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Two-dimensional electrophoresis analysis of proteins from epitokous forms of the polychaete *Perinereis cultrifera* from the English Channel and the Mediterranean Sea

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Abstract: We compared the general protein patterns of different populations of the epitokous form of the polychaete *Perinereis cultrifera* from the English Channel (Normandy and the north coast of Brittany) and the Mediterranean Sea (Algerian coast near the Tunisian boarder) using high resolution two-dimensional gel electrophoresis (2-DGE). Data sets were converted to similarity coefficients using the Dice coefficient, based on more than 150 protein spots. UPGMA dendrogram reveals a clear distinction between the three populations, individuals from the same population clustering together. The genetic distances between populations from the English Channel and the Mediterranean are large compared to those within populations. As a consequence, our results indicate a restricted gene flow between our sampled populations. In addition, the general protein pattern of individuals from the Mediterranean contains 5 exclusive spots whereas no specific spots were detected between the two populations of the English Channel which share one exclusive spot by comparison to the mediterranean form.

Résumé : *Electrophorèse bidimensionnelle des protéines de formes épitoques de l'annélide polychète* Perinereis cultrifera *de Manche et de Méditerranée.* Nous avons comparé les profils électrophorétiques des protéines générales de différentes populations de la forme épitoque de l'annélide polychète *Perinereis cultrifera* présente en Manche (Normandie et côte nord Bretagne) et en Méditerranée (littoral algérien à proximité de la frontière tunisienne) obtenus par électrophorèse bidimensionnelle hautement résolutive. Les données ont été converties en coefficients de similarité de Dice basés sur l'analyse de plus de 150 taches protéiques. Un dendrogramme UPGMA a montré que les trois populations étudiées se distinguent les unes des autres car les individus provenant d'une même population se regroupent. Les distances génétiques entre les populations provenant de la Manche et de la mer Méditerranée sont importantes en comparaison des distances génétiques intra-populations. Nos résultats indiquent donc un flux génique restreint entre les trois populations étudiées. Par ailleurs, les schémas de distribution des protéines totales des individus provenant de Méditerranée contiennent 5 taches protéiques exclusives. Aucune tache protéique spécifique n'a été détectée entre les deux populations originaires de la Manche ; cependant, elles partagent une tache protéique exclusive commune.

Keywords: *Perinereis cultrifera*, two-dimensional electrophoresis, proteins, genetic variation, English Channel, Mediterranean Sea.

Introduction

The polychaete *Perinereis cultrifera* (Grube, 1840) occurs along the northwestern coasts of Europe and the

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Mediterranean. This species was also described in the Indian Ocean and the Pacific (Durchon, 1957). The spawning season, mode of reproduction, age at maturity and biometric characteristics differ according to the location of populations. Reproduction in the English Channel and the Atlantic is of an epitokous type (Herpin, 1925; Durchon, 1951; Cazaux, 1965; Scaps et al., 1992; Scaps et al., 2000) as in the Mediterranean Sea at Salammbô near Tunis, on the Algerian Mediterranean coast at Annaba near the Tunisian boarder and in the Venice Lagoon in Italy (Ansaloni et al., 1986; Zghal & Ben Amor, 1989; Rouabah & Scaps, in press). In the English Channel and the Atlantic, reproduction occurs from May to June and sometimes July (Herpin, 1925; Fage & Legendre 1927; Durchon, 1951). At Salammbô, sexually mature individuals can be found in May (Zghal & Ben Amor 1989). At Annaba, spawning occurs at the end of April early May (Rouabah & Scaps, in press)**.** *P. cultrifera* reproduces in March in the Venice Lagoon (Ansaloni et al., 1986). On the north coasts of Brittany and at Annaba in the Mediterranean, *P. cultrifera* has a 3-year life cycle but some individuals may reproduce in their fourth year in Brittany (Scaps et al., 1992; Rouabah & Scaps, in press). However, in the Bay of Alger, the reproduction has been described as atokous (Marcel, 1962), occurring throughout the year but being more intense from July to November; individuals have a 1.5-year life cycle.

Biometrical characteristics (weight, number of segments) revealed two types of epitokous form of *P. cultrifera* characterized by a large segment number (up to 120) but differing by their weight. In the English Channel and the Atlantic (Cazaux, 1965; Scaps et al., 1992) adults are very large (3.0 to 6.6 g) whereas in the Mediterranean Sea at Annaba and at Salammbô adults are very small (0.24 to 0.85 g). In addition, the atokous form of the Bay of Alger can be distinguished by a segment number below 80 (Marcel, 1962).

In a previous attempt to distinguish the two types of the epitokous form of *P. cultrifera* Scaps et al. (2000) reported morphological (number and morphology of paragnaths on the proboscis, number of teeth per half jaw) and biochemical (allozymes, general protein band patterns obtained after one-dimensional electrophoretic procedure) divergence and concluded that *P. cultrifera* is a complex of species. More recently, Maltagliati et al. (2001) reported evidence for morphological (number of paragnaths on the zones of the pharynx) and genetic (allozymes) divergence in *P. cultrifera* at a small spatial scale from two habitat types (a brackish-water habitat and an adjacent marine site) at Elba Island (Italy) and concluded that the two groups can be assigned to cryptic species.

The aim of this study is to assess inter- and intrapopulation genetic variation in epitokous forms of *P. cultrifera* from the English Channel and the Mediterranean Sea in the context of the presence of cryptic species. With this aim, high-resolution two-dimensional electrophoresis (2-DGE) analysis of proteins was performed. 2-DGE is a powerful technique for separating complex mixture of denatured proteins according to two independent criteria, charge and molecular weight (O'Farrell, 1975). Combined with non-specific protein stains, this technique allows the visualization in a single gel of a very large number of gene products that represent the most abundant proteins in a cell or tissue. 2-DGE allows the examination of a broad spectrum of proteins and has a great potential in systematics (Ohnishi et al., 1983). However, most studies on this issue have been focused on a few species (mainly mammals and species of the genus *Drosophila*). In this work, it is the first time that 2-DGE has been applied to polychaetes in order to analyse levels and patterns of genetic differentiation between populations.

Materials and methods

Collection of individuals and sampling sites

Adult worms were collected by hand in a restricted area from two localities in France and one in Algeria (Fig. 1) during spring of 2000. Individuals were collected from St-Cloud (M, Fig. 1) close to the town of Annaba near the Tunisian border (30 km) on the Algerian Mediterranean coast and from the St-Aubin-sur-Mer beach (N, Fig. 1) in Normandy and the headland of Corn Ar Gazel (B, Fig. 1) on the north coast of Brittany. The last two sites are located in the English Channel.

The nature of the habitat of the worms differs markedly. In St-Aubin, worms build U or Y-shaped burrows in calcareous cobbles essentially in the *Fucus serratus* zone. In the headland of Corn Ar Gazel, individuals occur essentially in the lower part of the *F. serratus* zone in heterogeneous muddy cobbled sand. Individuals build U-shaped galleries with a surface gutter network protected by a cobble, which acts as a roof (Scaps et al., 1998). In St-Cloud, worms were found with the *Rhodophyceae*. In the three locations individuals reproduce exclusively by epitoky.

Sample preparation

Worms were kept alive in an aquarium at 15 °C, continuously supplied with natural seawater and aeration. Worms were used to prepare samples within a period of two days following the collection.

The preparation of samples was carried out on excised metameres and we used the method described by Boyer et al. (1993) with minor modifications. Metameres were reduced to powder directly in liquid N_2 using a mortar and a pestle. One ml of extraction solution (10% w/v trichloroacetic acid, 0.07% v/v b-mercaptoethanol in cold acetone) was added. Proteins were precipitated at -20 °C for

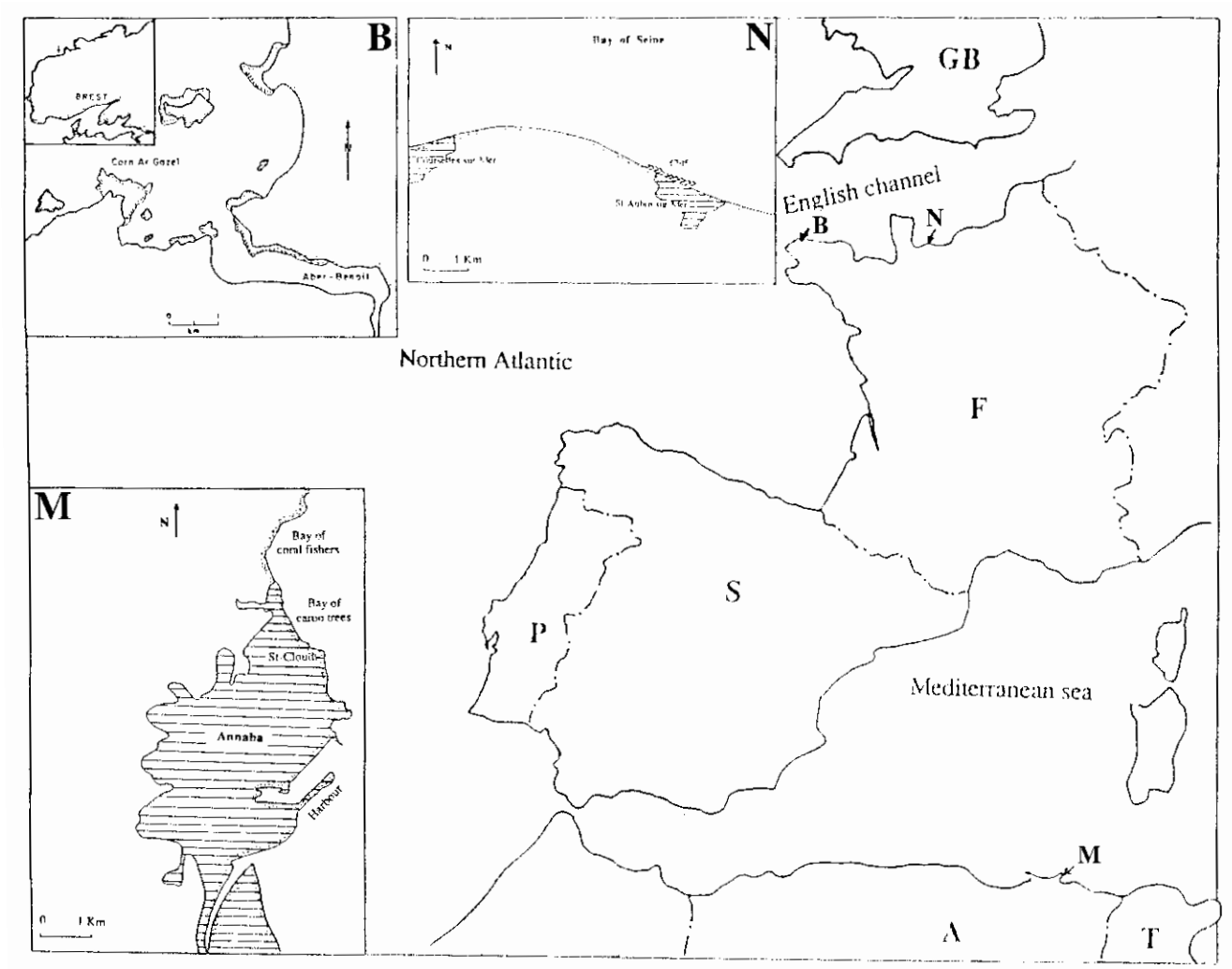

Figure 1. Map of western Europe and north Africa showing location of sampling sites around Annaba in the Mediterraneran Sea (M), St-Aubin-sur-Mer in Normandy (N) and the headland of Corn Ar Gazel on the north coast of Brittany (B).

Figure 1. Carte de l'Europe de l'ouest et de l'Afrique du Nord : localisation des sites de prélèvement près d'Annaba en Méditerranée (M), de St-Aubin-sur-Mer en Normandie (N) et de la pointe de Corn Ar gazel sur la côte nord de Bretagne (B).

45 min. After centrifugation at 30 000 x g for 20 min at 4 °C, the supernatant was discarded and the pellet washed twice with 1 ml b-mercaptoethanol (0.07% v/v) in cold acetone at -20 °C for 45 min, in order to remove any residual trichloroacetic acid. After centrifugation at 30 000 x g for 20 min at 4 °C, the b-mercaptoethanol solution was discarded and the pellet dried for at least 2 hours. To solubilize the proteins, the pellet was resuspended for 2 h in a lysis buffer (Damerval et al., 1986) (60 ml mg^{-1} dry weight pellet) and then centrifuged at 30 000 x g for 20 min at 4 °C. Supernatants were stored at -70 °C for further analysis.

Electrophoretic analysis

Two-dimensional electrophoresis (2-DGE) was essentially performed as described by O'Farrell (1975) with the modification of Hilbert et al. (1992) using the Protean II cell (Bio-Rad, Richmond, CA). Isoelectric focusing gels utilizing diacrylylpiperazine as the cross-linking agent in place of bisacrylamide were prepared. Ampholytes were added to a final concentration of 5.5% and consisted of 90% ampholytes (pH 3-10) and 10% ampholytes (pH 5-7). About 100 mg of protein were loaded on the basic end of the capillary tubes. The gels were 1 mm in diameter and 13.5 cm long. The isoelectric focusing (IEF) was performed at room temperature with a constant voltage of 1 200 V for

17.5 h, followed by 1 500 V for 0.5 h and power was limited to 3 W. IEF capillary gels were extruded from the glass tubes, equilibrated and loaded onto a 12% homogeneous SDS gel. Protein samples were electrophorised in the running buffer (24 mM Tris base; 192 mM glycine; 0.1% SDS). Electrophoresis in the second dimension was carried out with a constant voltage of 350 V until the dye front reaches the bottom of the gel (approximately 3 h).

After electrophoresis, gels were fixed overnight in 200 ml of a solution containing 50% ethanol, 12% glacial acetic acid and 200 ml 35% formaldehyde. Silver staining was performed according to Blum et al. (1987) and gels were dried in a Idea Scientific Tut's Tomb gel dryer. A mixture of protein standards of low molecular weight in the 17.5-76 kDa range (Bio-Rad) was used as reference.

Statistical analyses

A phenetic analysis of protein patterns was performed following the presence-absence criteria. Protein profiles in 2-DGE were evaluated visually by superimposing dried gels on a bench viewer. A zone of well-separated spots containing about 150 abundant proteins was selected in order to compare the gels. Protein patterns were compared between pairs of individuals and each spot considered as a distinct character.

The genetic similarity (F) in protein patterns among populations was estimated using the formula $2nxy/(nx + ny)$ where nx and ny are the total number of spots observed for population x and y, respectively, and 2nxy the number of identical spots in both patterns (Aquadro & Avise, 1981). F is an estimation of the Dice coefficient for similarity. Pairwise data of similarity coefficients were included in a matrix. Dendrogram constructed with the unweighted pair group method with arithmetic means (UPGMA) (Sneath & Sokal, 1973) was used to visualize the level of differentiation among individuals and populations of the two epitokous forms.

Genetic distance was calculated according to Aquadro & Avise (1981)'s equation D = 1-F.

Results

The general protein pattern of three individuals from Brittany, four individuals from Normandy and three from the Mediterranean Sea were analysed by 2-DGE in order to assess inter and intrapopulation genetic variation. Examples of 2-DGE-gel pattern are presented in Fig. 2, 3 and 4. Using 2-DGE, 400 protein spots could be separated. We selected about 150 spots located in the best resolution region of the gels in order to compare intra and inter-individual variability. Only spots showing clarity and good resolution were used. The overall pattern of the gels were similar enough to allow direct comparisons and identification of persistent polypeptides. The general protein pattern of individuals from the Mediterranean contains 5 exclusive spots (Fig. 2) found in all the individuals examined. No specific spots were detected between the two populations of the English Channel, which share one exclusive spot (Fig. 3 and 4). F values of each comparison between two individuals are shown in Table 1. These F values range from 0.952 (between individuals 1 and 3 from Normandy) to 0.716 (between individual 1 from the Mediterranean Sea and individual 4 from Normandy).

A dendrogram constructed from F coefficient is illustrated in figure 5. The dendrogram reveals a clear distinction between the three populations, individuals from the same population clustering together.

Mean genetic distances between the *P. cultrifera* populations calculated from F-values (Table 1) based upon 2-DGE data of total soluble proteins are 0.152 between Normandy and Brittany (range: 0.134-0.186), 0.216 between Brittany and the Mediterranean Sea (range: 0.171-0.252) and 0.241 between Normandy and the Mediterranean Sea (range: 0.206-0.284). These distances between populations are large compared to those within population. Mean genetic distances within populations from Normandy, Brittany and the Mediterranean Sea are 0.072 (range: 0.048-0.092), 0.067 (range: 0.056-0.078), 0.075 (range: 0.059-0.087) respectively.

Discussion

Complex of cryptic species are common in the polychaete family Nereididae. By karyotyping, Pesch et al. (1988) have found a diploid chromosome number of 18 for a Californian population of *Neanthes arenaceodentata* but 2n = 24 in a Connecticut population. In addition the morphology of chromosomes was different between the two populations suggesting that these populations represent different species. Weinberg et al. (1990) reported a complete premating isolation between populations of *Nereis acuminata* from the Atlantic and the Pacific coasts of North America and differences for tolerance to cold temperatures. Moreover, the Atlantic populations had a diploid chromosome number of 22 whereas in the Pacific populations 2n = 18, suggesting that million years of allopatry lead to different species. Fong & Garthwaite (1994), using ten allozyme loci, have compared three morphologically similar species in the polychaete genus *Hediste* (*H. limnicola* - *H. diversicolor* - *H. japonica*) respectively from the West Coast of North America, Europe and Japan. These authors have found that the three taxa are genetically distinct and constitute valid species. *H. limnicola* previously recognized as a self-fertilizing hermaphrodite is quite polymorphic in the four populations examined and these authors suggest that cross-fertilization

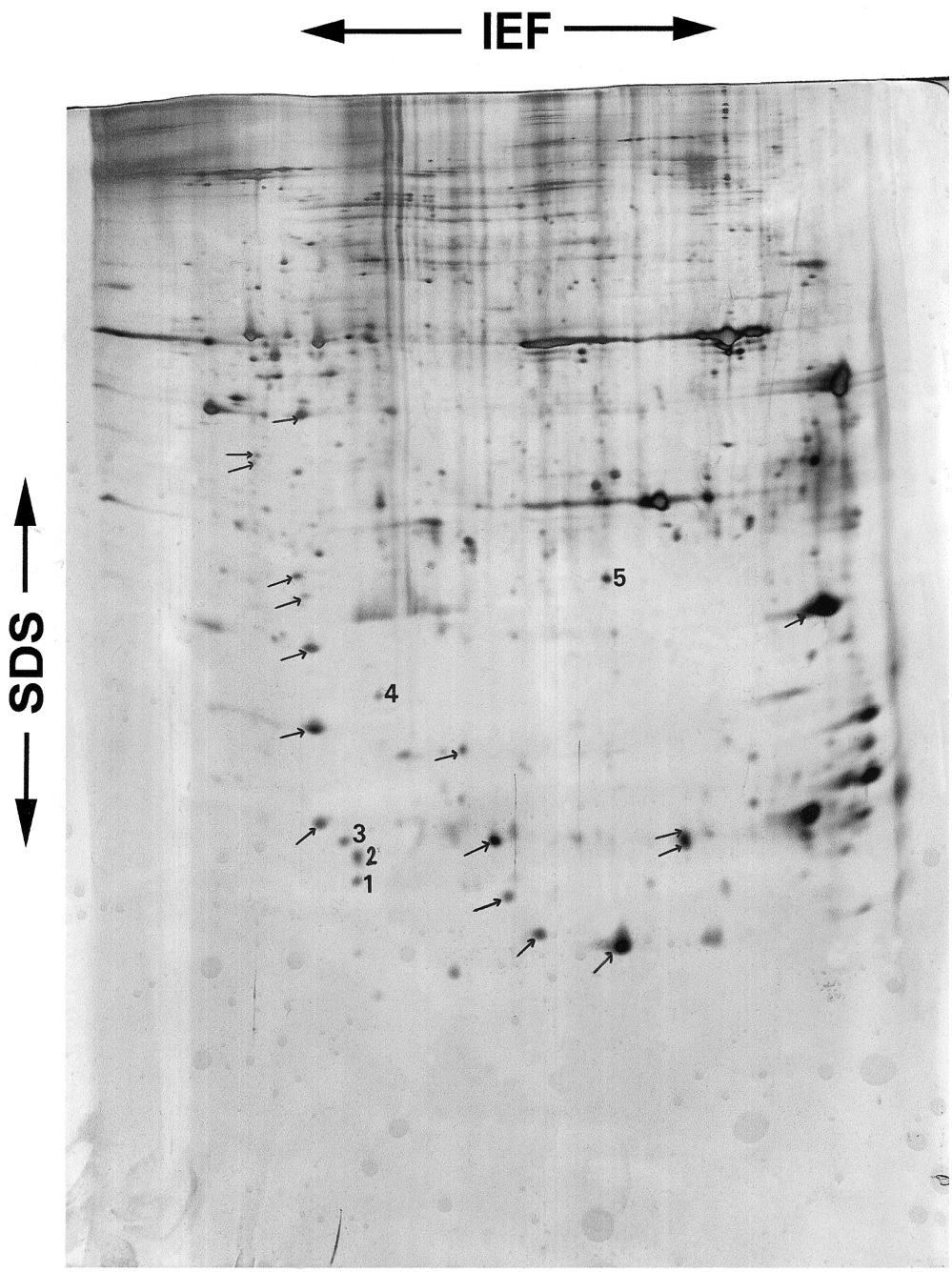

Figure 2. *Perinereis cultrifera.* Example of an electrophoretic analysis of the water-soluble protein fraction in an extract of metameres of an individual from the Algerian Mediterranean coast in which arrows indicate spots found in all the individuals examined. On the pattern 5 spots (1 to 5) present only in the Mediterranean Sea population are indicated.

Figure 2. *Perinereis cultrifera.* Exemple d'électrophorèse de la fraction protéique hydrosoluble d'un extrait de métamères d'un individu récolté sur la côte algérienne de la Méditerranée ; les flèches indiquent les taches trouvées chez tous les individus étudiés. Les 5 taches présentes uniquement chez la population de Méditerranée sont numérotées (1 à 5).

must occur in the field. More recently, Sato & Masuda (1997) have studied divergence among populations of small- and large-egg forms of the brackish water polychaete *H. japonica* complex by electrophoretic analysis. They found that the two forms show great genetic differentiation and no genetic evidence of hybridization in sympatric populations. These results indicate that the two forms are reproductively isolated and are two different species. In

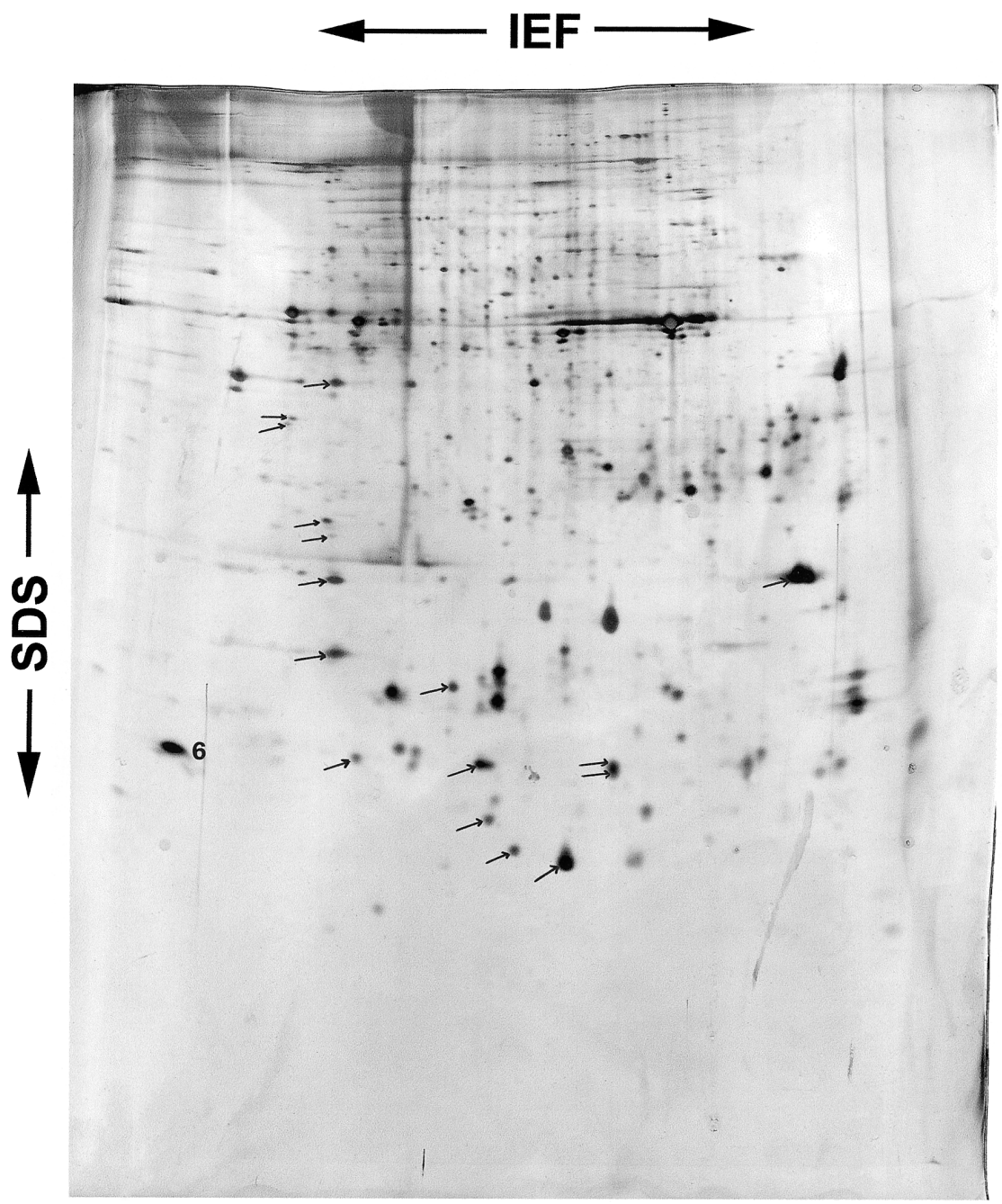

Figure 3. *Perinereis cultrifera.* Example of an electrophoretic analysis of the water-soluble protein fraction in an extract of metameres of an individual from the north coast of Brittany in which arrows indicate spots found in all the individuals examined. On the pattern 1 spot (6), present only in the English Channel populations, is indicated.

Figure 3. *Perinereis cultrifera.* Exemple d'électrophorèse de la fraction protéique hydrosoluble d'un extrait de métamères d'un individu récolté sur la côte nord de Bretagne ; les flèches indiquent les taches trouvées chez tous les individus étudiés. La tache présente seulement chez les populations de la Manche est numérotée (6).

addition, genetic variability among species in the polychaete family Nereididae has been shown by allozyme electrophoresis (Abbiati & Maltagliati, 1992, 1996; Hateley et al., 1992; Röhner et al., 1997; Scaps et al., 2001).

We assessed inter- and intrapopulation genetic variation in epitokous forms of *P. cultrifera* from the English Channel and the Mediterranean Sea in the context of the study of complexes of cryptic species in the polychaete family

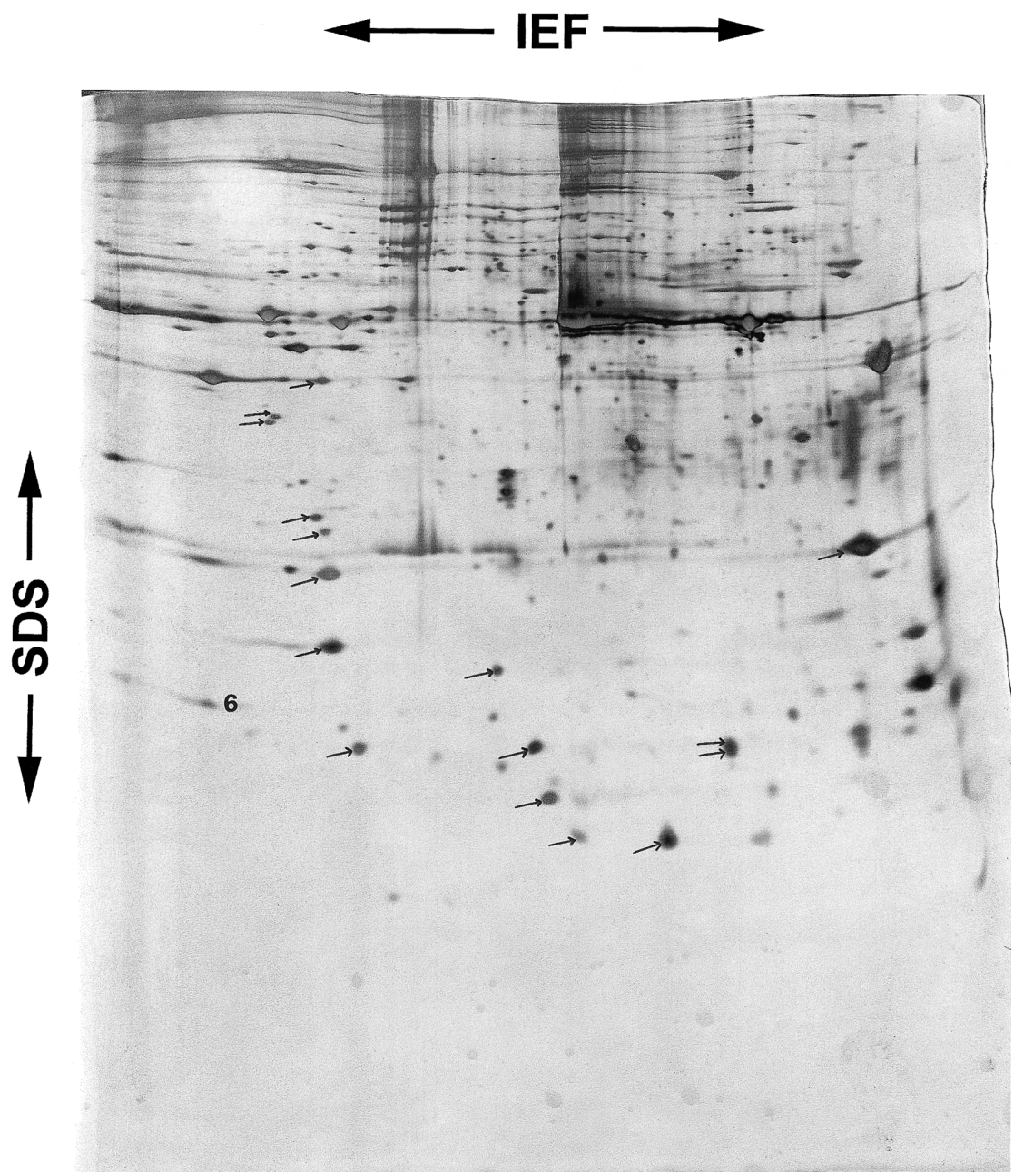

Figure 4. *Perinereis cultrifera.* Example of an electrophoretic analysis of the water-soluble protein fraction in an extract of metameres of an individual from Normandy in which arrows indicate spots found in all the individuals examined. On the pattern 1 spot (6), present only in the English Channel populations, is indicated.

Figure 4. *Perinereis cultrifera.* Exemple d'électrophorèse de la fraction protéique hydrosoluble d'un extrait de métamères d'un individu récolté en Normandie ; les flèches indiquent les taches trouvées chez tous les individus étudiés. La tache présente seulement chez les populations de la Manche est numérotée (6).

Nereididae. Allozyme and general protein patterns obtained after one-dimensional electrophoretic procedure have been used as taxonomic markers for separating polychaete cryptic species. Nevertheless, we are the first to apply high resolution 2-DGE to polychaetes in order to compare proteomics between two closely-related allopatric forms. Our results showed that individuals from the three populations cluster separetely and constitute three

Table 1. Similarity coefficients *F* of 10 individuals from the English Channel and the Mediterranean Sea based on protein spots. B: North coast of Brittany ; M: Mediterranean Sea ; N: Normandy.

Tableau 1. Indices de similarité *F* calculés pour les 10 individus provenant de Manche et de Méditerranée. B : côte nord de Bretagne ; M : Méditerranée ; N : Normandie.

	B1	B2	B3	N1	N2	N3	N4	M1	M2	M3
B1		0.932	0.922	0.829	0.814	0.849	0.789	0.748	0.767	0.734
B2			0.944	0.865	0.862	0.866	0.814	0.817	0.830	0.785
B3				0.848	0.839	0.855	0.815	0.800	0.806	0.773
N1					0.920	0.952	0.937	0.753	0.771	0.782
N2						0.934	0.908	0.771	0.776	0.794
N3							0.913	0.739	0.757	0.770
N4								0.716	0.721	0.758
M1									0.941	0.913
M2										0.920

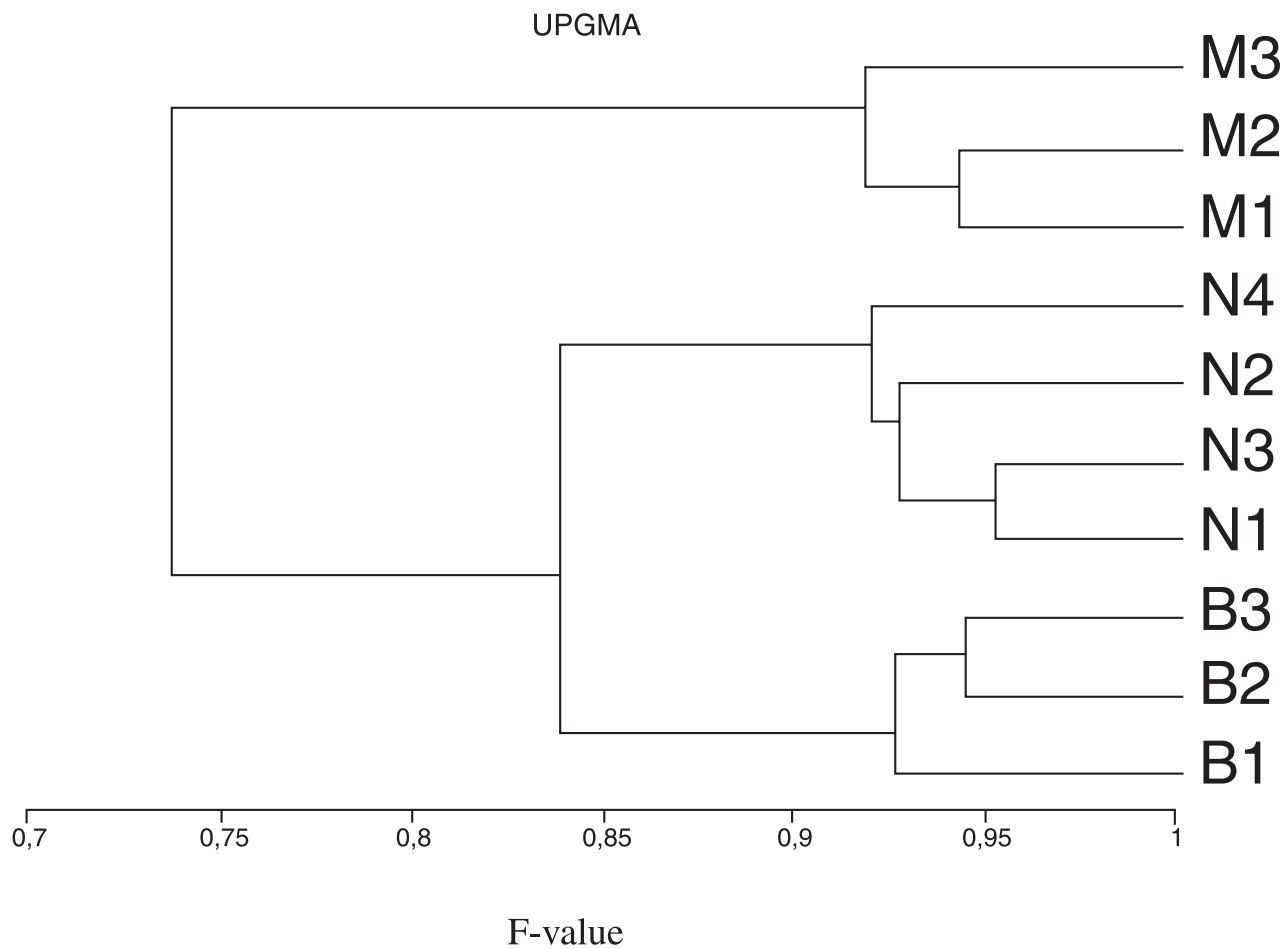

Figure 5. Similarity dendrogram, using similarity coefficient *F* and constructed with UPGMA of 10 individuals from the English Channel and the Mediterranean Sea based on protein spots. B: North coast of Brittany; M: Mediterranean Sea ; N: Normandy.

Figure 5. Dendrogramme de similarité utilisant l'indice de similarité *F* et construit par la méthode UPGMA de 10 individus provenant de Manche et de Méditerranée. B : côte nord de Bretagne ; M : Méditerranée ; N : Normandie.

'genetically'-distinct clades. In consequence our results indicate a restricted gene flow between our sampled populations. In fact, *P. cultrifera* is an intertidal poor disperser polychaete. It is reported to be a free-spawner. According to Cazaux (1970), *P. cultrifera* has a bentho-pelagic life cycle with a brief semi-pelagic phase. Eggs are large (egg diameter 350 µm), lecithotrophic and demersal. Hatching occurs at the 3-setiger erpochaete stage. Larvae exhibit a little developed ciliary crown and often crawl on the bottom. At the end of the semi-pelagic phase, animals become sedentary at the 4-setiger erpochaete stage. Then, erpochaeta loose their ciliary crown and thus are completely benthic. The juvenile, benthic worm of 10 or 11 segments has the same life style as the adult. This kind of life cycle does not favour a great dissemination of larvae. In consequence, the behaviour of *P. cultrifera* larvae promotes the geographic isolation of individuals.

In a previous attempt to distinguish the epitokous forms of *P. cultrifera* from the Mediterranean Sea and the English Channel, Scaps et al. (2000) reported morphological divergence. The total number of paragnaths and paragnath numbers in groups I, II right and left, III, VII-VII are significantly higher in individuals from the Mediterranean. Paragnaths in groups I-II are round and paragnaths in groups III-IV are conical for the Mediterranean form whereas paragnaths are larger and conical for the English Channel form. There are five teeth per half jaw for the Channel form and only three for the Mediterranean form. These authors also found biochemical differences between the two epitokous forms of *P. cultrifera*. The two types are fixed for different alleles at 5 loci, which were consistently resolvable, and each of the two types has a peculiar and unique protein-banding pattern, which is distinguishable by the identification of specific bands. Thus, general protein band patterns reveal 13 markers that discriminate the two types. Our study demonstrates that the general protein pattern of individuals from the Mediterranean contains 5 exclusive spots whereas no specific spots were detected between the two populations of the English Channel which share one exclusive spot. Given the larval development of this species which limits gene flow between populations, the congruence of the reported results from ecological, morphological, biological and biochemical investigations agrees with the assertion that *P. cultrifera* is a complex of cryptic species.

In order to resolve the problem of speciation in the genus *Perinereis*, the study must be extended to the atokous Mediterranean form of *P. cultrifera* and to *P. rullieri* (Pilato, 1974), a closely related atokous species which occurs in sympatry with *P. cultrifera* in the Venice Lagoon on the Italian Adriatic coast (Predevelli & Zunarelli Vandini, 1991) and which perhaps represents the atokous form of the Bay of Alger.

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