Supplementary Material on the Methodology Part XIV

FINAL REPORT ON MORPHOMETRIC ANALYSES OF SARDINE AND EUROPEAN HAKE

Transboundary population structure of Sardine, European hake and Blackspot Seabream in the Alboran Sea and adjacent waters: a multidisciplinary approach (TRANSBORAN)

Framework: CopeMed II project

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1. Introduction

Stock identification and the knowledge of population spatial structure provide a basis for understanding fish population dynamics and achieving reliable assessments for fishery management (Reiss et *al.*, 2009). Some studies suggested that an absence of knowledge of population spatial structure in fisheries management might be responsible for fishery collapses in the example of North Western Atlantic herring (Stephenson et *al*., 1999).

In the Mediterranean Sea, information on stock units of exploited fish and shellfish populations is limited. Stock assessments have traditionally been carried out at the level of the General Fisheries Council the Mediterranean (GFCM) geographical subareas (GSAs). These subareas were defined in the early 2000s based mainly on practical criteria like continuity of FAO-GFCM capture statistics and geographical borders of Mediterranean countries, rather than science-based evidence for stock boundaries (Lleonart and Maynou, 2003 and Quetglas *et al.*, 2012).

Therefore, a wide number of techniques was developed and applied to identify and discriminate stock units, such as tagging experiments or analyses of spatial variation in arrange of markers including genetic markers, morphological traits, life-history traits at various life stages, parasite load or infra-community structure, or concentration of come contaminants (Pawson and Jennings, 1996; Garcia et *al*., 2011; Cadrin et *al*., 2014; ICES, 2016; Pita et *al*., 2016 and ICES, 2018).

Geometric morphometrics is a powerful, cost-efficient study field to quantify differences in organism's body shape at different spatial scales (Benitez et *al*., 2014), to study specie's ecological and evolutionary aspects and to identify genus, species and local populations (Ibanez et *al.*, 2007). This technique may help to delimit geographic populations and fish stocks of marine species based on body shape variation (Cadrin, 2000; Sequeira et *al*., 2011 and Valentin et *al*., 2008).

This report provides the final step of morphometric analysis of sardine *Sardina pilchardus* and European Hake *Merluccius merluccius* in the Alboran Sea (Western Mediterranean), assigned to CNRDPA, within the framework of the FAO project Transboundary population structure of Sardine, European hake and Blackspot Seabream in the Alboran Sea and adjacent waters: a multidisciplinary approach (TRANSBORAN). This work aims to assess body shape variations between different populations of sardine and European hake targeted in five GFCM geographical subareas and Moroccan and Spanish Atlantic waters, using geometric morphometric analysis.

2. Materiel and methods

2.1. Sampling

Samples used in this analysis coming from different ports of GSAs 1, 3, 4 and 12 and Spanish and Moroccan Atlantic waters (Fig. 01). A total of 866 samples were collected for sardine in all the 17 ports initially proposed. For hake, 675 specimens were sampled in 15 out the 17 ports originally proposed excepted Al Hoceïma (ALH_GSA 3) and Cherchell (CHE_GSA 4).

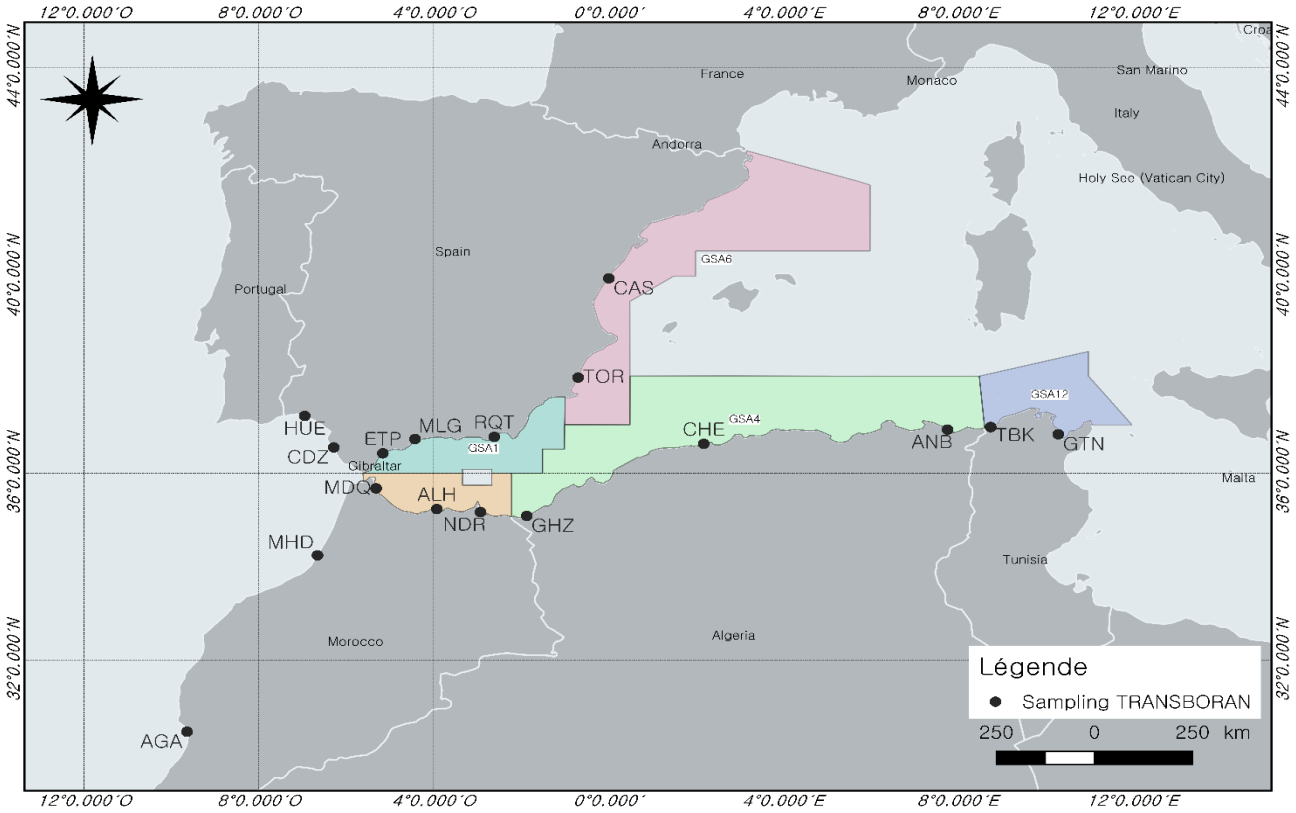

Figure. 01. Sardine and hake samples origins

2.2. Data preparation

A total of 866 sardines and 611 hake pictures collected from different institutions CNRDPA, INRH, IEO and INSTPM were received and visualised by eye. Photographs of the left side of body were collected with Eos reflex camera (55 mm zoom lens) fixed on 80 cm (reference) using needles to reduce arching artefacts. To avoid potential artefacts, 164 sardines and 73 hake pictures were excluded from analysis because they were not deemed appropriate to recover morphology.

2.3. Acquisition of morphometric data

To quantify the body shape of *Sardina pilchardus* and *Merluccius merluccius* samples, we digitized a set of 14 landmarks on sardine and 19 landmarks on hake pictures (Fig. 2 and 3). For both species 10 " *Helper points* " were used, these points are treated as sliding semi-landmarks to help in the alignment of other points, then removed as they do not provide additional information (Zelditch, Swiderski Sheets, 2004; Fruciano et al., 2016b). Landmarks were digitized as (x, y) coordinates on the top view pictures three times by the same operator and averaged to remove bias introduced by digitalisation error.

Figure 2. Landmarks used for sardine *S. pilchardus*

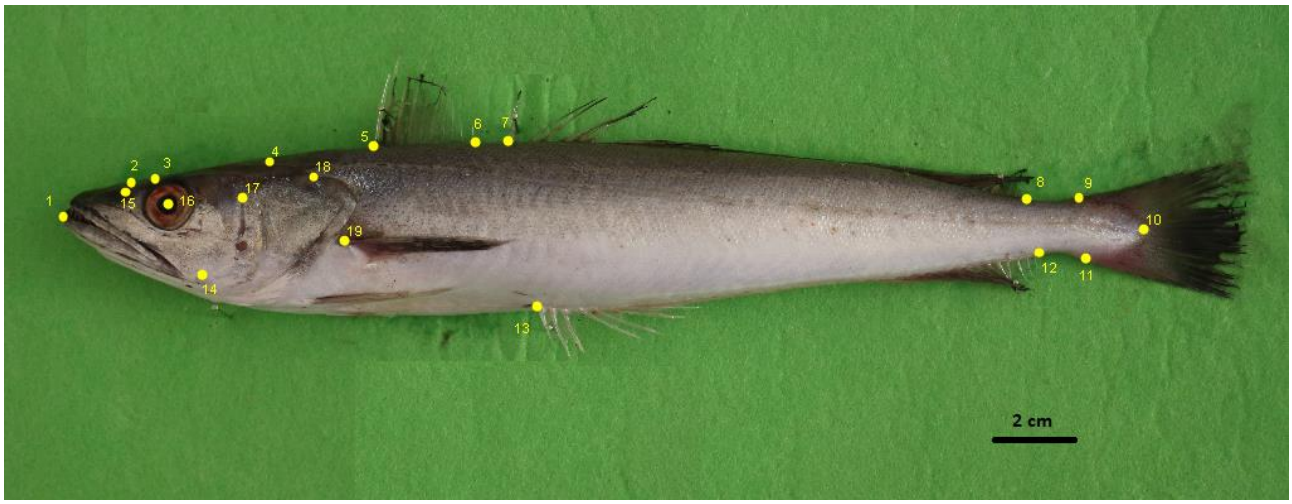

Figure 3. Landmarks used for European hake *M. merluccius*

2.4. Shape data analysis

The configurations of points thus obtained were subjected to a generalized Procrustes analysis with sliding of semi-landmarks with ten iteration and minimizing procrustes distances as criteria (Bookstein, 1997). After that, shape variation due to dorso-ventral arching of the fish body was modelled in this work as a shape change vector (Valentin et *al*. 2008) using a random subset of 10 images by sampling site showed different levels of dorso-ventral arching.

A total of 17 shape changes vectors of sardine and 15 for hake, one per site sample, were obtained, and then, a single shape change vector was computed by averaging them. This shape variation was then removed from the data set by projecting the shape variables constituting the data set to the multivariate subspace orthogonal using Burnaby's procedure (Burnaby, 1966).

To avoid the confusing effect due to sexual dimorphism in hake, we performed a between-groups principal component analysis using sex as grouping variable on the subset of fish sexed previously. The shape variation attributable to sexual dimorphism was then removed from the dataset by projecting shape variables in the subspace orthogonal to the first between-groups principal component (Burnaby, 1966).

To remove the allometric shape variation, a multivariate regression of shape variables on centroid size was carried out and regression residuals were used in subsequent analyses. Procrustean ANOVA and Pairwise permutation tests (10000 permutations) were performed to analyse difference in mean shape between samples. As an exploratory tool, we used Factorial Discriminant Analysis FDA.

3. Results

3.1. Hake body shape analysis

Hake displayed significant allometry ($p < 0.05$). All further analyses used regression residuals to remove allometric effects. The FDA revealed variation in hake's body shape among investigated ports, with the first three discriminate axes explaining a 66.2 % of the total observed variation (Table 01). Using GSAs as group, the first three axes explain 82.7% of the total discrimination (Table 02).

In general, excepting Tunisian samples (GTU and TBK), the factorial discrimination analysis with ports as a group reveals a very high degree of overlap among Alboran sampling sites (Fig. 4), but with some level of separation of specimens from north Alboran and specimens from south Alboran.

This pattern becomes clearer when performing an FDA using GFCM sub areas (GSAs) as a group, with scores along the first discriminant axe showing distinct distributions for GSA-01 and GSA-03 (Fig. 5) with an overlapping between them and GSA-04.

Table 1. Eigen values of Sardine FDA, considering sampling site used as factor

	F1	F2	F3	F4	F5	F6
Valeur propre	3.274	2.247	1.072	0.861	0.743	0.385
Discrimination (%)	32.874	22.560	10.767	8.640	7.462	3.868
% cumulé	32.874	55.434	66.201	74.841	82.303	86.171

Table 2. Eigen values of Sardine FDA, considering GFCM subareas used as factor

	F1	F2	F3	F4	F5	F6
Valeur propre	2.33	1.952	0.742	0.572	0.315	0.164
Discrimination (%)	38.348	32.134	12.214	9.418	5.183	2.704
% cumulé	38.348	70.482	82.696	92.113	97.296	100

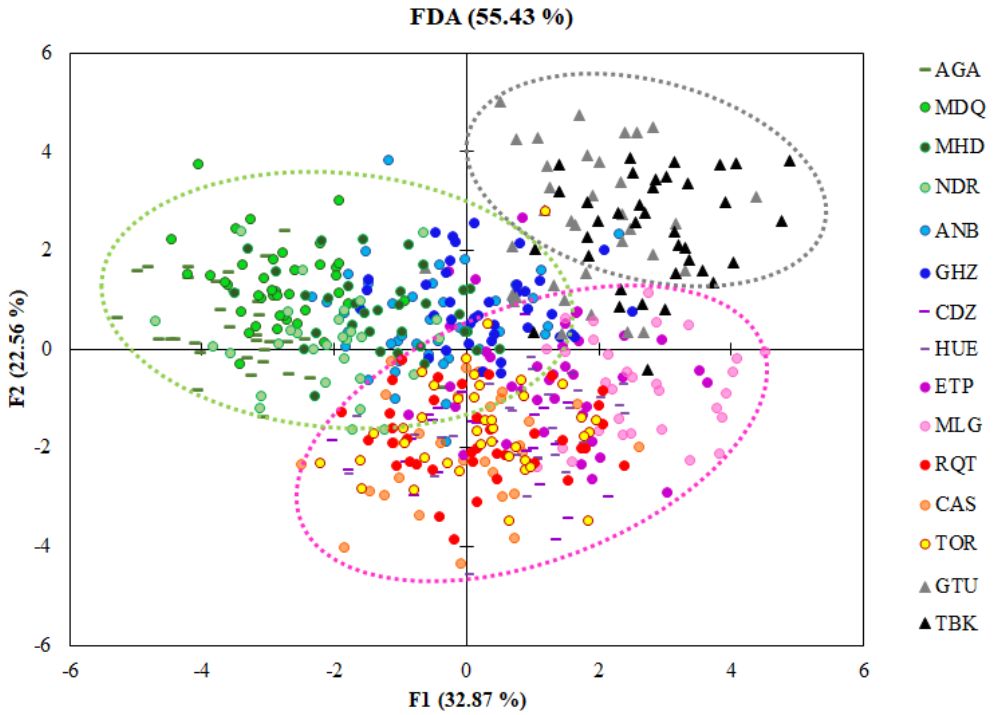

Figure 04. FDA of European hake *M. merluccius* body shape, considering sites as group

Cross validation posterior probability (Fig. 6) confirms the overlapping between GSAs. Posterior contribution was 67.6%, 75. 7%, 90.1%, 74.3% ,90.3%, 54.7% and 63.6% for GSA-1, GSA-3, GSA-4, GSA-6, GSA-12, Spanish Atlantic and Moroccan Atlantic waters respectively.

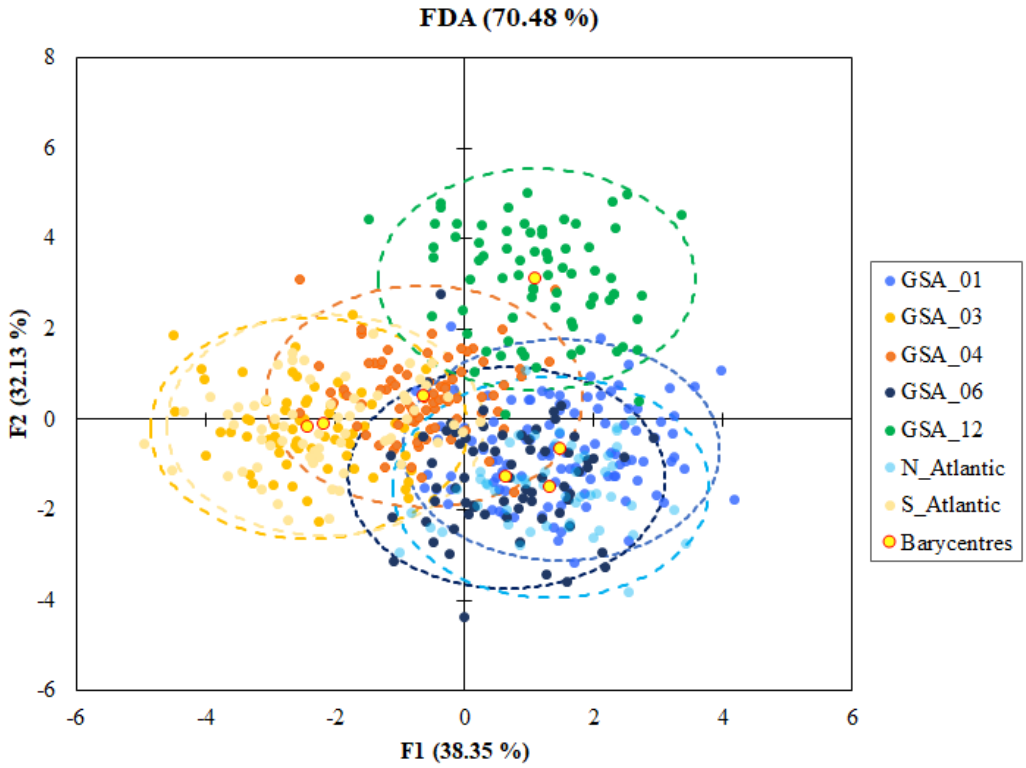

Figure 5. FDA of European hake *M. merluccius* body shape, considering GSAs as group

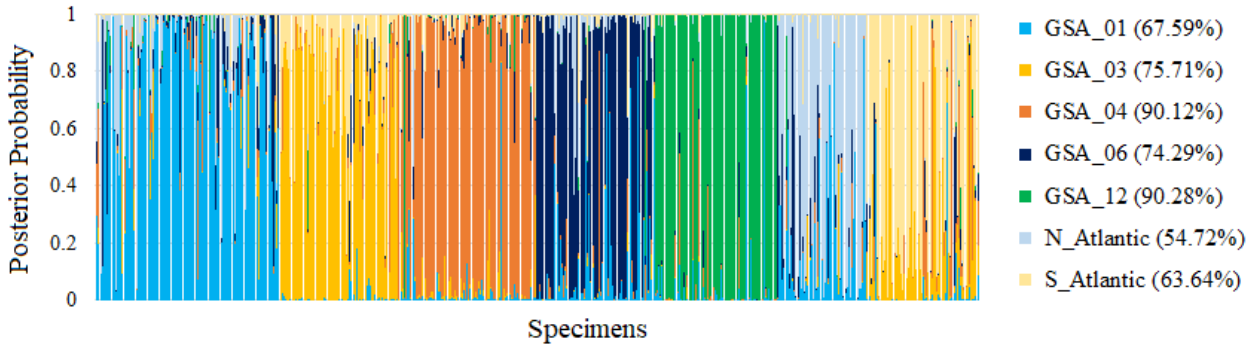

Figure 6. Classification probabilities after cross validation (HKE _GSAs as group)

Procrustes ANOVA based on the centroid size and body shape confirmed significant effects of the GFCM areas on body shape of European hake ($p < 0.05$). Tests of difference in mean shape between the 15 sampling sites reveal also significant differences among sampling ports (Table 01, Annexe).

Significant differences in mean shape between GFCM sub-geographical areas GSAs reflect the exploratory results obtained with discriminant analysis FDA. Pairwise comparisons among populations from West Mediterranean GSAs (Table 3) showed that GSA-01 was significatively different from the others excepting GSA-06. Hake of North Atlantic was significatively different from the other population of Mediterranean areas and South Atlantic.

Table 03. Morphological differentiation between *M. merluccius* sampling from different GSAs

P-Values / Distance	GSA_01	GSA_03	GSA_04	GSA_06	GSA_12	N. Atlantic	S. Atlantic
GSA_01	-	1.65E-04	1.48E-04	1.59E-05	8.74E-05	9.15E-05	8.04E-05
GSA_03	**<0.000**	-	1.78E-05	1.50E-04	7.80E-05	2.57E-04	8.50E-05
GSA_04	**<0.000**	0.639	-	1.32E-04	6.02E-05	2.39E-04	6.72E-05
GSA_06	0.650	**<0.000**	**<0.000**	-	7.15E-05	1.07E-04	6.45E-05
GSA_12	**0.016**	**0.044**	0.104	0.063	-	1.79E-04	7.03E-06
N. Atlantic	**0.017**	**<0.000**	**0.000**	**0.009**	**<0.000**	-	1.72E-04
S. Atlantic	**0.025**	**0.030**	0.075	0.098	0.854	**<0.000**	-

Procrustes distances and associated *P*-values are reported in the upper and lower triangle, respectively. Statistically significant ($P < 0.05$) comparisons are highlighted in boldface.

3.1. Sardine body shape analysis

The FDA revealed variation in sardine's body shape among investigated ports (Table 4), with the first two discriminate axes explaining a 54.08 % of the total observed variation (PC1 = 35.6 %; PC2 = 18.4 %; PC3 = 13.9 %). Using GSAs as grouping factor, the first three axes discriminate 71.3% of the total variation (Table 05).

Table 4. Eigen values of Sardine FDA, considering sampling sites as factor

	F1	F2	F3	F4	F5	F6
Valeur propre	3.983	2.066	1.563	0.972	0.852	0.445
Discrimination (%)	35.613	18.468	13.978	8.688	7.621	3.977
% cumulé	35.613	54.080	68.059	76.747	84.368	88.345

Table 5. Eigen values of Sardine FDA, considering GFCM subareas as factor

	F1	F2	F3	F4	F5	F6
Valeur propre	2.852	1.462	0.868	0.55	0.179	0.14
Discrimination (%)	47.131	24.163	14.346	9.095	2.955	2.31
% cumulé	47.131	71.293	85.64	94.735	97.69	100

In general, the FDA did not reveal clear differences among groups corresponding to different sites except for Eastern south-Mediterranean Sea (ANB, CHE, TBK and GTU) that was separated from the Atlantic sites and Estepona (Fig. 8) on the first discriminate axis. A clear distinction between ETP and the others ports of Alboran Sea was observed. The individuals from the other Alboran ports and eastern Spanish area showed a big overlapping between the two first groups.

Using GFCM subareas (GSAs) as grouping factor (Fig. 9), the variation explained by the two first axis (71.3%) showing an overlapping between GSA-6, GSA-12 and south Alboran Sea with minimum

degree. Some distinct distributions for GSA-01 and North Atlantic from GSA-03, GSA-4 and GSA-12 were observed on the first discriminant axis. However, on the second axis, sardine of GSA-12 was distinct from South Atlantic sardine's population.

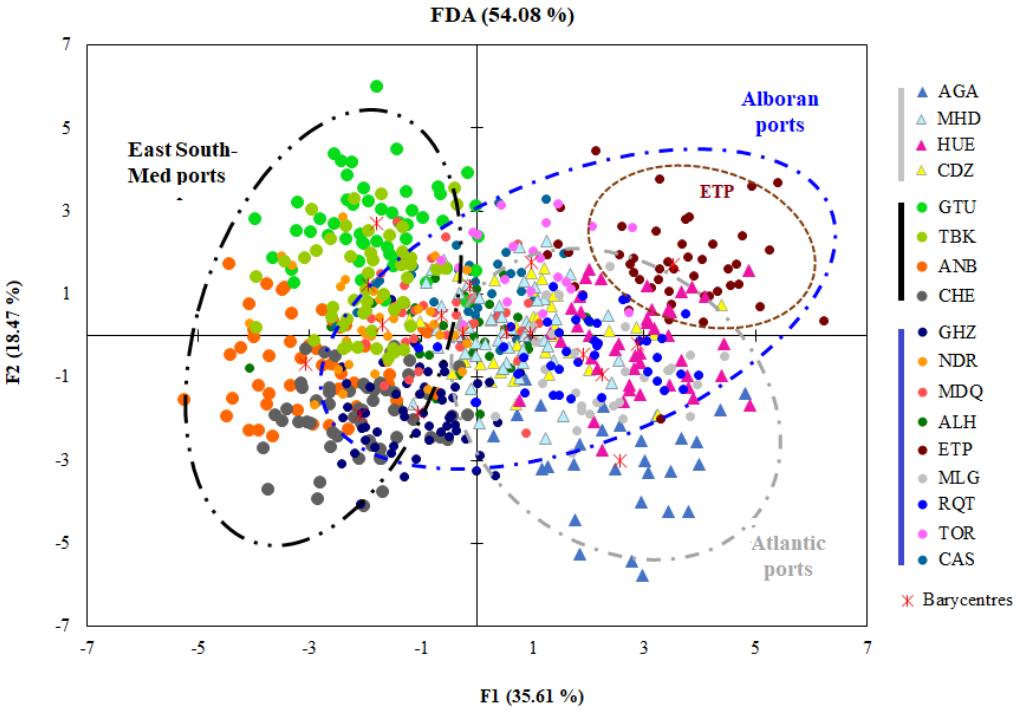

Figure 8. FDA of sardine in Alboran sea and adjacent waters ports

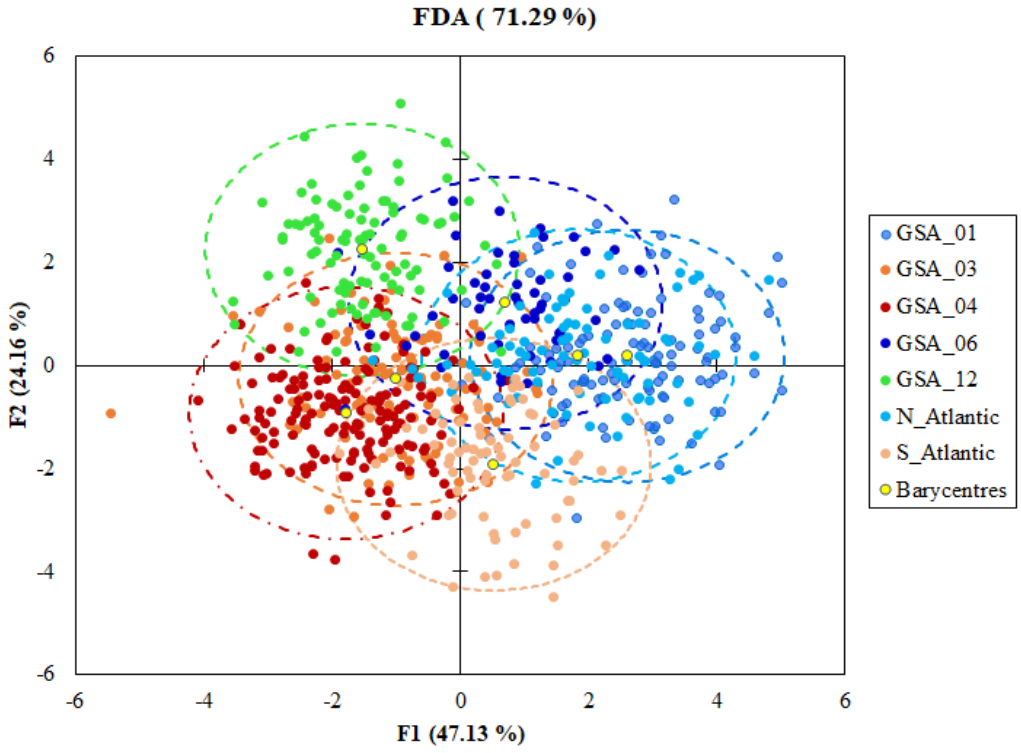

Figure 9. FDA of sardine in Alboran sea and adjacent waters with GSAs as group

Cross validation posterior probability (Fig. 10) confirms the overlapping between GSAs. Posterior contribution was 71.43%, 75. 45%, 88.61%, 69.23% ,85%, 60% and 70.5% for GSA-1, GSA-3, GSA-4, GSA-6, GSA-12, Spanish Atlantic and Moroccan Atlantic waters respectively. In comparison with hake, the largest overlapping present in Alboran sea (GSA-1, 3 6 and Atlantic waters).

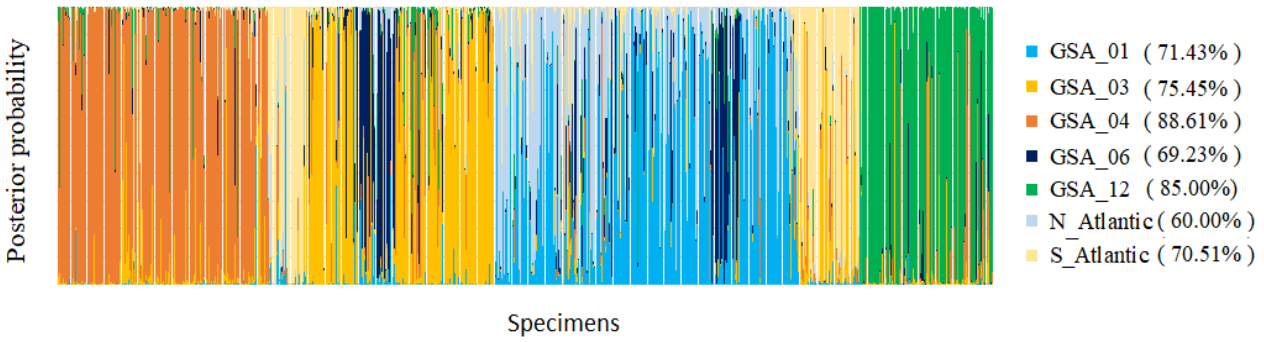

Figure 10. classification probabilities after cross validation (Sardine_ GSAs as group)

Procrustes ANOVA based on the centroid size and body shape confirmed significant effects of the site on body shape of *Sardina pilchardus* ($p < 0.05$). Pairwise comparisons among samples (Table. 02, annexe) and GSAs (Table 6) show a significant differences among GSA-01 and the other GSAs, samples of Atlantic waters are different from GSA-03, 04 and 12.

Table 06. Morphological differentiation between Sardine sampling from different GSAs

P-Values / Distance	GSA_01	GSA_03	GSA_04	GSA_06	GSA_12	N. Atlantic	S. Atlantic
GSA_01	-	1.04E-04	1.54E-04	9.46E-05	1.32E-04	4.19E-05	2.05E-05
GSA_03	**<0.001**	-	4.98E-05	9.35E-06	2.76E-05	6.20E-05	8.34E-05
GSA_04	**<0.001**	**0.018**	-	5.92E-05	2.22E-05	1.12E-04	1.33E-04
GSA_06	**0.002**	0.733	**0.039**	-	3.70E-05	5.26E-05	7.40E-05
GSA_12	**<0.001**	0.248	0.305	0.180	-	8.96E-05	1.11E-04
N. Atlantic	0.078	**0.011**	**<0.001**	0.061	**<0.001**	-	2.14E-05
S. Atlantic	0.414	**0.002**	**<0.001**	**0.017**	**<0.001**	0.421	-

Procrustes distances and associated *P*-values are reported in the upper and lower triangle, respectively. Statistically significant ($P < 0.05$) comparisons are highlighted in boldface.

In order to have more discrimination between sampling sites, a non-multidimensional scaling (nMDS) was carried out on sardine samples (Fig. 11) using primer 6 (version 1.0.6). Outsides the overlapping observed between different ports, they can be aggregated in three big groups, the first one is formed by GSA-12, the second one by south Atlantic ports, GSA-03 and GSA-04 and the third one associate North Atlantic ports with GSA-01 and GSA-06.

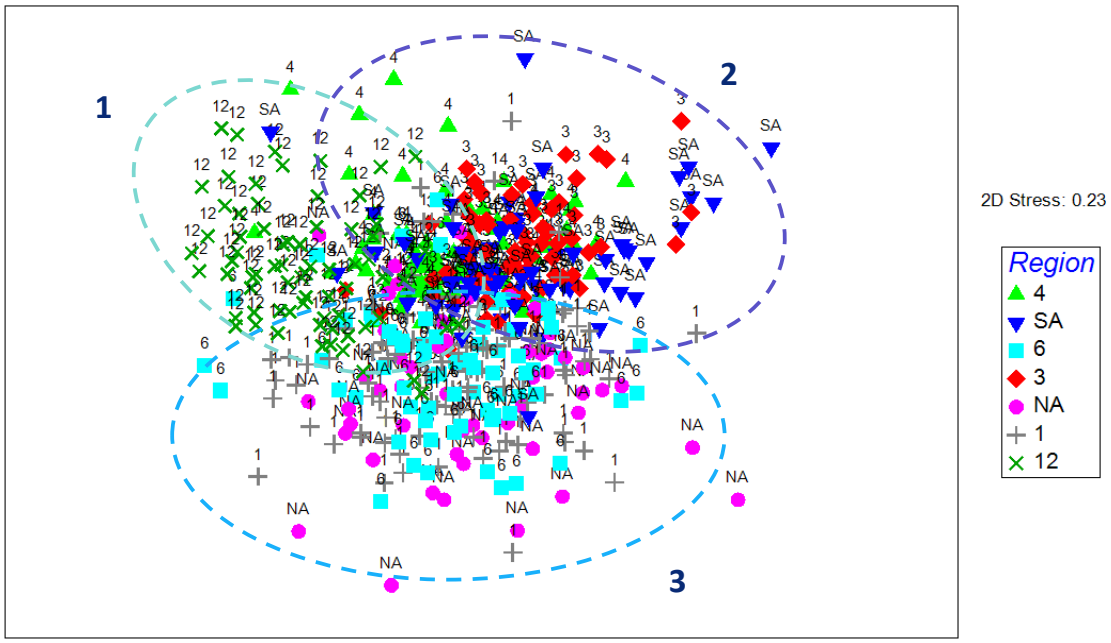

Figure 11. nMDS analysis of sardine shapes considering GSAs as grouping factor

Confusion matrix after cross-validation between sardine's populations from subareas (Fig. 12, Table 03 annexe) show that 26.92% of south Atlantic are affected to the south Alboran (GSA-3 and GHZ from GSA-4) group and 36.47% from North Atlantic to North Alboran (GSA-1 and 6).

The connection between sardine populations from North Alboran and south Alboran could be occur, 12% of sardines were affected to the populations of South Alboran and adjacent South Mediterranean water (7.02% to Gsa-3+GHZ and 4.85% to GSA-4+TBK).

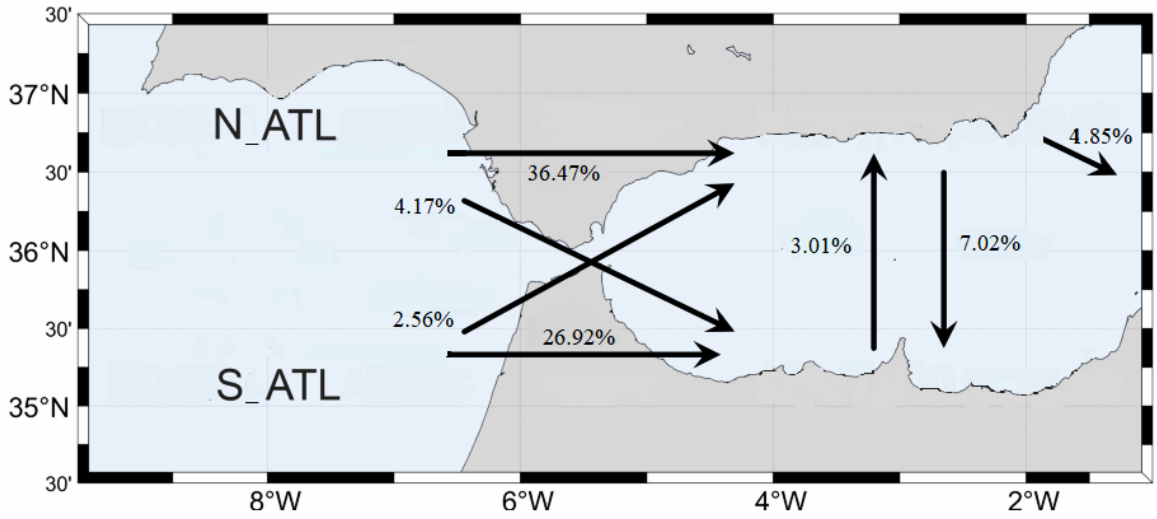

Figure 11. Confusion matrix of *S. pilchardus* after cross validation _subareas as group

4. Conclusion

Morphometric analyses have been used as alternative and robust tool for the discrimination of biological groups. In this study, sardines and hake population structure from Alboran Sea and adjacent waters were investigated using geometric-morphometric analysis to identify morphological groups or stock units.

On the basis of the output of this analysis, we have studied the morphometric connectivity of 17 selected regions of sardine and 15 for hake. The results of hake body shape variation did not reveal clear differences among groups corresponding to different ports of Alboran sea. However, a morphological pattern has been observed between north Atlantic - North Alboran hake samples and between South Atlantic - South Alboran samples.

For sardine, samples coming from Alboran ports presented morphometric similarities, except ETP sardines sampled in the North-west Alboran sea. The south Moroccan Atlantic sample (AGA) showed the greatest morphological differentiation with the other sites. This higher discrimination of Agadir sample, should therefore, be associated with the environmental factors of Moroccan Atlantic coasts.

In agreement with the different authors working on stock discrimination using morphometrics, the morphometric differences may be more related to the phenotypic plasticity than to the genetic variation.

These results about hake and sardine populations structure in West Mediterranean Sea would need to be confirmed by additional analysis including genetics results and environmental data.

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Annexe

Table 01. Pairwise test results for European hake between sampling sites

	AGA	ANB	CAS	CDZ	ETP	GHZ	GTU	HUE	MDQ	MHD	MLG	NDR	RQT	TBK	TOR
AGA															
ANB	0.878														
CAS	**0.003**	**0.001**													
CDZ	**0.000**	**0.000**	**0.005**												
ETP	**0.040**	**0.018**	0.292	**0.000**											
GHZ	0.273	0.326	**0.000**	**0.000**	**0.001**										
GTU	0.410	0.305	**0.029**	**0.000**	0.212	0.053									
HUE	**0.028**	**0.013**	0.476	**0.000**	0.781	**0.001**	0.151								
MDQ	0.243	0.277	**0.000**	**0.000**	**0.000**	0.923	**0.045**	**0.001**							
MHD	0.283	0.199	0.058	**0.000**	0.340	**0.027**	0.793	0.251	**0.027**						
MLG	**0.005**	**0.002**	0.816	**0.003**	0.417	**0.000**	0.051	0.631	**0.000**	0.092					
NDR	0.834	0.943	**0.002**	**0.000**	**0.028**	0.401	0.315	**0.018**	0.349	0.210	**0.004**				
RQT	0.197	0.124	0.074	**0.000**	0.419	**0.013**	0.641	0.299	**0.010**	0.858	0.119	0.144			
TBK	0.324	0.236	**0.038**	**0.000**	0.264	**0.033**	0.890	0.189	**0.028**	0.903	0.064	0.243	0.752		
TOR	0.097	0.051	0.144	**0.000**	0.662	**0.004**	0.411	0.486	**0.003**	0.592	0.222	0.068	0.707	0.492	

Table 02. Pairwise test results for Sardine between sampling sites

	AGA	ALH	ANB	CAS	CDZ	CHE	ETP	GHZ	GTU	HUE	MDQ	MHD	MLG	NDR	RQT	TBK	TOR
AGA	-																
ALH	**0.001**	-															
ANB	**0.001**	0.758	-														
CAS	**0.001**	0.118	0.058	-													
CDZ	**0.002**	0.169	0.077	0.814	-												
CHE	**0.001**	0.076	0.136	**0.002**	**0.003**	-											
ETP	0.189	**0.001**	**0.001**	**0.017**	**0.007**	**0.001**	-										
GHZ	**0.001**	0.437	0.67	**0.023**	**0.034**	0.256	**0.001**	-									
GTU	**0.001**	0.884	0.628	0.132	0.174	**0.034**	**0.001**	0.329	-								
HUE	**0.010**	**0.010**	**0.002**	0.398	0.274	**0.001**	0.094	**0.002**	**0.009**	-							
MDQ	**0.001**	0.694	0.466	0.207	0.327	**0.025**	**0.001**	0.231	0.777	**0.026**	-						
MHD	**0.001**	0.354	0.192	0.438	0.599	**0.002**	**0.001**	0.073	0.383	0.074	0.598	-					
MLG	**0.005**	0.053	**0.025**	0.749	0.551	**0.001**	**0.050**	**0.007**	0.051	0.658	0.112	0.24	-				
NDR	**0.001**	0.497	0.307	0.361	0.525	**0.013**	**0.002**	0.159	0.556	0.073	0.711	0.872	0.218	-			
RQT	**0.02**	**0.008**	**0.004**	0.312	0.181	**0.001**	0.171	**0.002**	**0.009**	0.802	**0.016**	**0.038**	0.487	0.050	-		
TBK	**0.001**	0.374	0.555	**0.018**	**0.023**	0.355	**0.001**	0.852	0.257	**0.002**	0.188	0.065	**0.005**	0.121	**0.001**	-	
TOR	**0.001**	0.436	0.605	**0.029**	0.050	0.536	**0.001**	0.834	0.332	**0.005**	0.271	0.115	**0.017**	0.172	**0.003**	0.931	-

Table. 3. Confusion matrix after cross validation _ *Sardina pilchardus*

From \ To	E. Med	N. Alboran	N. Atlantic	S. Alboran	S. Atlantic	S. Med
E. Med (GSA-12)	**85.00%**	2.00%	0.00%	8.00%	1.00%	4.00%
N. Alboran (GSA1 + GSA6)	2.92%	**76.02%**	9.94%	7.02%	3.51%	0.58%
N. Atlantic	3.53%	36.47%	**52.94%**	4.71%	1.18%	1.18%
S. Alboran (GSA-3 + GHZ)	4.82%	3.01%	1.20%	**74.10%**	4.22%	12.65%
S. Atlantic	0.00%	2.56%	3.85%	26.92%	**65.38%**	1.28%
S. Med (GHZ + ANB)	3.92%	0.00%	0.00%	17.65%	2.94%	**75.49%**

ANOVA_ HKE _GSAs

```
Analysis of Variance, using Residual Randomization
Permutation procedure: Randomization of null model residuals
Number of permutations: 10001
Estimation method: Ordinary Least Squares
Sums of Squares and Cross-products: Type I
Effect sizes (Z) based on F distributions

                 Df       SS        MS     Rsq       F       Z    Pr(>F)
log(Csize)        1 0.011591 0.0115915 0.03785 27.9164  7.5720 9.999e-05 ***
GSAs              6 0.072783 0.0121305 0.23768 29.2144 17.2818 9.999e-05 ***
log(Csize):GSAs   6 0.011751 0.0019585 0.03837  4.7168  9.0249 9.999e-05 ***
Residuals       506 0.210102 0.0004152 0.68610
Total           519 0.306228
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

ANOVA_ Sardine _GSAs

```
Analysis of Variance, using Residual Randomization
Permutation procedure: Randomization of null model residuals
Number of permutations: 1000
Estimation method: Ordinary Least Squares
Sums of Squares and Cross-products: Type I
Effect sizes (Z) based on F distributions

                 Df      SS       MS     Rsq        F       Z Pr(>F)
log(Csize)        1 0.03975 0.039746 0.11798 136.6866  9.6069  0.001 **
GSAs              6 0.09059 0.015099 0.26892  51.9249 14.9882  0.001 **
log(Csize):GSAs   6 0.00648 0.001080 0.01924   3.7155  6.2922  0.001 **
Residuals       688 0.20006 0.000291 0.59386
Total           701 0.33688
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```