Quantifying Acoustic Signal Divergence During Speciation by Reinforcement in *Pseudacris feriarum*

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INTRODUCTION

Speciation is the origin of new species through evolutionary divergence. Reinforcement, the evolution of behavioral reproductive isolation between species due to selection against hybridization, is one evolutionary force that can drive speciation. Prior studies have suggested that reinforcement between two chorus frog species, the Upland chorus frog (*Pseudacris feriarum*) and the Southern chorus frog (*P. nigrita*), can indirectly cause shifts in the male mating calls of *P. feriarum* populations. No studies to date, however, have examined these shifts in call structure across fine spatial scales. Here, we studied a transect of twenty *P. feriarum* populations spanning the Piedmont and Coastal Plain geographic regions near Macon, Georgia. Populations south of Macon have undergone reinforcement from interactions with *P. nigrita*, whereas populations north of Macon have not. By measuring and comparing call characteristics—such as wavelength, amplitude, pulse number, pulse rate, and frequency—across populations in the transect, this study provides valuable insights into the evolution of phenotypic variation and the divergence of mating behaviors during speciation.

METHODS

- Collection occurred during peak breeding seasons, with frogs being captured by hand and temporarily housed in aerated containers with appropriate moisture levels.
- Each recording session took place under controlled conditions within pre-constructed soundproof "frog boxes" for minimal background and external noise.
- Each frog box was fitted with a microphone and speaker to isolate male frog call recordings.
- Recordings capture the natural vocalizations of the studied populations and their responses to artificially broadcasted male calls known to elicit vocal reactions.
- Frog calls were recorded using the Sound Ruler application, which allowed for the precise measurement and analysis of acoustic properties.
- ~20 frogs of *Pseudacris feriarum* were used in the experiment
- Recorded calls were extracted and analyzed, contingent on key acoustic features, including wavelength, amplitude, pulse number, and pulse rate.
- These data points were then systematically compiled into a comprehensive spreadsheet for subsequent integration into a Genome-Wide Association Study (GWAS), aiming to identify genetic markers associated with call traits.
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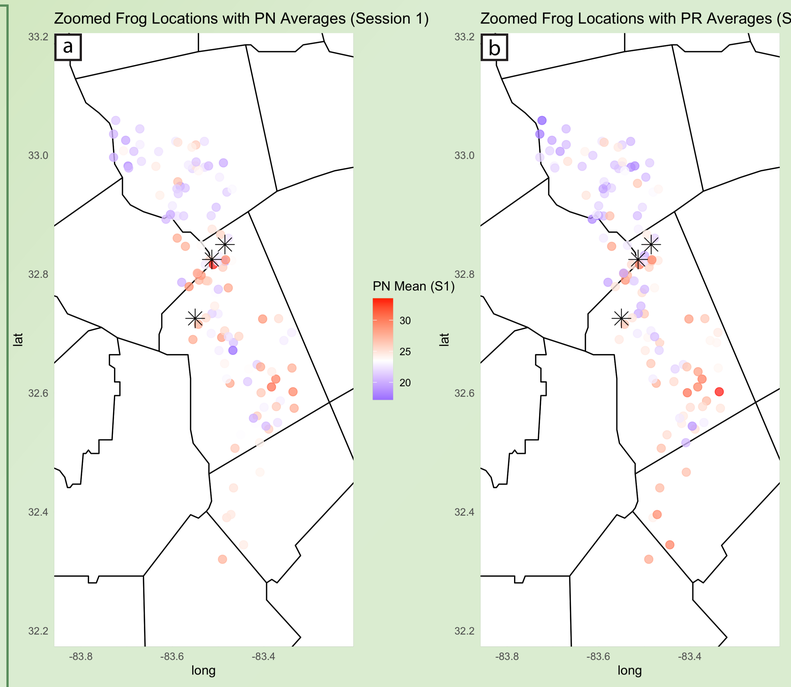

Figure 2. Geographic visualization of *P. feriarum* populations, displaying average pulse number (PN) and pulse rate (PR) across different locations, with warmer colors indicating higher values.

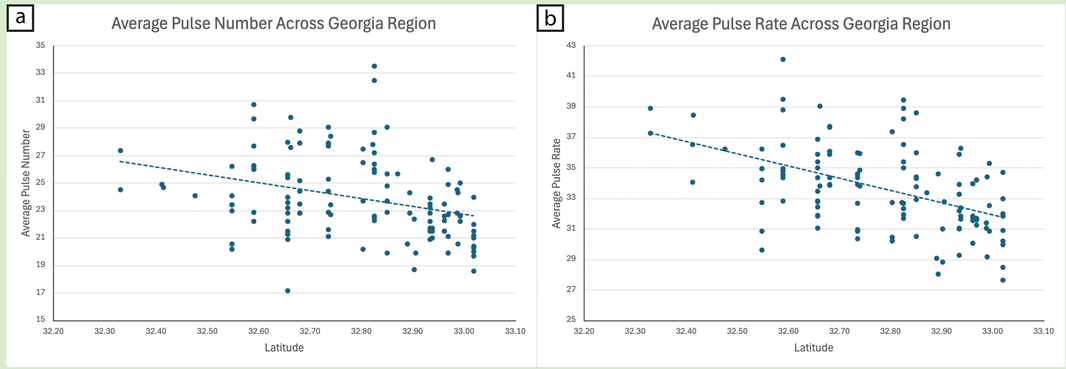

Figure 3. Scatterplots illustrating the relationship between latitude and acoustic traits, demonstrating the pattern of higher pulse numbers and rates in sympatric populations where P. nigrita is present

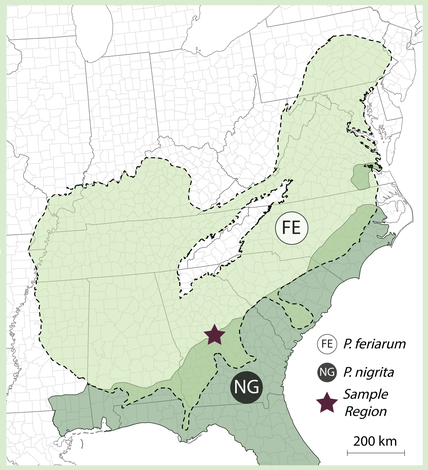

Figure 1. Map with the distribution of *P. feriarum* and *P. nigrita* populations and highlighted sampling region.

Pseudacris feriarum *Pseudacris nigrita*

RESULTS

- Analysis of male *Pseudacris feriarum* calls reveals distinct differences between sympatric (with *P. nigrita*) and allopatric populations.
- Sympatric populations (southern, ~32.3°–32.85° latitude) exhibit higher pulse numbers and pulse rates than allopatric populations (northern, >32.9° latitude).
- The maps of the average pulse rate (Fig. 2a) and pulse number (Fig. 2b) show a color gradient with deeper red hues corresponding to higher pulse numbers and pulse rates in sympatric zones and deeper blue hues corresponding to lower pulse numbers and pulse rates in allopatric zones.
- Sympatric call divergence is consistent with the pattern predicted from reinforcement. This divergence) has reduced the hybridization risk with *P. nigrita.*
- Allopatric calls suggest lower energetic investment in calls since hybridization pressure is absent.
- Scatterplots of pulse rate and pulse numbers (Fig. 3a and Fig. 3b) show more substantial variation with the trendline displaying elevated values in sympatric populations.
- The transition zone near Macon, GA (~32.8° latitude), indicates a potential boundary for reinforcement-driven acoustic divergence.
- Future genome-wide association studies (GWAS) will explore the genetic basis of acoustic signals in male frogs.

ACKNOWLEDGEMENTS

We humbly acknowledge the support and contributions that made this research possible. We extend our heartfelt thanks to Dr. Emily Lemmon for her exceptional mentorship and invaluable insights on speciation. We thank Dr. Alan Lemmon for providing us with advanced data analysis tools and coding expertise essential for managing and interpreting extensive datasets. Our sincere gratitude goes to Carlie Anderson, whose guidance in understanding both speciation and Lemmon lab methodologies greatly-enhanced our study. Finally, we appreciate Oliva Gonya for introducing us to the frog colony, an experience that was pivotal to the direction of our research.

DISCUSSION

- The observed pattern of call divergence in sympatric populations of *P. feriarum* indicates that reinforcement is driving the evolution of reproductive isolation in regions where *P. nigrita* co-occurs. Specifically, male *P. feriarum* in these sympatric zones produce calls with higher pulse numbers and increased pulse rates, likely as a strategy to distinguish their mating signals from those of *P. nigrita* and reduce hybridization risk. This shift, though energetically costly, aligns with prior research (Lemmon 2009; Lemmon & Lemmon, 2010), which suggests that divergent call structures act as a key mechanism for reproductive isolation. In contrast, allopatric *P. feriarum* populations, free from the selective pressure of *P. nigrita*, exhibit lower pulse numbers and slower pulse rates, implying that such costly modifications are unnecessary in the absence of interspecies competition.
- These findings emphasize the critical role of acoustic divergence in speciation. Future research on this system should prioritize larger sample sizes, long-term monitoring, and the integration of genome-wide association studies (GWAS) to pinpoint loci linked to call variation. Additionally, playback experiments could assess whether female *P. feriarum* show preferences for these divergent call traits, offering direct evidence that reinforcement drives speciation in this system.

RESOURCES

*These authors contributed equally to this work