

# Distribution and hybridization of two sedentary gobies (*Pomatoschistus microps* and *Pomatoschistus marmoratus*) in the lagoons of southern France

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**Summary:** *Pomatoschistus marmoratus* and *Pomatoschistus microps* are small sedentary gobies inhabiting the lagoons of European Mediterranean and Atlantic coasts. Along the French Mediterranean coast their respective geographical distribution is not precisely known, in part because they are cryptic species. In this study, 512 gobies of both species were caught as 17 samples in 12 lagoons of the Gulf of Lion on the French Mediterranean coast. They were genotyped at six microsatellite loci and investigated statistically using multidimensional analyses, Bayesian assignment (*Structure*) and *NewHybrids* classification. This allowed the contrasted distribution of each species (*P. microps* in the east, *P. marmoratus* in the west) to be described, with several exceptions. Neither geographic structure nor isolation by distance was detected among differentiated populations of each species. The suggested mechanism is a deep sedentary behaviour associated with foundations following extinctions. The two species are sympatric or even in syntopy in five or six sampled lagoons producing rare fertile hybrids.

**Keywords:** *Pomatoschistus*; microsatellites; Mediterranean; hybridization; exclusive competition.

**Distribución e hibridación de dos gobios sedentarios (*Pomatoschistus microps* y *Pomatoschistus marmoratus*) en las lagunas del sur de Francia**

**Resumen:** *Pomatoschistus marmoratus* y *Pomatoschistus microps* son pequeños gobios sedentarios que habitan las lagunas de las costas mediterráneas y atlánticas. Sus distribuciones geográficas respectivas no se conocen con precisión a lo largo de las costas mediterráneas francesas, en parte porque son especies crípticas. En este estudio, se capturaron 512 gobios de ambas especies en 17 muestras de 12 lagunas del Golfo de León, en la costa mediterránea francesa. Han sido genotipados en seis loci de microsatélites e investigados estadísticamente con análisis multidimensionales, asignación bayesiana (*Structure*) y clasificación de *NewHybrids*. Esto permitió describir la distribución contrastada de cada especie (*P. microps* en el este, *P. marmoratus* en el oeste), con varias excepciones. Ni la estructura geográfica ni el aislamiento por la distancia se detectaron entre poblaciones diferenciadas de cada especie. El mecanismo sugerido es un comportamiento sedentario fuerte asociado con las migraciones posteriores a las extinciones. Las dos especies son simpátricas o incluso en syntopia en 5 o 6 lagunas muestreadas y producen raros híbridos fértiles.

**Palabras clave:** *Pomatoschistus*; microsatélites; Mediterráneo; hibridación; competencia exclusiva.

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## INTRODUCTION

Gobies are teleost fish of the order Perciforms and the family Gobiidae (Miller 1986). Nearly 2000 species of this family have been identified worldwide (Miller

1984, Nelson 2006) in all aquatic environments: continental, brackish and marine. In the Mediterranean, Miller (1986) considered 44 species described in marine and lagoon environments. This number climbed to 60 species in 25 genera (Quignard and Tomasini 2000)

and then to 62, 26 of them endemic to the Mediterranean (Ahnelt and Dorda 2004, Engin and Seyhand 2017).

The monophyletic “sand gobies” clade, morphologically recognizable by the head canals and vertebrae characteristics, includes the genus *Pomatoschistus* Gill, 1863, among four paraphyletic genera. Thirteen *Pomatoschistus* species have been described, all less than 10 cm in length, the smallest being *P. nanus* (Engin and Seyhand 2017). Based on the pairwise distance matrix estimated with nuclear DNA (ITS1 locus) and mitochondrial DNA (12S and 16S fragments), Huysse et al. (2004) showed that *P. microps*, *P. marmoratus* and *P. knerii* clustered separately and in parallel with the *P. minutus* complex. *P. microps* and *P. marmoratus* are considered to have appeared in the Mediterranean (Gysels et al. 2004). However, recent molecular data suggest another story for *P. microps*. Namely, this species could have originated from Portuguese ancestral populations in glacial refuges. The species might have colonized northern Europe first and then the Mediterranean Sea (Tougaard et al. 2014).

*P. microps* is present from the Moroccan Atlantic coast to the Baltic and to the Mediterranean North West (Miller 1986). Gene flow is almost non-existent between the Atlantic and the Mediterranean populations, which are clearly differentiated into two lineages (Gysels et al. 2004) with different life-history characters (Healey 1972, Bouchereau and Guélorget 1997). Locally, there is evidence for slight genetic differentiation between close lagoons (Berrebi et al. 2009). It is the most abundant goby in Mediterranean lagoons with *P. minutus* (Quignard et al. 1984, Bouchereau et al. 1989a).

*P. marmoratus* is distributed on the Atlantic west coast of the Iberian Peninsula, the north coasts of the western and eastern Mediterranean, the Black Sea, the Azov Sea and Lake Qarun, Egypt, where it was introduced via the Suez Canal (Miller 1986), as well as along the southern coasts of the Mediterranean, but limited to the Tunisian lagoons (Mejri et al. 2009). Overall, a west/east genetic structure is hinged on either side of the Sicilian-Tunisian strait (Mejri et al. 2011). An intra-lagoon genetic structure has been observed in the Mar Menor coastal lagoon, SE Spain (González-Wangüemert and Vergara-Chen 2014), together with a high gene flow along the coast (Vergara-Chen et al. 2010). It is a sedentary species of Mediterranean lagoons little known because of the difficulty of discriminating it from the well-known *P. microps*.

Both species live primarily on sandy substrates. *P. microps* is a consumer of meiofauna, i.e. mainly planktonic and benthic crustaceans such as copepods and amphipods (De Casabianca and Kiener 1969, Leclerc et al. 2014). They live in very shallow lagoon zones, mainly on the shores, thus constituting a source of food for many species of the macrofauna. This gives them a key role in energy transfer from meiofauna to fish and birds (Pampoulie 1999, Ray 2005). Each species is a batch spawner with one or two spawning seasons (Bouchereau et al. 1989b). Because it is strictly sedentary, the fertility and growth of these species of gobies

are greatly affected when environmental conditions become unfavourable (Pampoulie et al. 2000).

Based on discriminating allozymic markers, *P. marmoratus* has been shown to represent over 90% of gobies of the Thau lagoon, while Mauguio Lagoon seems to be exclusively inhabited by *P. microps* (Berrebi et al. 2005). These two lagoons will be considered as references for each species in the present study.

Both species are called cryptic because it is very difficult to determine which is which using morphological criteria (Webb 1980). The safest test able to distinguish the two species morphologically is based on the presence (in *P. marmoratus*) or absence (in *P. microps*) of the mucosal occulo-scapular channel, which necessitates unstable staining protocols to be observed (Huysse et al. 2004, Rigal et al. 2008).

The relatively close positions of *P. microps*, Kroyer, 1838 (common goby) and *P. marmoratus*, Risso, 1810 (marbled goby) in the phylogeny and the fact that the two species coexist in sympatry in lagoons of southern France explains why they hybridize occasionally in a southern France lagoon complex: the Vaccarès and Impériaux lagoons (Berrebi et al. 2005), corresponding to stations 15 and 16 of the present survey (Table 1). This discrete hybridization between small and cryptic species is neither really understood nor explained and deserves a detailed regional investigation on the distribution of the two species, their hybrids and the ecological conditions of their hybridization. Because southern France is the only zone where *P. microps* and *P. marmoratus* are in sympatry (together with *P. minutus*), and because this is the first zone where hybridization between these two species has been recorded, the present investigation aimed to determine precisely the reciprocal distribution of the two species and of their hybrids on the French coasts of the Mediterranean Gulf of Lyon. For this study 12 lagoons were selected along the French Mediterranean coast, from the Spanish border to Marseille (Fig. 1), for a total of 512 fishes. Gobies were genotyped at six microsatellite loci and the data were analysed using several statistical methods.

## MATERIALS AND METHODS

### Sampling

The French part of the Mediterranean coast constitutes the Gulf of Lyon, where both species were captured in 12 lagoons in 2004. This January-May sampling campaign was the shortest possible. Beach seine nets were used by scientists and fyke nets by professional fishermen. The bigger Thau lagoon (19×8 km) was visited at six localities in March (Table 1 and Fig. 1) and the salinity was measured in each place (Table 1). After laboratory observation and sometimes rapid microsatellite genotyping at one or several strictly diagnostic loci (Pmar-03, Pmar-08, Pmin-08, Pmin-10, Pmin-11, according to Berrebi et al. 2006), the species *P. minutus* was removed from the 17 collected samples. Finally, 512 gobies were clearly determined as belonging to the two sedentary species *P. microps* and *P. marmoratus* and constituted the sample for this study. The

Table 1. – Details concerning the samples numbering 512 individuals in total belonging to two sedentary goby species and on salinity at the six Thau stations. The numbers in the first column refer to the map (Fig. 1). Salinity was measured during sampling only in Thau lagoon.

Map number	Lagoon name	Locality	WGS84 decimal degrees		N	Month 2004	Thau salinity
			North	East			
1	St Cyprien	-	42.6118	3.0367	25	May	-
2	Canet	-	42.6686	3.0221	30	May	-
3	Salses-Leucate	-	42.8588	3.0045	28	May	-
4	La Palme	-	42.9626	3.0144	25	May	-
5	Bages-Sigean	-	43.0991	3.0036	41	May	-
6		Marseillan 1	43.3575	3.5356	17	March	11.5
7		Marseillan 2	43.3445	3.5324	18	March	29
8		Mèze	43.4281	3.6130	26	March	22.2
9	Thau	Bouzigues	43.4478	3.6525	29	March	32
10		ULM	43.4008	3.6546	18	March	27.1
11		Listel	43.3837	3.6182	28	March	29.4
12	La Peyrade	-	43.4242	3.7378	82	February	-
13	Vic	-	43.4848	3.8205	19	January	-
14	Mauguio	-	43.5697	4.0048	50	June	-
15	Vaccarès	-	43.5396	4.5092	29	February	-
16	Impériaux	-	43.4848	4.4749	29	January	-
17	Berre	-	43.4342	5.0975	18	April	-

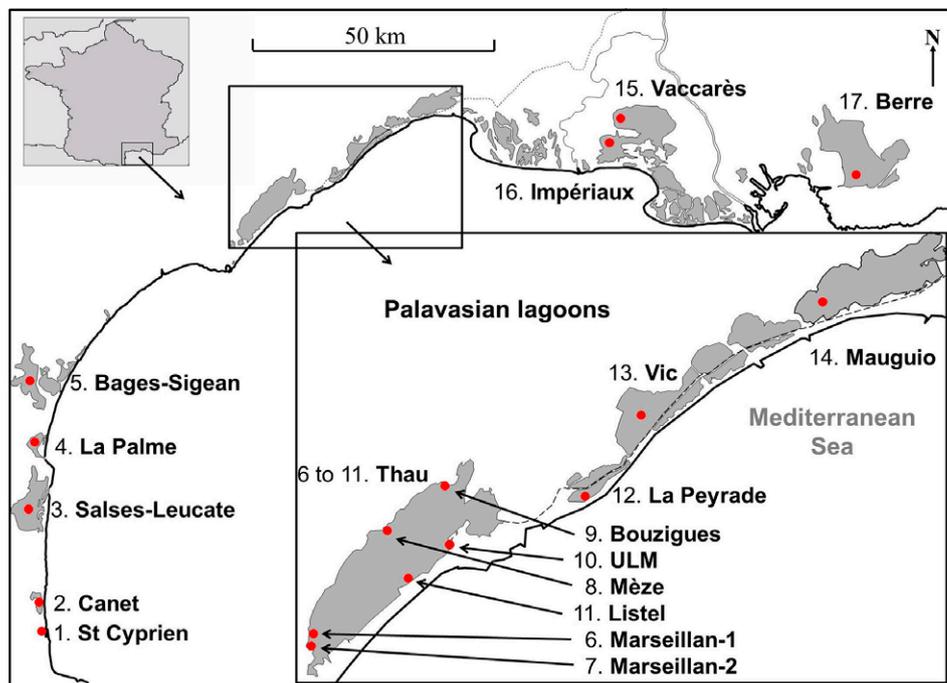


Fig. 1. – Geographic position of the 17 sampling sites. In grey, lagoons. Dotted line: Canal du Rhône à Sète allowing exchange between all Palavasian lagoons.

fish were individually preserved in 90% ethanol until dissection and DNA extraction. Table 1 gives detailed information about the samples and Figure 1 indicates the position of each sampling site.

### The lagoon ecology

Eleven lagoons and one small river mouth from the Spanish border to Marseille were sampled (Fig. 1). These sites are all more or less brackish but display high ecological diversity. A tentative classification of the 12 water bodies, highlighting ecological categories according to the area and salinity is given in Table 2. They can be divided into “big lagoons” exceeding 5000 ha in area (Berre, Thau, Vaccarès and Salses-Leucate) and with a mean depth of 2 to 6 m, and “small lagoons” of 45 to 3700 ha and a mean depth 0.3 to 1.3 m. Apart from size and water volume, salinity is a very

important parameter for lagoon inhabitants, generally fluctuating according to marine or continental influences that depend on marine or continental water input (rivers, rains), essentially driven by the seasons. The water exchanges and salinities given in Table 2 are only indicative because they always fluctuate. Haloclines have been described (Poizat et al. 2004) but are not considered in this simplified description. Because only *P. marmoratus* cannot bear null salinity (Rigal et al. 2008), the minimum salinity is another important ecological parameter. Therefore, Canet, Mauguio and Vaccarès are three clearly unfavourable lagoons for *P. marmoratus*. In the Vaccarès-Impériaux lagoon complex, constituting a large part of the Camargue region or Rhône Delta, the Impériaux lagoon in the south, where *P. marmoratus* can be found (Berrebi et al. 2005), is saltier and more euryhaline than the Vaccarès lagoon in the north.

Table 2. – Short description of the ecological characteristics of the lagoons (according to Labat 1976, Quignard et al. 1983, Pampoulie 2001, Ifremer 2002, 2003).

Map number	Lagoon name	type	sea exchanges	continental water	surface ha	mean depth (m)	salinity ‰
1	St Cyprien	river mouth	high	high	very small	unknown	unknown
2	Canet	lagoon	nearly null	very high	1200	0.5	5-43
3	Salses-Leucate	lagoon	medium	high	5400	2	25-40
4	La Palme	lagoon	medium	low	600	0.5	10-46
5	Bages-Sigean	lagoon complex	medium	medium	3700	1.3	13-41
6 to 11	Thau	lagoon	high	medium	7500	5	11.5-32
12	La Peyrade	lagoon	low	low	45	0.3	20-43
13	Vic	lagoon	low	low	1150	1.2	10-40
14	Mauguio	lagoon	low	high	3200	0.8	3-37
15	Vaccarès	lagoon complex	low	medium	6300	2	5
16	Impériaux	lagoon complex	medium	low	1100	1	23-39
17	Berre	lagoon	high	low	15500	6	20

### Microsatellite genotyping

DNA was extracted from fin tissue samples by the Chelex/proteinase K protocol of Estoup et al. (1996). Among the 14 loci tested, six microsatellite markers allowing cross-priming for three *Pomatoschistus* species were retained. Details of each locus are given in Table 3. The 5' end of one of the two primers was covalently linked to fluorescein, Cy3 or Cy5 labels. Polymerase chain reactions (PCR) were performed in an Eppendorf Mastercycler programmable thermocycler with a 10 µL reaction total volume containing 0.1 U of Taq polymerase (Sigma-Aldrich), 2.5 mM MgCl<sub>2</sub>, 0.4 mM of each dNTP (Invitrogen), 1 µL of 10X reaction buffer and 0.75 µM of each primer (Eurofins MWG). The thermal cycle was composed of an initial denaturation (94°C, 5 min); then of 34 repetitions of denaturation (94°C, 45 s), annealing (45 s at the temperatures given in Table 3 for each locus) and extension (72°C, 45 s); and then a final extension (72°C, 5 min).

Amplified DNA fragments were separated according to their size in an 8% polyacrylamide denaturing gel (Bio-Rad). The PCR products were visualized on a Hitachi FMBIO-II fluorescent imaging system scanner. Allele size was determined by comparison with a fluorescently labelled ladder of known size (100–600 bp, Promega), with the help of an image analysis software FMBIO ANALYSIS 8.0 (Hitachi).

The genotype matrix was then constructed and used as the basis for all of the following statistical analyses.

### Statistical methods

Assignment tests were performed using the *Structure* software (Pritchard et al. 2000). The model for sorting individuals is based on minimizing deviations from Hardy-Weinberg equilibrium (HWE) and link-

age disequilibrium between loci inside the randomly built subgroups (clusters). The admixture ancestry and correlated allele frequencies models were chosen. The calculation estimates the rate of admixture (Q) for each individual (here interpreted as an introgression rate). Each run consisted of 100000 burn-in steps and 200000 MCMC steps, repeated ten times. Assignments to K subgroups (K, the number of subgroups, was tested between 1 and 12 because of the 12 lagoons involved) were evaluated with the Evanno et al. (2005) delta K method through *Structure Harvester* (Earl vonHoldt 2012). The possible bias should be the large HWE deviation. For the best K, the ten runs obtained on the 512 individuals/17 samples were processed by CLUMPP software (Version 1.1.2) in order to obtain a consensus histogram.

In order to consolidate the results and obtain information on the status of potential hybrids (F1, F2, backcrosses of various classes), the same data were analysed with *NewHybrids* software, version 1.1 beta (Anderson and Thompson 2002). According to Anderson (2008), *NewHybrids* is applicable to the present situation, where there are only two diploid species that seem to be hybridizing, with some samples containing both pure and hybrid individuals. In order to provide references for pure species, the whole sample of 512 individuals was analysed in the same run. Any possible bias should be the differentiation among populations illustrated by factorial correspondence analyses (FCAs).

FCAs (Benzécri 1973) were performed using the *Genetix* 4.04 program (Belkhir et al. 2004), providing the overall genetic structure of the samples. This method is well adapted to genotype data. The classes are alleles and the correspondences that are calculated are the co-occurrence of two given alleles in the same individual or in a group of individuals. Clusters (clouds) detected on the diagram correspond to homogeneous

Table 3. – General characteristics of the seven loci used to study the spatial and intraspecific variability of *P. marmoratus* and *P. microps*. Allele sizes were determined on individuals analysed in the first step of assignment and with 0.90 < Q < 1, determining pure individuals of each species.

Locus	Allele sizes		TM (°C)	Repeated sequence	References
	<i>P. microps</i>	<i>P. marmoratus</i>			
Pmic-02	118-244	134-256	54	(CA) <sub>3</sub> CT (CA) <sub>3</sub> CT (CA) <sub>15</sub>	Berrebi et al. 2006
Pmic-03	180-214	178-220	65	(CA) <sub>3</sub> CT (CA) <sub>3</sub> CT (CA) <sub>15</sub>	Berrebi et al. 2006
Pmic-07	194-222	192-260	54	(GT) <sub>6</sub> GC (GT) <sub>2</sub> GC (GT) <sub>5</sub>	Berrebi et al. 2006
Pmar-03	246-390	304-376	58	(AC) <sub>8</sub> GC (AC) <sub>12</sub>	Berrebi et al. 2006
Pmar-05	212-278	210-312	54	(GT) <sub>6</sub> GC (GT) <sub>16</sub>	Berrebi et al. 2006
Pmin-05	142-188	118-186	52	(GT) <sub>31</sub>	Jones et al. 2001

genetic lineages, independent of the fish geographical origin. The inertia values (i.e. the proportion of the total information contained by an axis, expressed as a percentage) along each axis were shown to be equivalent to linear combinations of the monolocus fixation index ( $F_{st}$ ) values (Guinand 1996). More mathematical details of the method are given in She et al. (1987).

Population genetics parameters were calculated using *Genetix* software. These parameters were calculated for each sample. Some samples include the two species and some hybrids. Because hybrid detection depends on the software used and, for assignment, on the threshold chosen, the whole inhabitants of a given lagoon were included in the calculations. HWE can therefore be used to detect Wahlund effect. Genetic diversity was tested with observed heterozygosity,  $H_o$ , and with unbiased estimated heterozygosity,  $H_{nb}$  (Nei 1978), in order to limit the effect of small sample sizes. Parameter  $A$  (mean number of alleles per locus) was also calculated. Wright's  $F_{is}$  and  $F_{st}$  indices were determined through the  $f$  and  $\theta$  estimators of Weir and Cockerham (1984). Statistical significance of the estimated values was evaluated with 5000 permutations of alleles in each population ( $F_{is}$ ) or 5000 permutations of individuals among compared samples ( $F_{st}$ ). Bonferroni sequential corrections (Rice 1989) were applied to multiple tests.

The Mantel test was run with *Genetix*, using the genetic distance based on  $F_{st}/(1-F_{st})$ , as recommended by Rousset (1997) and the geographical distance (km) computed on the map as the coastline distances between the main openings to the sea of each sampled lagoon. The value of  $Z$ , the Mantel coefficient, between the two matrices of distances was calculated with the true

data, then the significance of each test was assessed by comparison with the series of pseudo-values produced by 5000 permutations of the populations' order of one of the two matrices of distances.

Finally, genetic relationships among populations based on Reynolds' genetic distance were graphically represented in Treeview software (Page 1996) according to a neighbour-joining (NJ) construction with a bootstrap (100 reconstructed matrices) testing the robustness of the branching as proposed in the *PhyIip* software (Felsenstein 2004).

One of the challenges of this analysis is the detection of hybrid individuals. Using assignment (*Structure* software), an individual is first considered to belong to a given species when the  $Q$  value is over 0.9, which means that more than 90% of its genome would belong to this species, though other thresholds are explored, mainly 0.8. When  $Q$  is below 0.9 (or 0.8), the individual is considered hybridized. *NewHybrids* gives probability for an individual to belong to a pure species or to a series of hybrid categories.

## RESULTS

### Species distribution

All genotypes are deposited in Dryad (DOI: <http://doi.org/10.5061/dryad.70rxwdbv7>).

The first information sought with the *Structure* software was the number of subgroups contained in the whole sampling of 512 gobies. The assignment tests were performed for  $K=1$  to  $K=12$ . The procedure of Evanno et al. (2005) designated  $K=2$  as the more informative

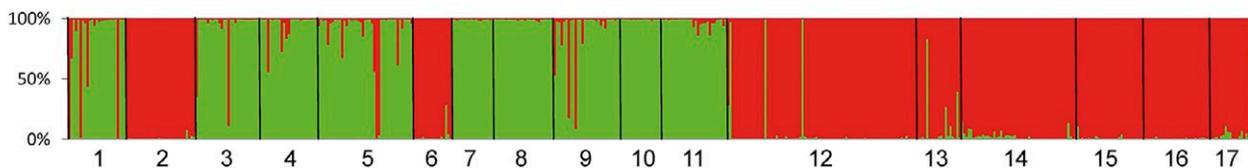


Fig. 2. – CLUMPP output consensus histogram of the 512 sampled individuals for  $K=2$ . Green represents *P. marmoratus* genotypes and red *P. microps*. Numbers below the diagram correspond to the sample order (first column of Table 1).

Table 4. – Number of individuals of each species or category of hybrid according to the two softwares used: *NewHybrids* and *Structure*. For *Structure* estimations, values between parentheses consider significant an assignment below 0.8 ( $Q$ ). Other values consider a 0.9 threshold.

	Sample n°	<i>Structure</i>					<i>NewHybrids</i>				
		<i>marmoratus</i>	<i>microps</i>	F1	<i>marmoratus</i> backcross	<i>microps</i> backcross	Total hybrids	<i>marmoratus</i>	<i>microps</i>	F2	Total hybrids
St Cyprien	1	19 (20)	3	1	2 (1)	0	3 (2)	21	3	1	1
Canet	2	0	30	0	0	0	0	0	30	0	0
Salses-Leucate	3	26	0 (1)	0	0	2 (1)	2 (1)	26	2	0	0
La Palme	4	21 (23)	0	1	3 (1)	0	4 (2)	23	0	1	1
Bages-Sigean	5	34 (35)	2	1	4 (3)	0	5 (4)	37	2	1	1
T-Marseillan 1	6	0	16	0	0	1	1	0	17	0	0
T-Marseillan 2	7	18	0	0	0	0	0	18	0	0	0
T-Mèze	8	26	0	0	0	0	0	26	0	0	0
T-Bouzigues	9	24	1 (2)	1	2	1 (0)	4 (3)	25	2	2	2
T-ULM	10	18	0	0	0	0	0	18	0	0	0
T-Listel	11	26 (28)	0	0	2 (0)	0	2 (0)	28	0	0	0
La Peyrade	12	3	78	0	0	1	1	3	79	0	0
Vic	13	0 (1)	15 (16)	0	1 (0)	3 (2)	4 (2)	1	18	0	0
Mauguio	14	0	48 (50)	0	0	2 (0)	2 (0)	0	50	0	0
Vaccarès	15	0	28 (29)	0	0	1 (0)	1 (0)	0	29	0	0
Impériaux	16	0	29	0	0	0	0	0	29	0	0
Berre	17	0	18 (19)	0	0	1 (0)	1 (0)	0	18	0	0
Total		215 (222)	268 (275)	4	14 (7)	12 (5)	30 (16)	226	279	5	5

partition. The following partitions ( $K > 2$ ) brought no better likelihood and no more information (Appendix 1). The ten runs performed for  $K = 2$  were strictly identical: the assignment and the goodness-of-fit values were very similar among runs, indicating that the structure was stable and the run length sufficient (confidence interval estimated to 1/1000). Figure 2 shows the consensus histogram using CLUMPP software. Berrebi et al. (2005) and Rigal et al. (2008) described the unique presence of *P. microps* in Mauguio Lagoon and of *P. marmoratus* in most of Thau lagoon, allowing identification of the two assignment clusters to one species each. The alternate presence of each species along the 12 lagoons corresponds to what was expected (Fig. 2: *P. microps* in red and *P. marmoratus* in green). The *NewHybrids* analysis gave exactly the same distribution (Table 4).

The *Structure* and *NewHybrids* softwares retained 268 (with  $Q > 0.9$  but 275 with  $Q > 0.8$ ) and 279 pure *P. microps* individuals, respectively. They were present in 11 lagoons of the 12 sampled ones. Very small numbers of *P. microps* were present in the St Cyprien, Salses-Leucate, La Palme and Bages-Sigean lagoons. However, it dominated or was exclusive in the Canet, La Peyrade, Vic, Mauguio, Vaccarès, Impériaux and Berre lagoons (Table 4). *P. marmoratus* was designated as a pure species in 215 (222) and 226 individuals by the two softwares, respectively. It was observed in seven lagoons with both softwares and was dominant in five of the seven (St Cyprien, Salses-Leucate, La Palme, Bages-Sigean, Thau). A very small number of individuals of *P. marmoratus* were found in the lagoons of La Peyrade and Vic (Table 4). Until now, the lagoon of Thau was thought to harbour only *P. marmoratus* (Berrebi et al. 2005). The present investigation detected the presence of pure *P. microps* in a tiny part of the lagoon, very close to a pure population of *P. marmoratus* (locations Marseillan 1 and 2 respectively).

The genetic discrimination of the two cryptic species revealed that their geographic distributions exhibit opposite patterns: *P. microps* in the east, *P. marmoratus* in the west. Two instructive exceptions were recorded: *P. microps* in Canet lagoon and at the Marseillan 1 station of Thau lagoon (Fig. 3). There were also intruders: (i) isolated individuals of *P. microps* in the west included three individuals in the St Cyprien estuary (=12%), one in the Salses-Leucate lagoon (4%), two in Bages-Sigean lagoon (5%) and two at Bouzigue station in Thau lagoon (7%); (ii) *P. marmoratus* in the east included three individuals in the La Peyrade lagoon (4%) and one in the Vic lagoon (5%).

### Hybrid detection

While false hybrid detection cannot be rejected, two programs were used to ascertain the presence of hybrids. Obviously the assignment method is more generous, detecting 16 to 30 hybrids in the whole sample (for  $Q < 0.8$  or  $0.9$  respectively), while *NewHybrids* detected just five of them, all in the F2 category. Using assignment, among hybrids, two categories were distinguished: F1 for  $0.4 < Q < 0.6$ , and backcrosses for the remaining hybrids. All *NewHybrids* detected hybrid individuals were among the 16 or 30 assignment hybrids. *NewHybrids* recognized fewer hybrids: five hybrid individuals distributed in four lagoons (Table 4). These five hybrids were all assigned to the F2 category. *Structure* and *NewHybrids* were used together to search for convergence of results obtained by two radically different methods. This was the case for species distribution, but hybrid detection was very disparate, with *Structure* giving three to six times more hybrids than *NewHybrids*. However all hybrids recognized by *NewHybrids* were among those found with *Structure*.

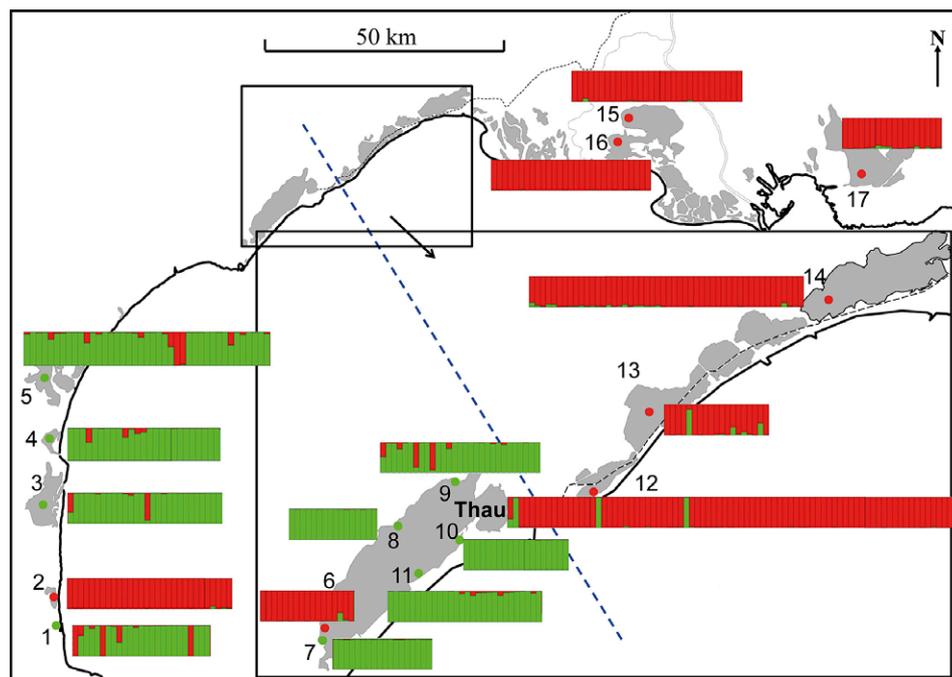


Fig. 3. – Geographic distribution of *P. microps* in red and *P. marmoratus* in green.

Table 5. – Population parameters applied to entire lagoon samples. N, number of individuals; Hyb., proportion of hybrid individual ( $q > 0.1$ ); He, expected heterozygosity; Hnb, unbiased heterozygosity (Nei 1978); Ho, observed heterozygosity; A, mean number of allele by locus; Fis, f estimator of Fis (Weir and Cockerham 1984); signif., significance of departure of the Fis estimator from zero (5000 permutations and Bonferroni correction). The p value is  $p < 0.01$  for a highly significant departure (\*\*) and  $p < 0.001$  for a very highly significant departure (\*\*\*)

Map	Lagoon name	Locality	Dominant spp.	N	Hyb.	He	Hnb	Ho	A	Fis	signif.
1	St Cyprien	-	<i>P. marmoratus</i>	25	0.120	0.712	0.727	0.571	9.5	0.218	***
2	Canet	-	<i>P. microps</i>	30	0	0.606	0.616	0.483	9.7	0.220	***
3	Salses-Leucate	-	<i>P. marmoratus</i>	28	0.071	0.817	0.832	0.596	14.2	0.287	***
4	La Palme	-	<i>P. marmoratus</i>	25	0.160	0.805	0.823	0.704	10.8	0.147	***
5	Bages-Sigean	-	<i>P. marmoratus</i>	41	0.125	0.777	0.787	0.572	15.2	0.276	***
6		Marseillan 1	<i>P. microps</i>	17	0.059	0.577	0.595	0.575	6.8	0.034	ns
7		Marseillan 2	<i>P. marmoratus</i>	18	0	0.773	0.796	0.671	9.8	0.161	***
8		Mèze	<i>P. marmoratus</i>	26	0	0.772	0.787	0.617	9.3	0.220	***
9	Thau	Bouzigues	<i>P. marmoratus</i>	29	0.138	0.812	0.827	0.661	13.7	0.204	***
10		ULM	<i>P. marmoratus</i>	18	0	0.715	0.737	0.640	8.2	0.135	**
11		Listel	<i>P. marmoratus</i>	28	0.071	0.796	0.811	0.609	13.3	0.253	***
12	La Peyrade	-	<i>P. microps</i>	82	0.012	0.639	0.643	0.515	14.7	0.200	***
13	Vic	-	<i>P. microps</i>	19	0.222	0.713	0.733	0.555	10.2	0.248	***
14	Mauguio	-	<i>P. microps</i>	50	0.040	0.653	0.660	0.479	11.3	0.276	***
15	Vaccarès	-	<i>P. microps</i>	29	0.034	0.553	0.563	0.417	9.0	0.263	***
16	Impériaux	-	<i>P. microps</i>	29	0	0.640	0.652	0.474	10.2	0.275	***
17	Berre	-	<i>P. microps</i>	18	0.056	0.677	0.698	0.529	8.5	0.247	***

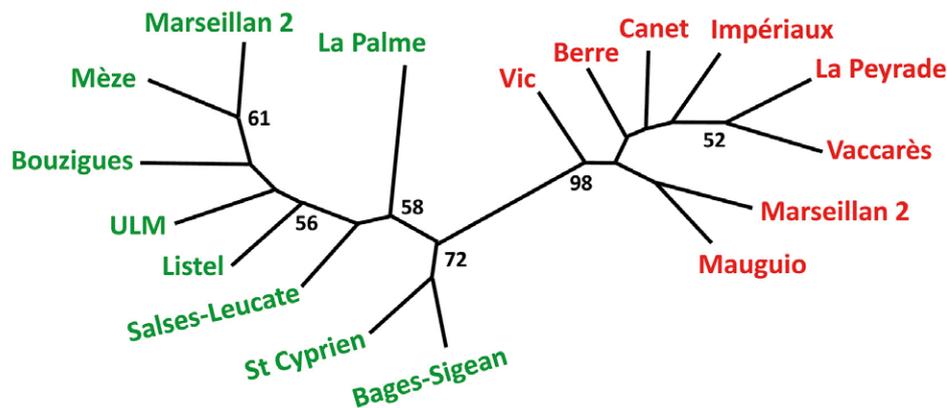


Fig. 4. – Neighbour-joining tree construction excluding hybrid and minority species individuals. Green represents *P. marmoratus* genotypes and red *P. microps*.

### Organization of species populations

Heterozygosity was slightly lower in *P. microps* (mean 0.63,  $0.59 < \text{Hnb} < 0.68$ ) than in *P. marmoratus* (mean 0.77,  $0.69 < \text{Hnb} < 0.81$ ). The interspecific difference in number of alleles was in the same direction: mean values were 8.9 for *P. microps* and 9.6 for *P. marmoratus* (Table 5). Nearly all populations showed a significant departure from HWE, even after removing intruders and hybrids. The Mantel test, used to determine the effect of isolation by distance, gave no significant results for either species. The NJ construction confirmed the populations' differentiation, with a slight geographic organization for *P. marmoratus* but not for *P. microps* (Fig. 4).

The organization of populations inside each species is described by FCA. This method was applied separately for each species, only on pure species individuals. An individual is considered here to belong to a species when the Q value is over 0.9, which is a stringent selection removing from the calculation 13 individuals whose genome is dominated by *P. microps* and 17

whose genome is dominated by *P. marmoratus*. With *P. microps* individuals, there was considerable multidimensional overlapping of the lagoon populations (Fig. 5), with three differentiated populations (all others are in the common centre of the diagram). Thus, Canet, Mauguio and Impériaux populations seem to represent partly differentiated groups. With *P. marmoratus*, only a big cloud is constituted, gathering all populations except the Thau-ULM (sample 10) into one (projection not shown).

Intraspecific variability among the lagoons can be tested through Fst estimations (Table 6). Among the eight *P. microps* samples, only two comparisons showed non-significant Fst: La Peyrade/Vaccarès (samples 2/12) and Impériaux/Berre (16/17). Among the nine *P. marmoratus* samples, most lagoons hosted a differentiated population, except seven comparisons giving a non-significant Fst, involving consecutive lagoons (stations 3/4) or consecutive stations of the Thau lagoon (stations 7/8 and 8/9) and suggesting rare local exchanges, as already observed (Berrebi et al. 2009).

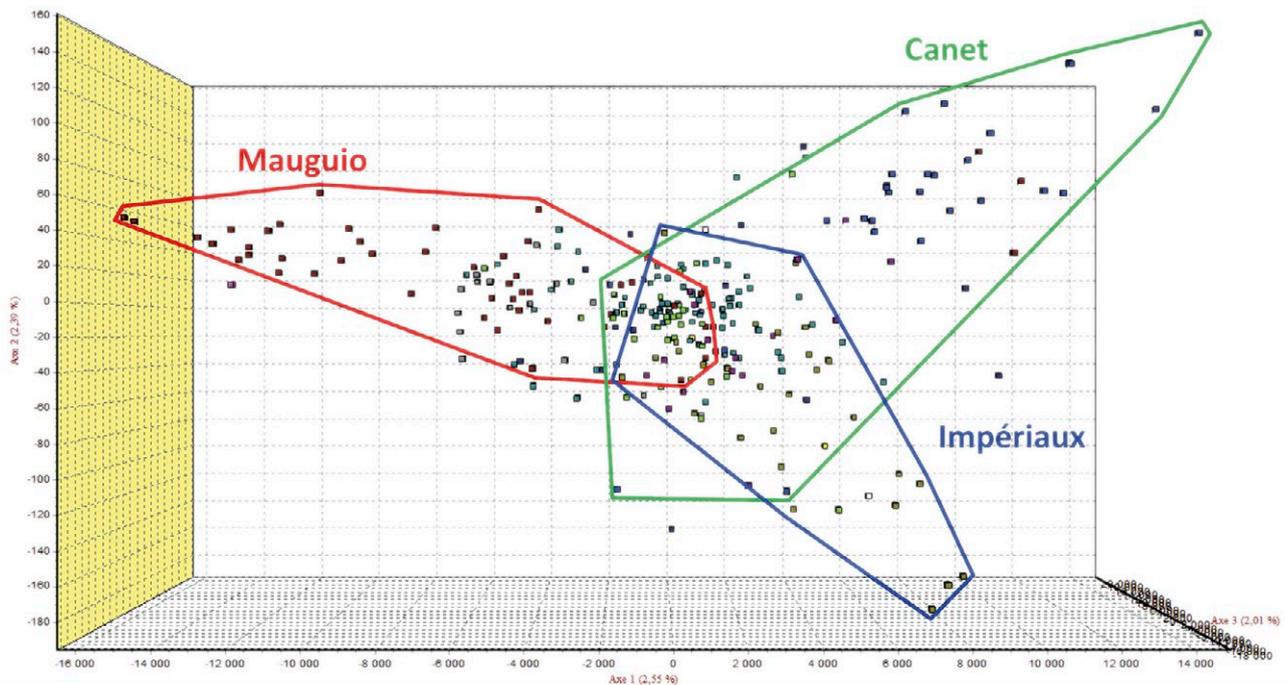


Fig. 5. – FCA of *P. microps* individuals. Most individuals are gathered in the middle of the diagram except for three differentiated populations.

Table 6. – Fst estimation by pair of population. Only a few tests were non-significant (values in bold) after Bonferroni correction at the  $p < 0.05$  level.

Map	Lagoon name	Locality	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	St Cyprien	-	<i>P. marmoratus</i>	0	0.173	0.087	0.059	<b>0.011</b>	0.157	0.131	0.129	0.102	0.081	0.109	0.146	0.112	0.183	0.182	0.129	0.089
2	Canet	-	<i>P. microps</i>		0	0.233	0.224	0.194	0.152	0.264	0.268	0.236	0.299	0.260	0.088	0.113	0.153	0.103	0.095	0.092
3	Salses-Leucate	-	<i>P. marmoratus</i>			0	<b>0.014</b>	0.051	0.234	0.059	0.059	0.026	<b>0.034</b>	0.025	0.226	0.157	0.195	0.251	0.211	0.171
4	La Palme	-	<i>P. marmoratus</i>				0	<b>0.017</b>	0.214	0.047	0.049	0.026	<b>0.019</b>	0.026	0.207	0.142	0.189	0.241	0.196	0.155
5	Bages-Sigean	-	<i>P. marmoratus</i>					0	0.178	0.092	0.090	0.057	<b>0.026</b>	0.062	0.175	0.124	0.182	0.207	0.160	0.115
6	Marseillan 1	-	<i>P. microps</i>						0	0.249	0.210	0.201	0.283	0.250	0.058	0.086	0.044	0.099	0.099	0.090
7	Marseillan 2	-	<i>P. marmoratus</i>							0	<b>0.029</b>	<b>0.015</b>	0.074	0.041	0.240	0.181	0.197	0.281	0.237	0.200
8	Mèze	-	<i>P. marmoratus</i>								0	<b>0.017</b>	0.068	0.043	0.241	0.190	0.178	0.279	0.246	0.207
9	Thau	Bouzigues	<i>P. marmoratus</i>									0	0.037	<b>0.013</b>	0.209	0.154	0.158	0.241	0.206	0.161
10	ULM	-	<i>P. marmoratus</i>										0	0.040	0.274	0.222	0.244	0.316	0.265	0.209
11	Listel	-	<i>P. marmoratus</i>											0	0.249	0.183	0.213	0.276	0.235	0.190
12	La Peyrade	-	<i>P. microps</i>												0	0.044	0.058	<b>0.017</b>	0.031	0.028
13	Vic	-	<i>P. microps</i>													0	0.073	0.054	0.059	0.061
14	Mauguio	-	<i>P. microps</i>														0	0.082	0.114	0.100
15	Vaccarès	-	<i>P. microps</i>															0	0.041	0.052
16	Impériaux	-	<i>P. microps</i>																0	<b>0.018</b>
17	Berre	-	<i>P. microps</i>																	0

DISCUSSION

The efficiency of the six microsatellite markers used was high enough to assign each individual to one of the two pure species or to different classes of hybrids, as seen in Figure 2 and Table 4. The presence of two species (*P. microps* and *P. marmoratus*) in the sampled lagoons has been demonstrated earlier. They were discovered and described using microsatellites (Berrebi et al 2005), mtDNA (Berrebi et al 2009, Tougard et al 2014), adaptation (Rigal et al 2008) and morphology (Travers et al 2011). Therefore, the detection by assignment and *NewHybrids* of two taxa in the samples corresponds necessarily to these two species, despite the partially overlapping allele sizes recorded between them (Table 3).

Hardy-Weinberg equilibrium departure

The Fis estimator used to test genotypes against HWE detected an imbalance for both species in almost all lagoons (Table 5). As revealed by *Micro-Checker*, null alleles can be present in all loci and explain the deviations. Other technical artefacts, such as large allele drop-outs were not expected. Several explanations have been proposed, in addition to artefacts such as scoring errors or null alleles: the Wahlund effect, inbreeding, family structure and natural selection. According to Pogson et al. (1995), the Wahlund effect may be a cause when there are significant Fis deviations at more than one locus. In contrast, natural selection is less probable, as microsatellite markers are assumed to be neutral and therefore little affected by natural selection (Côrte-Real et al. 1994). Therefore, the general HWE

departure appears to have a biological cause rather than being the result of artefacts. The Wahlund effect is probably the first cause in lagoons where intruders and hybrids have been found, but this disequilibrium has also been detected in lagoons inhabited by one species only (Canet and Impériaux lagoons for *P. microps*; Marseillan 2, Mèze and ULM stations in Thau lagoon for *P. marmoratus*). These significant deviations from HWE due to heterozygote deficit were documented by González-Wangüemert and Vergara-Chen (2014) in all five populations and eight microsatellites in *P. marmoratus* in Mar Menor coastal lagoon in SE Spain. Large deviations have also been recorded in *P. minutus* in the Mediterranean (Boissin et al. 2011). This imbalance for these species has been observed by some authors (Stefanni et al. 2003, Pampoulie et al. 2004) but not by others (Jones et al. 2001, Gysels et al. 2004). In fact, departure from HWE is not rare in marine organisms (García De León et al. 1997, De Innocentiis et al. 2001).

### Hybridization

When we selected the number of hybrids according to different levels of Q value, we found for the whole sampling 30 hybrids with  $Q > 0.1$ , 16 with  $Q > 0.2$ , 9 with  $Q > 0.3$  and 3 with  $Q > 0.4$ . By comparison, *NewHybrids* detected five hybrids corresponding to assignment with a threshold between 0.3 and 0.4. This discrepancy is due to different software methods: *Structure* calculation is based on the frequency of each allele in each species and individual, while *NewHybrids* reconstitutes the possible genealogy among individuals after assigning each allele to a species. Both techniques confirm the presence of hybrids, but their number depends on the statistical method. When hybrids were detected, the introgression rates observed in each individual generally differed from 0.50 (Fig. 2). In other words, backcrosses (according to *Structure*) or F2 (according to *NewHybrids*) had taken place. These findings allow us to conclude that hybrids are fertile.

### Macro- and microevolution in lagoonal *Pomatoschistus* gobies

The history of the present lagoons and therefore of their sedentary goby populations went through two successive phases. First the lagoons, at least those between Mauguio and Thau known as Palavasian lagoons near the small city of Palavas, were isolated from the sea by a sand bar (lido), giving the lagoonal zone its particular ecology with alternate continental and marine influences. Second, the formation of each lagoon happened later, due to both marine influence and anthropic actions. The principal process was isolation of the lagoon zone from the sea. Several sedimentological investigations have attempted to date this isolation. The more conclusive are those of Sabatier et al. (2010a) and Raynal et al. (2010), which both place closure of the Palavasian coastal lagoons by the sand barrier at around  $1220 \pm 120$  AD (after correction). This estimation was based on the transition of gastropod shells along the sediment core, from the marine sea snail *Bit-*

*tium reticulatum* (Cerithiidae) to the lagoonal sea snail *Hydrobia acuta* (Hydrobiidae). Their density shifted at about 1.80 m depth in the present sediments. This stratum was dated by Sabatier et al. (2010b) taking into account a  $^{14}\text{C}$  reservoir age correction of  $618 \pm 30$   $^{14}\text{C}$  yr. for the Palavasian lagoons.

The next step was dated from ancient maps of the coast. Among others (in Lenthéric 1876), those of Johannes Solivet 1570, the Marcator map of 1585 and the Nolin geographer publication of 1692 all show the Palavasian lagoons from Thau to Mauguio as a continuous ria isolated from the sea by a sand bar that is well constituted but pierced by several passages. Fragmentation of this continuum to constitute the present small lagoons has not been dated, but according to the Vidal map, it happened after 1744.

According to phylogenetic studies, the two species belong to the same evolutionary branch (Huysse et al. 2004, Mejri et al. 2011, Vanhove et al. 2012). The date of their separation has been estimated to be from 1.37 (Vanhove et al. 2012) or 2.5 (Huysse et al. 2004) to 10 Mya according to Thacker et al. (2019). While very divergent, these dates are long before the isolation and formation of the Gulf of Lion lagoons.

The genetic structure of sedentary sand gobies can therefore be described as a macroevolution, separating *P. microps* and *P. marmoratus* millions of years ago and a microevolution beginning by settlement of the two species on the Mediterranean coasts of southern France. The ria structure of the coast lasted approximately from 1200 to 1750, i.e. 5.5 centuries, during the period when sedentary gobies settled in the Palavasian lagoon. Inter-lagoon differentiation has lasted two centuries only. The progressive fragmentation, first between the Palavasian lagoons and the more eastern and southwestern lagoons, then between the Palavasian lagoons themselves, explains the observed intraspecific interpopulation differentiation (Berrebi et al. 2009, present investigation).

### Distribution, differentiation and adaptation of *P. microps*

*P. microps*, the common goby, is known to be very abundant in the French Mediterranean lagoons (Quignard et al. 1984, Bouchereau et al. 1989a). As confirmation, it was present at most sites sampled here, though differences in abundance are to be noted. Thus, in some lagoons it was almost the only sedentary species of the genus (with *P. minutus*): i.e. Canet, La Peyrade, Mauguio, Vaccarès, Impériaux, Berre and the Marseillan 1 station in Thau lagoon. It was predominant in Vic lagoon. Its presence was anecdotal at the other stations, except St Cyprien (12%). Salinity is an environmental factor that exerts selection pressure on aquatic animals and especially fish (Poizat et al. 2004). Bouchereau et al. (1989b) and De Casabianca and Kiener (1969) described the enormous resistance of *P. microps* to extreme environmental conditions and variations, as in the lagoons of Mauguio and Palo (Corsica). Rigal et al. (2008) demonstrated that *P. microps* is able to survive in absolutely fresh water, whereas *P. marmoratus* cannot.

Despite the phylogenetic tree which showed low bootstrap values for *P. microps* branches and no geographic logic (Fig. 4), and despite the absence of IBD according to the Mantel test, the multidimensional analysis, performed on pure *P. microps* individuals only ( $Q > 0.9$ ), was able to distinguish several populations (Fig. 5). A major genetic form, positioned at the centre of the graph, gathered populations from five main lagoons: La Peyrade, Vic, Vaccarès and Berre, together with the Marseillan 1 station of Thau lagoon. The *P. microps* populations of the Canet, Mauguio and Impériaux lagoons showed a slightly different genetic composition from that of all other lagoons. These results reflect a structure in meta-populations of the *P. microps* species in the Gulf of Lions, as a consequence of recent simultaneous lagoon isolation affecting the differentiation of gobies populations with no geographic logic, as observed on mitochondrial sequences (Berrebi et al. 2009). There is no specific information published about the pelagic larval duration (PLD) of sedentary gobies (González-Wangüemert and Vergara-Chen 2014), although congeneric species have a PLD of 30 to 39 days under laboratory conditions (Fonds 1973). In the few studied *Pomatoschistus* species, very limited migratory behaviour has been observed (Gysels et al. 2004, Berrebi et al. 2005). Their poor swimming abilities are possibly partly due to the shape of their pelvic fins, which constitute a suction disc typical of fixed animals (Miller 1986, Bardin and Pont 2002).

#### Distribution, differentiation and adaptation of *P. marmoratus*

The marbled goby, *P. marmoratus*, showed a single large cluster in FCA analysis (diagram not shown), with only one sample differentiated from the remainder (the ULM station in Thau lagoon), with no clear explanation for this. Distribution of *P. marmoratus* on the French Mediterranean coast has been little studied (De Casabianca and Kiener 1969). The species has been reported in Thau lagoon and in the Vaccarès complex (Berrebi et al. 2005) and far in the southwest, in the Mar Menor lagoon in Spain (González-Wangüemert and Vergara-Chen 2014). The present study widens our knowledge of its range in the lagoons of the French Mediterranean coast. It was detected in seven lagoons and constituted the main sedentary *Pomatoschistus* species in five of them (St Cyprien, Salses-Leucate, La Palme, Bages-Sigean and Thau). In other lagoons, it was represented by a very small number of individuals, frequently between 1% and 5% and always below 10% (Table 4). The lagoons in which this species predominated are marinized (Table 2). The mouth of the Tech River (St Cyprien station) is strongly influenced by marine waters. At Bages-Sigean lagoon, salinity does not drop below 13.7‰ (Labat 1976). Rigal et al. (2008) demonstrated experimentally that the marbled goby cannot survive in a completely desalinated environment, as individuals die at the threshold of 1.4‰.

#### Differential distributions, historic events and competitive exclusion

The differential distribution of the two sedentary *Pomatoschistus* species is far from presenting a random pattern with *P. marmoratus* in the west and *P. microps* in the east. This geographic distribution suggests a historical event, i.e. opposite migrations with contact at the middle. However, this process cannot wholly explain why the two hypothetical directional migrations ceased between the Thau and La Peyrade lagoons (Fig. 3) or some exceptions in the geographic picture, i.e. a *P. microps* population in the west (Canet lagoon) and a *P. microps* population (Marseillan 1 station) within a *P. marmoratus* lagoon (Thau). Concerning this last surprising phenomenon, the resurgence of freshwater springs in the Thau lagoon (Bakalowicz et al. 2003, Elbaz-Poulichet et al. 2005, Plus et al. 2006) might be an explanation because *P. marmoratus* does not survive in freshwater (Rigal et al. 2008). Other surprising features are the very limited intruders in both directions and relatively few fertile hybrids.

Absence of *P. marmoratus* can be explained in lagoons where freshwater crises occur due to intense rains. These include the Canet, Mauguio, Thau, La Peyrade, Vic, Vaccarès-Impériaux and Berre lagoons, i.e. the whole eastern part of the sampled region. The absence of *P. marmoratus* should be linked to its incapacity to survive in freshwater conditions. Its preference for marine water has been known for a long time (De Casabianca and Kiener 1969). The absence of the common goby, *P. microps*, needs some more hypotheses to be explained. This species has been detected in the west as hybrids in La Palme and as individual intruders in the St Cyprien, Salses-Leucate, Bages-Sigean lagoon and at the Thau-Bouzigues station. However, as far as we know, these are not inhospitable environments for the common goby (Rigal et al. 2008). A possible explanation could be that the marbled goby is a better competitor in these lagoons for an unknown ecological reason, which would exclude *P. microps* from these sites.

#### CONCLUSIONS

A new picture of *Pomatoschistus* inhabitants of southern French lagoons is now available. Each Mediterranean coastal lagoon is a site of exclusion, competition or hybridization between two cryptic species of sand gobies. While composed of differentiated populations, neither species shows a geographic structure in the NJ tree (Figs 4 and 5) or IBD. This reinforces the idea that the species exhibit a very sedentary behaviour. This status makes them dependent on extinction/foundation processes. For example, a major dystrophic crisis in August 1988 resulted in the disappearance of resident fish populations in the Mauguio Lagoon, including *P. microps*. Recovery of the *P. microps* population took several years and was carried out from a few lagoon survivors or from external intakes from healthy biotopes (Bouchereau et al. 1990). These random recolonizations would explain the phylogeographic “disorder” observed for both species.

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## APPENDIX

Appendix 1. – DeltaK calculations according to the Evanno et al. (2005) method, through *Structure Harvester* online software (Earl vonHoldt 2012). Here K=2 (in bold type) is the uppermost hierarchical level of structure.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	Ln''(K)	Delta K
1	10	-14159.540000	1.719884	—	—	—
<b>2</b>	<b>10</b>	<b>-12338.000000</b>	<b>1.445683</b>	<b>1821.540000</b>	<b>1647.000000</b>	<b>1139.253722</b>
3	10	-12163.460000	5.117910	174.540000	39.600000	7.737534
4	10	-11949.320000	68.723628	214.140000	60.360000	0.878301
5	10	-11795.540000	19.917655	153.780000	46.620000	2.340637
6	10	-11595.140000	15.319530	200.400000	97.880000	6.389230
7	10	-11492.620000	20.518211	102.520000	45.360000	2.210719
8	10	-11435.460000	34.851083	57.160000	40.160000	1.152331
9	10	-11338.140000	20.132387	97.320000	44.780000	2.224277
10	10	-11285.600000	32.945334	52.540000	7.320000	0.222186
11	10	-11225.740000	25.346657	59.860000	60.220000	2.375856
12	10	-11226.100000	58.593430	-0.360000	—	—