NORTHWESTERN NATURALIST 94:81–84 SPRING 2013

MOLECULAR IDENTIFICATION OF A HITCHHIKING FROG

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Key words: biogeography, Cuba, Cuban Treefrog, DNA barcoding, introduced species, molecular data, *Osteopilus septentrionalis*, phylogenetic analysis, phylogeography, Washington

The introduction and expansion of non-native species into an ecosystem can be detrimental and result in the decline of native species abundance and the possible extinction of native species (Dorcas and others 2012). Most commonly, the mode of introduction is unintentional and human driven. Many intentional introductions historically have been motivated by individuals or groups who believe that the newly introduced species will be in some way beneficial to humans in its new location (Pimentel and others 2005). Conversely, unintentional introductions are most often a byproduct of human movements, and are thus unbound to human motivations. Introduced species can have negative impacts on native populations, including population declines through niche displacement, interspecific competition for food and habitat, direct predation, and competitive exclusion (Suarez and others 2005).

In this study, we used forensic molecular techniques to identity a stowaway frog that was shipped to Kirkland, Washington, in an Amazon.com package. The frog was deceased and of unknown origin, despite our best attempts to locate the original shipping location from Amazon and its subsidiary shipping companies. It was also severely desiccated, which made it difficult to identify to species using morphological characteristics alone (Fig. 1).

The specimen arrived in a shipment of stereo equipment from Amazon.com on 19 February 2011. The specimen (Fig. 1) has been deposited at the Burke Museum of Natural History and Culture (UWBM 3483). Tissue samples from the frog were removed from the 2nd tarsal of the left foot, in addition to 1 mm^2 of skin from the lower abdomen. We extracted DNA using a Qiagen DNEasy Blood and Tissue kit, using the spin-column animal-tissue protocol. DNA samples were diluted to a concentration of 10ng/µl prior to PCR amplification.

We targeted the mitochondrial DNA *16S* rRNA gene *(16S)*, because this gene has been widely used in amphibian systematics and is currently the most useful marker for molecular taxonomic identification and DNA barcoding in frogs (Vences and others 2005). We amplified and sequenced a 550-bp region of the *16S* gene using standard amphibian primers 16SA-L and 16SB-H (Vences and others 2005). The 20 µl PCR reactions included 13 µl of dH_2O, 0.2 µl of *Taq* polymerase (1 unit), 0.2 µl of 25 mM $MgCl_2$, 2.0 µl of 10× PCR buffer, 0.5 µl of each 16S primer (20 µM), 0.8 µl of 10 mM dNTP, and 1.0 µl of DNA. The PCR amplification program consisted of an initial denaturation step at 2 min at 94°C. This was followed by 29 cycles of denaturation (30 s at 94°C), annealing (30 s at 48°C) and extension (30 s at 72°C), and a final extension of 5 min at 72°C. PCR products were visualized using a 1% agarose gel stained with EtBr. A sample of *Lithobates pipiens* (Northern Leopard Frog) was used as a positive control. The PCR products were purified using ExoSAP-IT (USB). We sequenced using dye-labeled

FIGURE 1. Photograph of the desiccated *Osteopilus septentrionalis* (UWBM 3483) shipped in an Amazon.com package to Kirkland, Washington. (Photo credit: Duncan Reid).

dideoxy terminator cycle sequencing with BigDye v3.1 (Applied Biosystems), and products were cleaned using hydrated sephadex placed in a Milipore plate. Sequencing (both directions) was performed on an ABI 3730 automated DNA sequencer. We aligned and edited DNA sequences using Sequencher v4.2. The DNA sequence data are deposited on Genbank (Accession #KC170728).

We conducted a nucleotide BLAST search (BLASTn) of the unknown frog *16S* gene on Genbank. We downloaded a phylogenetically-informative cluster of DNA sequences using the PhyLoTA Browser (rel. 1.5). The sequences were aligned using Muscle v3.6 (Edgar 2004). The nucleotide substitution model was selected using JModelTest v0.1 (Posada 2008). Phylogenetic relationships were inferred using maximum likelihood and Bayesian inference. Maximum likelihood analyses were conducted using RAxML-VI-HPC v7.0.4 (Stamatakis 2006). The RAxML analyses used the GTRGAMMA model of nucleotide substitution. Support values were estimated from 1000 non-parametric bootstrap replicates. We conducted Bayesian phylogenetic analyses using parallel MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). We ran 2 separate analyses with different starting seeds for 2 million generations using 4 heated Markov chains (using default heating values). We assessed convergence by inspecting the cumulative posterior probabilities of clades using the online program Are We There Yet? (AWTY; Nylander and others 2008). Posterior probability values were obtained by summarizing the posterior distribution of trees (post burn-in) with a 50% majority-rule consensus tree.

The *16S* data from the unknown sample included 440 base pairs. The top hits from the nucleotide similarity search match with frogs of the Lophiohylini, and the unknown sequences shared 100% coverage and 99% sequence similarity (E-value = 0.0) with *Osteopilus septentrionalis* (Cuban Treefrog). The single specimen of *O. septentrionalis* in Genbank with *16S* data is

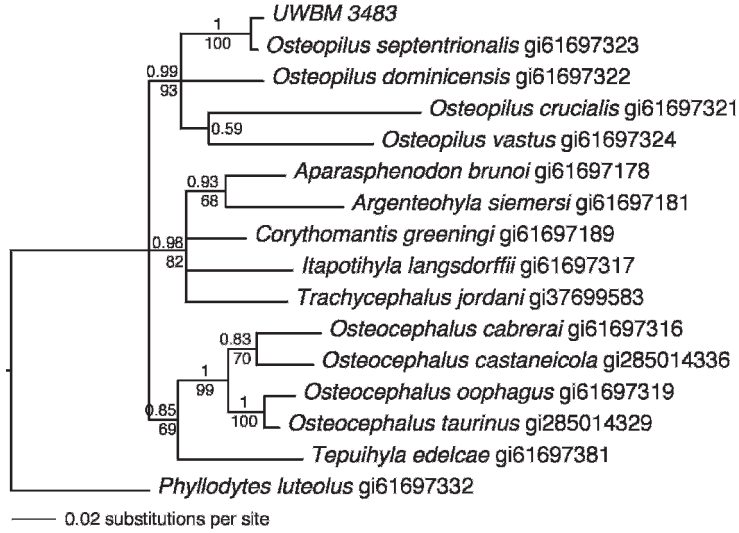

FIGURE 2. A phylogeny of *16S* mitochondrial DNA shows that specimen UWBM 3484 is an *Osteopilus septentrionalis*.

from Guantanamo Bay, Cuba (Accession Number: AY843712.1). The Cuba specimen differs from UWBM 3483 by 3 nucleotide substitutions (2 C-T transitions and 1 A-C transversion).

A phylogenetically-informative sequence cluster for the Lophiohylini, which contains the genus *Osteopilus*, was downloaded from PhyLoTA for phylogenetic analyses. The data matrix contained 16 species and 572 aligned nucleotide positions. The GTR+I+Γ nucleotide substation model was selected by JModelTest under all criteria (Akaike Information Criterion, Bayesian Information Criterion, or dynamical likelihood ration tests), and this model was used in the Bayesian phylogenetic analyses. The tree was rooted using *Phyllodytes luteolus* (Yellow Heart-tongued Frog) based on the results presented in Faivovich and others (2005).

The phylogenetic analyses of the Lophiohylini provided strong support for the placement of unknown sample UWBM 3483 (Fig. 2). Phylogenetic analyses using maximum likelihood and Bayesian analyses both support a clade containing *O. septentrionalis* and UWBM 3483 (bootstrap support = 100%; posterior probability = 1.0; Fig. 2). Monophyly of the genus *Osteopilus* is supported by a 93% bootstrap value and a 0.99 posterior probability, although the relationships among species are not fully resolved (Fig. 2).

Osteopilus septentrionalis is a neotropical and mostly arboreal frog in the family Hylidae that has become widely distributed in the southeastern United States. The native range of the species is Cuba, the Isle of Youth, the Cayman Islands, and the Bahamas (McGarrity and Johnson 2009), but *O. septentrionalis* is now considered an invasive species in the southern continental US. The 1st introduction of *O. septentrionalis* to the US is speculated to have occurred in Key West, Monroe County, Florida in 1931 (Barbour 1931), and introductions of *O. septentrionalis* are recorded throughout the state of Florida (Meshaka 2011), as well as in Georgia (Jensen and others 2008), Virginia (Mitchell and Reay 1999), Maryland (Meshaka 1996), and Colorado (Livo and others 1998). An ecological niche modeling study demonstrated that appropriate climates are available for *O. septentrionalis* across the entire southeastern US, and that the availability of suitable habitats is expected to increase under future climate warming scenarios (Rödder and Weinsheimer 2009). Across its introduced range, *O. septentrionalis* consumes a wide variety of invertebrate and vertebrate prey including beetles, roaches, isopods, and lepidopterans; other frogs including the Green Treefrog (*Hyla cinerea*), Squirrel Treefrog (*Hyla squirella*), Eastern Narrowmouth Toad (*Gastrophryne carolinensis*), Southern Leopard Frog (*Lithobates sphenocephalus*), and Southern Toad (*Anaxyrus terrestris*); and lizards including the Brown Anole (*Anolis sagrei*) and Common House Gecko (*Hemidactylus mabouia*) (Bartareau and Meshaka 2007; Meshaka 2011). According to Austin (1973), *O. septentrionalis* has had a negative effect on native frog populations.

Although here we report a failed cross-continental accidental introduction, *O. septentrionalis* is an opportunistic settler, and the expansion of this species into new regions could be further facilitated by an increase in suitable habitats as a result of climate change (Rödder and Weinsheimer 2009). This event also illustrates that the Pacific Northwest is not necessarily immune to this type of accidental introduction by non-native species that are successful at colonizing and surviving in appropriate habitats.

Acknowledgements.—We thank K Fries for donating the frog to the Burke Museum of Natural History and Culture.

LITERATURE CITED

AUSTIN DF. 1973. Range expansion of the Cuban Treefrog in Florida. Florida Naturalist 46:28.

BARBOUR T. 1931. Another introduced frog in North America. Copeia 1931:140.

BARTAREAU TM, MESHAKA WE JR. 2007. *Osteopilus septentrionalis* (Cuban Treefrog). Diet. Herpetological Review 38:324–325.

DORCAS ME, WILLSON JD, REED RN, SNOW RW, ROCHFORD MR, MILLER MA, MESHAKA WE JR, ANDREADIS PT, MAXXOTTI FJ, ROMAGOSSA CM, HART KM. 2012. Severe mammal declines coincide with proliferation of invasive Burmese Pythons in Everglades National Park. Proceedings of the National Academy of Sciences 109:2418–2422.

EDGAR RC. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32:1792–1797.

FAIVOVICH J, HADDAD CFB, GARCIA PCA, FROST DR, CAMPBELL JA, WHEELER WC. 2005. Systematic review of the frog family Hylidae, with special

reference to Hylinae: Phylogenetic analysis and taxonomic recommendations. Bulletin of the American Museum of Natural History 294:1–240.

JENSEN J, CAMP CD, GIBBONS JW, ELLIOTT M. 2008. The amphibians and reptiles of Georgia. Athens, GA: University of Georgia Press. 600 p.

LIVO LJ, HAMMERSON GA, SMITH HM. 1998. Summary of amphibians and reptiles introduced into Colorado. Northwestern Naturalist 79:1–11.

MCGARRITY ME, JOHNSON SA. 2009. Geographic trend in sexual size dimorphism and body size of *Osteopilus septentrionalis* (Cuban Treefrog): Implications for invasion of the southeastern United States. Biological Invasions 11:1411–1420.

MESHAKA WE JR. 1996. Vagility and the Florida distribution of the Cuban Treefrog (*Osteopilus septentrionalis*). Herpetological Review 27:37–40.

MESHAKA WE JR. 2011. A runaway train in the making: The exotic amphibians, reptiles, turtles and crocodilians of Florida. Monograph 1. Herpetological Conservation and Biology 6:1–101.

MITCHELL JC, REAY KK. 1999. Atlas of amphibians and reptiles in Virginia. Richmond, VA: Special Publication No. 1, Virginia Department of Game and Inland Fisheries. 132 p.

NYLANDER JAA, WILGENBUSCH JC, WARREN DL, SWOFFORD DL. 2008. AWTY (are we there yet?): A system for graphical exploration of MCMC convergence in Bayesian phylogenetics. Bioinformatics 24:581–583.

PIMENTEL D, ZUNIGA R, MORRISON D. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecological Economics 52:273–288.

POSADA D. 2008. jModelTest: Phylogenetic model averaging. Molecular Biology and Evolution 25: 1253–1256.

RÖDDER D, WEINSHEIMER F. 2009. Will future anthropogenic climate change increase the potential distribution of the alien invasive Cuban Treefrog (Anura: Hylidae). Journal of Natural History 43: 1207–1217.

RONQUIST F, HUELSENBECK JP. 2003. MrBayes version 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574.

STAMATAKIS A. 2006. RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688–2690.

SUAREZ AV, HOLWAY DA, WARD PS. 2005. The role of opportunity in the unintentional introduction of nonnative ants. Proceedings of the National Academy of Sciences 102:17032–17035.

VENCES M, THOMAS M, VAN DER MEIJDEN A, CHIARI Y, VIEITES DR. 2005. Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. Frontiers in Zoology 2:5.

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