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Classification Success of Species within the *Gila robusta* Complex Using Morphometric and Meristic Characters—A Reexamination

Julie Meka Carter[1], Matthew J. Clement[2], Andy S. Makinster[3,4], Clayton D. Crowder[3], and Brian T. Hickerson[3,5]

Three cyprinids often referred to as the *Gila robusta* complex, *G. robusta*, *G. nigra*, and *G. intermedia*, are morphologically similar and genetically indistinguishable at the currently recognized species level. Current taxonomy is based on purported morphometric and meristic differences that are detailed in a classification key; however, the ability of the key to reliably distinguish the species has recently come into question. Chubs were collected from locations in Arizona, and two analysis methods were used to predict species' identification success using the key: 1) correct assignment to species using cluster analysis and multinomial logistic regression; and 2) observer identification success by species. Cluster analysis and multinomial logistic regression correctly assigned only 62% and 74% of fish, respectively, to the assumed species designation. Identification success using both analysis methods was most successful for *G. robusta* (82% cluster; 82% regression), followed by *G. intermedia* (53% cluster; 80% regression), and *G. nigra* (49% cluster; 58% regression). Overall observer identification success was 54%, led by *G. intermedia* (68%), followed by *G. robusta* (63%) and *G. nigra* (33%). The high level of misidentification appears to be due to overlap in morphometric and meristic characters among assumed species groups. Although the three species are currently considered allopatric, sympatry was found in 88%, 76%, and 100% of locations in the cluster analysis, regression analysis, and observer analysis, respectively. These results indicate that the morphometric and meristic characters in the key do not consistently distinguish the three putative chub species. Because independent genetic analyses also fail to support the delineation of the three species, we consider *G. robusta* as a single polymorphic species a viable hypothesis. Furthermore, a recent formal taxonomic review of the three species conducted by the American Fisheries Society–American Society of Ichthyologists and Herpetologists Committee on Names of Fishes concluded the available morphological and genetic data (including a pre-publication version of this study) support recognition of only one species, *G. robusta*.

THREE cyprinids within the genus *Gila* (*G. robusta* [Roundtail Chub], *G. nigra* [Headwater Chub], and *G. intermedia* [Gila Chub]), also referred to as the *G. robusta* complex, occupy the Agua Fria, Bill Williams, Gila, Little Colorado, Salt, San Pedro, Santa Cruz, and Verde rivers and their tributaries in the lower Colorado River basin of Arizona and New Mexico (Rinne, 1976; DeMarais, 1986; Minckley and DeMarais, 2000). The taxonomic history of the *G. robusta* complex has evolved over time, from recognition as two subspecies of *G. robusta* (*G. r. robusta* and *G. r. intermedia;* Miller, 1945), as two unique species with one subspecies of *G. robusta* (*G. robusta, G. r. grahami, G. intermedia;* Rinne, 1969, 1976), two species with one intergrade (*G. r. grahami*) through hybridization between *G. robusta* and *G. intermedia* (DeMarais, 1986), and most recently three distinct species (*G. robusta, G. nigra, G. intermedia*; Minckley and DeMarais, 2000). Each of these taxonomic arrangements has been generally accepted at one time (Robins et al., 1980, 1991; Nelson et al., 2004; Page et al., 2013). The taxonomic ambiguity of the *G. robusta* complex has proved challenging for decades because the chubs are extremely similar in appearance, prompting multiple studies evaluating the genetic and morphological variation among species and populations within the complex (Rinne, 1969, 1976; DeMarais, 1986, 1992; Minckley and DeMarais, 2000; Gerber et al., 2001; Schwemm, 2006; Schonhuth et al., 2014; Brandenburg et al., 2015; Copus et al., 2016; Marsh et al., 2017; Moran et al., 2017).

A classification key was developed by Rinne (1969, 1976) that primarily relied on morphometric and meristic characters to distinguish among species of the *G. robusta* complex and *G. elegans* (Bonytail Chub). The recommendation for full species status for *G. intermedia* was proposed by Rinne (1969, 1976) based on the development of this key and its ability to distinguish *G. intermedia* from *G. robusta*. The key was modified by Minckley and DeMarais (2000) who proposed full species status for *G. nigra* based on morphological differences from *G. robusta* and *G. intermedia*, and described *G. nigra* as an intermediate form derived through past hybridization between *G. robusta* and *G. intermedia*. The key and corresponding distribution map of the *G. robusta* complex in the Gila River basin in Arizona and New Mexico in Minckley and DeMarais (2000) has been the accepted standard of the species' divergence and distribution, identifying them as allopatric in all cases. For the chubs in the Bill Williams and Little Colorado rivers in Arizona, which were not included in Minckley and DeMarais (2000), species identification by location is generally inferred from Rinne (1969, 1976). Although these studies were the impetus to the full species designations of *G. intermedia* and *G. nigra*, each documented considerable overlap in the morphometric and meristic characters used to distinguish the species (Rinne, 1969, 1976; DeMarais, 1986; Minckley and DeMarais, 2000). The

[1] Aquatic Wildlife Branch, Arizona Game and Fish Department, 5000 W. Carefree Highway, Phoenix, Arizona 85086; Email: jcarter@azgfd.gov. Send reprint requests to this address.
[2] Research Branch, Arizona Game and Fish Department, 5000 W. Carefree Highway, Phoenix, Arizona 85086; Email: mclement@azgfd.gov.
[3] Habitat, Evaluation, and Lands Branch, Arizona Game and Fish Department, 5000 W. Carefree Highway, Phoenix, Arizona 85086; Email: (CDC) ccrowder@azgfd.gov.
[4] Present address: 5230 Harris Creek Drive, Cumming, Georgia 30040; Email: amakinster50@gmail.com.
[5] Present address: University of Wyoming, Wyoming Cooperative Fish and Wildlife Research Unit, 1000 E. University Ave, Laramie, Wyoming 82071; Email: bhicker1@uwyo.edu.
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wide range of morphometric and meristic values for each species and the fact that some values in the key are based on population means makes the identification of individual chub specimens and even populations challenging.

Genetic studies have not been able to provide clarity to the taxonomic status of the *G. robusta* complex. Despite using nuclear, mitochondrial, and microsatellite DNA techniques, studies have been unable to corroborate the existence of three genetically distinct species in accordance with the species designations and distribution consistent with Minckley and DeMarais (2000; DeMarais, 1992; Gerber et al., 2001; Schwemm, 2006; Schonhuth et al., 2014; Dowling et al., 2015). One study found genetic variation to be greater among populations within species than among species, and variation to be greater within drainages than among drainages (Dowling et al., 2015). The lack of genetic markers within the *G. robusta* complex makes the reliability of the Minckley and DeMarais (2000) key (hereafter, the "key"), extremely important, especially in a conservation and management context.

The utility of the key is also important because chub populations have been discovered that were not known or were not presented in the key and associated species location map. Because populations of *G. robusta*, *G. nigra*, and/or *G. intermedia* occur within a number of the same drainages (e.g., Verde River, Salt River, and the Gila River), the key is the only tool currently available to purportedly identify individual fish and/or populations to species. A recent study that applied the key to museum specimens to determine the historical range of chub populations in New Mexico, including some used by Rinne (1969), identified multiple species at sample sites (Brandenburg et al., 2015), which contradicts the view that the chub species are currently allopatric (Minckley and DeMarais, 2000). Uncertainty in distinguishing among chub species has profound impacts on the ability to manage the species and implement conservation actions, particularly when Endangered Species Act (ESA) designations are applied. *Gila intermedia* was listed as Endangered with Critical Habitat in 2005, and in 2015 *G. robusta* and *G. nigra* were proposed to be listed as Threatened by the U.S. Fish and Wildlife Service (USFWS, 2005, 2015a).

The objectives of this study were to determine: 1) the ability of select morphometric and meristic characters in the key to accurately classify fish to species using cluster analysis and multinomial logistic regression; and 2) the effectiveness of the key to accurately identify fish to the current species designation by observers without knowledge of capture location or current species designation. The two morphometric ratios and three meristics recognized as being the most important for species identification were used for analyses (Rinne, 1969, 1976; Minckley and DeMarais, 2000). For the purposes of this study, we refer to current species designations per location as assumed species designations (Minckley and DeMarais, 2000; USFWS, 2015a, 2015b).

MATERIALS AND METHODS

Sampling.—Chub were collected from 14 wild and three captive localities (e.g., refuge population in pond, population in hatchery) within the Agua Fria, Bill Williams, Gila, Salt, San Pedro, Santa Cruz, and Verde river drainages in Arizona, with a goal to capture ten fish per location (Fig. 1). A total of 162 specimens >100 ml TL were captured. Due to damaged fish or incomplete measurements, 149 specimens were used in quantitative analyses with the following assumed species designations: 55 *G. robusta*, 43 *G. nigra*, and 51 *G. intermedia* (Table 1). A subset of the 149 specimens ($n = 89$) were identified to species by observers using the key (*G. robusta* $n = 30$, 5 capture locations; *G. nigra* $n = 29$, 5 capture locations; *G. intermedia* $n = 30$, 5 capture locations; Table 1).

Chub from all locations were used in the quantitative analysis and 15 locations were used in the observer analysis, including the 14 wild localities. Two captive localities were excluded because fish specimens were collected after the observer analysis was completed. We collected adult chub, defined as those greater than 100 mm in total length, to reduce variation of measurements due to allometric growth (Rinne, 1969, 1976). Chubs were captured between 7 October and 3 December 2014, using a combination of backpack electrofishing, collapsible hoop nets, and hook and line methods. After capture, all specimens were labeled according to the collection locality and assumed species designation, placed in individual bags, and frozen live (Benson McRae, 2007; Simon et al., 2010; González-Castro et al., 2012; Dodson et al., 2015). Specimens were transported to the Arizona Game and Fish Department laboratory, and each was randomly assigned an identification number for use in subsequent analyses. Each fish was thawed on ice prior to analysis.

Morphometric and meristic characters were recorded from the left lateral side of each specimen using metric rulers (mm) and stereomicroscopes. The characters used in the analyses were those from the key recognized as sufficient to identify *G. robusta* complex chub to species: head length (HL), least depth of caudal peduncle (LDCP), caudal peduncle length (CPL), principal dorsal-fin rays (DFR), principal anal-fin rays (AFR), and pored lateral line scales (LLS; Rinne, 1976; Minckley and DeMarais, 2000). For quantitative analyses, all measurements were made by one individual, and specimens were labeled only with identification numbers. We used Analysis of Variance (ANOVA) to test for significant differences among assumed species designations in mean measurements and counts of each character and Tukey HSD tests for pairwise comparisons ($\alpha = 0.05$).

Cluster analysis.—We conducted a cluster analysis to assess the number of distinct groups among chub collected for this study based on morphometric and meristic characters (Romesburg, 2004). Cluster analysis is an unsupervised learning technique that attempts to separate data points into groups of similar specimens by evaluating the latent structure in the data, without reference to known group membership (Fraley and Raftery, 1998). This approach does not presuppose that any of the previous taxonomic designations are correct. Instead, the analysis is a means to estimate an appropriate number of groups based on morphometric and meristic characters, as well as the distinctions between groups.

Prior to the hierarchical agglomerative cluster analysis (hereafter, cluster analysis), we reviewed and transformed the morphological data. First, we used boxplots to examine the data for outliers. As expected, measurements were strongly correlated with total length of specimens. Total length was used because it is easier to measure in the field than standard length and is a reliable measurement to use for length (Önsoy et al., 2011). To allow for comparisons, total length was also used in the quantitative analysis. Therefore, we standardized morphometric data according to:

$$Y'_{ij} = Y_{ij}\left(\frac{\overline{TL}}{TL_i}\right)^{v_j}$$

where Y'_{ij} is the adjusted size of character j for individual i, Y_{ij}

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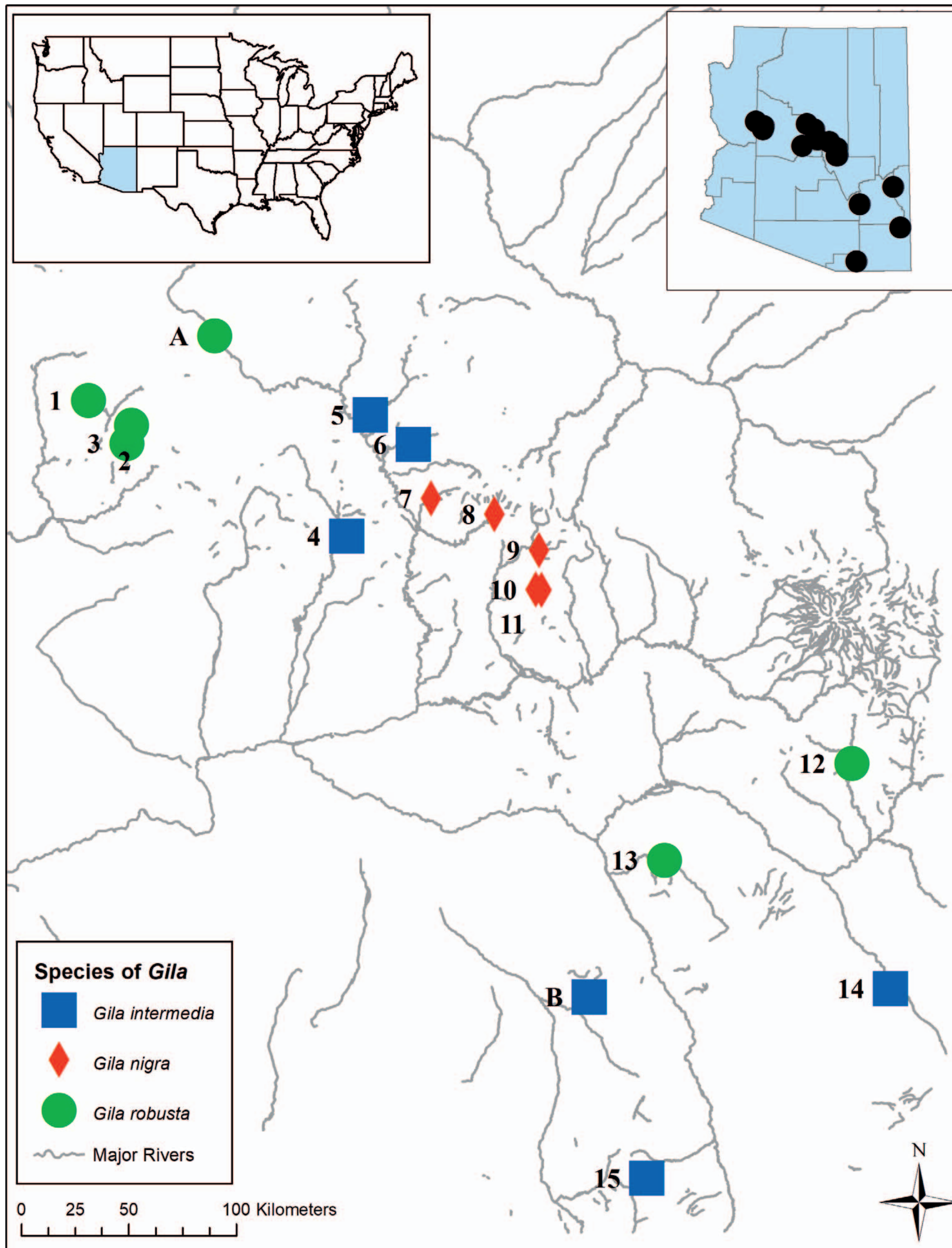

Fig. 1. Collection locations of *G. robusta*, *G. nigra*, and *G. intermedia* in Arizona; chub were collected from October to December 2014 (observer and quantitative analyses: 1 = Francis Creek; 2 = Wilder Creek; 3 = Boulder Creek; 4 = Silver Creek; 5 = Spring Creek [Verde River]; 6 = Walker Creek; 7 = Fossil Creek; 8 = East Verde River; 9 = Haigler Creek; 10 = Rock Creek; 11 = Spring Creek [Salt River]; 12 = Eagle Creek; 13 = Aravaipa Creek; 14 = Hot Springs Canyon; 15 = O'Donnell Canyon; quantitative analysis only: A = Verde River; B = Sabino Creek).

is the original character size, v_j is the linear regression slope relating the log of character j and the log of total length, TL_i is the total length of individual i, and $\overline{TL}$ is the mean total length for all individuals (Elliott et al., 1995). After standardizing morphometric data, we used a standard z-transformation to center and scale both morphometric and meristic data (Romesburg, 2004). Scaling data in this way is important so that distance measures are scale invariant, i.e., not dependent on the different measurement scales used for different morphometric and meristic characters (Romesburg, 2004). We also report the HL/LDCP and CPL/LDCP ratios for comparison with previous studies.

After standardizing, centering, and scaling the data, we used cluster analysis (Legendre and Legendre, 2012) to organize specimens into groups based on overall similarity of morphology. We selected cluster analysis because it is widely used in ecological studies (Legendre and Legendre, 2012) and because it avoids the restrictive distributional assumptions of other popular methods, such as k-means clusters (Fraley and Raftery, 1998). Under cluster analysis, all observations (fish specimens) are initially divided into clusters of size one, and then similar clusters are combined. Combination of clusters is guided by the dissimilarity of individual cases and a clustering algorithm (Romesburg, 2004). Dissimilarity of individuals can be measured by one of several distance measures. We used Euclidean distance, which measures the straight-line distance between data points, so that all measured characters contribute to the distance metric. We then used the complete linkage clustering algorithm (Legendre and Legendre, 2012). This algorithm evaluates the pairwise distance between each element in two clusters and uses the maximum distance between elements as the distance between the clusters. The two clusters with the minimum distance are combined and distances are recalculated. This approach yields relatively compact clusters. We explored additional clustering algorithms (e.g., single linkage, average linkage), but complete linkage provided the highest classification success. The clustering process yields a

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Table 1. Summary of data collected including capture locality, assumed species designation, watershed of capture location, the total number of fish collected and used in the quantitative analysis (Q; cluster; multinomial logistic regression), and the number of specimens used in the observer analysis (O; the number of observations of those specimens in parentheses). Measurement and count values include: total length range of specimens within each capture location in millimeters; HL/LDCP: mean and range of the head length divided by the least depth caudal peduncle; CPL/LDCP: mean and range of the caudal peduncle length divided by the least depth caudal peduncle; DFR: mean and range of dorsal-fin ray counts; AFR: mean and range of anal-fin ray counts; and LLS: mean and range of lateral line scale counts. Captive populations indicated by a *.

Locality	Species	Watershed	Analysis	Total *n*	TL (mm)	HL/LDCP	CPL/LDCP	DFR	AFR	LLS
Aravaipa Creek	*G. robusta*	San Pedro River	Q	10	129–169	3.1 (3.0–3.4)	2.6 (2.1–3.0)	8.9 (8–9)	8.9 (8–9)	82.2 (79–86)
			O	6 (34)	137–155	3.0 (2.6–4.1)	2.3 (1.5–3.5)	9.0 (8–10)	8.9 (8–10)	77.8 (67–88
Boulder Creek	*G. robusta*	Bill Williams River	Q	8	107–180	3.0 (2.8–3.2)	2.6 (2.3–2.8)	8.7 (8–9)	8.4 (8–9)	81.5 (75–87)
			O	6 (29)	114–167	2.9 (1.7–3.3)	2.4 (1.7–2.8)	8.7 (7–10)	8.1 (7–9)	79.0 (62–96)
Eagle Creek*	*G. robusta*	Gila River	Q	10	114–142	3.2 (3.0–3.5)	2.9 (2.3–3.2)	9.0 (9)	8.6 (8–9)	90.1 (85–97)
			O	6 (31)	120–144	3.2 (2.6–4.0)	2.7 (2.0–3.3)	8.9 (8–10)	8.4 (7–9)	85.8 (57–97)
Francis Creek	*G. robusta*	Bill Williams River	Q	8	121–195	3.1 (2.7–3.3)	2.6 (2.3–2.9)	9.0 (9)	8.5 (8–9)	84.7 (80–96)
			O	6 (33)	131–162	3.0 (2.4–3.4)	2.3 (1.7–2.9)	8.7 (7–9)	8.1 (7–9)	83.0 (64–99)
Verde River*	*G. robusta*	Verde River	Q	9	103–137	3.1 (2.9–3.4)	2.9 (2.6–3.2)	9.0 (8–10)	8.9 (8–9)	86.4 (79–93)
Wilder Creek	*G. robusta*	Bill Williams River	Q	10	114–157	3.3 (2.7–4.1)	2.7 (2.4–3.1)	8.9 (8–9)	8.6 (8–9)	89.0 (78–96)
			O	6 (31)	113–160	3.3 (2.7–4.0)	2.4 (1.4–3.2)	9.0 (8–10)	8.7 (8–9)	85.9 (75–94)
East Verde River	*G. nigra*	Verde River	Q	4	159–193	2.8 (2.6–2.9)	2.5 (2.4–2.6)	8.5 (8–9)	8.2 (8–9)	81.5 (80–84)
			O	5 (30)	159–205	2.8 (2.2–4.3)	2.4 (1.7–3.7)	8.8 (8–9)	8.2 (8–9)	79.0 (71–93)
Fossil Creek	*G. nigra*	Verde River	Q	10	120–176	3.1 (2.6–3.4)	2.6 (2.3–3.0)	9.0 (9)	8.4 (8–9)	85.1 (75–94)
			O	6 (29)	124–153	3.0 (2.6–3.8)	2.3 (1.8–3.0)	8.8 (7–9)	8.5 (7–9)	79.3 (53–89)
Haigler Creek	*G. nigra*	Salt River	Q	10	134–181	2.8 (2.4–3.1)	2.6 (2.0–3.0)	8.6 (8–9)	7.9 (7–8)	79.6 (66–89)
			O	6 (30)	138–160	2.8 (1.9–3.5)	2.4 (1.8–3.1)	8.6 (7–10)	8.1 (6–9)	80.0 (65–100)
Rock Creek	*G. nigra*	Salt River	Q	10	119–172	2.8 (2.5–3.1)	2.5 (2.1–3.1)	8.1 (8–9)	8.1 (8–9)	83.1 (78–95)
			O	6 (32)	121–163	2.8 (2.2–3.4)	2.4 (1.9–3.2)	8.0 (7–9)	8.0 (7–9)	81.9 (63–92)
Spring Creek	*G. nigra*	Salt River	Q	9	112–196	3.0 (2.7–3.2)	2.6 (2.4–2.8)	8.0 (8)	8.0 (8)	83.4 (75–92)
			O	6 (32)	125–155	2.9 (2.4–3.5)	2.3 (1.7–3.0)	8.1 (7–9)	8.2 (7–9)	80.7 (69–90)
Hot Springs Canyon	*G. intermedia*	San Pedro River	Q	10	126–182	2.9 (2.5–3.2)	2.6 (1.8–2.8)	8.0 (8)	7.8 (7–8)	71.4 (63–81)
			O	6 (31)	123–160	2.7 (1.6–3.4)	2.1 (1.3–2.5)	7.9 (7–9)	7.9 (6–8)	66.7 (50–76)
O'Donnell Canyon*	*G. intermedia*	San Pedro River	Q	3	116–124	3.2 (3.0–3.6)	2.7 (2.6–3.0)	8.0 (8)	8.0 (8)	71.3 (70–72)
	G. intermedia	San Pedro River	Q	5	130–151	2.7 (2.5–2.8)	2.5 (2.4–2.7)	8.0 (8)	8.0 (8)	72.2 (63–82)
			O	6 (24)	132–147	2.4 (1.7–3.1)	2.0 (1.4–3.1)	8.0 (7–9)	7.9 (7–9)	69.6 (60–76)
Sabino Creek	*G. intermedia*	Santa Cruz River	Q	8	109–135	2.8 (2.5–3.1)	2.2 (2.0–2.6)	7.9 (7–8)	7.9 (7–8)	69.0 (65–73)
Silver Creek	*G. intermedia*	Agua Fria River	Q	7	109–179	2.7 (2.5–3.1)	2.3 (1.9–2.6)	9.0 (8–10)	8.0 (8)	74.0 (65–81)
			O	6 (29)	105–180	2.7 (2.3–3.0)	2.1 (1.5–3.1)	8.8 (8–10)	8.0 (6–9)	75.0 (57–96)
Spring Creek	*G. intermedia*	Verde River	Q	9	109–150	2.9 (2.4–3.2)	2.6 (2.3–3.0)	8.1 (8–9)	7.9 (7–9)	76.3 (67–82)
			O	6 (25)	122–148	2.7 (2.1–3.2)	2.5 (1.8–3.0)	8.0 (7–9)	7.8 (6–9)	75.0 (54–87)
Walker Creek	*G. intermedia*	Verde River	Q	9	120–154	3.0 (2.6–3.5)	2.7 (2.4–2.8)	8.2 (8–9)	7.8 (7–9)	78.9 (66–88)
			O	6 (31)	131–150	2.9 (2.2–3.5)	2.5 (1.8–3.2)	8.3 (7–10)	8.1 (7–9)	77.0 (62–89)

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phenogram that displays both the sequence of cluster combinations and the distance between clusters. We also visually reviewed the phenograms and scatterplots to check the validity of the groupings (Hennig, 2007; Jackson et al., 2010).

We generated the hierarchical clustering phenogram using the "hclust" function in Program R (R Core Team, 2016). While the phenogram identifies which specimens share similar morphology, it does not identify the most appropriate number of clusters. As a first step, we visually reviewed the phenogram for relatively long branches, indicating distinct groups. For a more quantitative approach, we divided the phenogram into $K = 2, 3, \ldots 15$ groups and calculated the Calinski-Harabasz (CH) index for each division (Calinski and Harabasz, 1974). The CH index reflects the ratio of between-group variation to within-group variation in morphological characters and ranges from 0 to ∞. Therefore, the value of K with the highest CH index generates the most distinct groups. After identifying the most distinctive groups, we identified the morphometric and meristic characters that distinguished those groups.

While the CH index indicates which groupings are relatively distinct, it does not assure that the groups are meaningful. In particular, unsupervised classification methods can generate clusters from homogenous data sets (Ben-Hur and Guyon, 2003). Therefore, we also evaluated cluster stability. The idea behind cluster stability analysis is that meaningful groups should be robust to small changes in data sets (Lange et al., 2004). First, we used bootstrap resampling to generate small changes in the data set. Then, we used the Jaccard coefficient to assess stability. The Jaccard coefficient indicates the proportion of elements that two groups have in common and therefore ranges from 0 to 1 (Hennig, 2007). In this case, the original groups generated by the cluster analysis are compared to revised groups obtained by analyzing the bootstrapped data, with values >0.85 indicating stable clusters (Hennig, 2007). We bootstrapped the data and calculated the Jaccard coefficient 100 times using the "clusterboot" function from the "fpc" package (Hennig, 2015) in Program R. Finally, we generated a confusion matrix to compare the groups generated by the hierarchical cluster analysis to the assumed species designation. We also generated a confusion matrix to compare the cluster analysis results to the collection location to assess the possibility that there are several morphologically distinct, allopatric species, but they differ in distribution from the Minckley and DeMarais (2000) map.

Multinomial logistic regression.—We also used multinomial logistic regression to assess whether unknown specimens could be identified using morphometric and meristic characters and the assumed species designations of Minckley and DeMarais (2000). Multinomial logistic regression estimates the relationship between selected covariates (in this case morpho-meristic data) and the log-odds that a specimen belongs to one of the species of interest (Agresti, 2002). Log-odds yield probability estimates after an inverse-logit transformation, i.e., $p = \exp(\alpha)/(\exp(\alpha)+1)$, where p is probability and α is the log-odds. In contrast to the cluster analysis, multinomial logistic regression is a supervised learning method because the model is developed with reference to known group membership (Agresti, 2002), in this case the assumed species designation. Because multinomial logistic regression incorporates additional information (assumed species designation), identification success is expected to be higher than for cluster analysis. However, results will not be reliable if assumed species designations are not correct. Multinomial logistic regression is appropriate for a response variable that has more than two categorical outcomes, such as species classes. We selected multinomial logistic regression because it avoids the multivariate normality assumption of other popular methods, such as discriminant function analysis (Bull and Donner, 1987). For this analysis, we relied on the distribution map of Minckley and DeMarais (2000) for streams in the Gila River basin to determine the assumed species designations because it has been used as an authoritative source (USFWS, 2015a, 2015b) and because it represents a testable biological hypothesis: specimens of *Gila* from this study include three morphologically distinct, allopatric species. The assumed species designations for chubs from the Bill Williams and Little Colorado river drainages were inferred by Rinne (1969, 1976). If the hypothesis is correct and the key effectively distinguishes the three species, then multinomial logistic regression should show high agreement with species distribution shown on the map.

Prior to model-fitting, we centered and scaled all morphological data after size-correcting the morphometric data, as previously described. We developed a circumscribed list of regression models before performing model fitting and selection. The models we considered varied in the number of morphological features used for prediction, with a preference for including LDCP and LLS, based on the results of previous analyses of chub species (Douglas et al., 1998; Minckley and DeMarais, 2000). We used Akaike information criterion (AIC) to select the most parsimonious model from the model set (Burnham and Anderson, 2002). We used 10-fold cross validation to estimate prediction accuracy for the top model (Fielding and Bell, 1997) and checked goodness-of-fit using three Hosmer and Lemeshow tests (Hosmer and Lemeshow, 2000) to assess fit for each species individually. When necessary, we combined deciles to ensure that the predicted number of fish in a decile was >2. Finally, we reported on the morphological features that distinguished the species, and the overall correct identification rate.

Observer analysis.—In addition, we asked fisheries biologists to identify chub specimens using the key. We included this assessment because it has been argued that human observers have developed sophisticated pattern-recognition abilities that might outperform models that only consider morphological features that are relatively amenable to measurements and counts (Douglas et al., 1989). In addition, in practice, field-based investigations typically rely on fisheries biologists for identification of specimens, rather than on tools like multinomial logistic regression, and the ability to accurately identify species in a field setting is necessary for many management decisions and activities.

Ten professional fisheries biologists from state or federal agencies, or non-governmental organizations, participated in the observer analysis; each observer had at least ten years of experience working with the *G. robusta* complex in Arizona or New Mexico. Observers had no knowledge of the assumed species designations or capture locations but only the assigned specimen numbers. Observers were instructed to measure or count the morphological characters in the key to identify *G. robusta* complex chub to species. The morphometric and meristic characters reported in the results are identified as the most influential to distinguish among the species according to previous studies (Rinne, 1969; Minckley

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Table 2. Means (and standard deviations) of morphometric (size-standardized) and meristic characters for putative species of the *G. robusta* complex. Characters standardized to the mean size of 136.6 mm total length. Different superscript letters in the same row indicate significant differences from a Tukey HSD test ($\alpha = 0.05$). Character abbreviations are from Table 1.

Character	*G. intermedia*	*G. nigra*	*G. robusta*
HL	31.5 (2.0)[a]	30.3 (1.6)[b]	29.7 (1.8)[b]
CPL	27.3 (2.3)[a]	26.7 (2.4)[ab]	25.8 (1.9)[b]
LDCP	11.0 (0.9)[a]	10.5 (0.7)[b]	9.4 (0.7)[c]
DFR	8.2 (0.5)[a]	8.4 (0.5)[b]	8.9 (0.3)[c]
AFR	7.9 (0.4)[a]	8.1 (0.4)[b]	8.7 (0.5)[c]
LLS	73.6 (6.2)[a]	82.7 (6.2)[b]	85.8 (5.4)[c]

and DeMarais, 2000; Brandenburg et al., 2015): HL/LDCP, CPL/LDCP, DFR, AFR, and LLS. The observers were then asked to identify the specimen to species (observer analysis) according to their measurements and the key. As the key also used body color descriptions per species, observers were allowed to use body color to help identify specimens to species.

Five or six randomly selected specimens were chosen from 15 of the capture locations for the observer analysis. Each observer was assigned approximately 15 specimens from each assumed species designation, with at least one specimen from each capture location. The mean number of identifications per specimen was 5 (range 2–7), while the mean number of identifications per observer was 45 (range 34–49), yielding 450 total species identifications. We calculated the percentage of observer-identified species designations that matched assumed species designations based on Minckley and DeMarais (2000). We also used contingency *G* tests (Zar, 1999) to test if identification success varied by assumed species designation, by location, or by observer using Program R (α-values 0.05).

RESULTS

Analysis of variance.—The ANOVA indicated that mean values for morphometric (size-corrected) and meristic characters included a number of significant differences among purported species (Table 2). The mean values for the morphometric and meristic characters, and the relative rank of each species for each measure, were generally consistent with previously reported measurements (Rinne, 1969; Minckley and DeMarais, 2000).

Cluster analysis.—A subjective review of scatterplots of the standardized, centered, and scaled morphological data did not readily identify distinct groupings of the specimens (Fig. 2). Evaluation of the relative length of branches in the phenogram provided little evidence that the morphological data fell into distinct groups (Fig. 3). The CH index was highest when the phenogram was divided into two groups, indicating that there was more evidence for the presence of two groups than three to 15 groups. Because the CH index is not defined for a single group, we could not compare evidence for two groups to a single group. We also created

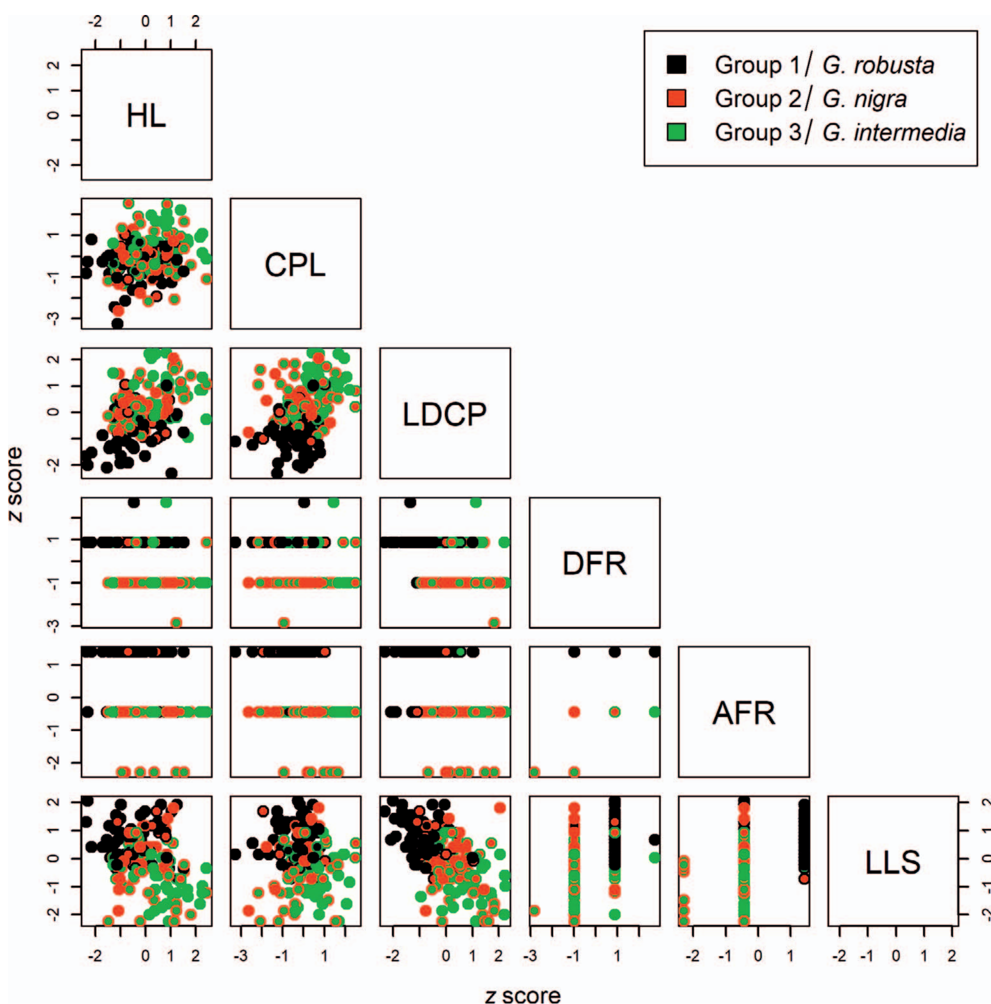

Fig. 2. Scatterplots of standardized, centered, and scaled morphological characters for species of *Gila* collected in Arizona. Circle borders are color-coded to show group membership from cluster analysis, while circle centers are color-coded to show assumed species designation. With only two total groups, Groups 2 and 3 were combined, while Group 1 was unchanged.

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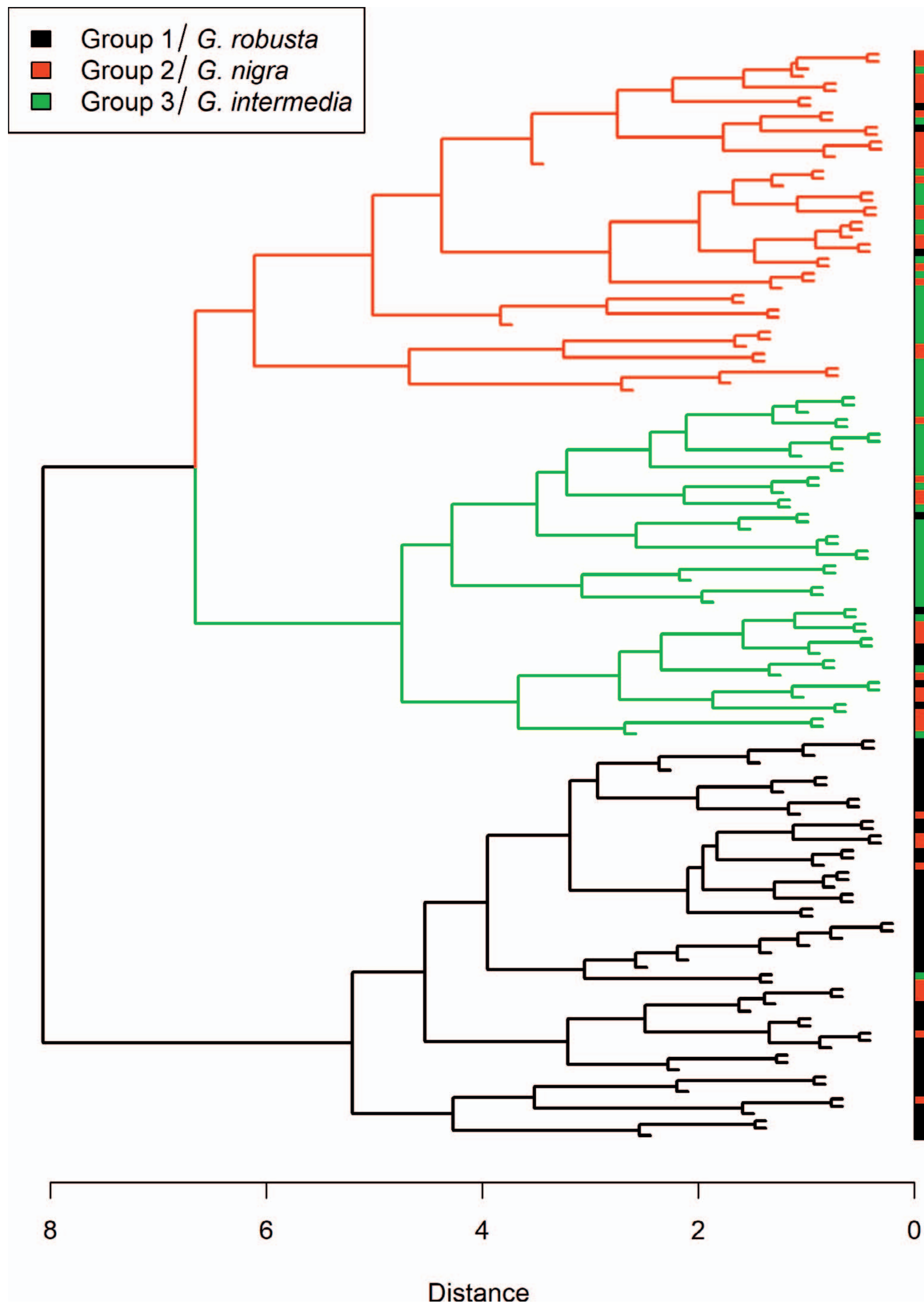

Fig. 3. Phenogram with branches color-coded to show three groups identified by cluster analysis. A two-group cluster analysis combined Groups 2 and 3, while Group 1 was unchanged. Text is color-coded to show the assumed species designation for the specimen.

three groups for comparison with the findings of Minckley and DeMarais (2000; Fig. 3). A scatterplot of the three clusters indicated that Group 1 (which included primarily *G. robusta*) was characterized by high counts for AFR, DFR, and LLS, and a low LDCP (Fig. 2). Groups 2 (primarily *G. nigra*) and 3 (primarily *G. intermedia*) were harder to distinguish, but Group 3 had slightly greater CPL and LDCP. With two total groups, as suggested by the CH index, Groups 2 and 3 were combined while Group 1 was unchanged.

We also evaluated the stability of the groups generated by the cluster analysis. High stability, sometimes defined as a Jaccard coefficient >0.85, indicates that the groups are robust (Hennig, 2007). With two groups, as indicated by the CH index, stability was poor, with Jaccard coefficients of 0.72 and 0.78 for the two groups. With three groups, as indicated by Minckley and DeMarais (2000), stability declined, with Jaccard coefficients of 0.73 (mostly *G. robusta*), 0.44 (mostly *G. nigra*), and 0.39 (mostly *G. intermedia*) for the three groups.

If we assume that that the three groups correspond to *G. robusta*, *G. nigra*, and *G. intermedia*, a confusion matrix indicated that 62% of specimens were placed in the group that corresponded to their assumed species designation, with higher success for *G. robusta* (82%) than for *G. nigra* (49%) and *G. intermedia* (53%, Table 3). For these groups, there was evidence of sympatry in 15 locations (88%). If we combine *G. nigra* and *G. intermedia*, as indicated by the CH index, then a confusion matrix indicated that 87% of specimens were placed in the appropriate group, with similar success for *G. robusta* (82%) and a *G. nigra*-*G. intermedia* group (89%). With two groups, evidence of sympatry was found in eight locations (47%).

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Table 3. Presumed and predicted species of *Gila* from hierarchical agglomerative cluster analysis.

	Predicted species		
Presumed species	*G. robusta*	*G. nigra*	*G. intermedia*
G. robusta	45	3	7
G. nigra	9	21	13
G. intermedia	1	23	27

Table 4. Presumed and predicted species of *Gila* from multinomial logistic regression.

	Predicted species		
Presumed species	*G. robusta*	*G. nigra*	*G. intermedia*
G. robusta	45	9	1
G. nigra	9	25	9
G. intermedia	0	10	41

Multinomial logistic regression.—We fit 12 multinomial regression models to the morphological data. The best supported model (lowest AIC) included LDCP, DFR, AFR, and LLS as predictors, indicating that HL and CPL contributed little to model fit. The three Hosmer and Lemeshow tests indicated that the model fit the data well for each species ($P > 0.78$). The model identified 74% of specimens to the assumed species designation, with higher success for *G. robusta* (82%) and *G. intermedia* (80%) than for *G. nigra* (58%, Table 4). Ten-fold cross-validation estimated a slightly lower correct identification rate of 73%. There was evidence of sympatry at 13 locations (76%) for both. The regression model characterized *G. intermedia* by low counts for LLS and separated *G. robusta* from *G. nigra* by a lower LDCP and higher AFR (Table 5, Fig. 4).

Observer-identification analysis.—The overall observer correct identification rate using the key was 54% ($n = 244/450$). Correct identification rate varied by assumed species designation ($G = 43.4$; df $= 2$; $P < 0.001$), with *G. nigra* identified at a lower rate than the other species. Specifically, *G. intermedia* was identified correctly 68% of the time and was most commonly misidentified as *G. nigra* (24%) and less so as *G. robusta* (8%). *Gila robusta* was identified correctly 63% of the time and was similarly misidentified as *G. nigra* (20%) and *G. intermedia* (17%). *Gila nigra* was only identified correctly 33% of the time, more commonly misidentified as *G. intermedia* (45%) and less so as *G. robusta* (22%). Overall, the misidentifications of observers resulted in at least two species present at all locations, suggesting sympatry (Fig. 5).

Among observers, correct identification rates ranged from 45% to 70%, which did not differ from random chance ($G = 10.5$; df $= 9$; $P = 0.32$). For *G. nigra*, correct identification rate did not vary by location ($G = 1.0$; df $= 4$; $P = 0.91$). However, both *G. intermedia* and *G. robusta* varied significantly in identification by location ($G = 40.7$; df $= 4$; $P < 0.001$; $G = 20.1$; df $= 4$; $P < 0.001$, respectively). For each species by location, correct identifications were over 90% at two

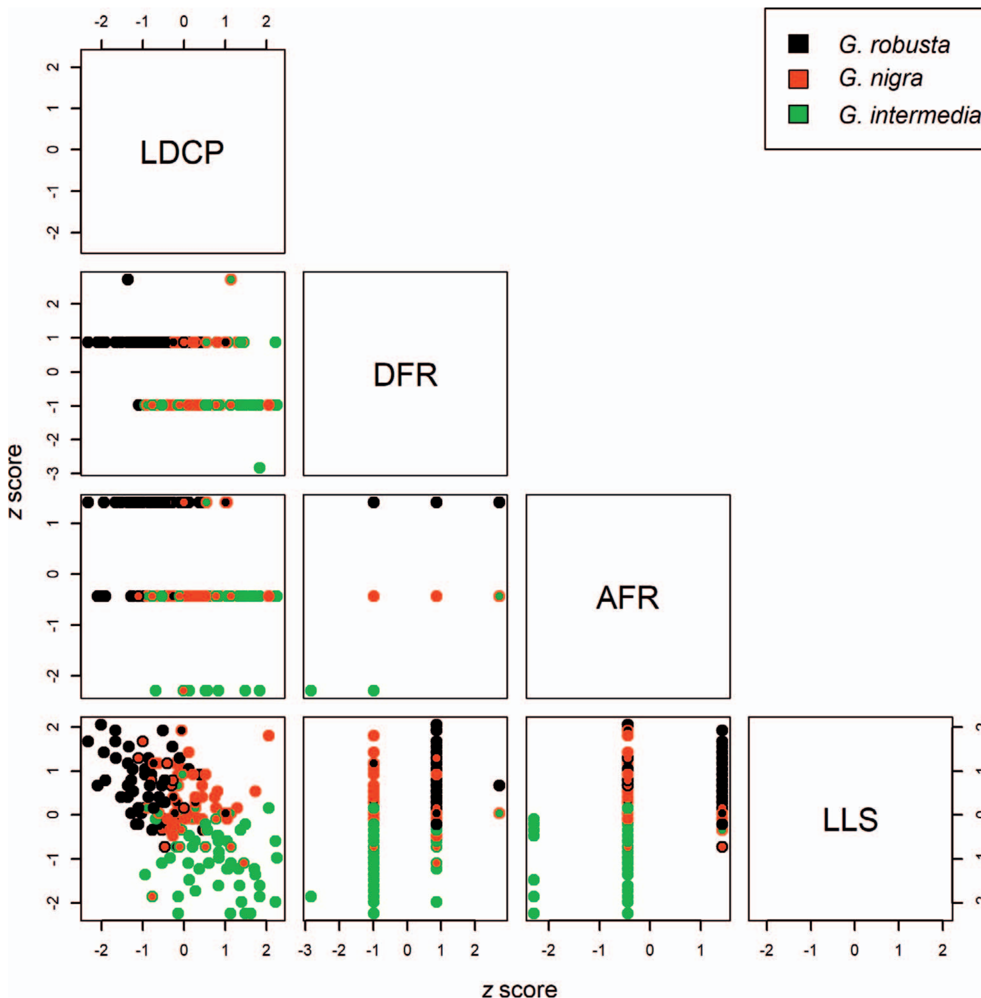

Fig. 4. Scatterplots of standardized, centered, and scaled morphological characters for fish within the *G. robusta* complex collected in Arizona. Circle borders are color-coded to show species prediction from multinomial logistic regression, while circle centers are color-coded to show assumed species designation.

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Table 5. Coefficient estimates (and standard errors) from multinomial logistic regression relating morphological characteristics to putative species. Coefficients express the increase in the log-odds that a specimen belongs to a species, relative to the reference species, *G. intermedia*.

Species	Intercept	LDCP	DFR	AFR	LLS
G. nigra	1.02 (0.40)	−0.58 (0.38)	0.22 (0.32)	0.66 (0.46)	1.78 (0.42)
G. robusta	0.24 (0.47)	−2.32 (0.57)	0.90 (0.43)	1.43 (0.53)	1.87 (0.55)

locations (correct identification: O'Donnell Canyon [96%; San Pedro Preserve] and Hot Springs Canyon [93%], *G. intermedia*), 51–85% at five locations (correct identification: Eagle Creek [87%], Wilder Creek [77%], Aravaipa Creek [56%], Francis Creek [51%], *G. robusta*; Silver Creek [65%], *G. intermedia*), and less than 50% at eight locations, meaning fish were classified as one of the other two species or a combination of both at the majority of locations (correct identification: Boulder Creek [41%], *G. robusta*; Fossil Creek [38%], Rock Creek [34%], Spring Creek [34%; Salt River drainage], East Verde River [31%], Haigler Creek [27%], *G. nigra*; Spring Creek [44%, Verde River drainage], Walker Creek [42%], *G. intermedia*).

DISCUSSION

The results of the quantitative and observer analyses in this study do not support the applied function of the Minckley and DeMarais (2000) key, finding that the two morphometric ratios and three meristics do not reliably distinguish *G. robusta*, *G. nigra*, and *G. intermedia*. The primary explanation for the relatively high levels of misidentification appears to be the overlap in the range of characters between each

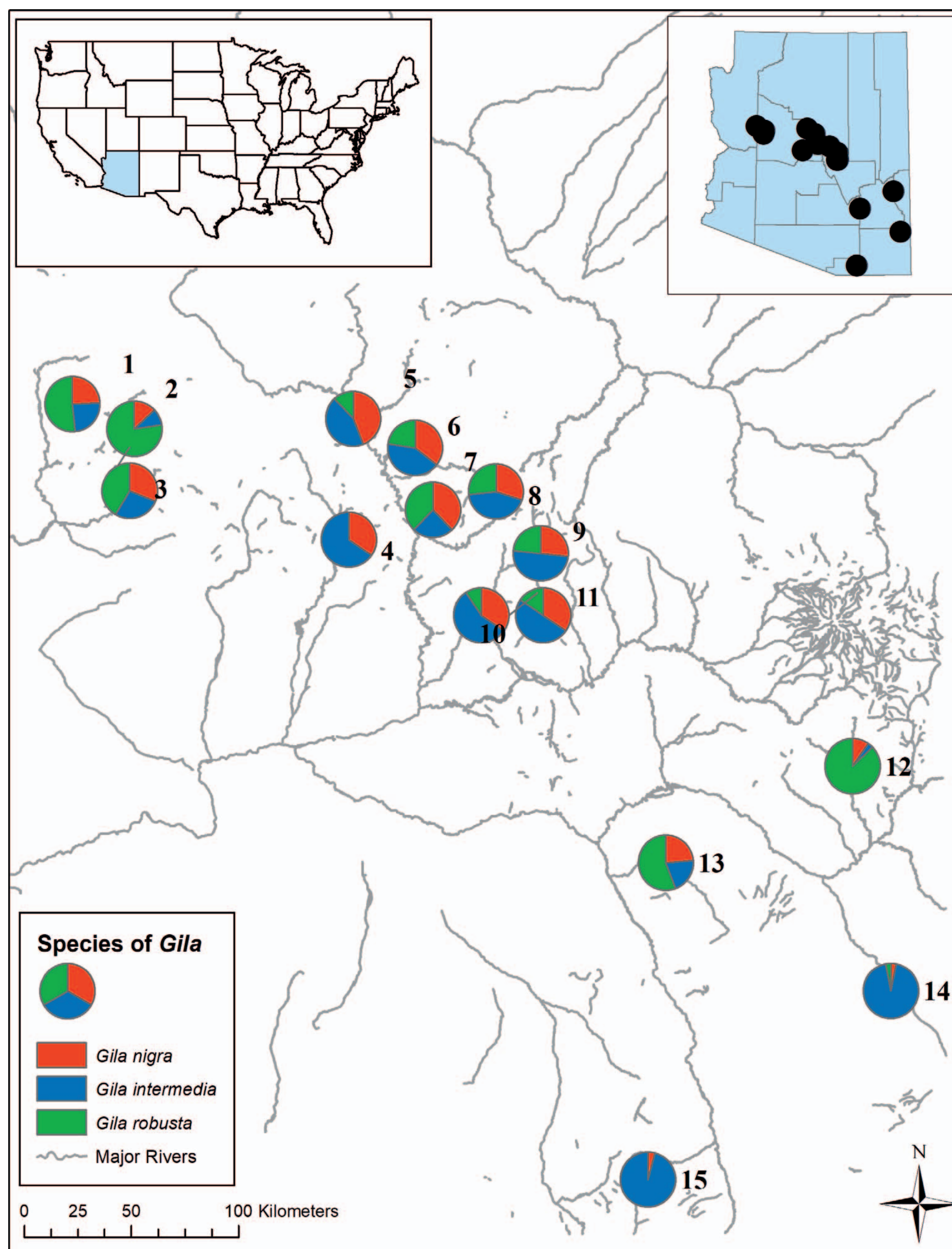

Fig. 5. Map with percent species (*G. robusta, G. nigra,* or *G. intermedia*) classification per collection location from the observer analysis ($n = 450$; 1 = Francis Creek; 2 = Wilder Creek; 3 = Boulder Creek; 4 = Silver Creek; 5 = Spring Creek [Verde River]; 6 = Walker Creek; 7 = Fossil Creek; 8 = East Verde River; 9 = Haigler Creek; 10 = Rock Creek; 11 = Spring Creek [Salt River]; 12 = Eagle Creek; 13 = Aravaipa Creek; 14 = Hot Springs Canyon; 15 = O'Donnell Canyon).

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assumed species group. We found the pattern of correct classifications to be similar among analysis methods, with an overall success rate of 62% for the cluster analysis, 74% for the multinomial logistic regression, and 54% for the observer analysis. The correct identification in all methods was highest for *G. robusta* (cluster = 82%; regression = 82%; observer = 63%), followed by *G. intermedia* (cluster = 57%; regression = 80%; observer = 68%) and *G. nigra* (cluster = 45%; regression = 58%; observer = 33%). Using the assumed species designations, sympatry among the species was evident in the results of each analysis type at 88%, 76%, and 100% of locations from the cluster, regression, and observer analysis, respectively. In the observer analysis alone, close to half of specimens were identified as a species other than what is currently recognized at those locations today. Specimens of *G. intermedia* and *G. nigra* were most commonly misidentified regardless of analysis type, and ultimately these results question the taxonomic designations of *G. intermedia* and *G. nigra* as independent morphological species.

Although variation in morphology was apparent in the ability of each analysis (cluster, regression, and observer) to identify specimens with better-than-random success, specimens still did not group as expected. Of particular interest is that the cluster analysis provided several results indicating a lack of distinct groups. The phenogram did not include relatively long branches that would indicate distinct groups. Similarly, the CH index increased as the number of groups declined, indicating at most two distinct groups. Finally, the stability analysis indicated that group boundaries were sensitive to small changes in the data, further corroborating the lack of distinct groups. Previously reported morphological data have indicated a lack of distinct boundaries between groups with, for example, typical CPL/LDCP ratios and LLS counts overlapping for *G. robusta* and *G. nigra* (Rinne, 1969, 1976; Minckley and DeMarais, 2000).

Minckley and DeMarais (2000) described group boundaries using expert knowledge, while we used quantitative analyses. Our analysis used HL, CPL, and LDCP as independent predictors of species identity, while Minckley and DeMarais (2000) used cutoff values for HL/LDCP and CPL/LDCP ratios to delineate species. We chose quantitative analyses because of the greater objectivity and repeatability, and retained three morphometric measures instead of using ratios because it increased our power to delineate groups. In contrast, using ratios reduces the amount of morphometric information. For example, two fish with the same HL/LDCP ratio could have rather different head lengths, but this would not be reflected in the metric. Furthermore, cutoff values constrain the species delineations to be perpendicular to that axis, while our modeling approaches allow non-perpendicular delineations. As a result, if the purported species can be reliably distinguished by their morphology, our approaches would be more likely to identify the morphological boundaries between the species.

It was recognized by both Rinne (1969, 1976) and Minckley and DeMarais (2000) that overlap existed in many of the morphometric ratios and meristics among the three species. Rinne (1969, 1976) identified two characters that best distinguished *G. robusta* from *G. intermedia* (LLS and HL/LDCP), but noted that there was substantial variation within these two characters and between the two species for other characters. The key used the mean population value of HL/LDCP and meristics to differentiate between *G. robusta* and *G. nigra*. We found that the overlap in values for the HL/LDCP ratio not only misidentifies individuals from a population recognized as one of the three species, but even the use of population means can classify the population as a species other than the assumed species designation. For example, the mean values of the HL/LDCP ratio from this study (observer = 3.0; quantitative = 3.1) and Rinne (1969; 3.1) indicate chub from Aravaipa Creek are *G. nigra* and *G. r. grahami*, respectively, and Rinne (1969) recognized that population as *G. r. grahami*. However, they were recognized as *G. robusta* by DeMarais (1986) and Minckley and DeMarais (2000). Similarly, the mean values of the HL/LDCP ratio from this study (observer = 2.8; quantitative = 2.8) and Rinne (1969; 3.0) indicated chub within the East Verde River are *G. intermedia*, yet they were recognized as *G. r. grahami* by Rinne (1969), as intermediate between *G. robusta* and *G. intermedia* by DeMarais (1986), and as *G. nigra* by Minckley and DeMarais (2000).

The overlap in meristics also impacts identification success. For example, the DFR count for chub from Aravaipa Creek from this study and in Rinne (1969) identified most chub as *G. robusta*, but 13% and 15% of fish, respectively, had DFR counts considered to be rare in the key for *G. robusta*, and could be identified as either *G. nigra* (*G. r. grahami* for Rinne, 1969) or *G. intermedia* (Table 1).The AFR counts for chub from Aravaipa Creek identify most chub as the assumed species designation, *G. robusta*, but 21% (this study) and 34% (Rinne, 1969) of fish, respectively, had rare AFR counts for *G. robusta* and could be identified as either *G. nigra* (*G. r. grahami* for Rinne, 1969) or *G. intermedia*. Chub from the East Verde River are considered *G. nigra* (Minckley and DeMarais, 2000; *G. r. grahami* for Rinne, 1969), but the DFR count identifies the majority of fish as *G. robusta* (83%, this study; 94%, Rinne, 1969). The AFR for chub from the East Verde River identified most of the individual chub as either *G. intermedia* or *G. nigra*, but 38% (this study) and 27% (Rinne, 1969) of fish could be identified as *G. robusta*. These levels of overlap are reflected in the identification success in the observer analysis, where just over half of the chub from Aravaipa Creek were identified as the assumed species designation, *G. robusta*, and the other half split between *G. nigra* and *G. intermedia*. Observers identified the majority of chub from the East Verde River as *G. intermedia*, rather than the assumed species designation of *G. nigra*. Using these two examples, identifying chub to species based on fin-ray counts from chub in Rinne's (1969) and our study is unreliable because "rarely" observed fin-ray counts can occur at a frequency that is not rare, resulting in "incorrect" or inconsistent identifications.

The keys created by Rinne (1969, 1976) and Minckley and DeMarais (2000) were the impetus to elevate *G. intermedia* and *G. nigra* to full species status, respectively. Minckley and DeMarais (2000) listed the following taxonomic options for the three assumed species: 1) they are a single polymorphic species (under *G. robusta*) with "ecological variants"; 2) they are polytypic with two subspecies of *G. robusta* (*G. r. robusta* and *G. r. intermedia*) with one intergrade (*G. nigra*), two species (*G. robusta* and *G. intermedia*) with *G. nigra* as a subspecies of one, or three allopatric subspecies of *G. robusta*; or 3) they are three separate species. While Minckley and DeMarais (2000) recommended the latter, we reexamine these taxonomic options based on the results of this and other studies.

In contrast to our results, Minckley and DeMarais (2000) argued that the *G. robusta* complex is composed of three "morphologically distinguishable" allopatric species (taxonomic option 3 above). Under their hypothesis, we would

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expect a cluster analysis to identify three stable, distinct groups that correspond to collection locations, which we did not observe in our data. We would also expect a regression analysis to successfully identify specimens to species. While our cross-validated correct mean identification rate of 74% (*G. robusta* 82%; *G. intermedia* 80%; *G. nigra* 58%) using logistic regression was significantly better than random, it was much lower than the 96 to 100% correct identification rates based on data of morphological differences reported for other groups of *Gila* spp. (*G. robusta* and *G. cypha*, McElroy and Douglas, 1995; *G. robusta*, *G. cypha,* and *G. elegans*, Douglas et al., 1998; *G. ditaenia*, *G. eremica*, *G. minacae,* and *G. purpurea*, Ballesteros-Córdova et al., 2016) and is more similar to the correct identification rates seen in fish populations or stocks rather than species (e.g., Poulet, 2008; Simon et al., 2010; Pannusa et al., 2015). It is possible there are three *potentially* morphologically distinguishable species, but we failed to identify the relevant morphological characters. However, the characters we measured were previously identified as relevant to distinguishing the species we investigated (Rinne, 1969, 1976; Minckley and DeMarais, 2000) as well as similar *Gila* spp. (Douglas et al., 1998). While the cluster analysis and regression analysis included a limited set of characters, the observer analysis allowed observers to use other features, such as body color, which was described per species in the key, and correct identification rates did not improve.

We considered the hypothesis that the *G. robusta* complex includes three morphologically distinct allopatric species but that some locations were attributed to the wrong species. Our results were not consistent with this possibility because the cluster analysis should still reveal three allopatric groups (taxonomic options 2, 3). Alternatively, if our data included three morphologically distinct sympatric species, we would expect the cluster analysis to identify three sympatric groups, while our regression analysis would include misidentifications due to incorrect assumed species designations. Again, the cluster analysis results did not support this hypothesis. Alternatively, if our data included three subspecies (taxonomic option 2), we would expect more ambiguous results from the cluster analysis and weaker identification success from the regression analysis, as we found. However, subspecies can typically only be maintained if they are allopatric (Mayr and Ashlock, 1991), and our results indicated that the groups were sympatric.

Rather than three morphologically distinct species (taxonomic option 3), it is conceivable that there are three morphologically similar species. As such, we would expect morphological analysis to have a limited ability to distinguish species. For example, three species of lamprey described by identification of haploid genotypes in their cytochrome *b* could only be distinguished morphologically 76% of the time (Mateus et al., 2013). Alternatively, our data could represent a single species, possibly with different ecotypes (taxonomic option 1). If habitat characteristics affect the phenotype through gene expression and microevolution (e.g., Yavno and Fox, 2013; Istead et al., 2015; Laporte et al., 2016), it could explain our ability to classify specimens with greater-than-random success. It is difficult to distinguish between the "three similar species" and the "one species" hypotheses using strictly morphological data. However, genetic studies found no species-level diagnostic markers and did not corroborate the three assumed species designations (DeMarais, 1992; Gerber et al., 2001; Schwemm, 2006; Dowling et al., 2015). Finally, Copus et al. (2016) recommended the recognition of only *G. robusta* after conducting a broad morphological analysis using type specimens and genetic analysis using reduced representation genomics with the ezRAD method. In conclusion, the results of this study indicate that the morphometric and meristic characters in the key do not consistently distinguish the three putative chub species. Because the available genetic analyses have not supported the three species hypothesis, we recommend that the Minckley and DeMarais (2000) taxonomic option 1, *G. robusta* as a single polymorphic species, be considered a viable hypothesis.

The lack of taxonomic clarity regarding chub populations and species recently prompted a formal taxonomic review of the *G. robusta* complex in the lower Colorado River basin by the American Fisheries Society–American Society of Ichthyologists and Herpetologists Committee on Names of Fishes (hereafter, the Committee). The Committee reviewed the available published and unpublished work (both morphological and genetic) on the *G. robusta* complex, including a pre-publication version of this study. The Committee concluded that the available morphological and genetic data support recognition of only one species, *G. robusta* (Page et al., 2017). This decision was reviewed and recognized by the USFWS by the withdrawal of a proposal to list *G. robusta* (lower Colorado River Distinct Population Segment) and *G. nigra* as Threatened species under the ESA (USFWS, 2017). The USFWS is now undergoing a reevaluation of the status of *G. intermedia*.

With the change in the taxonomy of *G. robusta*, the range-wide distribution of the species now encompasses drainages in six western states: Arizona, New Mexico, Colorado, Wyoming, Utah, and Nevada (*G. r. jordani*). For future management and conservation of *G. robusta* moving forward, we recommend incorporating *G. nigra* and *G. intermedia* into the two conservation agreements that were developed over ten years ago for *G. robusta* and *G. nigra* (UDNR, 2004; AGFD, 2006). The signatories to the agreements are implementing a conservation and management strategy to improve the status of the covered species. While state and federal wildlife agencies in Arizona and New Mexico have been managing *G. robusta*, *G. nigra*, and *G. intermedia* as separate species, the revised taxonomic status will allow fisheries managers to manage and conserve the species using a range-wide, watershed driven approach, while preserving genetic diversity, including identifying and protecting genetically distinct populations. We recommend that future characterization of populations of *G. robusta* use a combination of a broad morphological and genetic approach (e.g., reduced representation genomics), and for type specimens to be included in any morphological analysis.

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