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Assessment of Lineal Versus Landmark-Based Morphometry for Discriminating Species of Mugilidae (Actinopterygii)

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Mariano González-Castro, Ana Laura Ibáñez, Sandra Heras, María Inés Roldán, and María Berta Cousseau (2012) Assessment of lineal versus landmark-based morphometry for discriminating species of Mugilidae (Actinopterygii). *Zoological Studies* **51**(8): 1515-1528. Meristic and different morphometric approaches were employed to assess the discrimination of 7 species of Mugilidae fishes (*Mugil cephalus*, *M. liza*, *M. curema*, *M. hospes*, *Liza aurata*, *L. ramada*, and *Chelon labrosus*), but also to contribute to a better understanding of body-shape differences among this valuable species group. Three types of variables and their corresponding morphometric approaches were employed: 1) linear morphometrics measurements (LMMs); 2) interlandmark distances (IIDs); and 3) coordinate data (landmarks). Before the analyses, data exhibiting allometric growth were normalized. Data analysis included a one-way ANOVA (meristic data), a principal component analysis (PCA), and a cross-validated discriminant analysis (DA). The ANOVA showed significant differences in both lateral and transverse series scales. The PCA based on LMMs allowed the characterization of 6 groups, although some overlap between them was detected. The DA correctly classified 68.4% of the fishes according to their LMMs. The centroids of the 8 groups were separated for both the 1st and 2nd discriminant functions. The morphometric analysis based on IIDs yielded the best discrimination rates of the 3 approaches employed (96% for the DA). In the geometric morphometric analysis, the DA correctly classified 83.8% of the fishes according to their body shape. Although 8 groups were defined, some overlap among samples was detected. *Mugil hospes* was the best defined and most isolated species as observed in both the PCA and DA. Interestingly, the 3 morphometrics approaches employed separated *M. curema* specimens in 2 groups (Argentinean and Mexican samples). Moreover, European and Mexican samples of *M. cephalus* plotted separately in the PCA of the LMM- and IID-based approaches. These shape differences among *M. curema* of Argentina/Mexico and *M. cephalus* of Europe/Mexico reinforce the current hypothesis of a species complex, or even undescribed species as previously suggested by the authors.
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Key words: Mugilidae, Landmarks, Meristic characters, Morphometry, Multivariate analysis.

Members of the Mugilidae, called mullet, are ray-finned fishes that usually inhabit coastal marine and brackish waters in tropical and temperate seas (Thomson 1997, Nelson 2006). This family

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is mainly comprised of coastal marine fishes of considerable economic importance, shares similar phenotypes and comparable life histories, and as a result, reflects some taxonomic controversy (Harrison et al. 2007, González-Castro et al. 2008 2009). The taxonomic inconsistencies of the Mugilidae are reflected in the number of species recognized by several authors, i.e., Thomson (1997) only recognizes 62 species, while Nelson (2006) considers there to be 72 species.

Because of their economic importance, mullet have been broadly studied, in terms of age and growth (Ibáñez and Gallardo-Cabello 1996, Ibáñez et al. 1999, González-Castro et al. 2009, Kendall et al. 2009), reproduction (Vieira and Scalabrin 1991, Ibáñez and Gallardo-Cabello 2004, McDonough et al. 2005, Kendall and Gray 2008, González-Castro et al. 2011), and ecology (Cardona 2001, Vieira et al. 2008, Lebreton et al. 2011). With respect to the taxonomic identification of species and populations, studies on osteology (Harrison and Howes 1991), molecular genetics (Heras et al. 2009 and citations therein), karyotypes (Hett et al. 2011 and citations therein), and morphology (Menezes 1983, Thomson 1997, Harrison et al. 2007, Menezes et al. 2010) were conducted. However, due to the resemblance among species in relation to habitat and trophic and reproductive behaviors, morphological features can be similar or concurrent, and that is why identification is still a complicated task, particularly of juveniles specimens, especially in areas where several mullet species coexist. Features of diagnostic value commonly used to discriminate species of the Mugilidae include meristic characters (i.e., counts of scales, fin spines, and fin rays), the structure of the scales, the relative position of the nostrils, the presence/absence of adipose eyelids, the form of the preorbital, the number of pyloric ceca, the number and form of the gill rakers, measurements of body proportions (relative lengths of the head, snout, paired fins, body depth, etc.), and the position of the origin of the various fins (i.e., pre-dorsal 1 distance) (Thomson 1997).

In systematics research, the processes of interest are the speciation events. The traces left behind by these are genetic differences between organisms, which can be analyzed either directly at the level of nucleic acids or indirectly through visible modifications of morphological structures (Wägele 2005). While genetic studies provide the most accurate information on the specific identity of fishes, it is no less true that morphological and meristic characters are in part a reflection of their genetic structure, so cannot be ignored as valuable for species recognition (Wägele 2005, Harrison et al. 2007, Ibáñez et al. 2007, González-Castro et al. 2008). In addition, morphometric and meristic methodologies are recognized to be quicker, more practical, and less expensive than molecular studies, thus allowing many individuals to be screened in the field (Ibáñez et al. 2007).

Morphometric techniques have "evolved" in the last few decades, in parallel with the introduction of promissory methods for archiving forms of organisms. With the inclusion of the concept of "homologous landmark" (true anatomical points, identified by some consistent feature of the local morphology), Strauss and Bookstein (1982) proposed a geometric protocol for character selection, called the box-truss network. This protocol largely overcomes the disadvantages of traditional datasets (characters aligned along 1 axis, i.e., the longitudinal; coverage of forms that are highly uneven by region; repeated use of some morphological landmarks, etc). Moreover, it allows archiving the configuration of landmarks so that the form can be reconstructed (mapped) from a set of distances among landmarks. In this way, one can obtain landmark coordinates, the raw data necessary to perform the "new" geometric morphometric analysis (Rholf and Bookstein 1990, Cadrin 2000), which was called "a revolution in morphometrics" by Rholf and Marcus (1993). Considering the above, morphometric studies on the Mugilidae were performed either comparing only a few species or localities, or just employing a punctual methodology within the wide range offered by morphometry (i.e., ratios, linear measurements analyzed by principal component analyses (PCAs), box trusses, geometric morphometrics, etc.) (Corti and Crosetti 1996, Ibáñez and Lleonart 1996, Cousseau et al. 2005, Heras et al. 2006, González-Castro et al. 2008).

The aims of this study were a) to assess the effect of meristic and morphometric approaches on the identification rates of 7 representative species of Mugilidae: *Mugil cephalus* Linnaeus, 1758; *M. liza* Valenciennes, 1836; *M. curema* Valenciennes, 1836; *M. hospes* Jordan & Culver, 1895; *Liza aurata* (Risso, 1810); *L. ramada* (Risso, 1826), and *Chelon labrosus* (Risso, 1827); and b) to foster a better understanding of body shape differences and, consistently, to their taxonomy.

MATERIALS AND METHODS

Fish collection

In total, 135 specimens belonging to *Mugil*, *Liza*, and *Chelon* were collected from Argentina, Mexico, and Spain and analyzed (Table 1). Fish were frozen and transported to the laboratory, where they were measured, weighed, and macroscopically sexed after defrosting. Morphological species identification was based on Menezes (1983), Thomson (1997), and Harrison et al. (2007). Some of these specimens were also genetically identified previously (Table 1), as part of Heras et al. (2009).

Meristic data collection

Meristic data were considered for each specimen, following Cousseau et al. (2005). The meristic characters were compared between species, following Menezes (1983) and Thomson (1997). Since fish scale numbers are an important criterion for species discrimination, the mean, standard deviation, and range values for lateral (LSSs) and transverse series scales (TSSs) of the 9 groups analyzed were calculated. An analysis of variance (ANOVA) test (α = 0.05) was performed to evaluate significant differences in the number of LSSs and TSSs between species and localities. Tukey's honest significant difference (HSD) test was performed as a post-hoc multiple-comparisons test using SPSS vers. 13.0 (Nie et al. 2004).

Morphometry

Three types of variables were employed: 1) linear morphometrics measurements (LMMs); 2) inter-landmark distances (IlDs); and 3) coordinate data (landmarks). Accordingly, 3 different morphometric approaches were performed. Unfortunately, *C. labrosus* specimens showed several shape modifications after defrosting. Therefore, they were not included in the morphometric analysis.

Morphometric analysis based on LMMs

Five morphometrics variables were measured on the left side of fresh specimens (n = 125) to the nearest 0.5 mm with dial calipers, including the standard length (SL), head length (HL), snout length (Sn), pectoral fin length (PL), and predorsal 1 distance (pD1d). Methods for taking the morphometric measurements followed Cervigón (1980). Statistical and mathematical procedures for the LMM analysis followed Cousseau et al. (2005). The morphometric characters were organized by species. A normalization technique to scale the data that exhibited allometric growth was used according to Lleonart et al. (2000). The SL was used as the independent variable, while the remaining 4 variables were considered dependent variables. SL_0 represents a reference value of size (230 mm in this paper) to which all individuals were either reduced or amplified (Lombarte and Lleonart 1993, Ibáñez and Lleonart 1996,

Table 1. Species, collection sites, abbreviation group codes, and sample sizes of specimens used for this study

Species	Locality	Group code	n	N_G	Standard length (mm)			
					Range	Mean	S.D.	CV
Liza ramada	Ter Vell Lagoon, Spain	Lra	20	3	200-333	255.30	42.30	16.57
Liza aurata	Palamós, Spain	Lau	10	3	135-423	299.50	95.40	31.85
Chelon labrosus	Ter Vell Lagoon, Spain	Cla	6	6	141-334	194.50	70.60	36.30
Mugil cephalus	Palamós, Spain	Mce_E	3	3	339-423	375.30	43.13	11.49
	Ter Vell Lagoon, Spain	Mce_E	7	7	172-249	213.70	31.64	14.81
	Tamiahua Lagoon, Gulf of Mexico	Mce_M	11	-	240-372	303.50	46.70	15.39
Mugil liza	Mar Chiquita Lagoon, Argentina	Ml	26	18	223-425	335.50	73.40	21.88
Mugil curema	Mar del Plata coast, Argentina	Mcu_A	12	12	171-267	207.50	27.01	13.02
	Mar Chiquita Lagoon, Argentina	Mcu_A	18	18	205-249	228.58	13.26	5.80
	Cazones, Gulf of Mexico	Mcu_M	19	-	218-270	244.00	13.90	5.70
Mugil hospes	Alvarado, Gulf of Mexico	Mho	2	-	194-233	213.50	27.50	12.88
	Tamiahua Lagoon, Gulf of Mexico	Mho	1	-	-	226	-	-

N_G, number of specimens that were genetically identified previously in Heras et al. (2009); S.D., standard deviation; CV, coefficient of variability.

González-Castro et al. 2008). After transformation, a PCA was performed using MULTIVARIADO® software (Salomón et al. 2004). Finally, principal component scores (PCs) were submitted to cross-validated discriminant analysis (DA) using SPSS® vers. 13.0 (Nie et al. 2004), in order to build a predictive model of group membership based on the observed characteristics of each case. This procedure generates a set of discriminant functions based on linear combinations of the predictor variables that provide the best discrimination between groups.

Morphometric analysis based on IIDs

Twenty-one morphometric variables were taken as IIDs on the left side of 125 specimens, using digital calipers (to a precision of 0.05 mm). These variables were based on 12 landmarks obtained by the truss network (Bookstein et al. 1985), defined on the basis of the external morphology and homologous among the species, according to González-Castro et al. (2008) (Fig. 1). Statistical and mathematical procedures for the IID analysis followed Cousseau et al. (2005), Heras et al. (2006), and González-Castro et al. (2008). The morphometric characters were organized by species and location. As in the LMM analysis, the normalization technique of Lleonart et al. (2000) was employed. SL was used as the independent variable, while the remaining 21 variables were considered dependent variables. The same SL_0 (230 mm) was employed. After transformation, a new matrix was constructed containing the corrected matrices for each species, and the PCA was performed using MULTIVARIADO® software. PCs were submitted to the DA (SPSS® vers. 13.0) to build a predictive model of group membership based on the observed characteristics in each case.

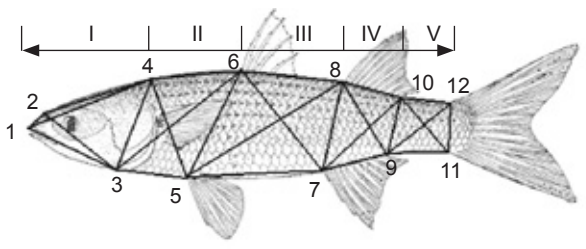

Fig. 1. Box truss (Roman numerals) showing the inter-landmark distances measured in the individuals analyzed, based on 12 anatomical landmarks (Arabic numerals).

Geometric morphometric analysis based on coordinate data (landmarks)

This analysis was performed on the Cartesian coordinates of 12 anatomical landmarks of specimens, reconstructed from distance measurements among the landmarks, based on the proposed box truss scheme (Fig. 1) using MORPHEUS® software (Slice 1994). The landmark coordinates of each specimen were scaled, translated, and rotated using the generalized Procrustes superimposition (GLS, also called GPA) (Rohlf 1999). Scaling, translation, and rotation were employed to minimize the Procrustes distance, which is the sum of squared distances between corresponding landmarks. Ibáñez and O'Higgins (2011), based on landmark coordinates, examined scale normalization and their impacts on species identification. They found that normalization adjustment failed to markedly improve classification beyond what was achieved using shape alone. For this reason, normalization was not applied to the geometric morphometric analysis.

The thin-plate spline (TPS) procedure was employed to compare shape differences among species, using both uniform and non-uniform shape components, and an upward/downward arching effect of the fishes' body was observed. This effect was not related to biological factors (size or species) or to the preservation technique (freezing), but was rather due to slight postural differences between fishes when IIDs were determined. This distortion associated with the specimen's posture was previously addressed in fishes by Valentin et al. (2008). Those authors proposed a method that effectively removes this artefact from the data, coupling a PCA-based model of the arching with Burnaby's orthogonal projection. This method also has the interesting property of making corrections directly on the landmark coordinates (Valentin et al. 2008). Then, the methodology of Valentin et al. (2008) was applied, and new unbiased coordinates were re-subjected to GLS and TPS (using TPSRELW Software vers. 1.46) (Rholf 2008). A PCA of the partial warps matrix was performed (usually called the relative warp (RW) analysis (RWA)), in order to describe major trends in shape variations. To examine the potential for differences in shapes for classifying unknown specimens, the relative warp scores were submitted to a DA (SPSS vers. 13.0; Nie et al. 2004). This was carried out using cross-validation.

RESULTS

Meristic

The ANOVA test showed significant differences ($p < 0.0001$; *d.f.* = 8) for both LSSs ($F = 109.7$) and TSSs ($F = 54.7$), for the species analyzed. The Levene test showed equality of variances for the 9 groups analyzed and for scales in both the lateral ($p = 0.641$) and transverse ($p = 0.274$) series. Mean and range values for the LSSs and TSSs of the 9 groups followed the expected values for each species, according to the identification keys employed (Table 2).

Tukey's HSD post-hoc test, performed for both LSSs and TSSs, showed 3 homogeneous subsets among the 9 groups analyzed for both variables (Table 3). The results obtained for the TSSs were less informative, because *M. hospes* appeared in 2 different subsets. It was noted that for both LSS and TSS, the Argentinean and Mexican samples of *M. curema*, and also the European and Mexican specimens of *M. cephalus* were statistically grouped in the same homogeneous subset, respectively (Table 3).

Morphometry

Morphometric analysis based on LMMs

The PCA of the correlation matrix, generated by the normalization procedure, produced 2 eigenvalues of > 1 (data not shown) and 4 PCs. Correlations between variables and components

Table 2. Mean, standard deviation (S.D.), range, and coefficient of variability (CV) values obtained for lateral and transverse series scales of the 9 groups of mugilids analyzed. Group codes are given in table 1

	Group code	Mean	S.D.	Minimum	Maximum	CV
Lateral series scales	Lra	43.67	1.23	42	46	2.82
	Lau	43.60	1.43	41	45	3.28
	Cla	44.00	0.89	43	45	2.02
	Mce_E	41.20	0.91	40	43	2.21
	Mce_M	41.18	1.16	39	43	2.82
	Ml	36.50	1.03	35	39	2.82
	Mcu_A	37.40	1.19	34	39	3.18
	Mcu_M	37.12	1.16	35	39	3.13
	Mho	38.00	1.00	37	39	2.63
Transverse series scales	Lra	13.86	0.57	13	15	4.11
	Lau	13.80	0.63	13	15	4.57
	Cla	13.67	0.51	13	14	3.73
	Mce_E	13.90	0.57	13	15	4.10
	Mce_M	14.27	0.47	14	15	3.29
	Ml	12.85	0.46	12	14	3.58
	Mcu_A	11.92	0.40	11	13	3.36
	Mcu_M	11.88	0.33	11	12	2.78
	Mho	12.33	0.58	11	13	4.70

Table 3. Tukey's honest significant differences post-hoc test performed for both lateral and transverse series scales. Group codes are given in table 1

		Lateral series scales		Transverse series scales	
		Group code	*p*	Group code	*p*
Homogeneous subsets	1	Ml; Mcu_A; Mho; Mcu_M	0.126*	Mcu_A; Mho; Mcu_M	0.549*
	2	Mce_E; Mce_M	1.000*	Ml; Mho;	0.369*
	3	Lra; Lau; Cla	0.998*	Lra; Lau; Cla; Mce_E; Mce_M	0.165*

* $p < 0.05$.

of > 0.59 were considered significant (data not shown). The PCA based on traditional morphometrics allowed the differentiation of six of the 8 groups analyzed, although some overlap between them was detected (Fig. 2). In this respect, *L. ramada* showed higher loadings for the variable HL, but also Sn/pD1d in a minor way (data not shown). Both *L. aurata* and *M. hospes* were characterized by higher loadings of PL and lower loadings of HL (data not shown). *Mugil hospes* constituted both the best defined and most isolated group of this analysis. Surprisingly, *M. curema* samples were basically divided into 2 groups (the 1st one comprised of *M. curema* specimens from Argentina (Mcur_A) and the 2nd one of *M. curema* from Mexico (Mcur_M); Fig. 2). Mcu_A showed lower loadings for the variables pD1d, Sn, and PL and, oppositely, Mcu_M showed higher loadings for these variables (data not shown). Most *M. liza* specimens were scattered in the PCA plot, and showed mid/high loadings for the variable pD1d (Fig. 2). Finally, *M. cephalus* specimens showed slight separation between European (Mce_E) and Mexican (Mce_M) populations (Fig. 2). Lower loadings of the variables pD1d, Sn, and HL/PL would characterize this group (data not shown).

DA. Variations in the 125 individuals of the Mugilidae classified by species and localities were explained by 4 canonical discrimination functions, of which the 1st 2 explained 84.3% (69.2% and 15.1%, respectively) of the total variance in the data, (Wilks' lambda = 0.042, $p < 0.0001$). The DA correctly classified 76.1% of the original grouped cases, whereas the cross-validated analysis correctly classified 68.4% of the fishes according to their LMMs (Table 4A). Moreover, the cross-validated analysis showed that misclassifications varied 0%-30% depending on the group analyzed (Table 4A). Only *L. aurata* and *M. hospes* showed that 100% of cross-validated cases were correctly classified. The centroids of the 8 groups were separated on both the 1st and 2nd discriminant functions (Fig. 3). However, all groups showed specimens overlap, with the exception of *M. hospes* (Fig. 3).

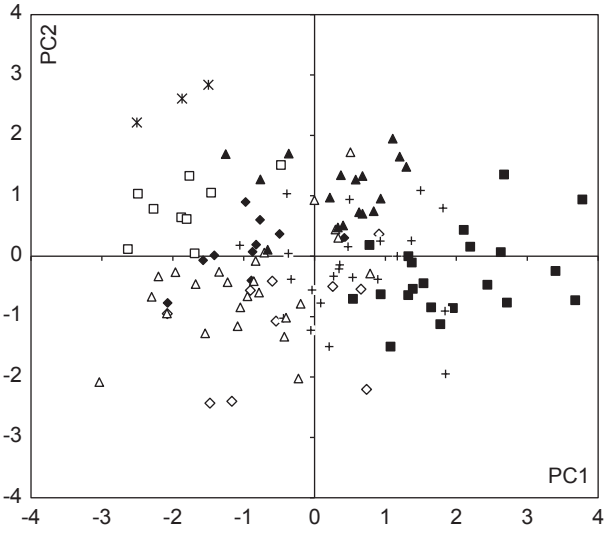

Fig. 2. Principal component (PC) analysis (PC1 vs. PC2) based on linear morphometric measurements. The 1st 2 PCs explained 78% of the variance in the data. *Liza ramada* (black squares); *L. aurata* (white squares); *Mugil cephalus* (Spain) (white diamonds); *M. cephalus* (Mexico) (black diamonds); *M. liza* (crosses); *M. curema* (Argentina) (white triangles); *M. curema* (Mexico) (black triangles); and *M. hospes* (asterisks).

Morphometric analysis based on IIDs

The 21 normalized IIDs, which were analyzed by the PCA of the correlation matrix, produced 4 eigenvalues of > 1 (data not shown). The 1st 4 PCs explained more than 76% of the variance in the data (41%, 15%, 12%, and 8%, respectively). Correlations between variables and components of > 0.59 were considered significant (data not shown). The PCA based on IIDs allowed graphic segregation of most of the 8 groups analyzed, with a slightly degree of overlap between them (Figs. 4, 5). In this respect, *L. ramada* was characterized by higher loadings for the 1-4 IID (which represents the HL) and lower values for the 1-3 and 2-3 (IIDs of the head) variables (data not shown), but also those IIDs that constitute the 2nd, 3rd, and 4th box trusses (Fig. 1). The congener species *L. aurata* showed somewhat-high loadings for the 10-11 and 9-10 IIDs (which were related to the caudal peduncle, or 5th box truss) and lower values for variables that constituted the 1st, 2nd, and 3rd box trusses (data not shown). With respect to *M. cephalus* samples, it was noted that European (Mcep_E) and Mexican (Mcep_M) samples plotted separately on the PCA. Mcep_E specimens were located in the lower left quadrant (Fig. 4) (except for 1 individual), with medium-high loadings for the 1-4 IID and lower values for the 9-10 and 10-11 variables (data not shown); in contrast, the Mce_M sample was basically located in the upper right quadrant (Fig 4), and had higher loadings for the 9-10 and 10-11 IIDs (which were part of the caudal peduncle in the 5th box truss) (data not shown). Moreover, the PC2 vs. PC3 plot showed complete separation between Mcep_M and Mcep_E specimens (data not shown). *Mugil liza* was

the species with the most dispersed grouping (Fig. 4). It showed higher loadings for the 1-4 and 1-2 variables (representing SL and HL) and lower ones for the 2-3 and 1-3 variables (data not shown) and also the IID that constituted the 3rd and 4th box trusses. Again, *M. hospes* was the best defined and most isolated species group in the PCA (Fig. 5), with higher loadings for the 1-3 and 2-3 IIDs (also for 7-9 and 7-10 in a minor way), and a lower one for the 3-4 variable (head height) (data not shown). As shown in the PCA of the LMM analysis, *M. curema* samples were basically separated into 2 groups (the 1st one comprised *M. curema* specimens from Argentina (Mcur_A) and the 2nd of *M. curema* from Mexico (Mcur_M)), with only minimal overlap between them (Fig. 5). Mcu_A showed higher loadings for the 1-3, 2-3, 7-9, and 7-10 IIDs (data not shown) but also moderately high loadings for variables constituting the 3rd box truss (Fig. 1). Mcu_M showed higher loadings for 2-3, 4-5 (data not shown), and the variables that constituted the 3rd box truss, but also moderately high loadings for variable 3-4 (head height) and those related to the 4th box truss (Fig. 1). Moreover, Mcu_A and Mcu_M respectively showed lower loadings for the 3-4 (head height) and 1-4 (HL) IIDs (data not shown).

DA. The DA for the 125 individuals of the Mugilidae classified by species and localities produced 7 significant canonical discrimination

Table 4. Percent values of the cross-validated discriminant analysis, based on the principal component (PC) scores of: (A) linear morphometric measurements, (B) inter-landmark distances, and (C) landmark coordinates. Group codes are given in table 1

		Predicted group membership (%)							
	Species	Lra	Lau	Mce_E	Mce_M	Ml	Mcu_A	Mcu_M	Mho
	Percent								
A) Linear morphometric measurements	Lra	70	0	5	0	25	0	0	0
	Lau	0	100	0	0	0	0	0	0
	Mce_E	20	10	40	0	30	0	0	0
	Mce_M	0	0	0	80	0	20	0	0
	Ml	8.7	4.3	21.7	0	47.8	8.7	8.7	0
	Mcu_A	0	0	4	8	0	72	16	0
	Mcu_M	0	0	0	5.9	5.9	5.9	76.5	5.9
	Mho	0	0	0	0	0	0	0	100
	Note: 68.4% of the cross-validated grouped cases were correctly classified.								
B) Inter-landmark distances	Lra	100	0	0	0	0	0	0	0
	Lau	0	100	0	0	0	0	0	0
	Mce_E	0	0	90	0	10	0	0	0
	Mce_M	0	0	0	90.9	0	9.1	0	0
	Ml	0	0	9.1	0	90.9	0	0	0
	Mcu_A	0	0	0	3.3	0	96.7	0	0
	Mcu_M	0	0	0	0	0	0	100	0
	Mho	0	0	0	0	0	0	0	100
	Note: 96.0% of the cross-validated grouped cases were correctly classified.								
C) Landmark coordinates	Lra	84.2	0	10.5	0	0	0	5.3	0
	Lau	0	75	0	12.5	12.5	0	0	0
	Mce_E	0	0	75	0	12.5	12.5	0	0
	Mce_M	0	0	0	90	0	10	0	0
	Ml	0	4.5	13.6	0	81.8	0	0	0
	Mcu_A	0	0	3.6	3.6	0	82.1	10.7	0
	Mcu_M	0	0	0	0	0	10.5	89.5	0
	Mho	0	0	0	0	0	0	0	100
	Note: 83.8% of the cross-validated grouped cases were correctly classified.								

functions, of which the 1st 2 explained 75.9% of the total variance in the data, (Wilks' lambda = 0.000; $p < 0.000$). The DA correctly classified 100% of the Mugilidae individuals to species and localities, whereas the cross-validated analysis correctly classified 96% of the fishes according to their body shape (Table 4B). Accordingly, group misclassifications where scarce, with the highest rate of 10% of Mce_E misclassified as *M. liza* (Table 4B). Eight groups were defined, and their centroids and individuals were separated on both the 1st and 2nd discriminant functions (Fig. 6). Minimal overlap was found among *L. aurata*, *M. cephalus* (Mce_M and Mce_E), and *M. liza* (Fig. 6). It was noted that *M. curema* samples (Mcu_M and Mcu_A) were entirely separated, which in fact showed that 100% and 96.7% of cross-validated cases had been correctly classified (Table 4B).

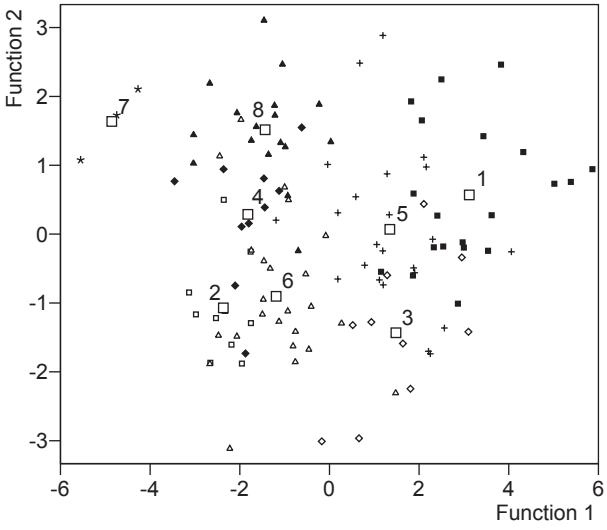

Fig. 3. Discriminant analysis performed on the principal component scores based on linear morphometric measurements. Centroid of each species group: large empty squares; (1) *Liza ramada* (black squares); (2) *L. aurata* (white squares); (3) *Mugil cephalus* (Spain) (white diamonds); (4) *M. cephalus* (México) (black diamonds); (5) *M. liza* (crosses); (6) *M. curema* (Argentina) (white triangles); (7) *M. curema* (México) (black triangles); (8) *M. hospes* (asterisks).

Geometric morphometric analysis based on coordinates (landmark data)

The 1st 4 RWs explained 68.24% (27.3%, 18.5%, 12.5%, and 10%, respectively) of the total variance for the GLS/RWA analysis of the body shape of the different mullet species studied. Patterns of morphological variations described by the 1st 3 RWs are shown in figures 7 and 8. Shape changes along the 1st RW were expressed by the depression (negative RW1 scores) or expansion (positive RW1 scores) of the body along the dorsoventral axis (i.e., the body height along by

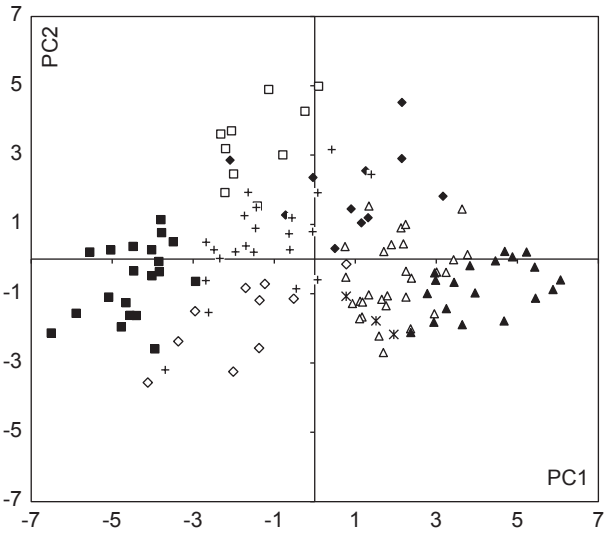

Fig. 4. Principal component (PC) analysis (PC1 vs. PC2) based on inter-landmark distances. *Liza ramada* (black squares); *L. aurata* (white squares); *Mugil cephalus* (Spain) (white diamonds); *M. cephalus* (México) (black diamonds); *M. liza* (crosses); *M. curema* (Argentina) (white triangles); *M. curema* (México) (black triangles); and *M. hospes* (asterisks).

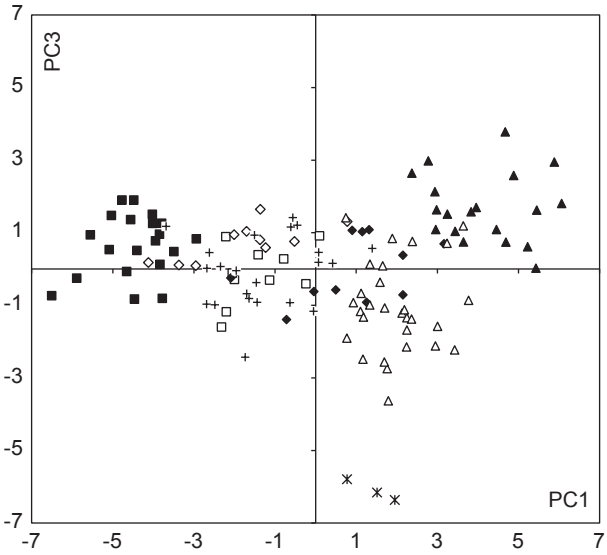

Fig. 5. Principal component (PC) analysis (PC1 vs. PC3) based on inter-landmark distances. *Liza ramada* (black squares); *L. aurata* (white squares); *Mugil cephalus* (Spain) (white diamonds); *M. cephalus* (México) (black diamonds); *M. liza* (crosses); *M. curema* (Argentina) (white triangles); *M. curema* (México) (black triangles); and *M. hospes* (asterisks).

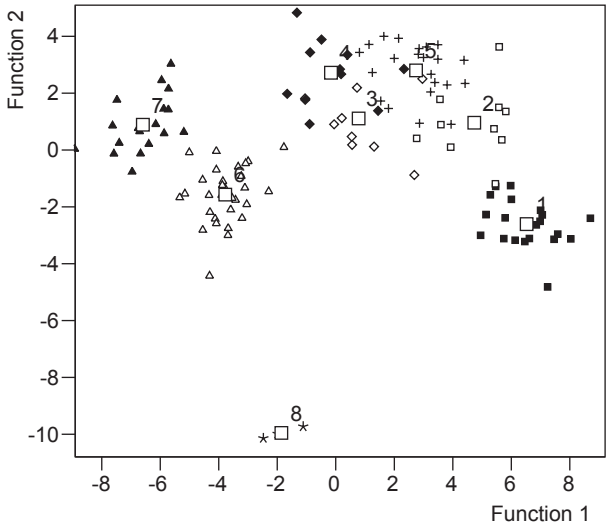

Fig. 6. Discriminant analysis performed on principal component scores based on inter-landmark distances. Centroid of each species group: large empty squares; (1) *Liza ramada* (black squares); (2) *L. aurata* (white squares); (3) *Mugil cephalus* (Spain) (white diamonds); (4) *M. cephalus* (México) (black diamonds); (5) *M. liza* (crosses); (6) *M. curema* (Argentina) (white triangles); (7) *M. curema* (México) (black triangles); and (8) *M. hospes* (asterisks).

the 2nd and 3rd box trusses) (Fig. 7). Accordingly, the head shape changed from sharp and flat (RW1-) typical of *L. ramada* specimens, to shorter (in length) but deeper (in height) (RW1+), as was observed in *M. curema* samples (Fig. 7). The shape of the caudal peduncle and 2nd dorsal-anal fin region (corresponding to the 4th and 5th box trusses) varied from shorter and less robust (RW1-) typical of *L. ramada* specimens to larger and more robust (RW1+) for *M. curema* samples. The shape of RW2 varied somewhat due to the displacement of landmarks 7, 10, and 12 which formed 2 types of caudal peduncle/anal fins: for the 1st 1 (RW2+), the base of the anal fin was wider and the caudal peduncle was straight, while the 2nd one was characterized by a shorter anal fin base and a curved peduncle (RW2-). Shape changes along RW3 were also expressed by depression/ expansion of the body height (especially on the 2nd and 3rd box trusses); however, in this case, the relative positions of landmarks 5 and 6 played important roles in the shape change (Fig. 8).

Data corresponding to the 20 RWs of the RWA were employed to perform the DA. The DA

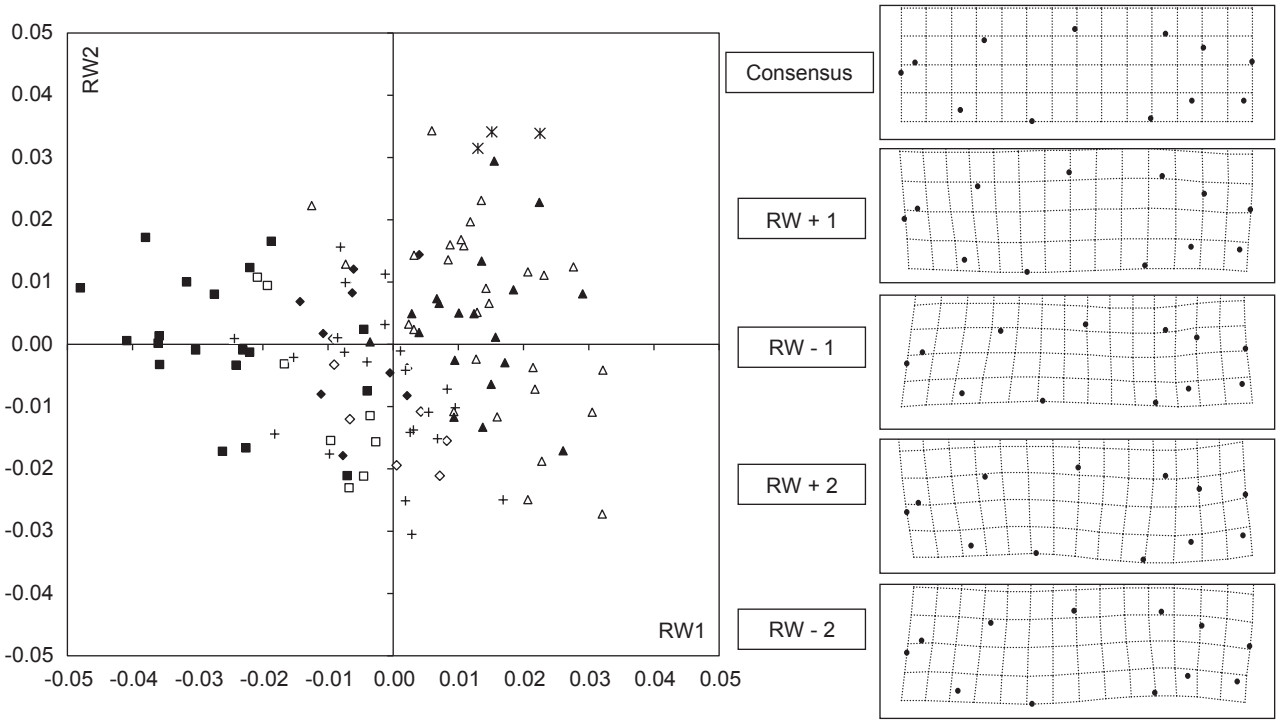

Fig. 7. Relative warp (RW) analysis (RW1 vs. RW2) based on landmark coordinates. Thin plate spline transformation grids for the extreme points of RW1/ RW2 are shown; these were superimposed on the shapes predicted when the average landmark configuration of all specimens was deformed into that of a hypothetical specimen positioned at the extreme of the RW of interest. Symbols: *Liza ramada* (black squares); *L. aurata* (white squares); *Mugil cephalus* (Spain) (white diamonds); *M. cephalus* (México) (black diamonds); *M. liza* (crosses); *M. curema* (Argentina) (white triangles); *M. curema* (México) (black triangles); and *M. hospes* (asterisks).

produced 7 significant canonical discrimination functions, of which the 1st 2 explained 66.2% of the total variance in the data (Wilks' lambda = 0.01 $p < 0.000$). The DA correctly classified 95.7% of the original grouped cases, whereas the cross-validated analysis correctly classified 83.8% of the fishes according to their body shape (Table 4C). Eight groups were defined, and their centroids and individuals were mainly separated on both the 1st and 2nd discriminant functions (Fig. 9). Some degree of overlap was found among Mcep_M, Mcep_E, and *M. liza* specimens, and also between *M. curema* (Mexican and Argentinean) samples. It was noted that the centroids of the *M. cephalus* groups (Mexico and Europe) slightly overlapped (Fig. 9). Nevertheless, misclassifications between Mcep_M and Mce_E were not found (Table 4C). Misclassifications (cross-validated analysis) ranged 3.6%-13.6% according to the groups analyzed. Only *M. hospes* specimens showed 100% of cross validated cases correctly classified (Table 4C).

DISCUSSION

Sufficient evidence was shown to accept the assumption that morphometry can discriminate among fish species and different populations (Minos et al. 1995, Cavalcanti et al. 1999, Sabadin et al. 2010, Díaz de Astarloa et al. 2011, Zhan and Wagn 2012). According to our results, the most important measures to take into account for discrimination purposes by species and by populations were the HL, caudal peduncle height, body height at the origin of the 2nd dorsal fin, PL, and anal fin length; these distances should be noted in mullet identification keys. However, to suggest some diagnostic characters which permit easy field identification among species, taxonomists need to combine different tools (osteology, meristic and morphometric characters, and pattern coloration) in order to achieve the highest identification rate (desirably 100%). With respect to the Mugilidae, Corti and Crosetti (1996) and more recently, Heras et al. (2006) and González-Castro et al. (2008) presented landmark-based taxonomic studies, employing the body shape to discriminate congeneric mullets, at the subspecific or specific taxonomical level. The present work is, to our knowledge, the 1st study to apply this methodology with several species of mullet, belonging to different genera of the Mugilidae. Moreover, the fact that 3 different morphometric approaches were used, contributed to a better understanding of the taxonomic differences related to the body shapes of these

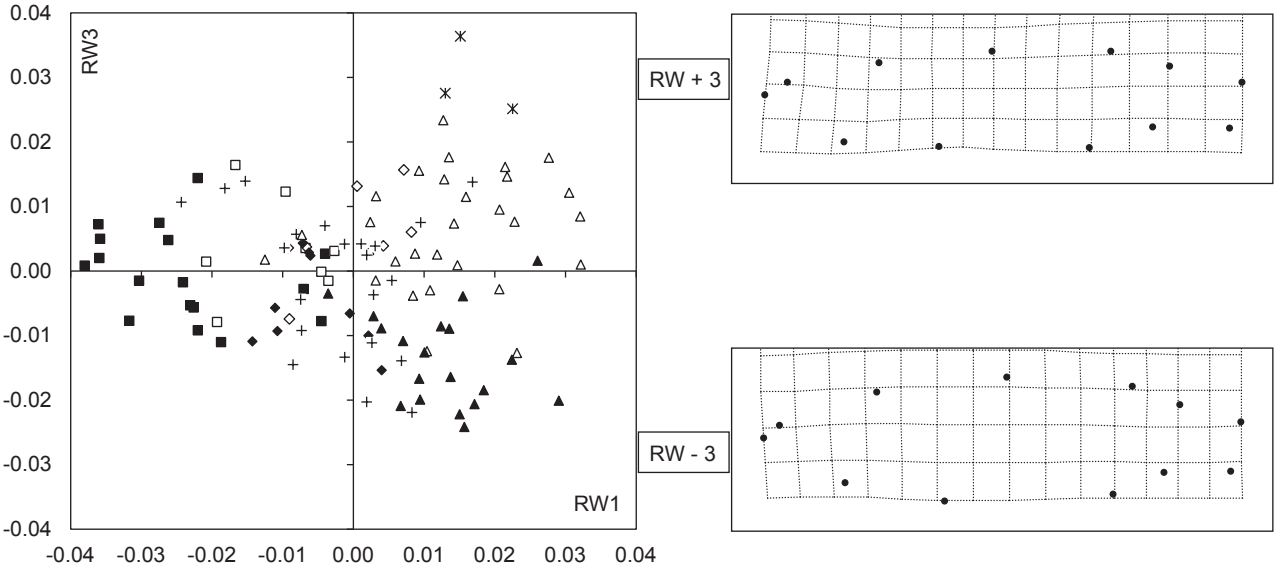

Fig. 8. Relative warp (RW) analysis (RW1 vs. RW3) based on landmark coordinates. Thin plate spline transformation grids for the extreme point RW3 are shown; these were superimposed on the shapes predicted when the average landmark configuration of all specimens was deformed into that of a hypothetical specimen positioned at the extreme of the RW of interest. Symbols: *Liza ramada* (black squares); *L. aurata* (white squares); *Mugil cephalus* (Spain) (white diamonds); *M. cephalus* (México) (black diamonds); *M. liza* (crosses); *M. curema* (Argentina) (white triangles); *M. curema* (México) (black triangles); and *M. hospes* (asterisks).

fishes, and allowed comparisons of the limits and scope of each methodology, since they were applied to the same individuals. In this sense, the morphometric analysis based on IIDs yielded the highest discrimination rates of the 3 approaches employed (96.0% of cross-validated grouped cases were correctly classified), followed by the landmark coordinate analysis (83.8%) and LMM analysis (68.4%). With respect to the results obtained, *Mugil hospes* was the best defined and most isolated species group as can be observed in the PCA plots (Figs. 2, 5, 7, 8), but also in the cross-validated DA based on PC scores, where the predicted group membership for this species was 100% for the 3 morphometric approaches employed (Table 4). This may be related to its shape, i.e., a long pectoral fin (evidenced by the higher loadings of PL in the LMM), short HL (evidenced by lower loadings of the HL variable in the LMM), lower head height (evidenced by the lower loadings for variable 3-4 in the IID analysis), an anal fin with a wide base (evidenced by the higher loadings for variable 7-9 in the IID analysis), and a depressed body height (especially on the 2nd and 3rd box trusses, as evidenced by the relative positions of landmarks 5 and 6 of the RWA in Fig. 8). Moreover, recently Ibáñez et al. (2011) contributed to the morphological diagnosis of this species, by differentiating *M. hospes* from *M. curema* of Mexico by the shape of the ctenii on their scales. *Liza ramada* was characterized by its long snout, long, sharp and flat head, and thin body (as evidenced by both the IID and landmark coordinate analyses). In contrast, *L. aurata* was evidenced by its short snout, long pectoral fin, short head, and robust caudal peduncle (5th box truss in the IID analysis).

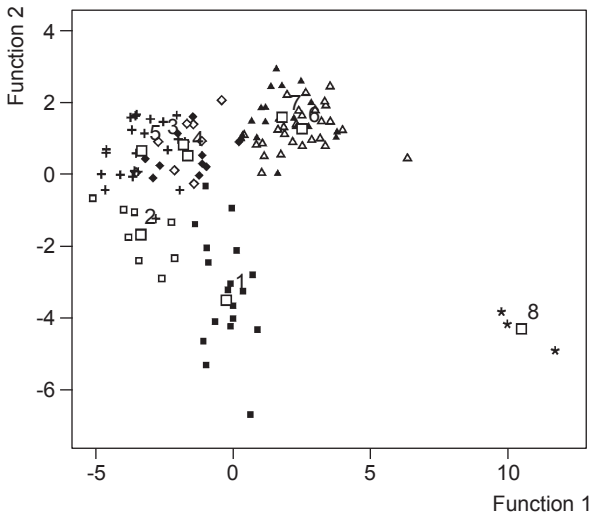

Fig. 9. Discriminant analysis performed on relative warp scores of landmark coordinates. Centroid of each species group: large empty squares; (1) *Liza ramada* (black squares); (2) *L. aurata* (white squares); (3) *Mugil cephalus* (Spain) (white diamonds); (4) *M. cephalus* (México) (black diamonds); (5) *M. liza* (crosses); (6) *M. curema* (Argentina) (white triangles); (7) *M. curema* (México) (black triangles); and (8) *M. hospes* (asterisks).

Despite the LMMs showing lower rates of grouped cases correctly classified in the DA (68.4%), notable discrimination was achieved with this method, especially taking into account that it only employed 4 variables, of which HL and PD1d were significant for species and population differentiation. Ibáñez and Lleonart (1996) found that group recognition between *M. cephalus* and *M. curema* particularly depended on differences in the ratio between the head and body. This dissimilarity was pointed out by Jordan and Everman (1896) in the specific descriptions. Accordingly, in a landmark-based morphometric study of *M. curema* and *M. cephalus*, Heras et al. (2006) showed that *M. curema* had more-robust middle and caudal segments of the body in lateral view, and IIDs of the 1st box truss (that represents the head shape) were also important measurements for group separation. In the present study, *M. curema* species (from Mexico and Argentina) were characterized by having a short but deep head. Also, their 4th and 5th box trusses were larger and more robust than those of the other studied species. SL and HL, but also pD1d, were useful variables in differentiating among Mexican and Argentinean samples.

Populations of several species of mullet were discriminated in the Gulf of Mexico and Aegean Sea using fish-scale shapes (Ibáñez et al. 2007). Discrimination between regions can be explained by the life history of the mullet, as they migrate to the ocean to spawn (Ibáñez and Gutiérrez-Benítez 2004), but they do not widely migrate, and the larvae are confined for 2-3 mo to surface currents (Ditty and Shaw 1996). Similar reproductive behaviors are shown by *M. cephalus*, *M. curema*, *C. labrosus*, and *L. saliens* in the Mediterranean Sea (Koutrakis 2004). These distances seems sufficient to maintain around 70%-80% stock integrity (Ibáñez et al. 2007); accordingly, wide support of non-contact populations such as *M. cephalus* (European and Mexican populations) and *M. curema* (Mexican and Argentinean populations) reflect broad shape differentiation. However, studies performed in the last few years

changed the point of view with regard to the taxonomic status of *M. curema*. This species not only notoriously increased its distribution range (González-Castro et al. 2006, Heras et al. 2006), but also 3 different haplogroups were identified (Fraga et al. 2007, Heras et al. 2009). Two of them belong to described *Mugil* species (*M. rubrioculus* and *M. curema*) but the 3rd one, which was morphologically identified as *M. curema* in Heras et al. (2009), presented genetic distances (when compared to the 2 other haplogroups) that are typical of *Mugil* interspecific distances (i.e., *M. curema/M. cephalus*). In this respect, morphometric differences found in the present paper between *M. curema* from Argentina and *M. curema* from Mexico suggest they may constitute different species: one of them would be the "true" *M. curema*, and the other would be the *M. curema* type 3 according to Heras et al. (2009), which has not yet been described.

On the other hand, the meristic analysis of scales counts performed in the present work was insufficient to discriminate species or populations. More meristic information is needed to discriminate among groups since there is some overlap in the scale counts. Depending on the kind of fishes under study, achieving a standard or neutral posture for each individual is not straightforward. The body of a fish is usually not a rigid structure, and a specimen's shape can be influenced by its posture during landmark capture. This issue was recently discussed by Valentin et al. (2008), who detected an upward or downward arching effect in the morphometric dataset of a multidisciplinary study on redfish (genus *Sebastes*) in the northwest Atlantic Ocean (Campana et al. 2007). Those authors proposed an approach, coupling a PCA-based model of the arching with Burnaby's orthogonal projection to remove such artefacts from the data. In the present work, the same kind of arching effect was encountered for the approaches based on landmark coordinate data. Valentin et al.'s (2008) methodology was applied, and the arching effect was removed, yielding satisfactory results as evidenced by the RWA and correct classification rates in the DA (Figs. 7-9).

The results for *M. liza* are interesting since it was the species with greater overlap, especially with other *Mugil* species. The present results should foster a fertile debate and definitively point out close resemblances among species; nonetheless, the morphometric analysis clearly discriminated between species as was also demonstrated by González-Castro et al. (2008) using mitochondrial gene cytochrome *b*, and landmark-based morphometric and meristic data. Harrison (1993) and Thomson (1997) considered *M. platanus* a synonym of *M. cephalus*. Nevertheless, González-Castro et al. (2008), based on genetic, meristic, and morphometric analyses, demonstrated that *M. platanus* and *M. cephalus* are both valid allopatric species. Recently, Fraga et al. (2007), Heras et al. (2009), and Menezes et al. (2010) showed that *M. platanus* should be considered a junior synonym of *M. liza*, which is the criterion applied in the present paper. Sometimes, morphological and genetic traits of fishes do not match, as was shown for species of the Loricaridae of Taiwan by Wu et al. (2011). However, we took in account that the mitochondrial genome did not have a unique evolutionary history, as it is genetically considered to be a single locus. There are several mechanisms that could lead to incongruence between gene trees and the species tree, most notably incomplete lineage sorting of ancestral polymorphisms and introgression resulting from interspecific hybridization (Maddison 1997, Bossu and Near 2009). The accuracy of mitochondrial (mt)DNA gene trees is compromised by hybridization that leads to introgression of mitochondrial genomes, particularly among closely related species (Bossu and Near 2009); Near et al. (2011) showed that Bayesian phylogenies inferred from mtDNA and nuclear genes revealed that heterospecific mtDNA was present in approximately 12.5% of all darter species (Percidae: Etheostomatinae). Thus, to confirm the present results (of *M. platanus* being a junior synonym of *M. liza*), it would be desirable to employ nuclear genetic markers. Also, assuming that *M. liza* is distributed from Cuba to Argentina, it would be expected hat at least 2 or 3 different populations would occur (González-Castro, unpublished data); progress in the knowledge of its life-cycles would be necessary for a better understanding of this intricate taxonomic issue.

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