one EOD parameter significantly different between sites, except between Tuira and Frijolito (Table S3). Interestingly, in the intradrainage comparison between Frijolito and Boquerón, both the descriptors of P2 duration, namely  $\tau$ -P2 ( $F_{1,40} = 22.79$ ,  $P < 0.001$ ) and peak power frequency ( $F_{1,40} = 16.82$ ,  $P < 0.001$ ), were found to differ significantly (Table S3). Fish tail width and body length had a comparable and significant multivariate effect on EOD waveform variation, whereas temperature had a smaller, but still significant effect, and sex of the second fish had none (Table S4a–d). Even after controlling for body length, tail width, and recording temper-

ature, the effect of locality was still highly significant, suggesting that it can predict most of the signal variation (Table S4a–c).

Interestingly, when accounting for phylogenetic relationships in our phylogenetic MANOVA, no significant differences in EOD properties among localities were detected (Pillai's trace = 1.3644,  $P = 1$ ). This result is not unexpected because all populations, except Frijolito and Boquerón, represent reciprocally monophyletic clades (Fig. S1). Signal evolution appears to “track” the evolutionary history of the populations closely, suggesting signal divergence in this species arose mostly as a consequence of neutral (or nearly neutral) rather than selective forces.

**Table 1.** Pairwise PERMANOVA comparisons of male *Brachyhyopomus occidentalis* signal waveforms from five localities in Panama.

| Pairwise population comparisons | <i>F</i>            | <i>P</i> value   |
|---------------------------------|---------------------|------------------|
| Guarumo-Tuira                   | $F_{1,44} = 127.14$ | <b>&lt;0.001</b> |
| Guarumo-Frijolito               | $F_{1,39} = 124.69$ | <b>&lt;0.001</b> |
| Guarumo-Boquerón                | $F_{1,39} = 89.21$  | <b>&lt;0.001</b> |
| Guarumo-Coclé                   | $F_{1,39} = 64.68$  | <b>&lt;0.001</b> |
| Coclé-Frijolito                 | $F_{1,40} = 17.59$  | <b>&lt;0.001</b> |
| Coclé-Boquerón                  | $F_{1,40} = 5.19$   | 0.014            |
| Coclé-Tuira                     | $F_{1,45} = 7.92$   | 0.008            |
| Tuira-Boquerón                  | $F_{1,45} = 5.13$   | 0.024            |
| Tuira-Frijolito                 | $F_{1,45} = 4.68$   | 0.028            |
| <i>Frijolito-Boquerón</i>       | $F_{1,40} = 14.67$  | <b>&lt;0.001</b> |

Significant pairwise differences are marked in bold, using Bonferroni-adjusted alpha levels ( $\alpha' = 0.05/10 = 0.005$ ). The intradrainage comparison is marked in italics.

### GENETIC VARIATION

Seventeen unique haplotypes were identified among 109 *B. occidentalis* sequences (Table S1). Phylogenetic analyses confirmed that all populations were reciprocally monophyletic and constituted highly supported clades except for Frijolito and Boquerón, which were the only ones sharing one haplotype (Fri1) that was also the most common and central haplotype in the network (Fig. S1). The largest divergence was observed between Guarumo and Tuira (mean *p*-distance = 0.06) and the lowest between Frijolito and Boquerón (mean *p*-distance = 0.018).

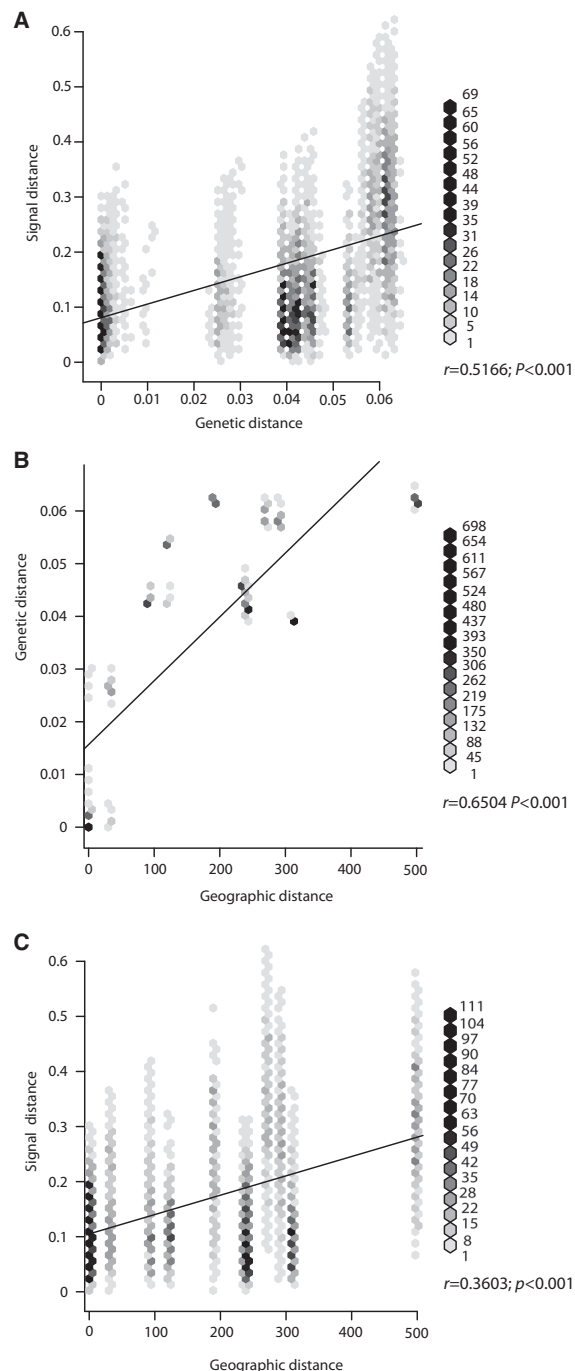
The nonsignificant results of the three neutrality tests, performed for the data overall and each population separately, are consistent with neutral evolution for the 5' portion of the COI gene (Table S2).

### CORRELATION BETWEEN SIGNAL, GENETIC, AND GEOGRAPHIC DISTANCES

Mantel tests showed a significant correlation between signal and genetic distances ( $r = 0.5166$ ,  $P = 0.001$ ; Fig. 3A), even after controlling for geographic distance (Partial Mantel test,  $r = 0.3983$ ,  $P = 0.001$ ). Geographic and genetic distances showed the highest correlation ( $r = 0.6504$ ,  $P = 0.001$ ; Fig. 3B). Signal and geographic distances were also significantly correlated ( $r = 0.3606$ ,  $P = 0.001$ ), although this correlation was the weakest (Fig. 3C).

### Discussion

In this study, we investigate whether stochastic forces play a major role in shaping electric signal variation in a species of weakly electric fish. We found that signal distances were highly correlated



**Figure 3.** Correlates of signal divergence in *B. occidentalis*. Bivariate binning into hexagonal cells was applied to all comparisons of signal, genetic, and geographic distances to show density distributions: darker colors indicate higher densities of points (see gray-scale codes to the right of each panel). Geographic distances were estimated as linear distances between recording sites after converting coordinates to the UTM geographic coordinate system. Genetic distances were estimated from mitochondrial COI sequences. (A) Pairwise signal and genetic distances ( $r = 0.5166$ ;  $P < 0.001$ ). (B) Pairwise genetic and geographic distances ( $r = 0.6504$ ;  $P < 0.001$ ). (C) Pairwise signal and geographic distances ( $r = 0.3603$ ;  $P < 0.001$ ).

with genetic and geographic distances and that signal parameters did not differ significantly after accounting for phylogeny. These results lead us to consider that divergent *B. occidentalis* signals may have evolved as a result of drift, whereby stochastic variation accumulated in isolated populations over time. At small geographic scales, significant intradrainage signal variation between genetically close populations suggests that additional local, microevolutionary forces may also contribute to shaping the evolution of electric communication signals.

It is noteworthy that previous studies investigating population divergence of communication signals rarely address drift as a fundamental alternative to selective hypotheses, and those that do rarely report evidence for neutral signal evolution (Soha et al. 2004; Nicholls et al. 2006; Pröhl et al. 2006, 2007; Ruegg et al. 2006; Rudh et al. 2007; Dingle et al. 2008; Huttunen et al. 2008; Tobias et al. 2010; Cadena et al. 2011). Nevertheless, a few studies have found strong correlations of neutral genetic distances and acoustic signal properties, notably in Neotropical singing mice (Campbell et al. 2010), Amazonian frogs (Amézquita et al. 2009), and greenish warblers (Irwin et al. 2008), thereby showing that intraspecific acoustic divergence can be largely stochastic. Our findings extend the role of drift in the evolution of communication systems to fish and electrocommunication.

Our patterns of signal and genetic divergence most likely evolved as a result of reduced gene flow between populations caused by the complex emergence of the Isthmus of Panama, which played a major role in the dispersal and evolution of Central American fauna and flora (Wallace 1876; Stehli and Webb 1985). Previous work showed that *B. occidentalis* colonized the land bridge of Panama from South America several times, starting in the late Miocene (Picq et al. 2014). Recent geological, fossil, and molecular data indicate that the emergence of the Isthmus, together with the dramatic biotic turnover across this land strip, were already ongoing at this time (Bacon et al. 2015; Montes et al. 2015). This supports our hypothesis that geological processes underlying the rise of the Isthmus, such as abrupt topographic changes and marine incursions, shaped the isolation of river basins, their fish populations, and the electric signals of these fish.

Interestingly, distant populations showed more genetic and signal divergence, a pattern of isolation by distance also found in the African electric fish *Paramormyrops kingsleyae* across Gabon (Gallant et al. 2011). Taken together, these results suggest that stochastic processes could play an important role in electric signal evolution in gymnotiforms and mormyriiforms, a surprising finding as signals used in mate attraction and courtship are usually expected to be subject to strong selection (Bradbury and Vehrencamp 2011).

Nonetheless, our study does not rule out a role for selection in signal divergence, including potential M-O processes, as neutral

and selective processes are non-exclusive explanations for the evolution of a given trait (Hansen and Orzack 2005). Ruling out M-O effects in the evolution of the *B. occidentalis* EOD is outside of the scope of the current study, because it would require a thorough exploration of all the potential selective forces acting on EODs in different populations. Even though our results are consistent with a potentially strong role of drift in driving signal variation, the remaining variation could likely be explained by the cumulative effect of various selective forces.

Indeed, a larger-than-expected signal distance was found in the within-drainage comparison, indicating that other forces are likely at play in the evolution of electric signals. One possibility is that higher predation by eavesdropping electroreceptive predators in some rivers could be driving the evolution of signals toward higher peak power frequencies, which are expected to be less detectable (Stoddard 1999). In some populations, sexual selection may be stronger than in others, driving signals toward lower peak power frequencies to increase attractiveness to females (Hagedorn and Carr 1985). It is also conceivable that the comparatively large within-drainage signal differences have arisen as a consequence of character displacement after secondary contact in the Chagres basin (Fig. S1), which may have accentuated divergence that had evolved in allopatry (Gerhardt 2013).

As we describe variation in signals between populations over a large geographic area, a major question is whether other morphological, environmental, experimental, or temporal factors besides geography are acting as potentially confounding sources of variation. Interestingly, we found that body length, tail width, and recording temperature explained part of the EOD variation in *B. occidentalis* (Table S4a–c). However, there are no data to suggest that the environment predicts signal variation: no correlation has been found in gymnotiforms or mormyriiforms between signal and habitat features, such as conductivity, water temperature, dissolved oxygen, substrate or vegetation type (Gallant et al. 2011; Crampton et al. 2013). Moreover, it seems unlikely that EOD variation could be driven by differences in reproductive timing, as we observed no particular temporal trend among Frijolito males recorded throughout the breeding season of 2011 (Fig. S2). The Mantel test performed on this dataset also showed no linear correlation between signal and temporal distances ( $r = -0.1576$ ,  $P = 0.991$ ).

Overall, our analysis of signal and genetic variation in *B. occidentalis* identified a positive correlation between signal and genetic distances, suggesting that electric signals may evolve mainly due to drift, whereby stochastic variation accumulated in isolated drainages. Nonetheless, other selective forces most likely also contributed to signal variation, especially in populations where differences in electric signals were greater than predicted from genetic-to-signal relationships. Based on our data, we propose that stochastic variation within species can provide the



raw material upon which sexual selection or adaptive divergence act and that weakly electric fish, with their easily recorded and quantified communication signals, are excellent models in which to explore these questions.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Appendix S1.** Recording setup description.

**Appendix S2.** Phylogenetic analyses of unique haplotypes of COI sequences.

**Appendix S3.** Signal analysis of 2011 Frijolito males through the breeding season.

**Table S1.** Genetic diversity of the COI gene for each of the five populations of *Brachyhyppopomus occidentalis* analyzed in Panama and for the entire dataset (all).

**Table S2.** Neutrality tests (Tajima's  $D$ , Fu's  $F_s$ ,  $R_2$ ) performed on each of the five populations of *B. occidentalis* analyzed in Panama and for the entire dataset.

**Table S3.** Pairwise population comparisons on the univariate ANOVA tests using Bonferroni-adjusted alpha levels ( $\alpha = 0.05/5/10 = 0.001$ ).

**Table S4a.** MANCOVA test performed on the five selected signal variables, with fish body length as covariate.

**Table S4b.** MANCOVA test performed on the five selected signal variables, with tail width as covariate.

**Table S4c.** MANCOVA test performed on the five selected signal variables, with water temperature as covariate.

**Table S4d.** MANCOVA test performed on the five signal variables, with sex of the stimulus fish as covariate.

**Figure S1.** Phylogenetic tree rendered by Bayesian Inference analysis of the mitochondrial COI gene for 109 *B. occidentalis* recorded males.

**Figure S2.** Plots of five extracted variables from males recorded throughout the breeding season of 2011 in the Frijolito river.