Report

Resolving the species status of Surprise Valley pyrg *(Pygulopsis gibba)* and Vineyard Pyrg (*Pyrgulopsis vinyardi)*

To

Stantec Environmental Consulting
1165 East Jennings Way, Suite 101
Elko, Nevada 89801
Ms. Diana Eck, project manager

Dr. Hsiu-Ping Liu
Department of Biology
Metropolitan Stat University of Denver

Dr. Robert T. Dillon, Jr.
Freshwater Gastropods of North America Project
Charleston, SC

22 October 2021

Diana Eck with Stantec sent 30 snail samples collected from 23 miles northeast of Lovelock, NV, on June 23, 2020, to delineate the species status of Surprise Valley pyrg (*Pyrgulopsis gibba*) and Vineyard Pyrg (*Pyrgulopsis vinyardi*).

Based on Hershler (1995 and 1998), the primary distinction between *P. gibba* and *P. vinyardi* was in their penial morphology. The dorsal aspect of the *P. vinyardi* penis is marked by three glands (dg1, dg2, and dg3, Hershler 1998, Figure 39). However, the dorsal aspect of the *P. gibba*penis does not demonstrate a dg1 or a dg2, and sometimes not even a dg3 (Hershler 1995, Figure 12.)

Specimens were dissected and identified as P. *gibba* or *P. vinyardi* based on the presence/absence of dg1 and dg2. DNA was extracted from dissected specimens, amplified using Folmer primers, and sequenced. Phylogenetic analyses were performed to determine whether there was concordance between penial morphology and genetic variation.

Materials

Among 30 snail specimens, 15 snails were identified as males. Five of those 15 males demonstrated both dg1 and dg2, matching *P. vinyardi* (labeled as V1-V5). Three males did not demonstrate either dg1 or dg2, matching *P. gibba* (labeled as G1-G3). And seven males demonstrated either dg1 or dg2, intermediate between *gibba* and *vinyardi* (labeled as I1-I7).

Genetic Analysis Methods

Genomic DNA was isolated from 15 individual snails using a CTAB protocol (Bucklin, 1992). For the mitochondrial COXI gene, COIL1490 and COIH2198 (Folmer et al., 1994; COIL1490 5′GGTCAACAAATCATAAAGATATTGG3′ and COIH2198 5′TAAACTTCAGGGTGACCAAAAAATCA3′) were used to amplify a 709 base pair (bp) fragment via polymerase chain reaction (PCR). Amplifications were conducted in a 25 µL total volume, containing 5 µL of Colorless GoTaq Flexi Reaction Buffer (Promega), 0.5 µL of dNTPs (200 µM for each dNTP), 1.25 µL of each primer (10 µM), 1 unit GoTaq G2 DNA polymerase (Promega), 1 µL of DNA or 1 µL of 1:20 diluted DNA and 13.8 µL of PCR grade water.

The temperature profile for the PCR reaction consisted of an initial 2 min denaturation step at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 45°C, 2 min at 72°C, and a final extension step at 72°C for 7 min. All PCR products were evaluated for successful amplification using 1.5% agarose gel electrophoresis. The unsuccessfully amplified specimens were further tested by using a different amount of template DNA. The amplified PCR product was incubated at 37°C for 30 min and then at 85°C for another 15 min with 2 µL of ExoSAP-IT (ThermoFisher Scientific) to cleave nucleotides one at a time from an end of excess primers and to inactivate single nucleotides.

Sequencing reactions were run in both directions with the same primer pairs used for amplification at Eton Bioscience Inc (San Diego, CA). Sequences were determined for both strands and were edited and aligned using Sequencher™.

BLAST analysis was used to find similarities between newly obtained sequences and sequences in GenBank. Phylogenetic analyses were performed using Bayesian inference. The phylogenetic analysis included the newly sequenced specimens, previously sequenced specimens from the same locality (23 miles northeast of Lovelock, NV; field label SV-Strm3, Liu label DE 22A-E), *P. gibba* sequences retrieved from GenBank (DQ364016, AY426359, AY426413), and *P. vinyardi* sequences retrieved from GenBank (EU700482) for comparative purposes. The tree was rooted with *Pyrgulopsis saxatilis* (AY627934) and *Marstonia lustrica* (MK895920). MrModeltest2 (Nylander, 2004) was used to obtain an appropriate substitution model (using the Akaike Information Criterion) and parameter values for the analyses. Bayesian inference was performed using MrBayes v. 3.2.7a (Ronquist et al., 2012). In the Bayesian analysis, Metropolis-coupled Markov chain Monte Carlo simulations were performed with four chains for 3,000,000 generations and Markov chains were sampled at intervals of 10 generations to obtain 300,000 sample points. The default settings were used for the priors on topologies and the HKY + G + I model parameters selected by MrModeltest2 as the best fit model. At the end of the analysis, the average standard deviation of split frequencies was 0.001 and the potential scale reduction factor was 1, indicating that the runs had reached convergence. The sampled trees with branch lengths were used to generate a 50% majority-rule consensus tree, with the first 25% of the samples removed to ensure that the chain sampled a stationary portion.

Results

DNA was extracted from 15 dissected specimens. Homologous nucleotide sequences were obtained from 13 specimens (see Table 1). BLAST analysis indicated newly obtained sequences are similar to *Pyrgulopsis gibba* and *P. vinyardi* (see Table 1).

A Bayesian tree is shown in Figure 1. Three *Pyrgulopsis gibba* sequences and one *P. vinyardi* sequence previously deposited in GenBank do not form a reciprocal monophyletic clade. Furthermore, all specimens identified as *P. gibba* (G1-G3) via penial morphology do not form a monophyletic clade by themselves or with the *gibba* sequences obtained from the GenBank. Similarly, specimens identified as *P. vinyardi* (V2-V5) do not form a monophyletic clade by themselves or with the *vinyardi* sequence obtained from the GenBank.

Discussion/Conclusion

The phylogenetic species concept is based on the monophyly and defines a species as the smallest set of lineages or populations that can be recognized by a unique combination of derived traits. Phylogenetic analysis showed specimens identified as *P. gibba* or *P. vinyardi* do not form a monophyletic clade.

P. gibba and *vinyardi* are genetically similar, uncorrected genetic distance averaged 0.7% (ranged from 0.5-1.1%) based on previously deposited sequences in GenBank. The mean 0.7% genetic distance is consistent with intraspecific variation in *Pyrgulopsis* (Liu et al. 2018).

Given non-monophyly, scant genetic differentiation, and the evolutionarily labile nature of morphology, we concluded that *P. gibba* and *P. vinyardi* should be synonymized into one species.

Table 1. Specimen codes, Sample sizes (N), and Blast analysis results.

Stantec ID	Liu ID	N	Blast analysis	Comments
SV-Strm3	DE22	5	DE22A: 99.04% EU700482 *P. vinyardi*, 98.63% DQ364016 *P. gibba* DE22B: 99.20% EU700482 *P. vinyardi*, 98.48% DQ364016 *P. gibba* DE22C: 98.94% DQ364016 *P. gibba*, 98.72% EU700482 *P. vinyardi* DE22D: 98.94% DQ364016 *P. gibba*, 98.72% EU700482 *P. vinyardi* DE22E: 99.20% EU700482 *P. vinyardi*, 98.48% DQ364016 *P. gibba*	
SV-Strm3	G1-3	3	G1: 99.20% EU700482 *P. vinyardi*, 98.38% DQ364016 *P. gibba* G2: 98.71% EU700482 *P. vinyardi*, 98.93% DQ364016 *P. gibba* G1: 98.71% EU700482 *P. vinyardi*, 98.93% DQ364016 *P. gibba*	Dissected specimens missing dg1 and dg2
	V2-5	4	V2: 99.16% EU700482 *P. vinyardi*, 98.41% DQ364016 *P. gibba* V3: 98.71% EU700482 *P. vinyardi*, 98.93% DQ364016 *P. gibba* V4: 98.55% EU700482 *P. vinyardi*, 98.78% DQ364016 *P. gibba* V5: 98.55% EU700482 *P. vinyardi*, 98.78% DQ364016 *P. gibba*	Dissected specimens with both dg1 and dg2
	I1-6	6	I1: 99.20% EU700482 *P. vinyardi*, 98.48% DQ364016 *P. gibba* I2: 98.71% EU700482 *P. vinyardi*, 98.93% DQ364016 *P. gibba* I3: 99.20% EU700482 *P. vinyardi*, 98.48% DQ364016 *P. gibba* I4: 99.20% EU700482 *P. vinyardi*, 98.48% DQ364016 *P. gibba* I5: 98.71% EU700482 *P. vinyardi*, 98.93% DQ364016 *P. gibba* I6: 98.71% EU700482 *P. vinyardi*, 98.93% DQ364016 *P. gibba*	Dissected specimens with either dg1 or dg2

Figure 1. Bayesian tree based on the COI dataset. Posterior probabilities for nodes are provided in blue when >= 95% and in red when < 95% for relevant clades.