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Haplotypic confinement in two cryptic and closely-related species of sedentary gobies, *Pomatoschistus microps* and *P. marmoratus* in French Mediterranean lagoons

Patrick BERREBI[1,2,*], Pascal RODRIGUEZ[1], Clare ROONEY[1], Silje d'ALOYA[1] and Ghislaine CATTANEO-BERREBI[1]

[1] *Laboratoire Ecosystèmes Lagunaires (UMR CNRS-UM2 5119), Université Montpellier 2, cc 093, place E. Bataillon, 34095 Montpellier cedex 05, France*

[2] *Institut des Sciences de l'Evolution (UMR CNRS-UM2 5554), Université Montpellier 2, cc 065, place E. Bataillon, 34095 Montpellier cedex 05, France; *e-mail: patrick.berrebi@univ-montp2.fr*

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A b s t r a c t . Two sedentary gobiids (the cryptic species *Pomatoschistus microps* and *P. marmoratus*) have been analysed in several Mediterranean lagoons of southern France and in Corsica. In order to determine the level of population isolation between neighbouring lagoons, mtDNA was screened using RFLP (7 endonucleases) of an amplified 2 kb region (including the D-loop and Cyt b). The mtDNA diversity of the genus is high (42 haplotypes for 125 individuals) so that a detailed haplotype tree has been constructed using two additional outgroup species. The main result is that almost no common haplotypes are shared between populations of the same species inhabiting neighbouring lagoons. A high level of isolation between neighbouring lagoons during several centuries is deduced, at least for *P. microps* populations.

Key words: Gobiidae, mtDNA RFLP, isolation, lagoon

Introduction

All around Europe, from the north Atlantic to Morocco and in the Mediterranean, the species of the gobiid genus *Pomatoschistus* are adapted to coastal and lagoon environments, playing an important role in energy flows from benthic invertebrates to the piscivorous fauna (D o o r n b o s & T w i s k 1987). This genus contains 10 euryhaline species in the Mediterranean, 5 of them endemic (M i l l e r 1986, Q u i g n a r d & T o m a s i n i 2000).

These small species (maximum length 90 mm), which are difficult to distinguish morphologically (M i l l e r 1973, W a l l i s & B e a r d m o r e 1984), occur in the Mediterranean lagoons of southern France, where two main taxa are currently recognised: *P. microps* and *P. minutus* (Q u i g n a r d & Z a o u a l i 1981). A third species, *P. marmoratus*, has been rarely recorded. Q u i g n a r d & Z a o u a l i (1980) were the first to report the presence of *P. marmoratus* on the mainland Mediterranean French coast, only in the Bage lagoon, and C r i v e l l i (1981) recorded this species in a Vaccarès sub-lagoon (Camargue, Rhône Delta), according to morphological analyses (dermal papillae of the head) carried out by the Museum d'Histoire Naturelle de Paris. C a s a b i a n c a & K i e n e r (1969) and B o u c h e r e a u & L a m H o a i (1997) recognised *P. marmoratus* in Corsica. Because specific distinction between *P. microps* and *P. marmoratus* needs special staining of dermal papillae or molecular analyses, not possible in the field, we consider these two species as cryptic. The review by M i l l e r (1986) summarised the presence of *P. marmoratus* along the north coasts of the Mediterranean as far as the Middle East.

* Corresponding author

The three species involved can be classified in two distinct ecological categories: the sedentary species (*P. microps* and *P. marmoratus*) conduct their entire biological cycle including reproduction in lagoons (P a m p o u l i e et al. 2000), while *P. minutus* spawns along the coast after a migration to the sea at the end of autumn, followed by a migration of the juveniles to the lagoons in early spring (B o u c h e r e a u et al. 1991).

G y s e l s et al. (2004) developed a detailed mtDNA and allozymic phylogeny of *P. microps* all around European coasts. They demonstrated mainly a cline of variation from the south to the north, compatible with a pattern of isolation by distance established after the last glaciations, plus several recolonisation events from ancient refugia in the north Atlantic. They also demonstrated a surprising relation between French Mediterranean lagoons and Norway (i.e. the shared H14 haplotype), explained by a possible retention of ancestral polymorphism due to a former broad distribution of the H14 haplotype displaced by a recent range expansion of the Atlantic haplotype H1.

More recently, B e r r e b i et al. (2005) confirmed, with allozymes, the presence in French Mediterranean coasts of both species of sedentary species, *P. microps* and *P. marmoratus* and highlighted their cryptic morphological differences, deducing that the respective distribution ranges of theses species, as it is published, are doubtful when not checked by molecular analyses. They described two unknown patterns: first, *P. marmoratus* is the only species found in the Thau lagoon, while *P. microps* occupies alone the rest of the investigated lagoons (exclusion); second, in Vaccarès/Imperiaux lagoons complex, hybridisation occurs between the two sedentary species (B e r r e b i et al. 2005).

The aim of this survey is the analysis of the mtDNA diversity (through RFLP) of the sedentary *Pomatoschistus* species in lagoons of southern France in order to estimate the level of population isolation between neighbouring lagoons of *P. microps*, and *P. marmoratus* respectively (with a very much smaller sample size of the latter, due to its scarcity along theses coasts). The sympatric migratory species *P. minutus* and the exotic (Italy) species *P. tortonesei* are also analysed in order to test the specificity of the marker.

Materials and Methods

S a m p l i n g

The main study area is located along the French Mediterranean coast, between the Spanish and the Italian borders, and in Corsica (Fig. 1). On the mainland, numerous brackish water lagoons and estuaries, including the Rhône Delta complex, constitute a transition area between the inland freshwater system and the sea. At a distance of about 350 km from the sampled French mainland, the island of Corsica also has several similar lagoons on its eastern coast.

Among the more than 50 regional lagoons, three of them, ecologically distinct, were sampled on the mainland (Fig. 1) and a fourth in Corsica. On the mainland, the following sites were sampled from east to west: the Vaccarès lagoon (6400 ha, remote from the coast, inside the Rhône Delta, with a low salinity), the Mauguio lagoon (3200 ha, linked to the sea by a channel, ecologically very variable due to its shallow waters) and the Thau lagoon (7500 ha, deep and nearly marine stable conditions). In Corsica, the Biguglia lagoon (1790 ha, similar to the Mauguio lagoon) was also sampled. Fishes were captured in February 2002 (Corsica) and April 2002 (mainland) with a beach seine or bought from lagoon fishermen who use fyke nets locally called “capetchades”.

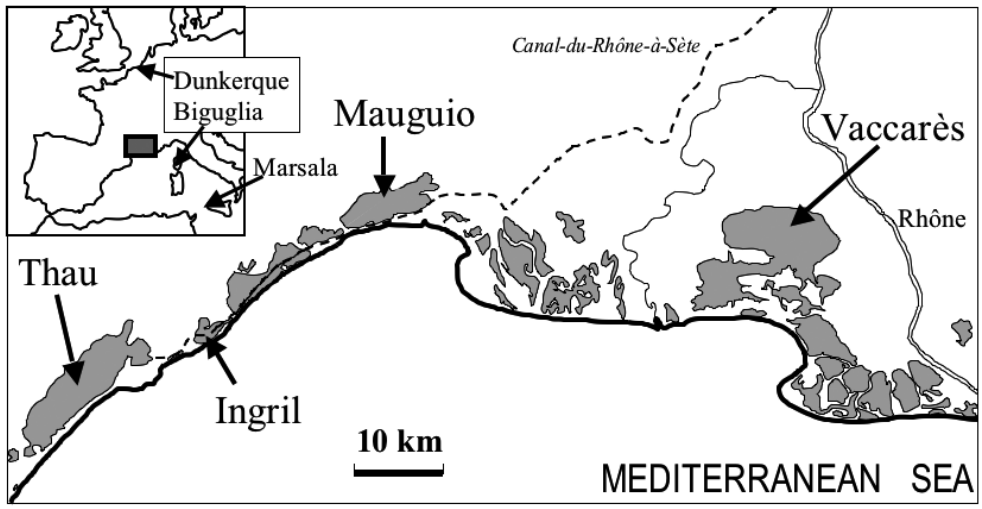

Fig. 1. Distribution of the samples.

Table 1. Description of the samples analysed at the seven localities. Proportion of the different species observed in each sample according to the phylogenetic analysis (see Fig. 2).

Locality	Sampling dates	Samples size	Capture gear	Samples composition
Thau Lagoon	April 2002	22	seine	22 *P. marmoratus*
Mauguio L.	Feb. to April 2002	19	fyke net	17 *P. microps* + 2 *P. minutus*
Vaccarès L. (Rhone D.)	Feb. to April 2002	27	fyke net	20 *P. microps* + 2 *P. marmoratus* + 5 *P. minutus*
Biguglia L. (Corsica)	February 2002	34	fyke net	34 *P. microps*
Dunkerque (English Ch.)	October 2001	11	-	11 *P. microps*
Ingril L.	October 2001	5	fyke net	5 *P. minutus*
Marsala L. (Sicily)	February 2002	7	fyke net	7 *P. tortonesei*
	total	125		

Complementary samples were added as taxonomic references for Atlantic populations of *P. microps* (Brey-Dunes, English Channel, near Dunkerque). Other species *P. minutus* (Mauguio, Vaccarès and Ingril lagoons, see Fig. 1) and *P. tortonesei* from Sicily (Stagnone di Marsala) were used as outgroups.

The fishes were generally transported to the laboratory on ice, and frozen until enzyme and DNA extractions. Their species was defined according to allozymic method (Berrebi et al. 2005). Table 1 provides all the sampling information on the 125 fishes analysed.

Screening of mitochondrial polymorphism

The total DNA was extracted using the phenol:chloroform technique of Sambrook et al. (1989). A fragment of mtDNA (2 kb including the D-loop and Cyt b) was amplified by polymerase chain reaction (PCR). The primers used, HN20 and VGLU, were defined respectively by Bernatchez & Danzman (1993) and Briolay et al. (1998).

Double-stranded DNA was amplified in 66 μL reaction volumes per fish (permitting three endonuclease digestions) containing 4 μL of 25mM $MgCl_2$, 6.6 μL of 10x reaction buffer, 3.3 μL of 10 mM dNTP, 6.6 μL of 5 ppm of each primer, 0.7 μL of 5U/μL Taq polymerase SIGMA, 31.7 μL of chemical water SIGMA, and 6.6 μL of DNA template at roughly 50–100 μg/mL. PCR amplification was as follows: one preliminary denaturation at 95°C (2 min), followed by strand denaturation at 94°C (1 min), annealing at 50°C (1.5 min) and primer extension at 72°C (2.5 min), repeated for 36 cycles and a final extension at 72°C (10 min). An approximately 2 kb amplified fragment including the control region (D-Loop) as well as the gene coding for cytochrome b, was obtained.

The screening for restriction fragment length polymorphism (RFLP) on the PCR product of mtDNA was carried out with seven enzymes (Alu I, Taq I, Tru9 I, Hsp92 II, Rsa I, Nde I and Hae III). For each fish, 15 μL of the PCR reaction containing amplified DNA was digested with 0.15 μL BSA, 1.5 μL enzyme buffer, 12.2 μL chemical water and 0.38 μL of endonuclease. The digested DNA was then electrophoretically separated on 2% agarose gels in 0.5 TBE (Tris-borate-EDTA) buffer containing ethidium bromide and visualised under UV light and documented photographically.

Data analysis

Distinct single endonuclease patterns were identified by a specific letter in order of appearance. Each haplotype was defined by a multi-letter code that corresponds to the seven restriction profiles (Table 2).

A disruptive matrix (composed only of 0 and 1) was constituted using the GENERATE option of the REAP computer program (McElroy et al. 1992) and the phylogenetic relations between haplotypes were estimated by a Wagner parsimony unrooted tree using the MIX option in the PHYLIP 3.5c (Felsentein 1993) computer package. Trees were visualised using the TREEVIEW 1.4 program (Page 1996). A bootstrap test was carried out on 100 replicates with the SEQBOOT and CONSENSE options in PHYLIP 3.5c.

The inter-population divergence was calculated using the D option of the REAP program. For this, only homospecific samples of large enough size (>10) were involved. The intra-sample haplotype diversity (HD) and nucleotide diversity (ND) were calculated using the DA option of the REAP program.

Results

All seven endonucleases were found to be informative and detected restriction fragment length polymorphisms. The addition of fragment sizes for each pattern did not give exactly the same amplified size and it therefore seems likely that doubled bands were sometimes present and bands of less than 200 bp were not detected.

The haplotype frequencies and haplotype and nucleotide diversities of the samples are listed in Table 2. In almost all localities, the variability was high. A total of 42 haplotypes was observed among the 125 fishes analysed, giving an overall mean of one haplotype for every three fishes: 26 haplotypes for 82 *P. microps*, 5 for 24 *P. marmoratus*, 8 for 12 *P. minutus* and 3 for 7 *P. tortonesei*. The haplotype diversity (HD), calculated only for the samples of more than 10 fishes (Table 2), was higher in *P. microps* (between 0.65 and 0.91) than in *P. marmoratus* (0.34). The same observation was found for the nucleotide diversity (ND), that was higher in *P. microps* (between 0.05 and 0.18) than in *P. marmoratus* (0.05).

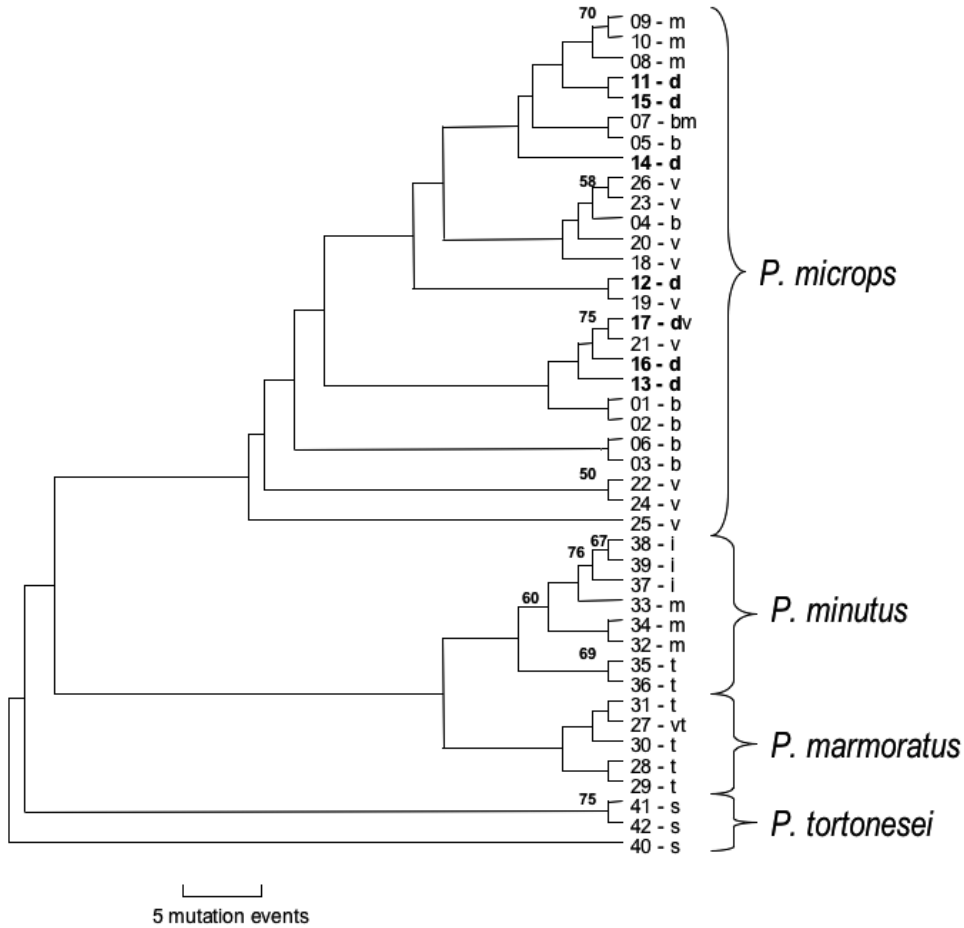

Fig. 2. Wagner parsimony tree of the 42 haplotypes defined according to the profiles of 7 restriction enzymes listed. Only bootstrap values over 50% are indicated. Each OTU is indicated by a code including (i) the haplotype number (see Table 2) and (ii) a letter indicating the station (b = Biguglia lagoon, Corsica; d = Dunkerque; m = Mauguio; t = Thau, v = Vaccarès, s = Sicily, Stagnone di Marsala). Atlantic haplotypes are in bolt letters.

No shared haplotype was observed between species. In the best-sampled species (*P. microps*), there was no haplotype shared between any sample pair with two exceptions: Biguglia/ Mauguio (haplotype AAABCCA) and Vaccarès/Dunkerque (haplotype AAACCCA).

The parsimony tree (Fig. 2) gives a picture of the mitochondrial diversity: each species is represented by numerous haplotypes, their number being more or less proportional to the sample sizes. One of the surprising observations is that, while each of the four species haplotypes are grouped into monophyletic clusters, these clusters are generally not significant according to bootstrap values.

P. microps, the best sampled species, is subdivided into several groups of haplotypes, resulting in 'star-burst' phylogeny lacking internal structure in this part of the tree; only end nodes show bootstrap values over 50%. However, it can be noticed that Atlantic haplotypes (underlined in Fig. 2) are not separated from Mediterranean ones and that even in branches well supported by bootstrap values (see haplotypes 17 and 21, Fig.2), specimens from Dunkerque (Atlantic) and Vaccarès (Mediterranean) are associated.

Table 2. Number of observed mtDNA haplotypes recorded in each sample. Each letter corresponds to a restriction profile, using respectively Alu I, Taq I, Tru9 I, Hsp92 II, Rsa I, Nde I and Hae III endonucleases. The haplotypes are sorted according to the species to which they were attributed after the phylogenetic analysis (Fig. 2). Haplotype diversity (HD) and nucleotide diversity (ND) are calculated by species when the sample size is over ten.

	haplotype	Biguglia	Mauguio	Dunkerque	Vaccarès	Thau	Ingril	Marsala
P. microps								
AAAABCA	1	2	-	-	-	-	-	-
AAAACCA	2	5	-	-	-	-	-	-
AAAACCB	3	9	-	-	-	-	-	-
AAAACCF	4	5	-	-	-	-	-	-
AAABBCA	5	3	-	-	-	-	-	-
BAAACCB	6	1	-	-	-	-	-	-
AAABCCA	7	9	2	-	-	-	-	-
AAABBCC	8	-	1	-	-	-	-	-
AAABBCE	9	-	5	-	-	-	-	-
AAABCCE	10	-	9	-	-	-	-	-
AAABBCB	11	-	-	1	-	-	-	-
AAABEBA	12	-	-	2	-	-	-	-
AAACBBA	13	-	-	2	-	-	-	-
AABBBFA	14	-	-	1	-	-	-	-
AACBBCB	15	-	-	1	-	-	-	-
ABACCCA	16	-	-	1	-	-	-	-
AAACCCA	17	-	-	3	2	-	-	-
AAAAEBF	18	-	-	-	4	-	-	-
AAABABA	19	-	-	-	1	-	-	-
AAABBCF	20	-	-	-	5	-	-	-
AAACECA	21	-	-	-	3	-	-	-
ADAADBB	22	-	-	-	1	-	-	-
ADAECCF	23	-	-	-	1	-	-	-
ADAEDBB	24	-	-	-	1	-	-	-
BDAADBB	25	-	-	-	1	-	-	-
BDAECCF	26	-	-	-	1	-	-	-
P. marmoratus								
BBBADBB	27	-	-	-	2	18	-	-
BBBABAB	28	-	-	-	-	1	-	-
BBBABBB	29	-	-	-	-	1	-	-
BBBADAB	30	-	-	-	-	1	-	-
BBBBDBB	31	-	-	-	-	1	-	-
P. minutus								
BBAABDC	32	-	-	-	1	-	-	-
CBAAADC	33	-	-	-	1	-	-	-
CBAABDC	34	-	-	-	3	-	-	-
CBBABDA	35	-	1	-	-	-	-	-
CBBBBDA	36	-	1	-	-	-	-	-
CBACBDC	37	-	-	-	-	-	1	-
CBACBDD	38	-	-	-	-	-	1	-
DBACBDC	39	-	-	-	-	-	3	-
P. tortonesei								
CCBBCAG	40	-	-	-	-	-	-	2
DCBBCBG	41	-	-	-	-	-	-	1
DCBCCBG	42	-	-	-	-	-	-	4
***P. microps* HD/ND**		0.83/0.11	0.66/0.05	0.91/0.18	0.89/0.11	-	-	-
***P. marmor.* HD/ND**		-	-	-	-	0.34/0.05	-	-

Discussion

Species distribution

In the Thau sample, the only sedentary species found was *P. marmoratus*, as previously indicated by Berrebi et al. (2005). Because this sample was caught by a beach seine, it represents only a very local sample and may not be representative of the whole Thau lagoon. In the Mauguio lagoon, among the non-migrant gobiids, only *P. microps* occurred. The Biguglia lagoon (Corsica) also seems to only harbour *P. microps*. Finally, the Vaccarès lagoon complex harbours both *P. microps* haplotypes (90% of the analysed individuals) and *P. marmoratus* (10%), where these species currently hybridise (Calatayud 2003, Berrebi et al. 2005, Trebuchon 2006). The species composition of Mauguio, Vaccarès and Biguglia lagoons are probably reliable because the samples were caught using fyke nets which collect moving fish during a 24 hour period, constituting representative random samples of the lagoons.

The Thau and Mauguio lagoons are separated by only 28 km of linear sandy coast and linked by a brackish channel: the "*Canal du Rhône à Sète*" (indicated by a dotted line in Fig. 1). The Vaccarès lagoon is about 50 km east of the Mauguio lagoon. This apparent general ease of migration from one lagoon to the other, and the contrasting distribution of the two non-migrant species *P. microps* and *P. marmoratus*, lead to some expectations.

On the one hand, the inter-specific distribution pattern can be explained by adaptation, because of the divergent ecological characteristics of each sampled lagoon as classically observed in various organisms (Le Loeuff & Zabi 1993). For non-migrant *Pomatoschistus* species, the Thau lagoon, harbouring only *P. marmoratus*, is also very different from the other three lagoons by its area and its depth (up to 8 metres) providing almost marine buffered conditions. The other lagoons are smaller, shallow, and so very unstable. *P. marmoratus* could be adapted to ecological conditions close to marine ecology and *P. microps* to unstable ecosystems. The osmoregulatory capacity is a good candidate factor (Rigal et al. 2008).

However, on the other hand, the distribution of the two species could be due to competition, resulting in the exclusion of one of the species in the Thau, Mauguio and Biguglia lagoons. Casabianca & Kiener (1969) suspected such a phenomenon between the two sedentary species, although their species identification, only on morphological characters, is doubtful. In this survey, only *P. microps* was observed in Corsica, though the sampling was limited to one lagoon.

When ecological conditions are intermediate or when exclusion is impossible, hybridisation occurs.

Intra-specific haplotypes distribution

Each of the four species analysed forms monophyletic clusters in the parsimony tree (Fig. 2), but except for the terminal nodes, the whole structure is not significant according to bootstrap values. This is a surprising result because the logical specific organization of the tree is unlikely to be entirely due to chance.

The haplotype organisation of *P. microps* is more detailed because of the sample size (82) and the subsequent high number of haplotypes (26). Berrebi et al. (2005) have partly analysed the same samples using allozymes. They detected significant intraspecific Fst values only between Mauguio and Vaccarès lagoons samples. While suspected, the sedentary

behaviour of this species has not really been demonstrated. The present haplotypes analysis indicates a very strong isolation (Table 2) with few haplotypes shared between neighbouring lagoons. This suggests a remarkable sedentary behaviour in this species. The parsimony tree shows no clear lagoon clustering except for several pairs of sibling profiles probably locally and recently diverging. Moreover, a cluster groups together the Atlantic/Mediterranean shared haplotype (No.17, AAACCCA) plus a Vaccarès haplotype (No.21, AAACECA) and a Dunkerque haplotype (No.16, ABACCCA).

The other observation is that the Dunkerque haplotypes are generally widespread throughout the *P. microps* tree among haplotypes of several Mediterranean lagoons. These features can be explained by a common origin of the different lagoon populations (ancestral polymorphism) but with no recent exchange occurring.

Gysels et al. (2004) analysed numerous Atlantic and Mediterranean samples of *P. microps*. As here, they observed shared Mediterranean and North Atlantic (Norway) haplotypes separated by divergent West Atlantic populations, also interpreted as due to ancestral polymorphism.

Pomatoschistus populations are common in northern seas (Baltic and North seas) showing a cold water affinity (Stefanni et al. 2003). During the last glacial event maximum (18000 years ago), the present North Sea surface temperature conditions were located around the Gibraltar Straits CLIMAP, 1976). As for *Platychthys flesus* (Berrebi 1988, Borsa et al. 1997), *P. microps* populations were probably flourishing during this period at the "entrance" to the Mediterranean. When the temperatures increased, the cold-affinity species retreated to the north, splitting into Mediterranean and Atlantic branches. This can explain the presence of Atlantic and Mediterranean *P. microps* haplotypes in several sub-branches, with the haplotype No.17 (AAACCCA) that occurs in both the Vaccarès lagoon (10%) and in the coastal Atlantic sample from the North Sea (27%).

More recently, we know that the southern French lagoons were not separated a few centuries ago, and at least the Thau and Mauguio lagoons formed a single complex (maps of Languedoc coasts by Jean de Beins, 17th century and Jacques-Nicolas Bellin, 18th century). Because almost no haplotypes are presently shared between neighbouring lagoons populations, it can be deduced that no significant exchange of migrants occurred between lagoons for centuries, except perhaps following re-foundations.

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