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RESEARCH ARTICLE

Avoidance of extinction through nonexistence: the use of museum specimens and molecular genetics to determine the taxonomic status of an endangered freshwater crayfish

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Abstract We investigated the endangered status and taxonomic status of the freshwater crayfish *Procambarus ferrugineus*, a crayfish species considered for the candidate list of the Endangered Species Act. This species has a narrow distribution from central Arkansas, USA and is codistributed with its presumed sister species, *Procambarus liberorum*. We sampled extensively throughout the ranges of both primary burrowing species and collected mitochondrial DNA from a hypervariable fragment of the 16S gene from 109 individuals across 22 sites. We also collected data from a variable region of the 12S gene from a subset of the resulting 16S haplotypes. Due to our inability to sample what we considered *P. ferrugineus* in the field, we included museum specimens from the United States Natural History Museum of both *P. ferrugineus* and *P. liberorum*. Analyses of the resulting data suggested that these two species are indeed the same and we therefore synonymize them under the name of priority—*P. liberorum*. Additionally, our sampling discovered three new cryptic species from southwestern Arkansas all from the genus *Procambarus*. Nested clade phylogeographic analysis coupled with population genetic analyses suggested that *P. liberorum* has had three rounds of range expansion throughout the inferred evolutionary history. Using IUCN Red List criteria for conservation assessment, we conclude that the species *P. liberorum* should be considered stable, but with special concern because of habitat fragmentation and urbanization, small restricted range, and a moderate level of genetic diversity. *Procambarus reimeri* should be considered endangered due to its limited geographic range and the potential for a decline in suitable habitat. The three potentially newly discovered species should be considered data deficient until more information is obtained on their distributional limits and habitat requirements. Our study highlights the importance of thorough geographic and taxonomic sampling coupled with the utility of collecting data from museum specimens to reach robust taxonomic and conservation conclusions for endangered species.

Keywords Endangered Species Act · Nested clade phylogeographic analysis · Crayfish · Arkansas · Population genetics · Conservation · Species diagnosis

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Introduction

For better or worse, species are the central currency of much of conservation biology. For example, the International Conservation Union (IUCN) keeps a Red List of endangered species (2001), which is often used to define "hot spots" of global and regional conservation efforts (Myers et al. 2000; Orme et al. 2005). Likewise, many local, regional, and national governments are focused on single species approaches to conservation. For example,

most governmental conservation action in the United States is dictated by the implications of the Endangered Species Act (Clegg 1995). Given such emphasis on "the species," it is critical to evaluate the validity of these taxonomic entities through a variety of means using a variety of ecological, morphological, and molecular data (Paquin and Hedin 2004) coupled with explicit and diagnosable species concepts (Sites and Crandall 1997; Sites and Marshall 2003). In some cases of highly endangered species, however, relevant data may not be available because of the rarity of the species. In such cases, museum specimens become essential in determining species status, historical population structure and levels of gene flow (Thomas et al. 1990; Graham et al. 2004; Wandeler et al. 2007), and even habitat reconstruction and climate change insights (Rowe 2007). Indeed, often such samples can be obtained while preserving the external morphology of the specimens themselves (e.g., Gilbert et al. 2007). We present such a case and demonstrate the utility of combining molecular phylogenetic investigation with museum samples to obtain relevant data on species status for endangered species. We demonstrate this approach by investigating the species status of the freshwater crayfish *Procambarus ferrugineus*.

Freshwater crayfishes are a highly endangered group of organisms with high alpha biodiversity in the eastern United States (Crandall 1997). With over 360 described species from North America alone (Crandall and Buhay 2008), freshwater crayfish share the dubious distinction of having more species imperiled than not (a distinction held only by one other group—the freshwater mussels) (Conservancy 1996). One such imperiled species is *P. ferrugineus*.

Originally, Hobbs and Robison (1988) described *P. ferrugineus* from collections at two sites in Lonoke County in central Arkansas along the Coastal Plain physiographic province. The presumed sister taxon, *Procambarus liberorum*, was described earlier by Fitzpatrick (1978) from Washington County in northern Arkansas in the Ozark Mountains. Both species were originally placed in the *gracilis* group of the subgenus *Girardiella* within the genus *Procambarus* (Hobbs and Robison 1988).

Procambarus ferrugineus was differentiated from *P. liberorum* by a combination of the following characters: (1) *P. liberorum* had a prominent angular excision in the basal third of the opposable margin of the dactyl while *P. ferrugineus* either lacked such a prominent angular excision or had a weak excision in the basal third of the opposable dactyl; (2) the dorsolateral surface of the palm in *P. ferrugineus* was described as tuberculate while the dorsolateral surface of the palm in *P. liberorum* was punctuate; and (3) the first form male of *P. liberorum* was mostly red while the first form male of *P. ferrugineus* was mostly brown or tan, although inexplicably in the color notes, Hobbs and Robison (1988) described the carapace of the holotype as brick red. However, Hobbs and Robison (1988) noted that *P. ferrugineus* was similar to *P. liberorum* in possessing a very narrow areola. Furthermore, as additional specimens were collected by Robison from the intervening areas between the Ozark Mountains and the Coastal Plains in Lonoke County, doubts about the specific distinctiveness of *P. ferrugineus* were raised. In a letter to Robison–Hobbs suggested that "he had erred in describing *P. ferrugineus*, as the original morphological characters differentiating the Coastal Plain *P. ferrugineus* from the mountain dwelling *P. liberorum* were beginning to blur."

In an effort to better understand the conservation status and taxonomic limits of these endemic species, we embarked on a study to sample and compare *P. ferrugineus* with its presumed sister taxon *P. liberorum* as well as to other related species found in similar habitats and geographic locations. *Procambarus reimeri* was thought to be a close relative of both *P. liberorum* and *P. ferrugineus*, and this species occurs in just one Arkansas County (Polk Co.) in similar burrow habitats. To determine the distinctiveness of these taxa, as well as their respective conservation statuses, we collected specimens from throughout the state of Arkansas and applied molecular genetic approaches for determining species validity and conservation needs. Unfortunately, our attempts to collect *P. ferrugineus* failed and we were forced to consider the possibility that this taxon was now extinct. However, we also acquired new localities for *P. liberorum* that encroached upon the distributional area of *P. ferrugineus*. We also sampled new localities south of the known range of both *P. liberorum* and *P. ferrugineus* but these individuals could not be confidently identified as either species or *P. reimeri*. Thus we suspected that *P. liberorum* and *P. ferrugineus* might actually be synonymous and might have a larger distribution than previously thought. We therefore collected museum specimens from the United States National Museum of Natural History (Smithsonian Institution), Washington DC from both species in the hopes of extracting quality DNA with which to compare with our field samples. The role of natural history museums in biodiversity studies has recently been highlighted (Graham et al. 2004) and this study provides a concrete example of the utility of such collections to literally save large sums of monies on would be misguided conservation and search efforts.

Materials and methods

Specimen collection

Specimens were collected by excavating burrows and extracting crayfish by hand. Sampling design in species

boundary studies is critical (Morando et al. 2003) and includes sampling extensively throughout the species distribution. As *P. liberorum* is the presumed sister taxon to *P. ferrugineus*, we sampled extensively throughout its historical distribution, especially in locations close to the limited distribution of *P. ferrugineus* (Table 1) and in new areas previously not recorded for either species. Sampling locations are shown in Fig. 1 with respect to the individual species distributions. Gill tissue was dissected from each specimen upon collection and stored in 100% EtOH for DNA extraction (see below). The remaining specimen was stored in 70% EtOH as voucher material and deposited in the crayfish collections at either Southern Arkansas University or the Monte L. Bean Life Science Museum at Brigham Young University.

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted using standard methods and the 16S mtDNA gene was amplified for all individuals (Table 1) during PCR with primers 16sf-cray: GACC GTGCKAAGGTAGCATAATC and 16s-1492r: GGTTA CCTTGTTACGACTT (Crandall and Fitzpatrick 1996) which amplify an approximately 500 base pair hypervariable region of the mitochondrial 16S ribosomal gene. The 16S mtDNA is the most variable gene for freshwater crayfishes (Fetzner and Crandall 2001) and has been used extensively and successfully for both population genetic and species diagnosis studies in freshwater crayfish (e.g., Fetzner and Crandall 2003; Buhay and Crandall 2005). The 12S mtDNA gene (Mokady et al. 1999) was also amplified using primers 12sf: 5′ GAAACCAGGATTAGATACCC 3′ and 12sr: 5′ TTTCCCGCGAGCGACGGGCG 3′ from one individual per sampled locality to provide deeper among-species phylogenetic relationships (Table 1). The 12S gene is approximately 400 base pairs and is slightly less variable than 16S (Buhay et al. 2007). Cycle-sequencing reactions were run with purified PCR products and the Big Dye Ready-Reaction kit on a Perkin Elmer Thermocycler. Reactions were cleaned using Millipore plates and then sequenced using an ABI3730XL automated DNA sequencer. There are cases where nuclear and mtDNA sequences can give conflicting signals (e.g., Shaw 2002; Evans et al. 2003), but these tend to be where lineage sorting due to recent radiation is problematic or where there is the potential for hybridization (see Posada and Crandall 2001). Neither of these processes is suspected in this case and the decision was made by the authors to do more thorough sampling across the geographic distribution of the target species rather than half the samples and develop a nuclear marker for those sampled, especially since sampling is critical to the Nested Clade Phylogeographic Analysis.

Sequence alignment and phylogenetic analyses

Resulting sequences were aligned using BioEdit (Hall 1999). A phylogenetic analysis of the *P. liberorum*, *P. ferrugineus*, closely-related species, and outgroup taxa was then performed on the unique 16S haplotypes (determined by TCS, see below) and the combined (since the loci are non-independent) 16S + 12S dataset using Maximum Likelihood (ML) (Felsenstein 1981) with a model of evolution selected from 56 alternatives using the AIC criterion as implemented in the software ModelTest 3.06 (Posada and Crandall 1998; Posada and Buckley 2004). Confidence in the resulting nodes was assessed using the bootstrap approach (Felsenstein 1985) with 1,000 pseudoreplications. ML runs were performed using the software PhyML 2.4.6 (Guindon and Gascuel 2003). Bayesian analyses using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) were run for 20 million generations over eight chains with the starting parameters determined by ModelTest. Tracer (Rambaut and Drummond 2003) was used to determine the burnin and a consensus tree was constructed from the remaining trees. Multiple independent identical ML and Bayesian runs were done to ensure convergence on similar results. Nodal support for the Bayesian analyses was assessed using the posterior probability (PP) generated from a consensus tree of the sampled trees past burnin (Huelsenbeck et al. 2001).

Phylogeographic analyses and species diagnoses

Nested Clade Phylogeographic Analysis (NCPA: Templeton 1998a, 2001, 2004) was employed to examine significant associations between genetic variation and geographic location for the *P. liberorum* and *P. ferrugineus* individuals (109 individuals from 22 geographic locations). This approach sheds light on historical and contemporary evolutionary patterns and processes, including range expansion, restricted gene flow, and fragmentation. Using the program TCS (Clement et al. 2000) with the 16S dataset, a haplotype network was constructed for the 95% confidence level. The network is a graphic representation of the base pair differences between unique haplotypes. GeoDIS 2.4 (Posada et al. 2000, 2006) was then used to test for significant associations between the haplotypes and their geographic localities using the latitude–longitude coordinates over 5,000 permutations. For the museum specimens used from the Smithsonian, we approximated the coordinates based on the locality descriptions, but for the recent collections, we gathered location information using Global Positioning System (Garmin XL) devices at each site in decimal degrees. The 2005 inference key, available from http://darwin.uvigo/es/software/geodis.html, was used to tease apart contemporary and historical

Table 1 *Procambarus* individuals with respective locality information, specimen voucher numbers, and GenBank accession numbers for 16S and 12S data

Species	State: County	Site name	Individual vouchers with 16S data	16S Haplotype	GenBank 16S data	Individual vouchers with 12S data	GenBank 12S data	Latitude	Longitude
liberorum	AR: Franklin	Bee Rock	JC2300–2305	1	EF012312	JC2303	EF012292	35.66762	–93.8927
liberorum	AR: Crawford	Corner of White Rock Mtn. and Mineral Hill	JC2307–2316	1	EF012312	JC2316	EF012293	35.6761	–93.92035
liberorum	AR: Crawford	Roadside south of White Rock Mtn.	JC2317–2321, JC2323–2327, JC2329–2330	1	EF012312	JC2319	EF012294	35.68327	–93.96545
liberorum	AR: Crawford	Reimer's Site at White Rock Mtn.	JC2332–2337, JC2340–2341	1	EF012312	JC2332	EF012295	35.68686	–93.96696
liberorum	AR: Crawford	Natural Pond/Swamp	JC2344–2346, JC2348–2350	1	EF012312	na	na	35.69403	–93.994
liberorum	AR: Washington	Fayetteville	KC2948, KC2950	1	EF012312	na	na	36.0456	–94.1498
liberorum	AR: Washington	Fayetteville	KC2949	2	EF012313	KC2949	EF012300	36.0456	–94.1498
liberorum	AR: Crawford	Natural Pond/Swamp	JC2343	3	EF012314	JC2343	EF012296	35.69403	–93.994
liberorum	AR: Crawford	Reimer's Site at White Rock Mtn.	JC2338	4	EF012315	na	na	35.68686	–93.96696
liberorum	AR: Crawford	Roadside south of White Rock Mtn.	JC2322	5	EF012316	na	na	35.68327	–93.96545
liberorum	AR: Franklin	Bee Rock	JC2306	6	EF012317	na	na	35.66762	–93.8927
liberorum	OK: Le Flore	Roadside off Hwy 128	JC2555	7	EF012318	na	na	34.875	–94.5
liberorum	AR: Franklin	Cherokee Prairie	KC2932–2933, KC2935	8	EF012319	KC2933	EF012298	35.343	–93.9986
liberorum	AR: Franklin	Flanagan Prairie	KC2941–2946	8	EF012319	KC2944	EF012299	35.36	–93.9986
liberorum	AR: Logan	Caulksville	JC2666–2668	8	EF012319	na	na	35.299	–93.85
liberorum	AR: Johnson	Lamar	KC3037	9	EF012320	na	na	35.4358	–93.3906
liberorum	AR: Perry	Hollis	KC3002–3008	10	EF012321	KC3004	EF012301	34.861	–92.903
ferrugineus	AR: Perry	Roadside near Hollis	USNM260008	10	EF012321	USNM260008	EF012309	34.85	–93.1
ferrugineus	AR: Perry	Roadside near Hollis	USNM260022	10	EF012321	USNM260022	EF012311	34.85	–93.1
liberorum	AR: Franklin	Cherokee Prairie	KC2934	11	EF012322	na	na	35.343	–93.9986
liberorum	AR: Franklin	Flanagan Prairie	KC2947	11	EF012322	na	na	35.36	–93.9986
liberorum	AR: Scott	Waldron	JC2615–2617, JC2619–2621, KC2921–2924	12	EF012323	KC2922	EF012297	34.89	−94.08
liberorum	OK: Le Flore	Roadside off Hwy 128	JC2553	13	EF012324	na	na	34.875	–94.5
liberorum	AR: Faulkner	Ballfields	KC3021–3025	14	EF012325	KC3021	EF012303	35.10054	–92.41853
liberorum	AR: Pope	Scottsville	USNM219236	15	EF012326	USNM219236	EF012306	35.45	–93.02
liberorum	AR: Faulkner	Vilonia	JC2218	16	EF012327	na	na	35.085	–92.1972
*ferrugineus**	AR: Lonoke	Highway 70	USNM218843	17	EF012328	na	na	34.675	–91.915
liberorum	AR: Faulkner	Vilonia	JC2219–2220	18	EF012329	na	na	35.085	–92.1972
liberorum	AR: Faulkner	Vilonia	JC2217	19	EF012330	JC2217	EF012291	35.085	–92.1972
liberorum	AR: Johnson	Clarksville	USNM260306	20	EF012331	USNM260306	EF012308	35.488	–93.442
liberorum	AR: Johnson	Lamar	KC3040–3041	20	EF012331	KC3040	EF012304	35.4358	–93.3906

Table 1 continued

Species	State: County	Site name	Individual vouchers with 16S data	16S Haplotype	GenBank 16S data	Individual vouchers with 12S data	GenBank 12S data	Latitude	Longitude
liberorum	AR: Johnson	Lamar	KC3038–3039, KC3042–3043	21	EF012332	na	na	35.4358	–93.3906
liberorum	AR: Perry	Roadside	USNM260016	22	EF012333	USNM260016	EF012305	35.036	–93.035
liberorum	AR: Perry	Petit Jean	KC3009–3012	22	EF012333	KC3010	EF012302	35.071	–92.979
ferrugineus	AR: Conway	Roadside ditch	USNM260006	22	EF012333	USNM260006	EF012310	35.109	–93.011
liberorum	AR: Madison	Roadside near Crosses	USNM260303	na	na	USNM260303	EF012307	35.895	–93.922
Closely-related species									
sp nov 1	AR: Lafayette	Lewisville	JC2208–2209, JC2211–2213	1	EF012336	na	na	33.4128	–93.5731
sp nov 1	AR: Lafayette	Lewisville	JC2207	2	EF012337	JC2207	EF012286	33.4128	–93.5731
sp nov 1	AR: Hempstead	Patmos	JC2202–2203	3	EF012335	JC2202	EF012285	33.5057	–93.4866
sp nov 1	AR: Lafayette	Lewisville	JC2210	3	EF012335	na	na	33.4128	–93.5731
sp nov 2	AR: Montgomery	Caddo Hills	JC2542	1	EF012338	na	na	34.42	–93.6333
sp nov 2	AR: Clark	Wingfield Creek	JC2543	1	EF012338	JC2543	EF012290	34.1917	−93.2533
sp nov 2	AR: Montgomery	Caddo Hills	JC2541	2	EF012339	JC2541	EF012289	34.42	–93.6333
sp nov 2	AR: Hempstead	Blevins	JC2187	3	EF012340	na	na	33.8718	–93.5701
sp nov 2	AR: Hempstead	Blevins	JC2185–2186	4	EF012341	JC2186	EF012288	33.8718	–93.5701
sp nov 3	AR: Hempstead	Grandview Prairie	JC2175–2177, KC2959–2962	na	EF012334	JC2177	EF012287	33.8035	–93.7836
reimeri	AR: Polk	Irons Fork Road	KC3013–3017	1	EF012343	KC3014	EF012284	34.6494	–94.1225
reimeri	AR: Polk	Mena	KC2262–2263, KC2266–2268	2	EF012342	KC2262	EF012283	34.5873	–94.2302
curdi	TX: Marion	Black Cypress Bayou	KC968–969	na	EF012344	KC968	EF012281	32.7941	–94.3249
nigrocinctus	TX: Angelina	Moccasin Creek	KC1013–1015	na	EF012345	KC1013	EF012282	31.4332	–94.6127
Outgroup species									
tenuis	AR: Polk	Trib to Cedar Creek	JC2281–2283	1	EF012349	na	na	34.6833	–94.2
tenuis	OK: Le Flore	Little Powell River	KC2867	2	EF012348	na	na	34.6463	–94.537
tenuis	OK: Le Flore	Little Powell River	KC2852	3	EF012346	na	na	34.6463	–94.537
tenuis	OK: Le Flore	Little Powell River	KC2854	4	EF012347	na	na	34.6463	–94.537
ouachitae	AR: Pulaski	Fourche Creek	KC2996	1	EF012355	na	na	34.656	–92.422
ouachitae	AR: Sevier	Trib to Cedar Creek	KC3036	2	EF012356	na	na	33.95	–94.3193
clarkii	AL: Tuscaloosa	Marrs Spring, Univ. Alabama	JC828	1	EF012350	na	na	33.2	–87.55
clarkii	TX: Fort Bend	CR 359 just north of Fulshear	KC1206	2	EF012352	KC1206	EF012280	29.697	–95.8977
clarkii	NM: Eddy	Black River	JC1985	3	EF012351	na	na	32.1586	–104.2889
acutus	AR: Franklin	Downs Prairie	KC2940	1	EF012353	na	na	35.3416	–94.043
acutus	AR: Pulaski	Fourche Creek	KC2987	2	EF012354	na	na	34.656	–92.422

* = paratype

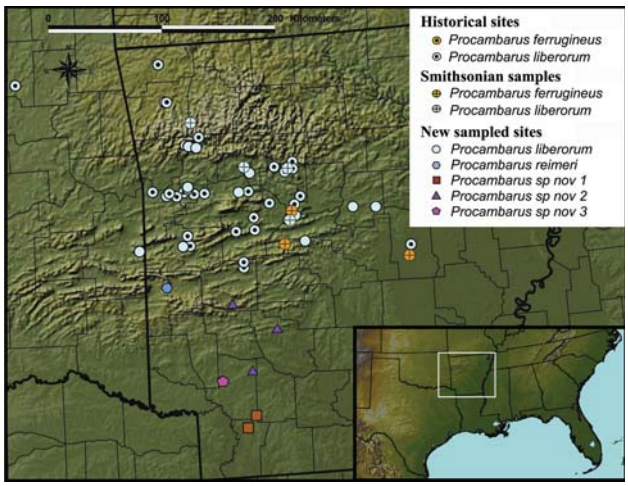

Fig. 1 Distribution of *Procambarus* species included in this study. State boundaries for Arkansas and Oklahoma shown as bold lines, while county boundaries are shown as thin lines. Historical records of *P. liberorum* and *P. ferrugineus* are marked as circles with dots in the middle, and historical collections from the Smithsonian Museum (United States National Museum of Natural History) that we obtained genetic data from are circles marked with crosshairs. Open circles depict newly-discovered sites as a result of this study and genetic data was gathered for those samples. Closely-related species are represented as follows: *P. reimeri*, hexagon; *P. sp. nov. 1*, square; *P. sp. nov. 2*, triangle; *P. sp. nov. 3*, pentagon

processes. The NCPA is also central to testing both hypotheses of species using the Cohesion Species Concept (Templeton 2001) as well as Evolutionarily Significant Units (Crandall et al. 2000). Here we use Templeton's Cohesion Species Concept to test the hypothesis that *P. liberorum* and *P. ferrugineus* form distinct species (Templeton 1998b, 1999, 2001).

Genetic diversity and demography

The current genetic diversity (θ_π) (Tajima 1983) estimate was obtained from the program DNASP 4.0 (Rozas et al. 2003) using data from the hypervariable region of the 16S gene. Current genetic diversity estimates are based on pairwise nucleotide differences between sequences. Since endangered status assessments use as one of the key criteria the stability of the population size across time, contrasting these estimates provides a robust way of testing for stability in genetic diversity across evolutionary timescales and thereby contributing key insights into the conservation status of the species in question.

We also explored the historical population dynamics of the *P. liberorum* complex using the Bayesian skyline plot model (Drummond et al. 2005) as implemented in BEASTv1.3 (http://beast.bio.ed.ac.uk/Main_Page). This coalescent-based demographic model uses standard Markov Chain Monte Carlo (MCMC) sampling procedures to estimate a posterior distribution of effective population size through time directly from sequence data under a best-fit substitution model. The hyperparameter m was set to 1/4 of the sequences in each data set. Two independent MCMC analyses 2×10^7 steps long were performed sampling every 1,000th generation, with the burn-in set at 2×10^6 generations. Strict and relaxed clock evolutionary models were tested for consistency across assumptions (Drummond et al. 2006). All the Bayesian MCMC outputs generated by BEAST were analyzed in Tracer v1.3 (Rambaut and Drummond 2003).

Results

Phylogenetic analyses

Our resulting nucleotide sequences were deposited in GenBank under accession numbers EF012312–EF012356 for the 16S fragment and EF012280–EF012311 for the 12S data (Table 1). Phylogenetic relationships among *P. liberorum*, *P. ferrugineus*, and their closely-related species were determined using Bayesian and Maximum Likelihood approaches for both the 16S haplotype dataset and the combined 16S and 12S dataset representing one individual from most of the sampled localities (Table 1). ModelTest parameters for the 16S dataset (45 sequences, 490 base pairs) were as follows: GTR + I + G model of evolution (–lnL = 1489.8899, AIC = 2999.7798), base frequencies (A = 0.3461, C = 0.1029, T = 0.3647, G = 0.1863), proportion of invariable sites (I) = 0.5343, gamma distribution shape parameter (G) = 0.7362, Rmat = (0.9033 13.8691 2.8519 0.000 7.1326). ModelTest parameters for the combined 16S + 12S dataset (32 sequences, 878 base pairs) were as follows: TVM + I + G model of evolution (–lnL = 2268.9863, AIC = 4555.9365), base frequencies (A = 0.3744, C = 0.1431, G = 0.1229, T = 0.3596), proportion of invariable sites (I) = 0.6120, gamma distribution shape parameter (G) = –6267), Rmat = (0.5792 7.3728 1.0593 0.0000 7.3728).

For Bayesian analyses, starting parameters were number of substitution types (nst) = 6 and rates = invgamma. The first 4,000 trees were discarded as burnin and the consensus tree was estimated using the remaining 36,000 trees. For PhyML runs, starting parameters were determined by ModelTest with the initial tree determined by BIONJ with optimization. The TVM model is not an option in PhyML, therefore, for the combined 16S + 12S dataset, we used the GTR + I + G model using the ModelTest maximum likelihood estimated parameters with the exception of the transition/transversion ratio parameter.

Using the 16S data, *P. liberorum* and *P. ferrugineus* appear to be synonymous as they form a monophyletic group with a posterior probability of 89% and ML

bootstrap support of 91% (Fig. 2). The sister relationship to *P. liberorum/P. ferrugineus* was unresolved, but the analyses did reveal the existence of three cryptic taxa, two of which have high bootstrap (BS) and posterior probability (PP) support and the third is represented by a single haplotype (although there were seven individuals with this same distinctive haplotype). *Procambarus reimeri*, *Procambarus curdi*, and *Procambarus nigrocinctus* fell out as closely-related species, while *Procambarus tenuis* was sister to that unresolved assemblage (Fig. 2).

The combined 16S + 12S gene dataset was analyzed using both ML and Bayesian approaches and revealed some similar trends to the 16S haplotype analyses, but clarified the deeper relationships between some of the *Procambarus* species. Again, *P. liberorum* and *P. ferrugineus* fell out in the same clade with strong support of 98% PP/91% BS (Fig. 3). *Procambarus reimeri* was recovered as the sister species to *P. liberorum/P. ferrugineus* with 99% PP/73% BS support. *Procambarus sp nov 1* and *P. sp nov 2* were also strongly supported clades each with 100% PP/100% BS. The phylogenetic positioning of *P. sp nov 3* was unresolved yet the haplotype clearly represents a distinct evolutionary lineage.

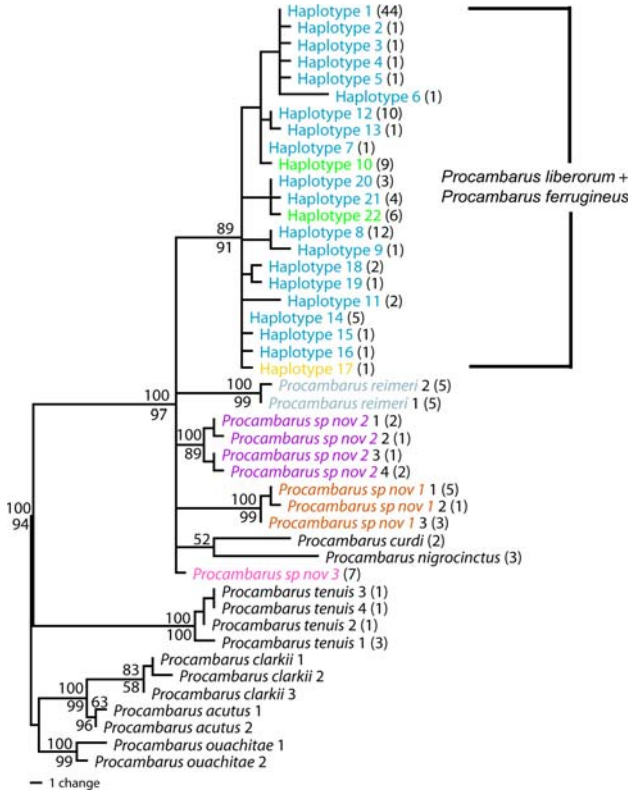

Fig. 2 Phylogenetic tree using 16S haplotypes showing relationships of *Procambarus* species. Numbers of individuals represented by each haplotype are given in parentheses. Haplotype numbers for *P. liberorum* and *P. ferrugineus* match those in the parsimony network. Bayesian posterior probabilities given above nodes >50% and ML bootstrap support for nodes >50% given below. Topology was identical with both Bayesian (–lnL = 1636.969) and ML (–lnL = 1501.168) analyses. *Procambarus clarkii*, *Procambarus acutus*, and *Procambarus ouachitae* were used to root the tree

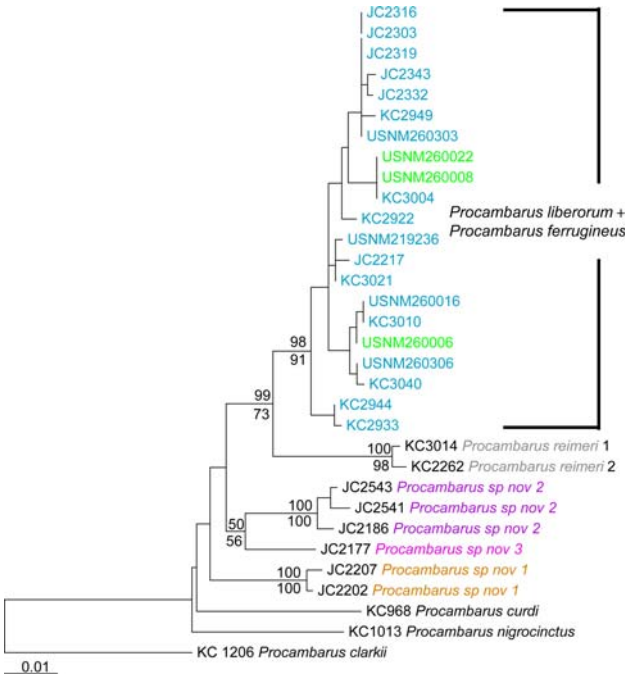

Fig. 3 Phylogenetic tree using combined 16S and 12S representing one individual for most sampled sites of *Procambarus* species. Bayesian posterior probabilities given above nodes >50% and ML bootstrap support for nodes >50% given below. Topology was identical with both Bayesian (–lnL = 2308.12) and ML (–lnL = 2268.163) analyses. *Procambarus clarkii* was used to root the tree

Phylogeographic analyses

A total of 22 haplotypes for *P. liberorum/P. ferrugineus* were recovered using TCS for 109 individuals collected from 22 localities throughout the range of the species complex (including new records that extend the range). The sister species based on the 16S + 12S phylogenetic analyses, *P. reimeri*, was used to root the TCS network which was set at the 95% confidence limit (nine mutational steps) (Templeton et al. 1992). *Procambarus reimeri* most closely connected to haplotype 17 at 11 steps, outside the confidence interval.

The total network cladogram was comprised of 11 one-step clades, 6 two-step clades, and 2 three-step clades (Fig. 4). Only 12 of these clades had both genetic and geographic variation, which was examined using the program GeoDIS (Table 2). Of these clades, only six showed significant variation for which Templeton's 2005 inference key was used to determine the current and historical processes contributing to the variation (Table 3). Contiguous Range Expansion (CRE) and Restricted Gene Flow with Isolation by Distance (RGF w/ IBD) were the inferred

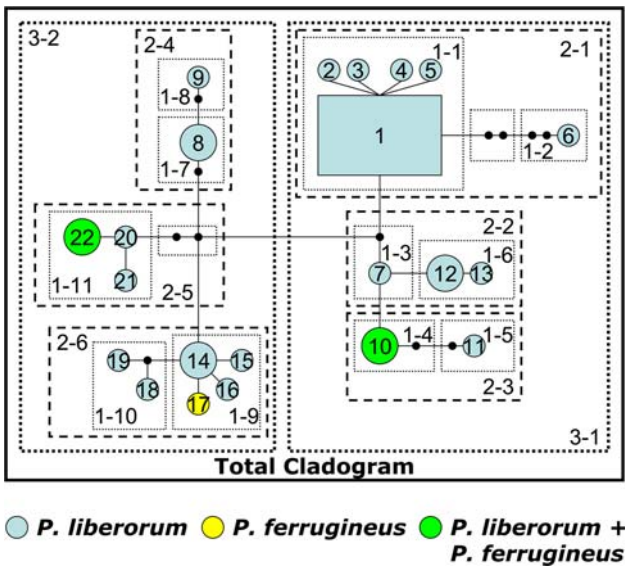

Fig. 4 Haplotype network of 16S data and corresponding nesting levels for *P. liberorum* and *P. ferrugineus*. *Procambarus reimeri* was used to root the network. The 95% connection limit was determined to be nine steps. Haplotypes containing only *P. liberorum* individuals were shaded blue, those containing only *P. ferrugineus* were shaded yellow, and those haplotypes representing individuals from both *P. liberorum* and *P. ferrugineus* were shaded green

patterns for four clades, while the total cladogram pattern was inconclusive. Clade 3-1 could be explained by either Long Distance Colonization (LDC) followed by fragmentation or Past Fragmentation (PF) followed by Range Expansion (RE), therefore, we performed a mismatch analysis of this clade (see below).

Genetic diversity and demography

We performed a mismatch analysis (Rogers and Harpending 1992; Harpending et al. 1998) of clade 3-1 to help determine whether colonization followed by fragmentation or fragmentation followed by range expansion can best explain the genetic-geographic patterns for the clade, as suggested by the inference key (Table 3). Examining deviations from neutrality can also help clarify past demographic events, so we determined Tajima's *D* (Tajima 1989) which if significantly negative, can provide information about possible bottlenecks associated with range expansion events, typical of large floods and washouts. An initial $\theta = 1.25965$, final $\theta = 1000$, and the expansion parameter $\tau = 2\mu t = 0.57994$ was used in DNASP for the mismatch analysis. The resulting plot showed a ragged bimodal distribution (Fig. 5), which is indicative of a population that is not expanding [raggedness = r = 0.2837; P ($r_{expected} < r_{observed}$) = 0.86877]. Tajima's $D = -1.40484$ ($P > 0.10$) suggested a constant population size. It appears that colonization followed by fragmentation best explains the pattern of diversity shown in clade 3-1, since we did not detect population expansion.

The genetic diversity estimates for *P. liberorum/P. ferrugineus* were $\theta_\pi = 0.00698 \pm 0.0004$ SD which is a moderate level of diversity (Nei 1987). The BEAST analysis indicates a moderate increase in population size over the last two million years (Fig. 6), consistent with the NCPA analysis suggesting Contiguous Range Expansion at all three nesting levels (clades 1-9, 2-3, and 3-2). The analysis also demonstrates that the species is not in decline. Similar results were obtained for both the strict and relaxed clock approaches.

Discussion

Conservation issues often center around taxonomic issues (the validity and/or distinctiveness of a particular taxon) due to much of conservation legislation and indeed much of the conservation science being focused on species or on evolutionarily significant units (ESU) within a species. Thus we need concrete, testable, and repeatable criteria with which to assess species boundaries, species status, and to define conservation units (like ESUs) within species. Here we have taken a broad sampling approach to examining the species status of a presumed endangered species *P. ferrugineus* to investigate the evolutionary distinctiveness of this taxon relative to closely-related taxa. Our analyses clearly show that the currently described species of *P. liberorum* and *P. ferrugineus* form a monophyletic group and distinct evolutionary lineage relative to other closely related species of *Procambarus*. However, our recent field sampling did not encounter populations of *P. ferrugineus*. Therefore, this study required museum specimens to represent the species to distinguish between the hypotheses of synonymy versus the alternative that *P. ferrugineus* was indeed distinct but could not be found in the field due to recent extinction. Ample museum specimens preserved in a fashion that allowed reasonable DNA isolation (70% EtOH) allowed us to test these hypotheses. We used the Cohesion Species Concept to test the hypothesis that *P. liberorum* and *P. ferrugineus* are distinct species. A Cohesion Species is first and foremost an evolutionary lineage concept, that is, to designate two populations as distinct species, they must form distinct evolutionary lineages. Thus, the first null hypothesis to be tested is that the individuals sampled from these two presumed species are derived from a single evolutionary lineage versus the alternative that they form distinct evolutionary lineages. We can test this hypothesis formally using the NCA framework by testing if these prior categories correspond to phylogenetic lineages (e.g., Matos and Schaal 2000). As the NCA defines a nested set of

Table 2 Results of the Nested Clade Phylogeographic Analysis of *P. liberorum* and *P. ferrugineus* 16S haplotypes based on 5,000 replicates

0-step clades			1-step clades			2-step clades			3-step clades		
Haplotype	Dc	Dn	Clade	Dc	Dn	Clade	Dc	Dn	Clade	Dc	Dn
1	*10.43*	*11.76*	*1-1*	*12.87*	*12.71*	2-1	12.69 S	40.82 S	3-1	56.53 S	71.89
2	0.00	36.68									
3	0.00	5.63									
4	0.00	6.53									
5	0.00	6.95									
I-T	10.43	–2.18									
6			1-2	0.00	11.53						
			I-T	12.87	1.18						
7			*1-3*	*0.00*	*19.15*	*2-2*	*19.15 S*	*70.75 L*			
12	*0.00*	*12.76*	1-6	17.02	19.15						
13	0.00	25.54									
I-T	0.00	–12.77	I-T	–17.02	0.00						
10			*1-4*	*8.99 S*	*18.31 S*	2-3	29.83	84.03 L			
11			1-5	0.87	88.50 L						
			I-T	8.12	–70.19 S	I-T	3.32	22.00 L			
8			*1-7*	*6.99*	*7.65*	2-4	9.77 S	77.90 L	3-2	54.33	60.68
9			1-8	0.00	48.56						
			I-T	6.99	–40.91						
20	*2.56*	*37.12*	1-11			*2-5*	*27.18 S*	*26.00 S*			
21	0.00 S	31.34									
22	3.82 S	22.12 S									
I-T	0.27	11.31									
14	*0.00S*	*2.98 S*	*1-9*	*43.31*	*43.36*	2-6	38.48	72.53			
15	0.00	67.63									
16	0.00	21.33									
17	0.00	64.81									
I-T	0.00 S	–48.28 S									
18			1-10	0.00	17.33						
19											
			I-T	43.31	26.02	I-T	4.24	–49.43 S			

Clade (Dc) and nested clade (Dn) distances are given. An "s" indicates that the distance is significantly small at the 5% confidence level and an "L" indicates that the distance is significantly large. In clades with both tip and interior groups, the average I–T distance is given. Interior clades with geographic and genetic differences are italicized

hierarchical categories, we can apply an exact random permutation test to the nested design to test the null hypothesis of no association of these prior taxonomic categories with phylogenetic structure. Indeed, in this case, we fail to reject this null hypothesis. The museum specimens from *P. ferrugineus*, which included a paratype specimen, represented one distinct haplotype and two shared haplotypes with *P. liberorum*. Given the shared haplotypes coupled with the distinct haplotype of *P. ferrugineus* being clearly nested within the *P. liberorum* clade, we fail to reject the null hypothesis of distinct evolutionary lineages and therefore reject the hypothesis that *P. liberorum* and *P. ferrugineus* are distinct species. Thus, we hereby synonymize *P. ferrugineus* and *P. liberorum* with *P. liberorum* being the recognized species name due to priority. This example demonstrates the utility of the NCA in testing taxonomic hypotheses. Indeed, had we found distinct species, the NCA approach also immediately identifies diagnostic molecular characters for differentiating species that could effectively be used in barcode designations (Hebert et al. 2003). Finally, the NCA approach also defines the geographic areas associated with distinct lineages. The potential distributional area of species is an essential piece of information in IUCN and other conservation assessments and NCA provides a robust way of estimating this area.

Our study highlights the importance of museum specimens in conservation science. Because much of conservation

Table 3 Nested contingency results and inferred patterns

Clade	Chi-square	Probability	Inference chain	Inferred pattern
1-1	28.2438	0.1330	na	na
1-6	1.000	0.0920	na	na
1-9	24.0000	0.0210*	1–19–20–2–11–12 No	CRE
1-11	15.8889	0.0056*	1–2–3–4 No	RGF w/ IBD
2-1	6.1250	0.3532	na	na
2-2	5.4545	0.1660	na	na
2-3	11.0000	0.0348*	1–19–20–2–11–12 No	CRE
2-4	13.0000	0.0770	na	na
2-6	7.2188	0.0488*	na	na
3-1	144.0000	0.0000*	1–2–3–5–6–13 Yes	LDC possibly coupled with subsequent Frag
3-2	69.1209	0.0000*	1–2–11–12 No	CRE
Total	101.8324	0.0000*	1–2–11–17 No	Inconclusive

Inferences were made using Templeton's 2005 key. Abbreviations for the inferences are: CRE, contiguous range expansion; LDC, long distance colonization; RGF, restricted gene flow; IBD, isolation by distance; PF, past fragmentation; RE, range expansion; Frag, fragmentation; na, not applicable

Note: * $P < 0.05$

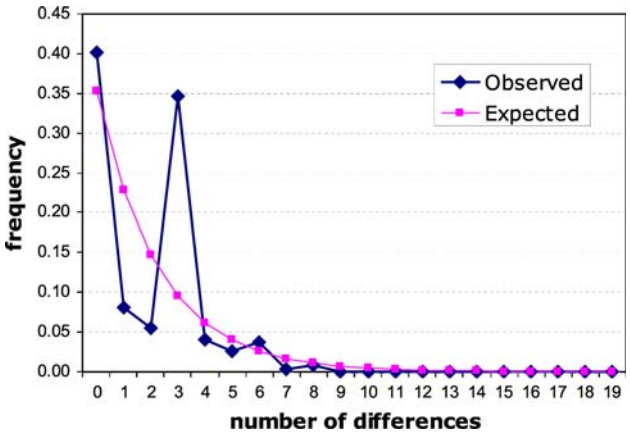

Fig. 5 Mismatch distribution for clade 3-1 of *P. liberorum* and *P. ferrugineus*. The observed frequency is represented by the diamonds with the thick solid line, while the expected frequency under the population growth model is represented by squares with a thin solid line

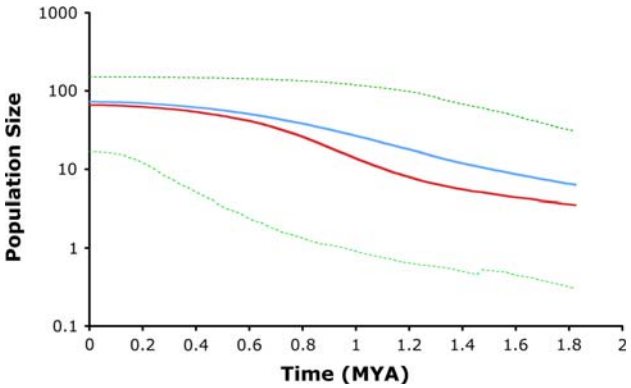

Fig. 6 Population dynamics analysis inferred using BEAST with two independent runs shown in solid blue and red lines and confidence intervals shown with dashed lines. Population size is measured in units of logNe*tau (see BEAST documentation for details)

biology is taxonomically oriented, when assessing the validity and distinctiveness of taxa, it is essential to have broad sampling throughout each species range. In addition, it is ideal to have material from type specimens or specimens collected from type localities to be sure of proper assignment of nomenclature. Finally, good sampling in terms of genetic markers, specimen localities, and number of specimens, is essential. For this study, we sampled broadly throughout the distribution of the species and even included specimens from beyond the known boundaries of the distribution. This broad sampling resulted in robust conclusions about the distinctiveness of the taxa under question and led to the discovery of previously unknown phylogenetic and taxonomic diversity.

During the course of this study, our sampling revealed three possible new lineages of freshwater crayfish closely related to *P. liberorum*. All three of these lineages are restricted to the southwestern corner of Arkansas with narrow distributions. The description of these new species is beyond the scope of this paper (as a formal species description is typically written for a very different audience and requires distinct data and presentation), but they will be further sampled for a subsequent publication, possibly including new species descriptions as warranted by the data. We are currently collecting nuclear genetic data as well as morphological data to support the distinct species hypotheses suggested by these results. Yet this result highlights the importance of broad sampling for the inclusion of the range of genetic diversity in species diagnoses.

The Interior Highlands which include the Ozark Mountains and the Ouachita Mountains are of considerable biogeographic significance as areas of endemism for both plants and animals (Matthews and Robison 1998). These mountainous regions have provided a safe haven for many

forms during geological epochs when most of the rest of the continent was not available for habitation (Robison and Allen 1995). As many as 100–150 species may be endemic to the Interior Highlands (Robison and Smith 1982; Allen 1995). Faunistically, the Ozark Mountains harbor many endemic vertebrates including 14 endemic fish species (Mayden 1985, 1988; Robison 1986; Turner et al. 1996) and five salamander taxa as well as numerous invertebrates such as crayfishes (Williams 1954; Hobbs and Robison 1988; Robison and Allen 1995), stoneflies (Stark et al. 1983; Ernst et al. 1986; Poulton and Stewart 1987), caddisflies (Frazer and Harris 1991; Moulton and Stewart 1996), and other insects (Allen 1990).

Procambarus liberorum appears to have originated in the headwaters of the White River in the Ozark Mountains and then migrated southward through the southern Ozarks through the Arkansas River drainage onto the north flank of the Ouachita Mountains (Arkansas River drainage) and then proceeded eastward through the Arkansas River Valley as far east as Lonoke County in the Coastal Plain province. The Arkansas River floodplain has been hypothesized to be a major barrier to dispersal southward for stream fishes from the Ozarks (Mayden 1985) yielding high endemism in the Ouachita Mountain region. However, this appears not be the case with our burrowing crayfishes which exhibit moderate genetic diversity, expanding population sizes, and extensive gene flow.

There is little in the way of life history information available regarding *P. liberorum*. This species has not been studied in any depth. Ovigerous females have been found, however, no females carrying eggs have ever been collected (Hobbs and Robison 1988).

Finally, we conclude by providing a conservation assessment of the species of key interest in this study. We used the IUCN (2001) Red List Criteria for endangerment as they provide a globally recognized framework for conservation assessment. At this point, the three potential *P. sp nov* must be considered data deficient given the lack of information on these populations and lack of formal description. Yet they clearly form distinct evolutionary lineages worthy of conservation and are highly restricted in distribution at the moment. Thus, they are most deserving of future study. *Procambarus reimeri* is considered endangered based on the IUCN criteria B (geographic range in the form of); B1 (extent of occurrence estimated to be less than 5,000 km^2 and); a—known to exist at no more than five locations and b—continuing decline projected in (i) area of occupancy and (ii) area, extent and/or quality of habitat. The last inference is based on the susceptibility of burrowing crayfish to habitat degradation coupled with the potential for such degradation at the current known locations of this crayfish. Finally, we consider *P. liberorum* to be stable and not immediately imperiled. However, while this species is more broadly distributed, encroaching urbanization, grassland habitat loss, and deforestation can be detrimental to this species' terrestrial gene flow routes and dispersal behaviors.

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