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Can members of the south-western *Gila robusta* species complex be distinguished by morphological features?

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The goal for this project was to re-examine key morphological characters hypothesized to differentiate *Gila intermedia*, *Gila robusta* and *Gila nigra* and outline methods better suited for making species designations based on morphology. Using a combination of meristic counts, morphological measurements and geometric morphometrics, morphological dissimilarities were quantified among these three putative species. Traditional meristic counts and morphological measurements (*i.e.* distances between landmarks) were not useful for species identification. Geometric morphometrics, however, identified differences among species, while also suggesting an effect of geographic location on morphological variation. Using canonical variate analysis for the 441 fish sampled in this study, geometric morphometrics accurately predicted true group membership 100% of the time for *G. nigra*, 97% of the time for *G. intermedia* and 91% of the time for *G. robusta*. These results suggest that geometric morphometric analysis is necessary to identify morphological differences among the three species. Geometric morphometric analysis used in this study can be adopted by management officials as a tool to classify unidentified individuals.

Key words: geometric morphometrics; *Gila intermedia*; *Gila nigra*; hybridization; species complex.

INTRODUCTION

The modern biological species concept was founded on the principle that differences in genetics among species can be reflected in differences in morphology (Mayr, 1963). Mayr (1963), however, pointed out that designating species based on morphological distinctness is fallacious. Indeed, the morphology-based species concept is inaccurate because it is a measure, not the cause, of true species rank. The degree of morphological differentiation among species is a by-product of the genetic differentiation that occurs during reproductive isolation (Mayr, 1963). In this view, organisms appear similar because they are genetically similar and morphological distinctness arises only after barriers to interbreeding arise. Donoghue (1985) suggested that it is only appropriate to study the primary cause of speciation (barriers to interbreeding) rather than incidental effects (such as morphology or ecology). While the conceptual framework of

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observing–documenting interbreeding may be the best way to make species designations, many times this is not feasible.

Hybridization poses both theoretical and practical problems to the modern biological species concept. Among vertebrates, fishes have the highest number of hybrid taxa because many reproduce through external fertilization (Dawley & Bogart, 1989). Hybridization violates reproductive isolation and creates a phenotype that is typically intermediate to the two parent species (Mayr, 1963). In some instances, however, hybridization zones can be difficult to identify because some hybrid individuals may be morphologically indistinguishable from the parent species (Mayr, 1984). If individuals within a hybrid population demonstrate morphological and genetic similarities to the parent species, are hybrids identifiable, or will they be mistakenly identified as a parental species? Are morphologically and genetically distinct hybrid populations considered a new species? These are all questions that are pertinent to the taxonomic status of the south-western fishes from the genus *Gila* Baird & Girard 1853.

The taxonomic designations of the south-western chubs (genus *Gila*; Baird & Girard, 1853*a*) have been complex and, at times, controversial. Members of the genus *Gila* have been classified as distinct species, subspecies of the roundtail chub *Gila robusta* Baird & Girard 1853, or as a species complex (*Gila robusta* complex, *sensu* Miller, 1945; Rinne, 1976; Robins *et al.*, 1980). *Gila robusta* was originally described by Baird and Girard in 1853 from specimens collected by S. W. Woodhouse, who was tasked with the exploration of the River Zuni (a tributary to the Little Colorado River) and its tributaries while serving as a surgeon to an expedition commanded by Captain Sitgreaves. Specimens collected on this trip were classified as a new genus: *Gila*. *Gila robusta* was originally described as, 'very much swollen anteriorly and tapering very suddenly from the dorsal fin to the insertion of the caudal. Eyes proportionally small, mouth tolerably large with a light greyish brown colour' (Baird & Girard, 1853*a*: 368). Shortly thereafter, Baird & Girard (1856) described the Gila chub *Gila intermedia* (Girard 1856) as a species belonging to an entirely different genus (*Tigoma*, which was later synonymized with *Gila*). Original specimens for what is now termed *Gila intermedia* were collected by J. H. Clark, under the leadership of J. D. Graham, during the exploration of the San Pedro and Gila Rivers. Baird & Girard (1853*b*) wrote of the new *Tigoma* genus: 'they all bear a general resemblance to *Gila*, from which they differ by a much smaller mouth and larger scales'. They also stated that 'the fins are much less developed, the inferior fins are especially quite small' (Baird & Girard, 1856: 206). Twenty years later, the headwater chub *Gila nigra* Cope 1875 was described by the famous naturalist and palaeontologist E. D. Cope of the Philadelphia Academy of Sciences (published in Cope & Yarrow, 1875). Cope described *Gila nigra* as having, 'a large head, lots of scales and a profile that is gradually descending then recurved to the upper lip' (Cope, 1875: 663). Unfortunately, the original descriptions of these three species contained only a few morphological and meristic characteristics, of which many overlap.

Given the morphological ambiguity among species, considerable effort has been made to resolve the taxonomy of *Gila* using genetic markers. DeMarais (1986, 1992) and Gerber *et al.* (2001) were unable to find species-specific markers, but suggested that *G. nigra* has resulted from several hybridization events between *G. robusta* and *G. intermedia*. Mitochondrial and nuclear markers (Schwemm, 2006) and microsatellite markers (Dowling *et al.*, 2015) demonstrate that genetic variation is partitioned by population, rather than by species and population-level variation may obscure species-level distinctions (Dowling *et al.*, 2015). Schönhuth *et al.* (2014) also did not find evidence

for monophyly of any of the species within the *G. robusta* species complex using one mitochondrial and several nuclear genes. Marsh *et al.* (2017) consider the *Gila robusta* complex as three cryptic species and used microsatellites to distinguish between *G. robusta* and *G. nigra*, document hybridization and identify the replacement of *G. nigra* with *G. robusta* in middle sections of a single stream. The American Fisheries Society and the American Society of Ichthyologists and Herpetologists Joint Committee on the Names of Fishes, however, recently investigated the taxonomic status of species in the *robusta* complex and, based on preliminary whole-genome sequence data (Copus *et al.*, 2016), determined the available genetic and morphological data supports a single species rather than three (Page *et al.*, 2016). Within this context, it is important to note that *G. robusta* and *G. nigra* are candidates for listing as species of concern under the United States Endangered Species Act (Congress, 1973), while *G. intermedia* is already listed as endangered. As a result, identifying both the true relationships and distinguishing features among these species may prove critical for management efforts.

Unfortunately, identifying these fishes remains difficult because they are so similar in both meristic and morphological characteristics (Rinne, 1976). Minckley & DeMarais (2000) proposed a dichotomous key using mean meristic and morphological data for *G. robusta*, *G. intermedia* and *G. nigra* in an effort to help naturalists, scientists and managers identify species in the field. Their key uses fin-ray counts, head length, caudal-peduncle length, caudal-peduncle depth, body colour and body-shape linear dimensions to outline distinct patterns among the three species to be used to identify populations of each species. Many of the meristic and morphological variables in the key, however, have overlap among the three species, which greatly complicates species identification (Farrington *et al.*, 2013; Crowder *et al.*, 2015).

The overarching goal of this study was to identify key morphological differences among *G. robusta*, *G. intermedia* and *G. nigra* and address the following questions. Can the morphometrics and meristics previously described as differentiating the three taxa (*G. robusta*, *G. nigra* and *G. intermedia*) be used to identify individuals? If not, can a more finely-tuned description of shape (*i.e.* geometric morphometrics) identify individuals from the three species? If individuals cannot be identified as belonging to a particular species, can body shape be used to identify species differences at the population level? To address these questions, photographs were taken of preserved specimens collected from the main drainages in the lower Colorado River basin between 1975 and 1998; these collections were made by W. L. Minckley and his colleagues, specialists reliable in the identification of species of *Gila*. From these photographs, traditional morphological variables that had been previously proposed as differentiating the three species were quantified. In addition, geometric morphometrics were used to quantify subtle variation in body shape in hopes of identifying previously unrecognised morphological differences among the three species. All of these analyses were conducted with the overarching goal of providing a new methodology that could potentially identify morphologically cryptic species in the field.

MATERIALS AND METHODS

Individual fish were photographed from a research collection held at Arizona State University, Tempe, AZ. Field location and species designations (as made by the individual who collected

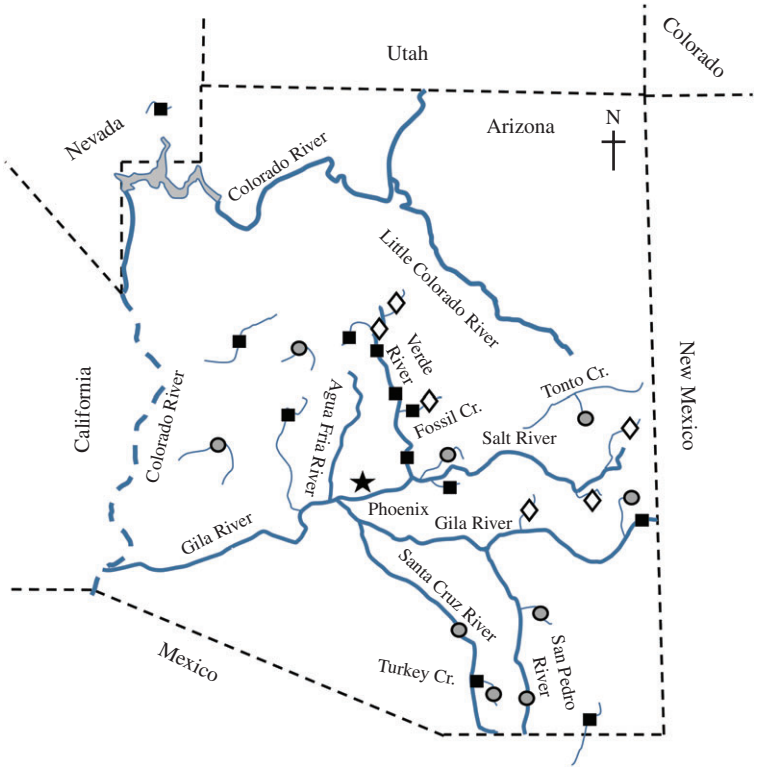

FIG. 1. Collection locations for the specimens used in this study and species identification as made by the person who collected the specimen. ■, *Gila robusta*; ◍, *Gila intermedia*; ◇, *Gila nigra*; ★, capital city of Arizona.

the specimen) were noted for each individual that was photographed (Fig. 1). A photograph of the lateral aspect of each fish was taken with a Cannon S95 digital camera (www.canon.com); an object of known dimension was placed in the background. This scaling object was later used to determine the number of pixels cm^{-2} in each photograph. Photographs were imported into ImageJ (Abràmoff *et al.*, 2004) and then used for subsequent morphological analyses. Collection locations for *G. robusta* were Moapa River, Sycamore Creek, Turkey Creek, Verde River, Fossil Creek, Virgin River, Yaqui River, Arivipa Creek, Gila River, Burrow Creek and Little Snake River. Collection locations for *G. intermedia* were Bass Canyon, Bonita Creek, Cienega Creek, Indian Creek, Monkey Springs, Santa Cruz River and Turkey Creek. Collection locations for *G. nigra* were Verde River, Fossil Creek, Gordon Canyon Creek, Gun Creek, Spring Creek and Webber Creek (Fig. 1).

Morphometric variables outlined by Rinne (1976), Minckley & DeMarais (2000) and several additional variables from the species descriptions of Cope, Baird and Girard were measured from the photos. These included: standard length (L_S), head length (L_H), peduncle length (L_P), predorsal length (L_{PD}; length from the snout to the origin of the pectoral fin), peduncle depth (D_P) and body depth (D_B) [Fig. 2(a)]. With these measurements, ratios were constructed for each individual to examine potential differences in body proportions among species. According to Minckley & DeMarais (2000), fin-ray counts and the ratios of L_H:D_P and L_{PD}:D_P are distinguishing features for *G. robusta*, *G. nigra* and *G. intermedia*. The following ratios were also quantified in the study reported here: L_S:D_B, L_S:D_P, L_S:L_{PD}, L_S:L_H and L_S:L_P. Sample sizes for this portion of the study were as follows: *G. robusta*, $n = 108$; *G. intermedia*, $n = 74$; *G. nigra*, $n = 48$.

For the geometric morphometric analysis, photographs of specimens from the same collections were used. Because large sample sizes are necessary to identify subtle shape changes,

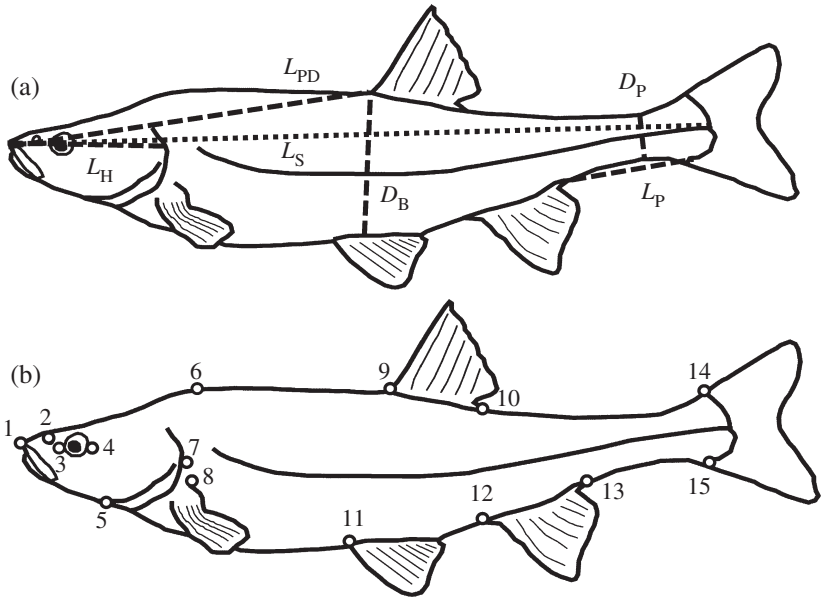

FIG. 2. Morphological measurements for *Gila* specimens used this study. (a) The key morphological measurements taken by Minckley & DeMarais (2000): L_H, head length; L_P, peduncle length; D_P, peduncle depth; L_S, standard length; L_{PD}, predorsal length; D_B, body depth. (b) The 15 landmarks used for the geometric morphometrics portion of *Gila*, as outlined in Armbruster (2012).

sample sizes for this portion of the study were as follows: *G. robusta*, $n = 216$; *G. intermedia*, $n = 150$; *G. nigra*, $n = 78$. TpsDig 1.5 software (Rohlf, 2004) was used to create two-dimensional coordinate data using 15 landmark points visible on each picture. The landmark points used in this study were previously established for cyprinids by Armbruster (2012) [Fig. 2(b)]. Landmark alignment was made using Procrustes superimposition in the MorphoJ software (www.flywings.org.uk/morphoj_page.htm); this superimposition method rescales landmarks to the same orientation, scale and locality (Klingenberg, 2011).

To evaluate shape differences among species with geometric morphometrics, a principal components analysis (PCA) was performed. Because PCA only assesses the axes of greatest disparity within the dataset, it may miss characteristics that explain interspecies differences due to developmental and phenotypic plasticity, but PCA is free from any *a priori* assumptions. A canonical variate analysis (CVA) requires designation of groups *a priori* and a CVA will maximize the differences between groups (Fisher, 1936; Owen & Chmielewski, 1985; Klingenberg & Monteiro, 2005). Whereas a PCA draws axes that define the maximum variation within a dataset, a CVA draws axes that maximize the discrimination of the assigned groups and provides the additional metric of whether or not individuals are correctly classified to their groups based on the data available. In this study, a CVA was run in MorphoJ and exported the Procrustes data from MorphoJ as a text file; these data were then transferred to JMP Pro 11.0.0 (www.sas.com). Two CVAs were run, the first with landmark data, then a CVA was run on the Procrustes data in JMP, enabling JMP to generate predicted group (species) designations for each specimen based on Procrustes distances. Specimens that were flagged as outliers were removed from the analysis. (For additional information on conducting geometric morphometric analyses in Cypriniformes using MorphoJ, see: http://www.auburn.edu/~armbrjw/gmguide/Geometric_Morphometrics_Guide/MorphoJ_Main_Window.html/).

Following the geometric morphometric analysis, average wireframe outlines of each species were examined. Wireframe outlines are generated by the MorphoJ programme; these illustrations depict the average values of shape for each axis for both PCA and CVA and identify which

TABLE I. Measurements of morphological variables previously proposed to distinguish species of *Gila* at the population level

Species	Sample size (n)	Measurement ± 95% C.I.				Morphological distinctions*		
		Dorsal rays	Anal rays	L_H:D_P	L_P:D_P	Dorsal and anal rays	L_H:D_P	L_P:D_P
G. intermedia	74	7·6 ± 0·2	8·1 ± 0·1	2·937 ± 0·075	1·474 ± 0·045	8 (7, 9)	<3·0	–
G. robusta	108	8·6 ± 0·1	8·3 ± 0·1	2·586 ± 0·061	1·627 ± 0·059	9 (8, 10)	>3·25	>2·3
G. nigra	48	8·3 ± 0·1	8·4 ± 0·1	2·859 ± 0·049	1·604 ± 0·041	8 (7, 9)	3·0–3·2	<2·4

D_P, peduncle depth; L_H, head length; L_P, peduncle length.
*From Minckley & DeMarais (2000). Commonly found fin ray counts with rarely found values in parentheses.

areas of the body have the greatest differences in shape along each PC and CV axis of variation. From these outlines, key areas were identified where body shape differs among species. The lengths of these key areas were calculated *via* Pythagoras theorem as the square root of the sum of the square of change in the X Procrustes coordinates and the sum of the square of change in the *y*-coordinates (Armbruster, 2012).

RESULTS

The individuals used in this study could not be identified to species using the dichotomous keys of Rinne (1976) or Minckley & DeMarais (2000). For example, the ratios of L_H:D_P and L_P:D_P do not differentiate the three species (Table I) because of the overlap in the ratios (Fig. 3). The ratios of L_H:D_P and L_P: D_P, measured here, are different than those reported in Minckley & DeMarais (2000) (Table I). In concordance with Minckley & DeMarais (2000), the present study shows that dorsal and anal-fin ray counts range between seven and nine for all species considered here. There is also substantial overlap, however, in the ratios and fin counts among species (Fig. 3). Thus, in contrast with the predictions of Minckley & DeMarais (2000), fin-ray counts and traditional morphometric ratios are not useful in differentiating species.

Principal components analysis tends to separate *Gila intermedia* from *G. nigra* along PC1, but *G. robusta* occupies almost all of the variation in morphospace quantified by the PCA. Although PC3 accounted for more than 10% of the total variability, it provided little separation among species (Fig. 4). Because there is an *a priori* hypothesis, however, that the species are distinct, a CVA (where group identity is established before the analysis is run) is likely to be more effective at parsing interspecies differences, relative to PCA. Indeed, the CVA reveals significant differences among all three species groups and the majority of specimens were correctly identified to species (Table II). The CVA accurately classifies *G. intermedia* to its respective group 97·3% of the time, while *G. intermedia* specimens are mistaken for *G. robusta* 2·0% of the time and *G. nigra* 0·7% of the time. *Gila nigra* is accurately classified 100% of the time. *Gila robusta* is correctly classified 91·2% of the time, while specimens of *G. robusta* are mistaken for *G. intermedia* 2·8% of the time and *G. nigra* 6% of the time.

After examining the wireframe outlines of average body shape for each species, most of the morphological variability was found in the posterior region of the body. To quantify body shape variation, distances between landmarks were calculated using

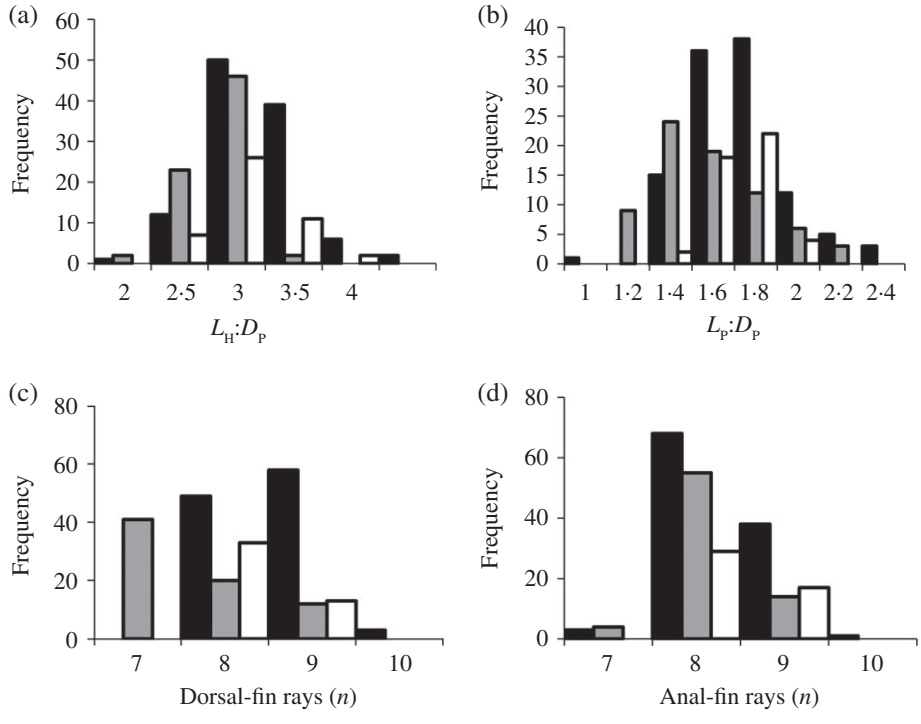

FIG. 3. *Gila* spp. frequency distributions of (a) the ratio of head length (L_H) to peduncle depth (D_P), (b) the ratio of peduncle length (L_P) to D_P, (c) dorsal and (d) anal fin-ray counts. ■, *Gila robusta*; ▩, *Gila intermedia*; □, *Gila nigra*

TABLE II. Observed and predicted *Gila* species designations based on a canonical variate analysis

	Predicted species		
Actual species	*G. intermedia*	*G. nigra*	*G. robusta*
G. intermedia	146	1	3
G. nigra	0	73	0
G. robusta	6	13	199

Procrustes coordinate values. When these distances are plotted, the greatest variability is in the distance between landmarks 12 and 13 (anal-fin base length). The next two most variable distances are between landmarks 13–15 (caudal-peduncle length) and 10–14 (insertion of dorsal fin to origin of caudal fin). When the distances between landmarks 12 and 13 are plotted with the distances between 10 and 14, it becomes evident that these distances tend to separate *G. intermedia* and *G. nigra* from one another and split *G. robusta* specimens into two distinct groups, those that are more similar (based on Procrustes distances) to *G. nigra* and those that are more similar to *G. intermedia* (Fig 5). Based on this finding, data for *G. robusta* were re-analysed with an additional

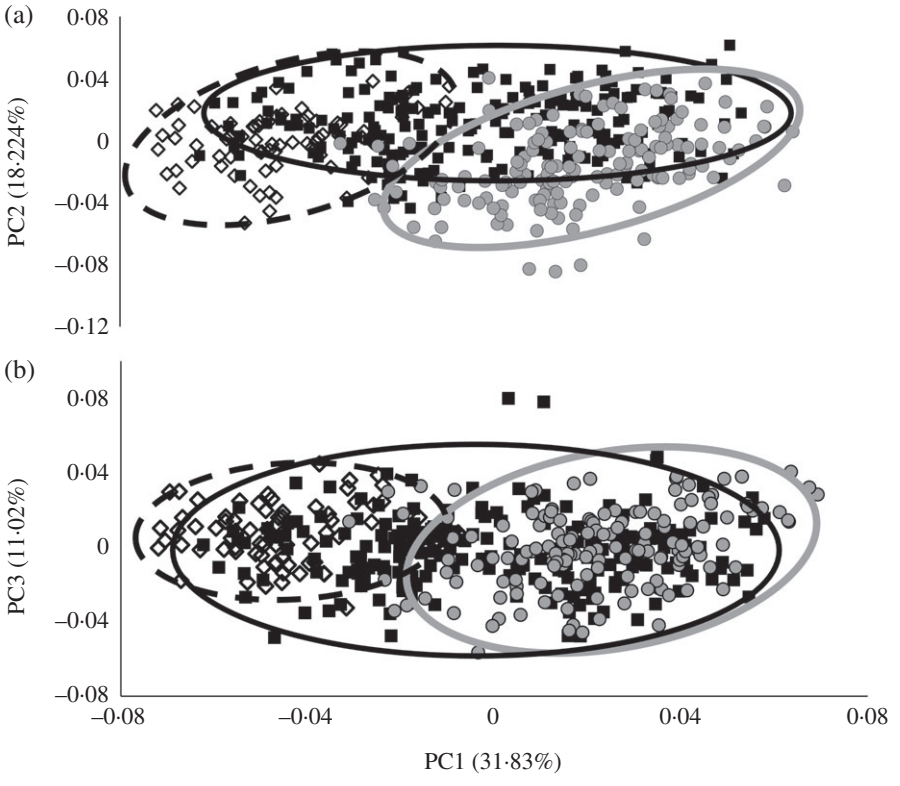

FIG. 4. Principal component analysis (PCA) of *Gila* spp. geometric morphometric variables. (a) PC1 *v*. PC2. (b) PC1 *v*. PC3. ◇, *Gila nigra*; ●, *Gila intermedia*; ■, *Gila robusta*; – – –, ——— and ———, respective 90% confidence ellipses.

CVA defining four hypothesized taxa (instead of the original three) with the prediction that there are two morphologically distinct populations of the *G. robusta* within the sample. The results of this second CVA suggest that there are indeed two distinct groups of *G. robusta* and that these groups are morphologically distinct from each other, in addition to being distinct from both *G. nigra* and *G. intermedia* (Fig. 6). In fact, the specimens of *G. robusta* collected from Moapa, Turkey Creek, Verde River, Virgin River and Yaqui River are distinct from all of the other *G. robusta* specimens examined here.

DISCUSSION

The failure of traditional morphological characteristics to identify species is a hurdle that may be overcome with geometric morphometric analyses. In species of *Gila*, because there is extensive morphological overlap in fin-ray counts L_P:D_P and L_H:D_P among species, individuals from historic collections cannot be accurately placed into their *a priori* species designations using metrics proposed by Rinne (1976) or Minckley & DeMarais (2000). In contrast, data from landmark points in MorphoJ and JMP

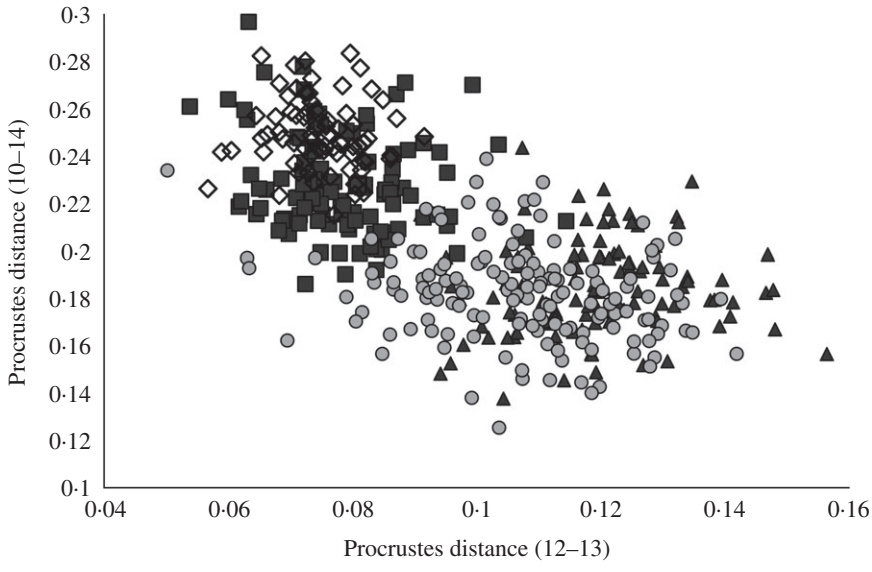

FIG. 5. Procrustes distances [see Fig. 2(b)] for *Gila* spp., including two morphs of *Gila robusta*. ▲, *G. robusta* (morph 1) from Moapa River, Sycamore Creek, Turkey Creek, Verde River, Virgin River and the Yaqui River (Fig. 1); ■, *G. robusta* (morph 2) from Arivipa Creek, Burrow Creek, Fossil Creek, Gila River and the Little Snake River; ◇, *Gila nigra*; ●, *Gila intermedia*.

were largely successful at predicting group identity of individual specimens using CVA. Using CVA, only 5·22% of the specimens examined were incorrectly assigned to species. It is possible that the original species designation was incorrect for those few individuals that were misidentified by the CVA. The high proportion of correct classification of the species by the CVA, however, suggests that all three species are morphologically cohesive.

The findings of this study suggest that managers could potentially use geometric morphometrics as a non-destructive tool to assess populations by taking photographs of wild-caught fish before releasing them back into the waterway. Photographs taken in the field could be examined at a later time to determine species identity. Alternatively, a tablet computer could be employed in the field to take photographs and measure landmark points and the values obtained for a given fish used to immediately assign it to a species (using the data from this paper to provide baseline values) before it is released back into the wild. Given that there appear to be regional differences in the species, however, only a few (<10) unknown specimens should be added to the dataset at a time. This precaution is critical because the CVA attempts to maximize distance among individuals, so a large population of new specimens could quickly become a distinct cluster of its own. The results of this study make it clear that, although it is certainly simpler to make traditional counts and measurements in the field, species of *Gila* present a situation where more complex tools appear to be necessary.

A goal of this study was to provide easily measured morphological variables that could differentiate the three species from one another. The one measurement from the geometric morphological analysis that was distinct across species was the dorso-ventral depth of the posterior region of the body. Following this finding from

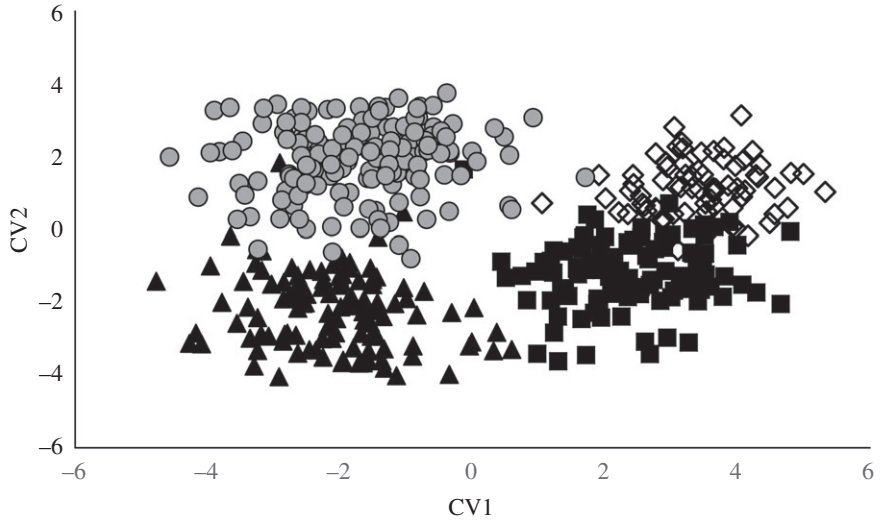

FIG. 6. Canonical variate (CV) analysis values for *Gila* spp., including two morphs of *Gila robusta*. ▲, *G. robusta* (morph 1) from Moapa River, Sycamore Creek, Turkey Creek, Verde River, Virgin River and the Yaqui River (Fig. 1); ■, *G. robusta* (morph 2) from Arivipa Creek, Burrow Creek, Fossil Creek, Gila River and the Little Snake River; ◇, *Gila nigra*; ●, *Gila intermedia*.

geometric morphometric analyses, a new ratio was created, one that separated *G. intermedia* from *G. nigra* and *G. robusta*: the standard length of the fish divided by the length of the chord (distance) from the origin of the dorsal fin to the origin of the pelvic fin. This variable was then measured in a subset ($n = 30$ per species) of individuals and ratios that were less than 3·8 were predominately found in *G. intermedia*, while ratios that were greater than 3·8 were predominately found in *G. robusta* and *G. nigra*. This metric accurately differentiated *G. robusta* and *G. nigra* from *G. intermedia* in 90% of the individuals in the data subset. In fact, for the specimens considered here, this ratio is better at separating these species than the variables previously proposed by Rinne (1976) and Minckley & DeMarais (2000), which could not be used to successfully categorize these individuals to species.

Unfortunately, there is no single diagnostic feature that consistently differentiates *G. robusta* from *G. nigra*. Features such as L_H, L_P, D_P, dorsal and anal fin base lengths accurately categorize over 75% of the individuals to the *a priori* species group, but some individuals from both species are incorrectly categorized using these variables. For example, *G. nigra* generally has a deeper caudal peduncle than *G. robusta and* this relationship is consistent across most specimens. There are waterways, however, where *G. robusta* (morph 2) closely resembles *G. nigra* (Fig. 5).

Rinne (1976) and DeMarais (1986) suggested that there was no effect of location on morphology for these three species of *Gila*. Shape data, however, obtained from the geometric morphometric analyses conducted in this study suggest there is geographic variation in morphology within species of *Gila*. In the two localities where there were multiple individuals representing different species from within the same waterway (Turkey Creek and Fossil Creek), individuals identified as *G. robusta* share shape characteristics with individuals identified as *G. nigra* and *G. intermedia* collected

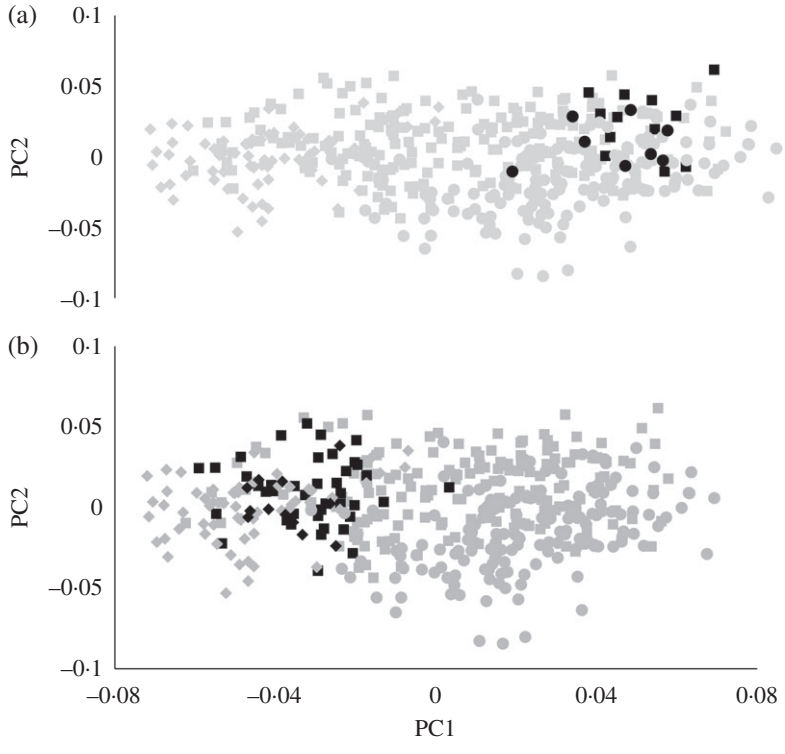

FIG. 7. Principal component (PC) analysis of *Gila* spp. geometric morphometric variables: (a) ■, *Gila robusta*; ●, *Gila intermedia*; *G. intermedia* from Turkey Creek. (b) ■, *Gila robusta*; ◆, *Gila nigra* from Fossil Creek. □, ○, ◇, Fishes from all other locations.

from the same locations (Fig. 7). Specifically, individuals identified as *G. robusta* from Turkey Creek have shortened posterior and anterior parts of the body, which is a characteristic of *G. intermedia*, when all fish in the study are considered together. In addition, individuals identified as *G. robusta* from Fossil Creek have a shallower caudal peduncle, a shape that was similar to that of individuals identified as *G. nigra* collected from the same location. It is possible these specimens were misidentified when added to the collection; if so, this indicates how difficult it is to identify the three species based on their morphology.

Previous studies have found that other fish species demonstrate phenotypic plasticity in response to variation in flow in riverine environments. McLaughlin & Grant (1994) and Imre *et al.* (2002) found that brook charr *Salvelinus fontinalis* (Mitchill 1814) reared in high flows had more streamlined bodies, shallower caudal peduncles and deeper caudal fins than *S. fontinalis* reared in a low-flow habitat. In the desert south-west U.S.A., there is great variability in flow among waterways. Average spring flow in the Verde River (where *G. robusta* exhibit a streamlined shape) is *c.* $3{\cdot}34\,m^3\,s^{-1}$, but average spring flow in the San Pedro River, where *G. intermedia* is found, is *c.* $0{\cdot}08\,m^3\,s^{-1}$. Individuals of *G. intermedia* are thought to occupy low-flow rivers and pool habitats, which could explain their relatively deeper bodies and broader caudal peduncles (Rinne, 1976). Conversely, individuals of *G. robusta* often occupy high-flow

habitats in rivers (*e.g.* Verde River), which could explain their shallower caudal peduncles and more streamlined bodies.

Previous studies have proposed multiple hybrid origins of *G. nigra* through interbreeding of *G. intermedia* and *G. robusta*; however, those same studies acknowledge that all three species do not currently co-occur (Rinne, 1976; DeMarais, 1986). How have multiple hybridization events created *G. nigra* if the two parent species do not co-occur? It is possible that the answer to this question lies in the unique characteristics of aquatic habitats in arid environments. Long periods of drought or minimal precipitation may create physically disjunct populations as rivers retreat to headwater regions. During wet periods that cause massive flooding events, previously disconnected tributaries can become reconnected to the main reaches of larger rivers. A physical connection between waterways allows for gene flow and the opportunity for hybridization between previously isolated species. The microsatellites that have been used to genetically differentiate these three species (and geographic populations; Dowling *et al.*, 2015) are rapidly evolving markers; thus, populations may become genetically distinct (through drift or selection) during dry periods when populations are physically disjunct, but genetic differences may deteriorate *via* interbreeding during wet periods as previously isolated populations become sympatric. Indeed Douglas *et al.* (1999) speculated that phenotypic variation in *G. robusta*, *G. intermedia* and *G. nigra* is the result of vicariance events that occurred in the late Miocene.

The findings of this study, in combination with the previous findings of minimal genetic differences among species raise the question: how are these three species morphologically different if they are not genetically different? There are two primary mechanisms that could result in *G. intermedia*, *G. nigra and G. robusta* occupying distinct morphospaces even if they are not genetically distinct. The first possibility is that the three species may represent morphotypes of a single species. Indeed the analysis used here (CVA) maximizes the morphological differences among groups, so populations may be statistically different, even though there are no biologically significant differences (*e.g.* no difference in swimming performance, reproductive behaviours, *etc.*) among the individuals. A second possibility is that microsatellite markers do not provide accurate information about relationships among species because the rapid mutation rate of microsatellite markers creates homoplasy, which obscures true genetic relationships (Dowling *et al.*, 2015).

According to the biological species concept, a subspecies is defined as having partial reproductive isolation from the parent species populations. Under the phylogenetic species concept, however, species level designations, as indicated by molecular markers, are the smallest monophyletic unit; therefore subspecies are not recognized under the phylogenetic species concept (Haig *et al.*, 2006). An ecotype was a term originally described by the Swedish botanist Turesson (1922) and this term has been widely used to describe genetically distinct populations that are adapted to their local environments. Among the three species that form the *G. robusta* complex, genetic distinctiveness may be driven by vicariance events and habitat may influence morphology in temporarily disconnected populations. Because these three species are not permanently isolated, however, and they are not clearly distinct in genetics or morphology, a subspecies or ecotype designation may be warranted. These alternative designations would not change the management status for these populations because the U.S. Endangered Species Act (ESA) of 1973 protects 'any distinct population of any species of vertebrate fish or wildlife, which interbreeds when mature' (Waples, 1991). Given that 25%

of the endangered species protected by the ESA are currently described as subspecies, protective status for *G. robusta*, *G. intermedia* and *G. nigra* can still be obtained at the subspecies level if managers pursue this designation.

Although Mayr (1963) suggested that morphological changes follow reproductive isolation, he also acknowledged that evolution of distinct body plans may be a first step in species evolution. Evidence for this pattern is provided by species flocks, like Darwin's finches, East African rift-lake cichlids and Lake Tana (Ethiopia) barbs, all of which show surprisingly little variation in genetics (Mayr, 1984). The three species of *Gila* may not have not been physically separate from one another long enough to acquire genetic differences. Alternately, hybridization may be occurring among these three species; in which case, genetic introgression could be acting to obscure genetic differences among the taxa. In light of this, it is difficult to decide if the three species should be retained as valid, recognized as a single species, or considered as subspecies. The morphological differences described here, however, in combination with the conservation concerns of the aquatic habitats of the south-west U.S.A., suggest that the three species should be recognised as distinct from one another and that further examination of these species is warranted.

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References

Abràmoff, M. D., Magalhães, P. J. & Ram, S. J. (2004). Image processing with ImageJ. *Biophotonics International* **11,** 36–43.

Armbruster, J. W. (2012). Standardized measurements, landmarks and meristic counts for cypriniform fishes. *Zootaxa* **3586,** 8–16.

Baird, S. F. & Girard, C. (1853*a*). Descriptions of some new fishes from the River Zuni. *Proceedings of the Academy of Natural Sciences, Philadelphia* **6,** 368–369.

Baird, S. F. & Girard, C. (1853*b*). Descriptions of new species of fishes collected by Mr. John H. Clark, on the US and Mexican boundary Survey, under Lt. Col. Jas. D. Graham. *Proceedings of the Academy of Natural Sciences, Philadelphia* **4,** 387–390.

Baird, S. F. & Girard, C. (1856). Descriptions of new species of fishes collected by Mr. John H. Clark, on the US and Mexican boundary Survey, under Lt. Col. Jas. D. Graham. *Proceedings of the Academy of Natural Sciences, Philadelphia* **8,** 205–207.

Cope, E. D. (1875). Report upon the collections of fishes made in portions of Nevada, Utah, California, Colorado, New Mexico and Arizona: during the years 1871, 1872, 1873 and 1874. *Geographical Geological Exploratory Survey West of the 100th Meridian* **5,** 660–672.

Cope, E. D. & Yarrow, H. C. (1875). Report upon the collections of fishes made in portions of Nevada, Utah, California, Colorado, New Mexico and Arizona, during the years 1871, 1872, 1873 and 1874. *Report upon United States Geographical Surveys West of the 100th Meridian* **5,** 635–703.

Copus, J. M., Foresman, Z., Montgomery, W. L., Bowen, B. W. & Toonen, R. J. (2016). Revision of the *Gila robusta* (Teleostei, Cyprinidae) species complex: morphological examination and molecular phylogenetics reveal a single species. *Report to the Joint ASIH-AFS Committee on Names of Fishes*. Washington, D.C.: Department of the Interior, Fish and Wildlife Service.

Dawley, R. M. & Bogart, J. P. (1989). *Evolution and Ecology of Unisexual Vertebrates*. Albany, NY: University of the State of New York, State Education Dept., New York State Museum.
DeMarais, B. D. (1986). Morphological variation in *Gila* (Pisces: Cyprinidae) and geologic history: lower Colorado river basin. Master's Thesis, Arizona State University, Phoenix, AZ. Available at http://www.nativefishlab.net/library/textpdf/12778.pdf/
DeMarais, B. D. (1992). Genetic relationships among fishes allied to the genus *Gila* (Teleostei: Cyprinidae) from the American southwest. PhD Thesis, Arizona State University, Phoenix, AZ.
Donoghue, M. J. (1985). A critique of the biological species concept and recommendations for a phylogenetic alternative. *Bryologist* **88,** 172–181.
Douglas, M. E., Minckley, W. L. & DeMarais, B. D. (1999). Did vicariance mold phenotypes of western North American fishes? Evidence from Gila River cyprinids. *Evolution* **53,** 238–246.
Dowling, T. E., Anderson, C. D., Marsh, P. C. & Rosenberg, M. S. (2015). Population structure in the roundtail Chub (*Gila robusta* complex) of the Gila River Basin as determined by microsatellites: evolutionary and conservation implications. *PLoS One* **10,** 1–21.
Fisher, R. A. (1936). The use of multiple measurements in taxonomic problems. *Annals of Eugenics* **7,** 179–188.
Gerber, A. S., Tibbets, C. A. & Dowling, T. E. (2001). The role of introgressive hybridization in the evolution of the *Gila robusta* complex (Teleostei: Cyprinidae). *Evolution* **55,** 2028–2039.
Haig, S. M., Beever, E. A., Chambers, S. M., Draheim, H. M., Dugger, B. D., Dunham, S., Elliott-Smith, E., Fontaine, J. B., Kesler, D. C., Knaus, B. J., Lopes, L. F., Loschl, P., Mullins, T. D. & Sheffield, L. M. (2006). Taxonomic considerations in listing subspecies under the US Endangered Species Act. *Conservation Biology* **20,** 1584–1594.
Imre, I., McLaughlin, R. L. & Noakes, D. L. G. (2002). Phenotypic plasticity in brook charr: changes in caudal fin induced by water flow. *Journal of Fish Biology* **61,** 1171–1181.
Klingenberg, C. P. (2011). MorphoJ: an integrated software package for geometric morphometrics. *Molecular Ecology Resources* **11,** 353–357.
Klingenberg, C. P. & Monteiro, L. R. (2005). Distances and directions in multidimensional shape spaces: implications for morphometric applications. *Systematic Biology* **54,** 678–688.
Marsh, P. C., Clarkson, R. W. & Dowling, T. E. (2017). Molecular genetics informs spatial segregation of two desert stream *Gila* species. *Transactions of the American Fisheries Society* **146,** 47–59.
Mayr, E. (1963). *Animal Species and Evolution*, Vol. 797. Cambridge, MA: Harvard University Press.
Mayr, E. (1984). Evolution of fish species flocks: a commentary. In *Evolution of Fish Species Flocks* (Echelle, A. A. & Kornfield, I., eds), pp. 3–12. Orono, ME: University of Maine at Orono Press.
McLaughlin, R. L. & Grant, J. W. (1994). Morphological and behavioural differences among recently-emerged brook charr, *Salvelinus fontinalis*, foraging in slow *vs*. fast-running water. *Environmental Biology of Fishes* **39,** 289–300.
Miller, R. R. (1945). A new cyprinid fish from southern Arizona and Sonora, Mexico, with the description of a new subgenus of *Gila* and a review of related species. *Copeia* **1945,** 104–110.
Minckley, W. L. & DeMarais, B. D. (2000). Taxonomy of chubs (Teleostei, Cyprinidae, Genus *Gila*) in the American southwest with comments on conservation. *Copeia* **2000,** 251–256.
Owen, J. G. & Chmielewski, M. A. (1985). On canonical variates ellipses and the construction of confidence ellipses in systematic studies. *Systematic Zoology* **34,** 366–374.
Rinne, J. N. (1976). Cyprinid fishes of the genus *Gila* from the lower Colorado River basin. *Wassman Journal of Biology* **34,** 65–107.
Robins, C. R., Bailey, R. M., Bond, C. E., Brooker, J. R., Lachner, E. A., Lea, R. N. & Scott, W. B. (1980). A list of common and scientific names of fishes from the United States and Canada. *American Fisheries Society Special Publication* **12,** 1–174.
Schönhuth, S., Perdices, A., Lozano-Vilano, L., García-de-León, F. J., Espinosa, H. & Mayden, R. L. (2014). Phylogenetic relationships of North American western chubs of the genus

Gila (Cyprinidae, Teleostei), with emphasis on southern species. *Molecular Phylogenetics and Evolution* **70,** 210–230.

Schwemm, M. R. (2006). Genetic variation in the *Gila robusta* complex (Teleostei: Cyprinidae) in the lower Colorado River. MSc Thesis, Arizona State University, Phoenix, AZ. Available at https://www.usbr.gov/lc/phoenix/biology/azfish/pdf/SchwemmMRtheGilaRrobustacomplex.pdf/

Turesson, G. (1922). The genotypical response of the plant species to the habitat. *Hereditas* **3,** 211–235.

Electronic References

Congress (1973). *The Endangered Species Act*. Washington, D.C.: U.S. Department of the Interior, Fish and Wildlife and Service. Available at https://www.fws.gov/endangered/esa-library/pdf/ESAall.pdf/

Crowder, C., Meka-Carter, J., Leavitt, D. & Hickerson, B. (2015). Does observer bias affect species identification of members of the roundtail chub complex? *In The Current* **1,** 1–4. Phoenix, AZ: Arizona Game and Fish Department. Available at https://www.azgfd.com/PortalImages/files/WildlifeNewsletters/InTheCurrent_Winter2015issue_Final.pdf/

Farrington, M. A., Brandenburg, W. H. & Kennedy, J. L. (2013). Determining the historical distribution of the *Gila robusta* complex (*Gila* chub, *Gila intermedia*, headwater chub, *Gila nigra* and roundtail chub, *Gila robusta*) using morphological analysis in the Gila River Basin, New Mexico. *Draft Final Report submitted to New Mexico Department of Game and Fish*. Available at http://www.bison-m.org/documents/49115_Brandenburg2015.pdf/

Page, L. M., Baldwin, C. C., Espinosa-Perez, H., Gilbert, C. R., Hartel, K. E., Lea, R. N, Mandrak, J. J., Schmitter-Soto, J. J. & Walker, H. J. (2016). Final report of the AFS/ASIH Joint Committee on the Names of Fishes on the taxonomy of *Gila* in the lower Colorado River basin of Arizona and New Mexico. *Report to the Arizona Game and Fish Department*. Available at www.inthecurrent.org/wp-content/uploads/2016/09/Final-report-to-Arizona-Game-and-Fish-Department.1-Sept.2016.pdf

Rohlf, F. J. (2004). *TpsDig–Thin Plate Spline Digitise, Version 1.4*. Stony Brook, NY: Dept. of Ecology and Evolution, State University of New York. Available at life.bio.sunysb.edu/morph/soft-tps.html

Waples, R. S. (1991). Definition of "species" under the Endangered Species Act: application to Pacific salmon. *NOAA Technical Memorandum NMFS* **194**. Available at https://docs.lib.noaa.gov/noaa_documents/NMFS/TM_NMFS_FNWC/TM_NMFS_FNWC_194_files/TM_NMFS_FNWC_194.htm/