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Use of otolith microchemical and morphological analyses for stock discrimination of *Sarpa salpa* on two Tunisian islands, Djerba and Kerkennah

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Abstract

Otolith morphological and microchemical analyses are relatively new scientific research methods used in fish stock evaluation and management. However, in Tunisia, only morphological methods have been used. The objective of this study was the *Sarpa salpa* stock discrimination of Djerba and Kerkennah by the otoliths morphological and microchemical analysis, while carrying out a fluctuating asymmetry analysis and a stock comparison of males and females for each population. The results revealed significant differences between the Djerba and Kerkennah populations, significant differences between the stocks of males and females in each population, and a highly significant fluctuating asymmetry for both populations. The results of the otolith morphological analysis were similar to those of the microchemical analysis. This result proves that both morphological and microchemical analyses are powerful tools for fish stock discrimination.

Introduction

Sarpa salpa, commonly called Salema, is a Sparidae that lives in shallow seas where seagrass beds like *Posidonia oceanica* and *Cymodocea nodosa* are abundant. The species prefers temperate and tropical areas; it is widely distributed in the eastern Atlantic, western Indian Ocean, some areas of the Black Sea, and the Mediterranean Sea (Criscoli *et al.*, 2006). This fish is a demersal herbivore (Verlaque, 1990) that feeds on algae, diatoms, and macrophytes (Havelange *et al.*, 1997). However, this fish is rarely eaten worldwide, with the exception of France and Tunisia. Indeed, it is very appreciated in the south of Tunisia, especially during its reproduction season when it stops feeding to spawn (Anato and Ktari, 1983). Because of those reasons, we chose to discriminate the *S. salpa* stock at the two most important consumer sites in Tunisia: Djerba and Kerkennah, using otolith morphology and microchemistry analysis.

Otoliths, mineralized concretions found in the inner ears of teleost fish, are composed of three pairs positioned in endolymph-filled cavities on either side of the brain. The Sagittae is the largest; it is distinguished by two sides (concave and convex), two projections (rostrum and antirostrum), and a furrow (Sulcus acusticus) (Lecomte-Finiger, 1999). The balance and hearing senses depend on these calcareous concretions for correct functionality (Popper *et al.*, 2005).

Otoliths are produced by adding calcium carbonate ($CaCO_3$), minor elements (Na, Sr, K, S, Cl, P), traces (Mg, Si, Zn, B, Fe, Hg, Mn, Ba, Cu, Al, Br, Li), and infratrace (Pb, As, Se, Ag, Co, Cd, U, Cs) to a protein matrix (otolin) (Campana, 1999). They record events in fish life history due to their metabolically inert composition (Campana and Neilson, 1985). Thus, they are used for many biological investigations, such as species identification (Rousset, 1983; Maisey, 1987); palaeontological studies (Nolf, 1993; Steurbaut and Nolf, 1998); growth analysis; age determination; and fish mortality analysis (Cardinale *et al.*, 2004).

In recent years, otoliths have become widely used in fish stock analyses. So, many researchers in Tunisia have used otolith morphology to discriminate stocks of various species (Messaoud *et al.*, 2011; Trojette *et al.*, 2015; Rebaya *et al.*, 2016, 2017; Mejri *et al.*, 2018a, 2018b; Ben Mohamed *et al.*, 2019; Jmil *et al.*, 2019; Bakkari *et al.*, 2020; Ben Labidi *et al.*, 2020a, 2020b; Bouriga *et al.*, 2021; Khedher *et al.*, 2021).

Although several scientists around the world have used microchemical analysis to discriminate fish stocks (Gordon *et al.*, 2001; Swan *et al.*, 2003; Veinott and Porter, 2005; Miyana *et al.*, 2016), no scientific research has used this method for fish stock evaluation. The fish stock evaluation allows the determination of the population's degree of isolation from other populations of the same species.

However, scientific research based on the fluctuating asymmetry of otolith shape has been used for stock discrimination in Tunisia (Mejri *et al*., 2020, 2022). Fluctuating asymmetry can be defined as deviations from a known ratio of morphological structure. As a particular type of phenotypic variability, the fluctuating asymmetry level is an indicator of optimal conditions for development and genetic coadaptation. Thus, fluctuating asymmetry acts as a measure of developmental state and stability in population biology (Zakharov and Trofimov, 2022). Thus, otolith fluctuating asymmetry is both appealing and simple to use, and it has been found to vary between populations, hence having the potential to be useful for fisheries evaluation (Diaz-Gil *et al*., 2015).

Fish stock is the basic unit used in the assessment of fisheries states and the application of management policies for the implementation of sustainable development (Tanner *et al*., 2015). In fact, in this study, we performed three different otolithometric analyses to discriminate the *S. salpa* stock captured from Djerba and Kerkennah, Tunisian islands. So, firstly, we used elliptical Fourier descriptors (EFD) to analyse the otoliths' morphology. Secondly, the otoliths' biometry was examined in terms of length, width, air, perimeter, and weight. Finally, a microchemical analysis was performed for K, Cs, and Pb. Those heavy metals have been chosen for the microchemical analysis given their importance in the Gulf of Gabes ecological analyses and especially given that most of the scientific research in Tunisia is interested in these elements (Chouba *et al*., 1996; Mkawar *et al*., 2007; Rabaoui *et al*., 2013).

The main goals were to discriminate the *S. salpa* stocks on the islands of Djerba and Kerkennah, to determine fluctuating asymmetry in both populations, to check sexual stock dimorphism in each population, and to test the feasibility of otolith morphological and microchemical analyses as a new tool for stock evaluation.

Materials and methods

Sample collection

120 adult specimens of *S. salpa* were collected from the two islands (Djerba and Kerkennah) (Figure 1) by artisanal fishing (gill nets) in October 2021. Therefore, 60 fish (30 males and 30 females) were studied from each station. In fact, using an ichthyometer and a balance, the total length (LT) and total weight (WT) of each specimen were recorded to the nearest mm and 0.01 g, respectively. Longmore *et al*. (2010) report that for otolith morphological and microchemical analysis, it is necessary to study mature individuals with similar size and weight ranges to avoid confounding effects of ontogenesis on otolith chemistry and

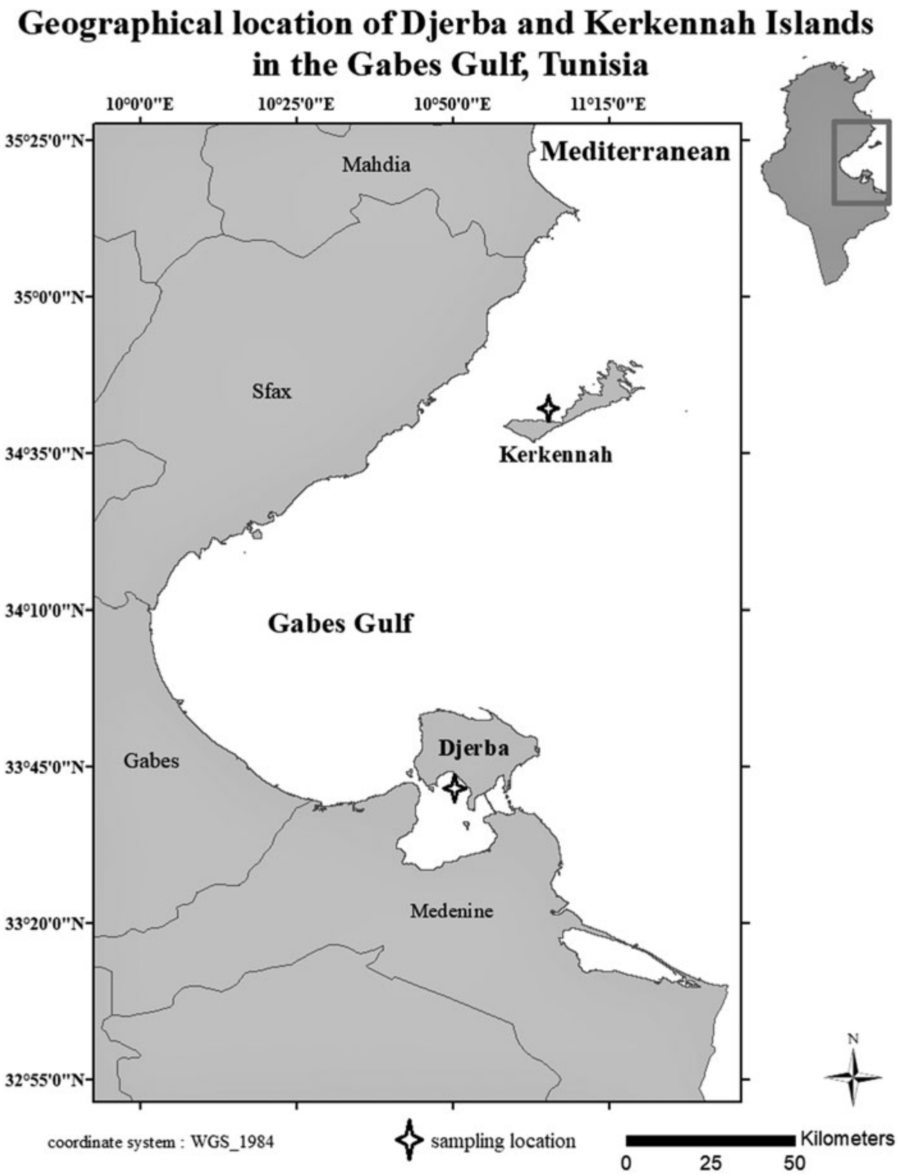

Figure 1. Sarpa *salpa* sampling sites on Djerba and Kerkennah islands, Tunisia.

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shape. Thus, our samples were selected based on their maturity and similar size. The variance comparison test of total length (LT) and weight was used to check the sample homogeneity. However, this homogeneity test is parametric, so the Shapiro–Wilks test for normality verification was first carried out for the lengths and the total weights.

Otolith extraction

Firstly, a small chisel was used to make a horizontal cut on the upper bone of the fish head. Then, Sagittae pairs were extracted with forceps. They are then cleaned with distilled water, dried, and finally stored in Eppendorf tubes.

Otolith morphometric analysis

Both right and left sagittal otoliths were placed on a microscope slide, and each sagittae was photographed using a digital camera (Canon IXUS 185, 20 MP). Otoliths were positioned with the sulcus facing up and the concave side above. Otolith photos previously converted into BMP format were processed with SHAPE software Ver. 1.3 (Iwata and Ukai, 2002). Firstly, a chain code employing a binary contour projection was used to create the otolith shape. Secondly, EFD were created from the chain code. The EFDs were normalized, and twenty harmonics were extracted for each otolith. For each harmonic, four coefficients (a, b, c, and d) were distributed. Thus, for each otolith, a total of 80 coefficients was obtained; therefore, the principal component analysis was carried out to summarize the information of the EFD in four principal coefficients.

The otolith morphology measurements for length (Lo), width (Wo), area (Ao), perimeter (Po), and weight (Mo) were determined using ImageJ (Figure 2). To avoid the statistical analyses being impacted by the change in pixel count, we used the identical otolith image in both the Shape and the ImageJ software. Additionally, right and left otolith weights were measured in grams using a balance precision of 0.01 mg. Using Shapiro–Wilks' test, the normal distribution of otolith morphological variables was tested. If the variables do not follow the normal distribution, a Box–Cox transformation (Box and Cox, 1964) is performed. Then, the comparison test between the variances of the two samples was carried out. Therefore, the MANOVA was performed on the otolith morphological variables (the four elliptical Fourier coefficients, the length, the width, the perimeter, the area, and the weight). Finally, the discriminant factor analysis (DFA) was applied to the otolith morphological variables and expressed with the tests of lambda Wilks and *p*-values of Fisher distances. These statistical tests were first used to compare the two populations, then to verify the fluctuating asymmetry of each population by comparing the morphological variables of the right and left otoliths of each population. The sexual dimorphism of the population was finally verified by comparing statistical variables between males and females at each site.

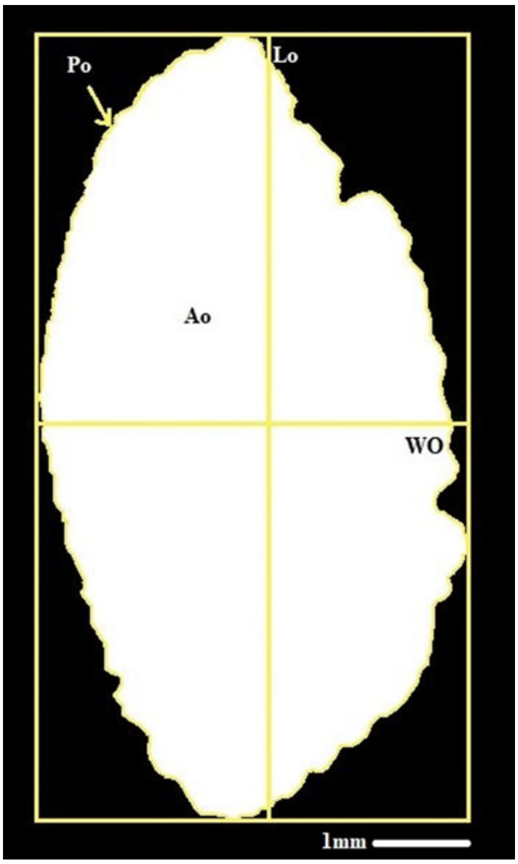

Figure 2. Left otolith of *Sarpa-Salpa* and its biometric parameter, scale bars: 1mm.

Otolith microchemical analysis

All otoliths were crushed to a fine powder in a mortar that had been cleaned with acetone to prevent contamination. Then 1 g of otolith powder was mineralized by adding 8 mL of HNO_3 solution (65%) and 2 mL of H_2O_2 solution. The resulting mixture was heated on a hot block at 125 °C ± 5 °C before being dissolved in 50 ml of ultrapure water (Milli Q). The results of this mineralization were then analysed by Flame Atomic Absorption Spectrometry (FAAS), the Analytik Jena (Novaa350) model. Acetylene is injected into the spectrometer's flame at a rate of 70 L/h with a 6 mm burner height. The wavelengths used to detect K, Cs, and Pb are 766.5 nm, 852.1 nm, 228.8 nm, and 283.3 nm, respectively. Therefore, we obtained the content of K, Cs, and Pb for 1 g of otolith, and since we measured the weight of the otolith on a precision balance, we calculated the content of each mineral element (K, Cs, and Pb). We then used a discriminant factor analysis (DFA) on all of the values to compare the two populations using microchemical analysis.

Results

Sample homogeneity

The *S. salpa* sampled at Djerba had an average total length of 170.66 ± 16.72 mm and a mean total weight of 68.76 ± 18.98g. However, the *S. salpa* collected in Kerkennah had an average total length of 169.85 ± 15.45 mm and an average total weight of 70.17 ± 16.24 g (Table 1).

The Shapiro–Wilks test showed that the sample followed the normal distribution with a *p*-value 0.78 (>0.05). The homogeneity test of variance comparison performed on the fish's total length (LT) and total weight (WT) proves the absence of a significant difference between samples (LT *p*-value = 0.22; PT *p*-value = 0.54). This allows us to eliminate the effect of size variation on the otolith's shape.

Comparison between the two populations

The MANOVA test performed on the otolith morphological variables showed a *p*-value <0.0001. This result was confirmed by Wilk's lambda test of DFA, which also gave a *p*-value of less than 0.0001 (Table 2). Otolith microchemical analysis shows that the contents of K, Cs, and Pb are higher in Djerba (K = 378.71 ppm, Cs = 223.33 ppm, and Pb = 63.72 ppm) than in Kerkennah

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Table 1. Biometric measurements of *Sarpa salpa*

		Sample size	Average total length in mm	Average total weight in g
Djerba sample	Females	30	173.2 ± 19.13	72.39 ± 20.3
	Males	30	168.12 ± 14.31	65.13 ± 17.5
	Total	60	170.66 ± 16.72	68.76 ± 18.98.
Kerkennah sample	Females	30	169.38 ± 14.28	68.94 ± 17.07
	Males	30	170.32 ± 16.62	71.4 ± 15.41
	Total	60	169.85 ± 15.45	70.17 ± 16.24

(K = 235.91 ppm, Cs = 1.23 ppm, and Pb = 50.66 ppm) (Table 3). The Wilk's lambda test of otolith microchemical analyses gave a *p*-value < 0.0001 (Table 2). This means that the otolith microchemical analysis confirms the morphological analysis. Both microchemical and morphological otolith analyses prove that Djerba and Kerkennah stocks are significantly different.

Fluctuating asymmetry and sexual dimorphism in the Djerba population

The Wilks lambda test performed on *S. salpa* sampled in Djerba revealed that both fluctuating asymmetry and sexual stock dimorphism are very significant (*p*-value <0.0001) (Table 2). The *p*-values for Fisher distances show that males have a higher fluctuating asymmetry (*p*-value <0.0001) than females (*p*-value = 0.0421). Furthermore, the *p*-value test for Fisher distances indicated that sexual dimorphism (*p*-value <0.0001) between left otoliths is more pronounced than that between right otoliths (*p*-value = 0.7345) (Table 4).

Fluctuating asymmetry and sexual dimorphism in the Kerkennah population

Otolith morphology analysis showed that the fluctuating asymmetry and sexual stock dimorphism of the Kerkennah population are very important (Wilks' Lambda *p*-value < 0.0001) (Table 2). However, males have a greater fluctuating asymmetry (*p*-value for the fisher distance = 0.0025) than females (*p*-value for the fisher distance = 0.0105). Furthermore, the Fisher distance *p*-value test for sexual dimorphism evaluation revealed a greater degree of significance between left otoliths (*p*-value = 0.0012) than between right otoliths (*p*-value = 0.0189) (Table 4).

Discussion

The results of otolith morphological and microchemical analysis showed a highly significant difference between the populations of Djerba and Kerkennah. The otolith fluctuating asymmetry was significant in both the populations of Djerba and Kerkennah, with a higher value in males than in females.

In the current study, the MANOVA results obtained from otolith morphological analysis for *S. salpa* demonstrate a significant difference between Djerba and Kerkennah. This finding is similar to those of Trojette *et al.* (2015), who discovered significant variations in *Diplodus annularis* populations between Djerba and Kerkennah. The results can be explained by physicochemical differences between Djerba and Kerkennah, such as temperature (Cardinale *et al*., 2004), which is higher in Djerba (from 15 °C to 27 °C) than in Kerkennah (14 °C to 24.5 °C), according to the National Institute of Meteorology of Tunisia.

Many authors indicate that otolith formation is influenced by a synergistic action of various factors (Vignon, 2012), including environmental conditions (Vignon and Morat, 2010), food resources (Gagliano and Mccormic, 2004; Mille *et al*., 2016; Ben Labidi *et al*., 2020a; Khedher *et al*., 2021; and Mejri *et al*., 2020), and genetic characteristics (Vignon and Morat, 2010).

A significant difference between the stock of males and females was found for both the Djerba and Kerkennah populations. According to Cardinale *et al.* (2004), sexual dimorphism has a considerable impact on the morphology of the otolith. The sexual dimorphism found in otoliths, according to Bakkari *et al.* (2020), can be explained by genetic or environmental stress. Panfili *et al.* (2005) also explain sexual dimorphism as a fluctuation of factors associated to growth, fertility, or even survival. Shuster (2009), on the other hand, defines otolith sexual dimorphism as a unique sound reception ability used to seek mating partners throughout the reproductive phase.

Both the Djerba and Kerkennah groups have considerable fluctuating asymmetry in otolith shape. Many authors have linked this imbalance to environmental stresses (Somarkis *et al*., 1997; Panfili *et al*., 2005; Diaz-Gil *et al*., 2015). Pollution, according to some authors, is the most important element influencing fluctuating asymmetry (Elsdon *et al*., 2008; Munday *et al*., 2011; Perry *et al*., 2015; Kontas *et al*., 2018). According to Wang (2002), heavy metals have a direct effect on otolith development. According to Rabaoui *et al.* (2013), the heavy metal content in the Gabes Gulf is extremely high.

Otolith microchemical analysis of *S. salpa* sampled from Djerba and Kerkennah confirms the presence of a significant heavy metal concentration of K, Cs, and Pb in the otolith composition, with different quantities within the two sites. This result

Table 2. Wilks' Lambda test for otoliths morphology, and microchemistry of *Sarpa salpa* sampled from Djerba and Kerkennah, Tunisia; executed on left and right otolith

	Otolith morphological analyses	Otolith microchemical analyses
Lambda	0.022	0.14
F (Observed value)	1.48	342.19
F (Critical value)	1.12	3.0700
DF1	539	2
DF2	1101.91	117
p-value	< 0.0001	< 0.0001
alpha	0.05	0.05

Table 3. Heavy metals analysis (K, Cs, and Pb) in the otoliths of *Sarpa salpa* sampled from the Tunisian islands of Djerba and Kerkennah

Sites	K in ppm	Cs in ppm	Pb in ppm	Cd in ppm
Djerba	378.71	223.33	63.72	<0.011
Kerkennah	235.91	1.23	50.66	<0.011

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Table 4. *P*-value of the Fisher distances test for sexual dimorphism and fluctuating asymmetry in males and females of *Sarpa salpa* from Djerba and Kerkennah, Tunisia

	S.D.F.L	S.D.M.R	S.D.M.L	S.K.F.R	S.K.M.R	S.K.M.L
S.D.**F.R**	0.0421	0.7345				
S.D.**F.L**			< 0.0001			
S.D.**M.R**			< 0.0001			
S.K.**F.R**					0.0189	
S.K.**F.L**				0.0105		
S.K.**M.R**						0.0025
S.K.**M.L**						0.0012

confirms and explains the significant difference between the morphological analyses. However, the chemical composition of the otolith is strongly connected to the chemical composition of the environment (Campana and Neilson, 1985); therefore, we may deduce that the chemical properties of the Djerba and Kerkennah habitats differ.

Overall, through this investigation, we were able to distinguish between two distinct stocks of *S. salpa*. The otolith morphology and microchemistry of the *S. salpa* Djerba differ significantly from those of the Kerkennah. The difference can be explained by variations in environmental conditions and pollution stress, as seen by the high concentrations of heavy metals on both islands. Sexual dimorphism was seen in both the Djerba and Kerkennah populations, which can be linked to genetic and environmental stress. The study offers new perspectives for investigating the ecological consequences of the high content of heavy metals in these two environments as well as the impact of human consumption of *S. salpa* collected from Djerba and Kerkennah. It also provides unique data on the *S. salpa* population in Kerkennah and Djerba, as well as the use of otolith microchemistry and morphology as a potential method for fish stock distinction.

Data availability statement. Data that support the findings of this study are available from the corresponding author, [Meriam Ben Ghorbel], upon request.

Authors' contributions. Meriam Ben Ghorbel carried out the sampling of *S. salpa* from Djerba and Kerkennah; she has done the otolith morphology, microchemical analysis, and statistical analysis. Also the article writing. Marwa Mejri corrects the article's redaction. Houeto Madel Floriane Adjibayo showed the working method of morphology analysis. Abdellah Chalh: advice for biostatistics. Jean-Pierre Quignard advises on otolith manipulations. Monia Trabelsi chose the subject of the research and coordinated between the researchers. Nawzet Bouriga coordinates the overall work.

Competing interests. None.

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