Marine Biology (1997) 129: 477–487

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G. V. Triantaphyllidis · G. R. J. Criel
T. J. Abatzopoulos · K. M. Thomas · J. Peleman
J. A. Beardmore · P. Sorgeloos

International Study on *Artemia*. LVII. Morphological and molecular characters suggest conspecificity of all bisexual European and North African *Artemia* populations

Received: 16 May 1997 / Accepted: 30 June 1997

Abstract A scanning electron microscopy (SEM) study of bisexual *Artemia* populations revealed that populations representing the species *A. franciscana*, *A. persimilis*, *A. urmiana*, *A. sinica* and a recently described species from Kazakhstan have a pair of spine-like outgrowths at the basal parts of their penes, whereas populations from southern Europe and North Africa (i.e. Mediterranean populations) lack these spine-like outgrowths. Allozyme and DNA polymorphisms, detected by allozyme starch gel electrophoresis and AFLP fingerprinting, respectively, suggested conspecificity of the studied populations from the broader Mediterranean basin. Male specimens from the collection of the Natural History Museum of London (UK) of the extinct *A. salina* population from Lymington lack spine-like outgrowths at the basal parts of the penes. This finding, based on a taxonomic character which is quite reliable, suggests conspecificity of *A. salina* from Lymington and the present bisexual *Artemia* populations from the Mediterranean basin, grouped under the binomen *A. tunisiana*.

Communicated by O. Kinne, Oldendorf/Luhe

G.V. Triantaphyllidis · P. Sorgeloos
Laboratory of Aquaculture and *Artemia* Reference Center, University of Ghent, Rozier 44, B-9000 Ghent, Belgium

G.R.J. Criel
Department of Anatomy, Embryology and Histology, Section of Human Anatomy and Embryology, University of Ghent, Godshuizenlaan 4, B-9000 Ghent, Belgium

T.J. Abatzopoulos (✉)
Faculty of Sciences, School of Biology, Department of Genetics, Development and Molecular Biology, Aristotle University of Thessaloniki, GR-54006 Thessaloniki, Greece

K.M. Thomas · J.A. Beardmore
School of Biological Sciences, University of Wales Swansea, Singleton Park, Swansea SA2 8PP, Wales, United Kingdom

J. Peleman
Keygene N. V., Agro Business Park 90, P.O. Box 216, 6700 AE Wageningen, The Netherlands

Introduction

The brine shrimp *Artemia* (Crustacea: Branchiopoda) is a well-studied organism, however, taxonomists are still puzzled about the evolution and the phylogenetic relationship of the populations that comprise the genus (for a review of the confusing names in the genus *Artemia* see Belk and Brtek 1995). Schlösser made the first description of the brine shrimp in 1755 on material collected from solar saltworks near Lymington, England, UK (Kuenen and Baas-Becking 1938). Linnaeus described the brine shrimp as *Cancer salinus* in 1758 and Leach renamed it as *A. salina* in 1819 (Artom 1931). In the following years many populations have been identified, and nowadays the genus *Artemia* is comprised of a complex of bisexual species and superspecies as well as parthenogenetic populations with various degrees of ploidy (Browne and Bowen 1991).

Artom (1905, 1906, 1907), Stella (1933), Stefani (1963), Stefani and Cadeddu (1967), Halfer Cervini et al. (1968), Piccinelli et al. (1968) and Barigozzi (1974), studying the *Artemia* populations in Italy, used the binomen *A. salina* for the bisexual populations that had 42 chromosomes. Ever since 1910 (Barigozzi 1974) and even recently (Sorgeloos and Beardmore 1995), most scientific papers referred to all brine shrimps as *A. salina* although in the meantime it was known that two distinct modes of reproduction occurred (parthenogenetic or zygogenetic) and that new bisexual species had been described: *A. franciscana* (Kellogg 1906) in the New World and *A. urmiana* (Günther 1890) in Lake Urmia, Iran. Piccinelli and Prosdocimi (1968) described a new species, *A. persimilis*, from the saltworks of San Bartolomeo, Cagliari (Sardinia) and Hidalgo (Argentina). This species has 44 chromosomes and a different adult morphology. It was considered to be sympatric with *A. salina*. Clark and Bowen (1976) showed that the

Tunisian population from Chott Ariana, Raoued, was cross fertile with the Italian population from San Bartolomeo, Cagliari, Sardinia, but both of them were not cross fertile with representative populations of *A. franciscana*, *A. persimilis* and *A. urmiana*. Clark and Bowen (1976) proposed dropping the term *A. salina*, rather than change its meaning, considering that confusion would result if it was restricted to the San Bartolomeo and Chott Ariana populations. Bowen and Sterling (1978) suggested that the binomen *A. salina* should be restricted to the extinct population at Lymington and the binomen *A. tunisiana* should be used when referring to the bisexual populations of the Mediterranean basin. Several researchers and/or participants in the two international symposia on *Artemia*, held in 1979 and 1985, have adopted this approach (Bowen et al. 1978, 1980, 1985; Abreu-Grobois and Beardmore 1980; Barigozzi 1980; Abreu-Grobois 1987; Barigozzi et al. 1987; Triantaphyllidis et al. 1994). Badaracco et al. (1987) deviated from the suggestion of Bowen and Sterling (1978) assigning to a population from Sfax, Tunisia, the name *A. tunisiana* and to a population from Trapani, Italy, the binomen *Artemia* sp., which might correspond to *A. salina*. A few years later Badaracco et al. (1991) referred to the same populations, from Sfax and Trapani, as *A. salina*. Based on crossbreeding experiments of Baratelli et al. (1990), Barigozzi (1989) proposed (1) to retain the name *A. salina* for the Italian populations, and (2) to group all North African bisexual populations under the name *A. tunisiana*. Mura (1990) suggested conspecificity of the Italian populations, the North African populations and the original population of *A. salina* from Lymington based on the morphology of the male frontal knobs. In the most recent review of taxonomy and population genetics of *Artemia*, Browne and Bowen (1991) restated the argument for abandoning the name *salina* for all but the extinct population at Lymington, England, and replacing it with *tunisiana* for all the Mediterranean biparental populations.

Apparently, there is taxonomic confusion and some scientists are referring to the same population as *Artemia tunisiana* and others as *A. salina*. Belk and Brtek (1995) clarified that the approach of Bowen and Sterling (1978) is not an option under the International Code of Zoological Nomenclature: i.e. if *tunisiana* and *salina* are names for the same taxon, *salina* has priority and *tunisiana* becomes a synonym (Belk and Brtek 1995).

Studies on morphology, cytology as well as electrophoretic data have been used to distinguish the various species in *Artemia* (Piccinelli and Prosdocimi 1968; Barigozzi 1974; Bowen and Sterling 1978; Amat 1980; Abreu-Grobois and Beardmore 1980; Abreu-Grobois 1983, 1987; Abatzopoulos et al. 1986, 1989, 1993; Hontoria and Amat 1992a, b; Triantaphyllidis et al. 1993, 1994, 1995; Pilla and Beardmore 1994). One of the morphological characters that has been employed to separate the various species is the presence or absence of "spines" from the penes (Piccinelli and Prosdocimi 1968; Barigozzi 1974, 1989; Beardmore and Abreu-Grobois 1983; Abreu-Grobois 1987). There is a controversy in these reports; Barigozzi (1974, 1989) supported that the New World species *A. franciscana* was practically identical to the Old World *A. salina*, having no spines at their penes, while Beardmore and Abreu-Grobois (1983) and Abreu-Grobois (1987) reported that *A. franciscana* had spines and *A. salina* did not. None of these reports gave a detailed description of these spines with the exception of Piccinelli and Prosdocimi (1968) who described in general the existence of small spines on the base of the penis in the *A. persimilis* individuals.

It is surprising that these spines have never been studied in detail and/or used as a tool for the characterization of the Mediterranean populations. Considerable effort has been invested in the study of frontal knob morphology (Mura et al. 1989a,b; Mura 1990) as a tool to discriminate Mediterranean populations from other species but with confusing and contradictory results in some cases (Thiery and Robert 1992). In the present paper, we attempt (1) to elucidate whether the bisexual Mediterranean populations belong to the same species or not, and (2) to shed light on the long debate and controversy concerning the use of either *A. salina* or *A. tunisiana* for the Old World bisexual *Artemia* populations. To approach this issue, we used scanning electron microscopy, starch gel electrophoresis of allozymes and a novel molecular fingerprinting technique (AFLP) which is based on amplified DNA fragments (Vos et al. 1995).

Materials and methods

The origin of the *Artemia* populations used in this study as well as the abbreviations used are given in Table 1. For the AFLP fingerprinting, we analysed cysts from the cyst bank of the *Artemia* Reference Center that were decapsulated according to Sorgeloos et al. (1986). The hatchable cysts were raised until adulthood to study their morphology and their allozyme polymorphisms. Two male specimens of *A. salina* from Lymington (Hampshire, England, UK), the habitat where the brine shrimp was described for the very first time, were kindly provided from the collection of the Natural History Museum of London (UK). The Lymington *A. salina* material was collected by J. Abethethy, registered in 1989 and had the number 1989.894–903. Samples of the population from San Pedro del Pinatar (Spain) were collected in their natural environment in May 1995, fixed in 70% ethanol and sent to Ghent for scanning electron microscopy analysis.

Culture conditions

The hatched nauplii were transferred to 1-liter cylindroconical glass tubes containing 0.25 μm-filtered Dietrich and Kalle medium (D & K) (Kalle 1971) which was prepared following the modifications of Vanhaecke et al. (1984). The initial number of nauplii per tube was 200, the salinity was 50‰ and the temperature was 25 ± 1 °C. Three replicates for each population were used. Initial animal density was one animal per 2 ml but from Day 8 onwards it was reduced to one animal per 4 ml. The alga *Dunaliella tertiolecta* Butcher and a yeast-based formulated feed were used as food following a feeding schedule described by Triantaphyllidis et al. (1995).

To study the effect of salinity and aging on the morphology of the spine-like outgrowths we cultured the URM population at salinities of 100, 140 and 180‰ of D & K medium following the

Table 1 List of the studied populations, their *Artemia* Reference Center (*ARC*) cyst bank code number, their species designation (if known) and abbreviations used. All the populations are bisexual with exception of the AIB and NAM populations which are parthenogenetic

Population	ARC cyst bank code number and species designation	Abbreviation
San Francisco Bay, California, USA	1209 (*A. franciscana*)	SFB
Argentina, unknown locality	– (*A. persimilis*)	PER
Yuncheng, Shanxi, P.R.China	1218 (*A. sinica*)	YUN
Yimeng, Inner Mongolia, P.R.China	1188 (*A. sinica*)	YIM
Inner Mongolia, unknown locality, P.R.China	1154 (*A. sinica*)	INM
Haolebaoji, Inner Mongolia, P.R.China	1215 (*A. sinica*)	HAL
Kazakhstan, unknown locality	1039 (*A.* sp.)	KAZ
Urmia Lake, Iran	1229 (*A. urmiana*)	URM
Aibi Lake, Xinjiang, P. R. China	1236 (*A. parthenogenetica*)	AIB
Swakopmund, Namibia	1186 (*A. parthenogenetica*)	NAM
Larnaca, Cyprus	1011	LARa
Larnaca, Cyprus	1149	LARb
Larnaca, Cyprus	1148	LARc
Wadi El Natrun, Egypt	1290	EGY
Abu-Khammash salt marches, Libya	–	LIB
Mégrine, Tunisia	1268	MEG
Sfax, Tunisia	1269	SFA
Chemmaiaa, Morocco	–	MAR
Carloforte, Sardinia, Italy	552	CAR
Sant' Antioco, Sardinia, Italy	560	SAS
Quartu, Sardinia, Italy	–	QUA
Salinas San Fernando, Cadiz, Spain	–	SAF
San Pedro del Pinatar, Murcia, Spain	–	SAP
Lymington, England, United Kingdom	(*A. salina*)	LYM

procedures described by Triantaphyllidis et al. (1995). Males from the three salinities were studied after 37 and 52 d by means of scanning electron microscopy.

Scanning electron microscopy study

For the scanning electron microscopy study, four to eight adult males were fixed overnight in a glutaraldehyde–paraformaldehyde mixture (Karnovsky 1965) diluted 3:1 with cacodylate buffer 0.2 *M*, pH 7.4, rinsed in cacodylate buffer and postfixed in 2% osmium tetroxide in cacodylate buffer. Dehydration in acetone was followed by critical point drying. After mounting the specimens they were gold coated and examined with an ISI-SR-50 scanning electron microscope (SEM).

Allozyme analysis

Adult individuals from the Mediterranean populations MEG, SFA, LARa, EGY and LIB as well as the SFB *Artemia franciscana* and the URM *A. urmiana* were isolated from the cultures, and prepared for allozyme analysis following the procedures described by Abreu-Grobois and Beardmore (1980). Standard horizontal starch gel (12.5%) electrophoresis was applied to whole adult *Artemia* homogenates. A total of 14 enzymes encoded by 20 loci were analysed: amylase (AMY-1; Enzyme Commission Number E.C. 3.2.1.1), aspartate aminotransferase (AAT-1, AAT-2; E.C. 2.6.1.1), esterases (EST-1, EST-4; E.C. 3.1.1.-), esterase-D (EST-D; E.C. 3.1.1.-), isocitrate dehydrogenase (IDH-1, IDH-2; E.C. 1.1.1.42), *l*-lactate dehydrogenase (LDH-1; E.C. 1.1.1.27), leucine aminopeptidase (LAP-2, LAP-3; E.C. 3.4.11.1), malate dehydrogenase (MDH-1, MDH-2; E.C. 1.1.1.37), NADP-malate dehydrogenase (MEZ-1; E.C. 1.1.1.40), peptidase (PEP-1, PEP-4; E.C. 3.4.11.-), glucosephosphate isomerase (GPI-1; E.C. 5.3.1.9), phosphoglucomutase (PGM-1; E.C. 5.4.2.2), phosphogluconate dehydrogenase (6PGD-1; E.C. 1.1.1.44) and superoxide dismutase (SOD-1; E.C. 1.15.1.1). Staining of gels followed the methods of Abreu-Grobois (1983) and Pilla (1992). Standard genetic distances and identities were computed according to Nei (1972), using the computer program BIOSYS-1 Release 1.7 (Swofford and Selander 1981).

AFLP analysis

AFLP is a new technique developed by Keygene N. V. (Wageningen, The Netherlands) and is based on the amplification of subsets of genomic restriction fragments, using polymerase chain reaction (PCR) (Vos et al. 1995). AFLP involves three steps: (1) restriction of the DNA and ligation of oligonucleotide adapters, (2) selective amplification by PCR of sets of restriction fragments, and (3) gel analysis of the amplified fragments. This method combines the accuracy and reliability of the restriction fragment length polymorphisms (RFLP) technique, with the power of the PCR technique (for details see Vos et al. 1995).

For AFLP analysis, cysts were hydrated in a 10‰ NaCl solution for 2 h and decapsulated following Sorgeloos et al. (1986). Total genomic DNA was isolated from approximately 1000 cysts. Cysts were ground with a plastic pestle in microcentrifuge tubes at 0 °C. The ground material was transferred to 2.2 ml Eppendorf tubes that contained 1 ml 1% w/v cetyl trimethyl ammonium bromide (CTAB) extraction buffer (100 m*M* Tris pH 7.5, 700 m*M* NaCl, 10 m*M* EDTA and 27.4 m*M* CTAB). Samples were incubated at 60 °C for 1 h and afterwards cooled down with ice to room temperature. The DNA was extracted by adding 450 µl chloroform/isoamyl alcohol (24:1) and mixing for 5 min. The samples were centrifuged for 15 min at 900 ×*g* at room temperature. Next, 500 µl of the aqueous phase were transferred into new 2.2 ml Eppendorf tubes and 500 µl of isopropanol added. After mixing well the DNA was precipitated by centrifugation for 10 min at 900 ×*g*. The pellets were washed with 76% ethanol that contained 10 m*M* NH_4OAc. The DNA was dissolved in 100 µl of TE buffer (10 m*M* Tris pH 8.0, 1 m*M* EDTA) at 4 °C overnight. The DNA concentration was determined by analyzing 5 µl of sample on a 1% agarose gel.

A total of 0.5 µg of DNA from each sample was digested with 5 units EcoRI and 5 units MseI restriction enzymes, following the incubation procedures described in detail by Vos et al. (1995). DNA digestion resulted in three classes of restriction fragments: (1) MseI–MseI fragments, which were the majority (~90%), (2) EcoRI–EcoRI fragments, which were the minority and (3) EcoRI–MseI fragments, which were about twice the number of EcoRI restriction sites. Double stranded AFLP adapters were li-

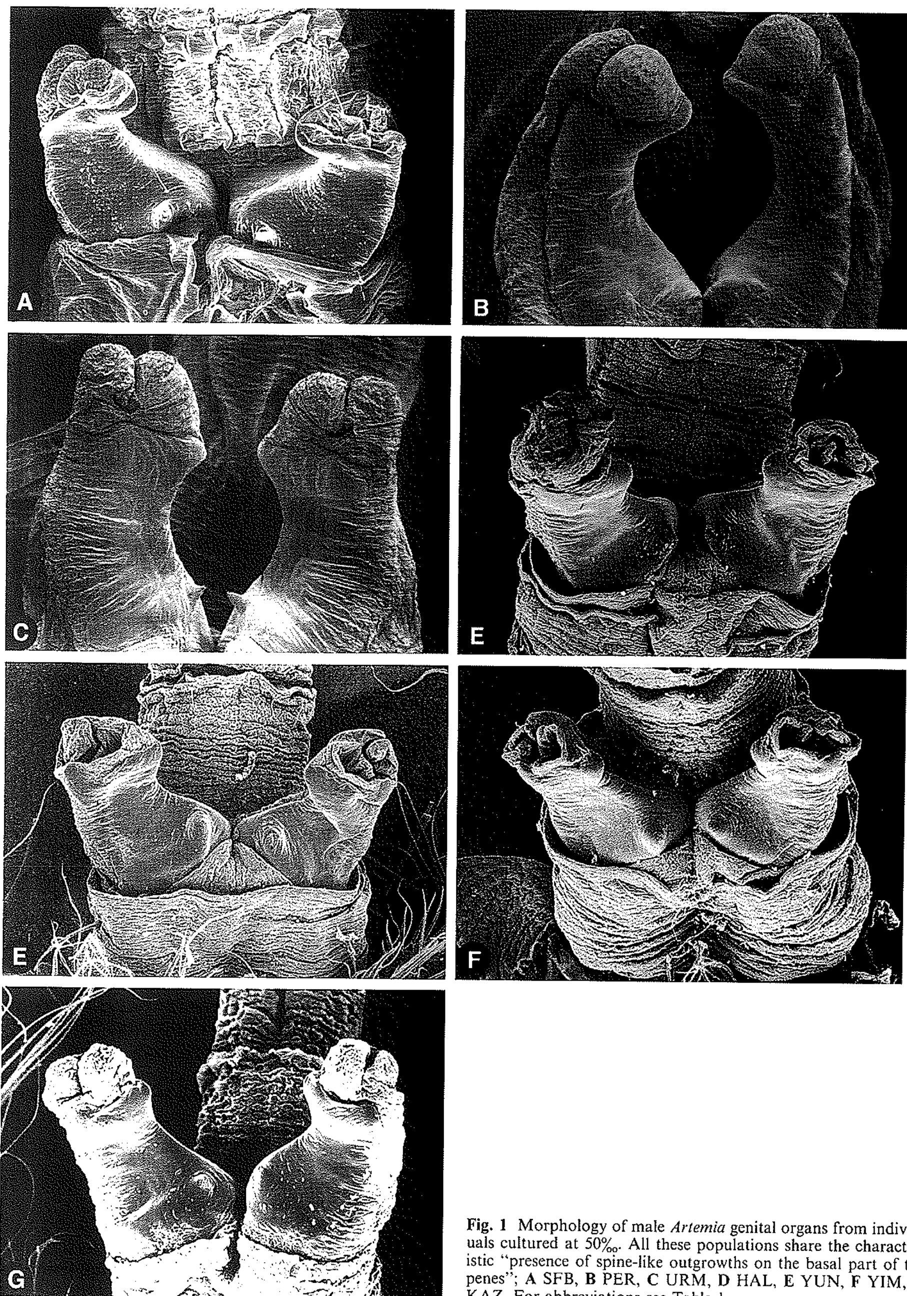


Fig. 1 Morphology of male *Artemia* genital organs from individuals cultured at 50‰. All these populations share the characteristic "presence of spine-like outgrowths on the basal part of the penes"; **A** SFB, **B** PER, **C** URM, **D** HAL, **E** YUN, **F** YIM, **G** KAZ. For abbreviations see Table 1

gated to the ends of the DNA-fragments to generate template DNA for amplification.

The structure of the EcoRI adapter was:
5′-CTCGTAGACTGCGTACC
CATCTGACGCATGGTTAA-5′.

The structure of the Msel adapter was:

5-GACGATGAGTCCTGAG
TACTCAGGACTCAT-5.

The sequence of the adapters and the adjacent restriction site served as primer binding sites for subsequent PCR amplification of the restriction fragments. AFLP primers for PCR amplification consisted of three parts, a core sequence (CORE), an enzyme-specific sequence (ENZ) and a selective extension (EXT). Only restriction fragments in which the nucleotides flanking the restriction site matched the selective nucleotides could be amplified: the ones with a rare cutter sequence on one end and a frequent cutter sequence on the other end. The following primers were used (the selective nucleotides are underlined):
one EcoRI primer (CORE ENZ EXT)

5′-GACTGCGTACC AATTC AAC-3′ (E32)

and four Msel primers (CORE ENZ EXT) (patented by Keygene, N. V.)

5′-GATGAGTCCTGAG TAA CAA-3′ (M47)
5′-GATGAGTCCTGAG TAA CAG-3′ (M49)
5′-GATGAGTCCTGAG TAA CTC-3′ (M60)
5′-GATGAGTCCTGAG TAA CTT-3′ (M62) .

The PCR conditions used were the same as in Vos et al. (1995). After amplification of the reaction products the samples were analyzed on a 5% denaturing (sequencing) polyacrylamide gel following the protocol described by Vos et al. (1995). The reference ladder for the fingerprints was purchased from Research Genetics, Inc. (Cat. No. 701001 and 701002) while Vent (exo) DNA polymerase was obtained from Biolabs (Cat. No. 257). Genetic distances were computed using the computer program NTSYS-pc, Version 1.80 (Rohlf 1993).

Results

The results of the scanning electron microscopy are shown in Figs. 1, 2 and 3. Figure 1 shows the morphology of male genital organs from seven populations. All these populations share a common characteristic: at the base of their penis they have a spine-like outgrowth. Although it seems that these outgrowths vary from one population to another in protruding more or less emphatically, it is difficult to assign a particular shape, size and/or type to each population or populations that belong to the same species. Higher magnification of these outgrowths (Fig. 2) shows a rosette of scale-like projections at their apical end. Culture of the URM population under three different salinities (100, 140 and 180‰) did not result in changes in the appearance of the spine-like outgrowths. In addition, aging does not seem to affect their appearance.

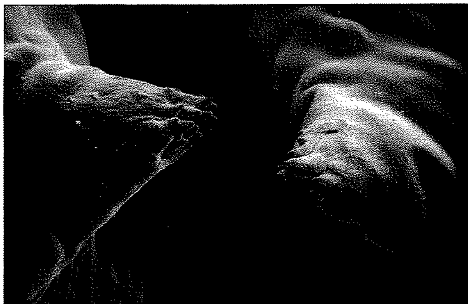


Fig. 2 *Artemia*. Detail of the spine-like outgrowths on the basal part of the penes

Figure 3 depicts the morphology of the male genital organs in six populations from the Mediterranean basin, as well as of a specimen from the extinct LYM population. All these populations lack the spine-like outgrowths which are characteristic for populations far from Europe and the Mediterranean basin.

Table 2 summarizes the results from the allozyme electrophoresis performed with various Mediterranean populations. The mean genetic distance (Nei's *D*) is found to be 0.033 (±0.025) suggesting conspecificity of the studied populations. Figure 4 depicts the dendrogram of the Mediterranean populations in comparison with the SFB and URM populations.

Figure 5 illustrates the AFLP fingerprint of various *Artemia* populations. The bisexual Mediterranean populations (Lanes 5, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 and 18) exhibited more similarities in their fingerprints than SFB, YIM, PER, URM, AIB and NAM populations (Fig. 5, Lanes 1, 2, 3, 4, 6 and 7, respectively). DNA polymorphism as revealed by AFLP was far greater than that of protein polymorphism. Table 3 shows the genetic distances of the studied populations based on calculations of Nei's coefficient. The mean genetic distance of the Mediterranean populations evaluated was 0.172(±0.094).

The dendrogram in Fig. 6 summarizes the results of the AFLP analysis. The populations from the Mediterranean are separated from all the other populations, suggesting that they comprise an entity that exhibits striking genetic differences from other populations of the New and eastern Old World. The Mediterranean populations are grouped in two subclusters: (1) the eastern Mediterranean basin group which contains the LARa, LARb, LARc and EGY populations from Cyprus and Egypt and (2) the western Mediterranean basin group formed by populations from Italy, Spain, Marocco, Tunisia and Libya.

The two samples from Wadi Natrun (Egypt) are from the same population but one consisted of Instar I nauplii and the second of decapsulated cysts.

Discussion and conclusions

Allozyme and DNA polymorphisms (AFLP) revealed that the bisexual Mediterranean populations present great genetic similarities and can be grouped into the

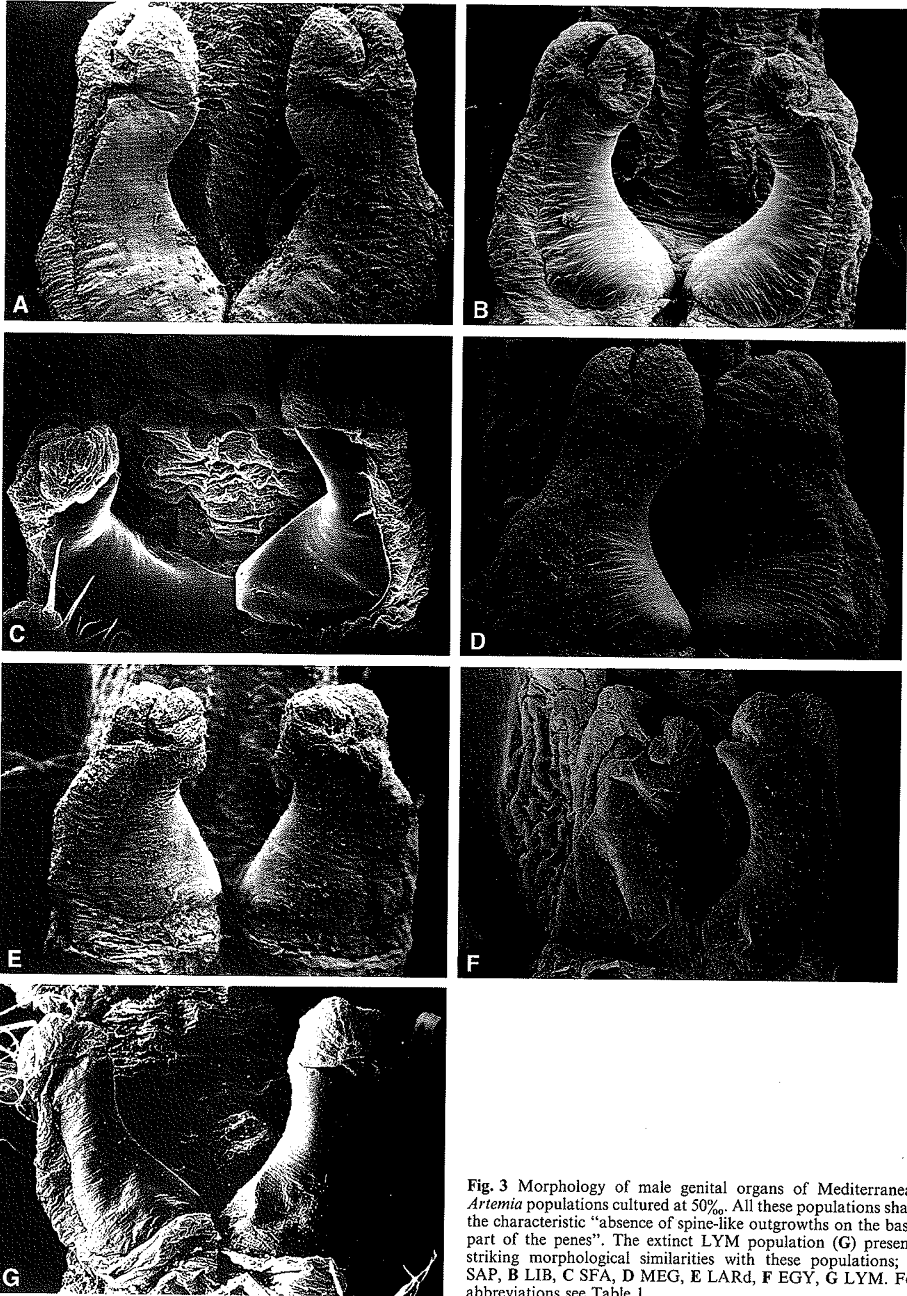


Fig. 3 Morphology of male genital organs of Mediterranean *Artemia* populations cultured at 50‰. All these populations share the characteristic "absence of spine-like outgrowths on the basal part of the penes". The extinct LYM population (G) presents striking morphological similarities with these populations; **A** SAP, **B** LIB, **C** SFA, **D** MEG, **E** LARd, **F** EGY, **G** LYM. For abbreviations see Table 1

Table 2 Matrix of pairwise Nei's (1972) genetic identity (*below diagonal*) and distance (*above diagonal*) between various Mediterranean *Artemia* populations. Population codes are the same as in Table 1. Data for QUA are from Pilla (1992)

Population	LARa	EGY	MEG	SFA	LIB	QUA
LARa		0.006	0.025	0.063	0.010	0.009
EGY	0.994		0.037	0.063	0.018	0.017
MEG	0.975	0.963		0.078	0.020	0.016
SFA	0.939	0.939	0.925		0.059	0.064
LIB	0.990	0.982	0.980	0.943		0.008
QUA	0.991	0.983	0.984	0.938	0.992	

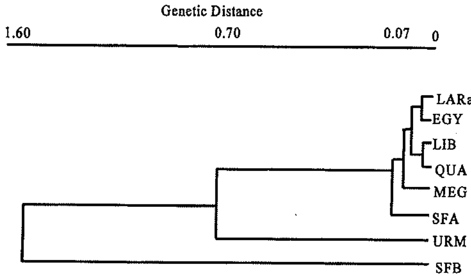


Fig. 4 UPGMA (Unweighted pair-group method with arithmetic averaging) dendrogram of Nei's genetic distance based on allozyme polymorphisms between Mediterranean *Artemia* populations, SFB *A. franciscana* and URM *A. urmiana*

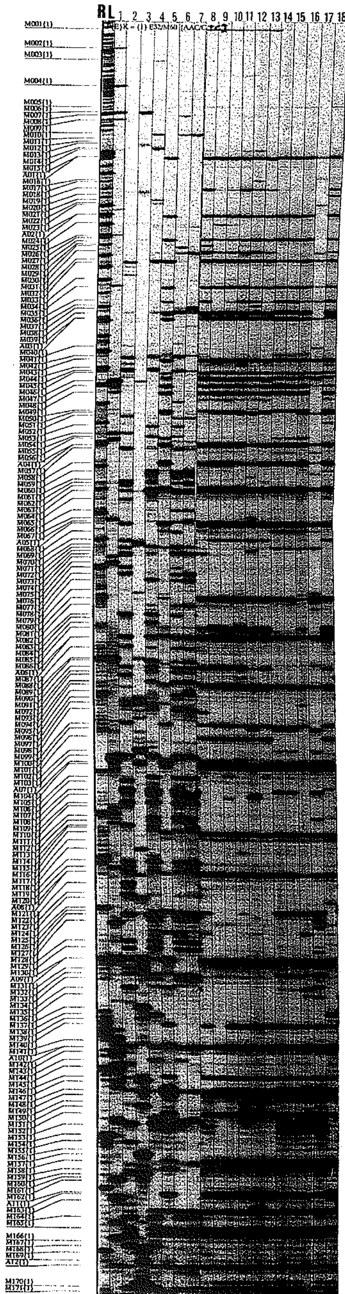
same species. The dendrograms based on allozymes, and especially AFLP, show that these populations are well separated from representatives of the bisexual species *Artemia franciscana*, *A. persimilis*, *A. urmiana* and *A. sinica* as well as from parthenogenetic populations. Moreover, AFLP shows that there is considerable genetic differentiation between the eastern and western Mediterranean populations, a differentiation that cannot be detected through allozyme polymorphisms. Our allozyme data are in agreement with Beardmore and Abreu-Grobois (1983) and Abreu-Grobois (1983, 1987) who studied bisexual Mediterranean populations from Spain, Italy, Tunisia and Cyprus. They found great genetic similarities between these populations (their mean genetic distance, Nei's D, was 0.091) and grouped them together as a species well separated from *A. franciscana*, *A. persimilis* and *A. urmiana*.

To our knowledge, this is the first time that the AFLP technique has been used to detect DNA polymorphism in *Artemia*. This technique has many advantages compared to the detection of allozyme polymorphisms: apart from being far more sensitive, since it detects polymorphisms on the DNA level, it presents a better description of the population since the sample is not limited to a few specimens but takes hundreds or thousands of individual cysts into account. The use of cysts eliminates the costs and difficulties of maintaining *Artemia* cultures in the laboratory. Also, it allows the use of old cyst collections that cannot be used for allozyme studies because they no longer hatch. This was the case with LARb, LARc, CAR, SAS, SAF and MAR populations. The use of decapsulated cysts or Instar I nauplii gave practically the same results, as demonstrated with the EGY population (see Table 3 and Fig. 6). We recommend use of cysts in cases where hatching percentage of the cysts is low, and consequently the hatched nauplii might not represent the entire population.

The first description of the spine-like outgrowths dates back to 1941 when Linder, defining the family Artemiidae and thus the genus *Artemia,* wrote: "from the rigid basal parts of the penes, which are thick at the base but distally tapering, arise a pair of medioventral, spine-like outgrowths". Wolfe (1971) studying the San Francisco Bay *A. franciscana* described that "the end of the penis is bifurcated and a spine is located on the medial surface of the penis, about midway between its tip and the junction of the two penes". Barigozzi (1974, 1989) used these spine-like outgrowths as one of many characters to discriminate the species *A. salina*, *A. franciscana* and *A. persimilis*. However, he inaccurately supported the thesis that *A. franciscana* was practically identical to the Old World *A. salina*, presenting no spines on their penes. Our results show that *A. franciscana* has spines on their penes, whereas the Mediterranean populations do not. Our results are in agreement with Beardmore and Abreu-Grobois (1983), Abreu-Grobois (1987), Linder (1941) and Wolfe (1971).

Our results show that the bisexual populations from the Mediterranean basin have a character (absence of spine-like outgrowths) that discriminates them very well from the other bisexual populations we studied. The morphology of the basal parts of the penes of the specimens from Lymington show great similarities with the Mediterranean populations studied here. This unique feature, and the fact that representatives of all the other known species have these spine-like outgrowths, suggests that the extinct Lymington population and the present bisexual Mediterranean populations are conspecific.

Unfortunately, the only comparison between the present Mediterranean populations and the extinct population from Lymington is the comparative morphological study of preserved specimens kept in the British Natural History Museum in London. The use of molecular DNA techniques incurs the risk and difficulty of how well the DNA from these fixed animals has been preserved. DNA is degraded by ethanol and other aldehydes (Ristow et al. 1995), and we have no information on the fixatives that these specimens have been exposed to (R. Huys personal communication).



Mura (1990), studying the frontal knob morphology of the Lymington population and comparing it with Italian and North African populations, also suggested conspecificity of the studied populations. Crossbreeding experiments (Clark and Bowen 1976; Browne 1983, 1988; Baratelli et al. 1988, 1990; Browne and Bowen 1991), morphological studies (Mura 1990) and molecular biology results (Badaracco et al. 1987, 1991) suggest that all the bisexual populations of the Mediterranean area belong to the same taxonomical entity, with the exception of a population from San Bartolomeo (Sardinia, Italy) which according to Piccinelli and Prosdocimi (1968) was a mixture of *Artemia salina* and *A. persimilis*. However, Barigozzi (1989) suggested that this population no longer exists. Furthermore, AFLP analysis of the samples of CAR and SAS populations did not reveal contamination or coexistence of two species as was suggested by Baratelli et al. (1990).

In Europe, and especially in the Mediterranean, only two species have been recorded: *Artemia salina* (= *A. tunisiana*) and *A. persimilis*. Our results show that *A. persimilis* can be easily distinguished from *A. salina* (from the spine-like outgrowths, the allozymes, AFLP and their chromosome number) and from the LYM population (from the spine-like outgrowths).

One provocative question with regard to the Lymington *Artemia* remains unanswered: what if this population belonged to an entirely different species, now extinct, that also had the characteristic "absence of spine-like outgrowths from the penis". Such a hypothesis does not seem to be likely, since no bisexual species has ever been reported in Europe other than *A. salina* or *A. persimilis*. The hypothesis that the population in Lymington was a mixture of two species cannot be verified either.

We believe that characters such as the frontal knobs (Mura 1989a, b, 1990), or variations in the size and the shape of the spine-like outgrowths of the penis (Torrentera and Dodson 1995) are of limited taxonomic value and are not very reliable characters in elucidating problems of classification of new populations (Thiery and Robert 1992). This is due to the fact that descriptions of lateral or dorsal views of the knobs may give different information according to the orientation of the electron beam of the scanning microscope (Torrentera and Dodson 1995). Also, the spines and mechanoreceptors of the frontal knobs vary with age and salinity (Criel and Van Horenbeeck unpublished data). On the contrary, we believe that characters such as presence or absence of a structure are independent from the

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Fig. 5 AFLP fingerprints of *Artemia* populations (*RL* 10 bp reference ladder; *Lane 1* SFB; *Lane 2* YIM; *Lane 3* PER; *Lane 4* URM; *Lane 5* EGY (sample of nauplii): *Lane 6* AIB; *Lane 7* NAM; *Lane 8* LIB; *Lane 9* CAR; *Lane 10* SAS; *Lane 11* SFA; *Lane 12* MEG; *Lane 13* SAF; *Lane 14* LARa; *Lane 15* LARb; *Lane 16* EGY (sample of cysts); *Lane 17* LARc; *Lane 18* MAR)

Table 3 Nei's genetic distance after AFLP analysis

	SFB	YIM	PER	URM	EGYn	AIB	NAM	LIB	CAR	SAS	SFA	MEG	SAF	LARa	LARb	EGYc	LARc	MAR
SFB																		
YIM	1.900																	
PER	1.660	1.869																
URM	1.947	0.558	2.102															
EGYn	1.656	1.611	1.612	2.101														
AIB	2.026	0.536	2.115	0.159	2.232													
NAM	2.023	0.595	2.270	0.253	2.295	0.146												
LIB	1.654	1.691	1.764	2.027	0.208	2.042	2.129											
CAR	1.569	1.657	1.709	2.053	0.226	2.123	2.062	0.110										
SAS	1.591	1.675	1.735	2.038	0.199	2.099	1.994	0.082	0.030									
SFA	1.534	1.767	1.819	2.035	0.215	2.093	2.128	0.050	0.067	0.044								
MEG	1.649	1.777	1.779	1.948	0.235	1.962	2.034	0.110	0.100	0.087	0.068							
SAF	1.622	1.700	1.729	2.081	0.197	2.148	2.035	0.079	0.045	0.009	0.041	0.088						
LARa	1.830	1.734	1.687	2.041	0.105	2.280	2.216	0.227	0.261	0.250	0.239	0.249	0.252					
LARb	1.825	1.731	1.684	2.038	0.102	2.277	2.274	0.224	0.258	0.247	0.236	0.254	0.249	0.003				
LARc	1.655	1.684	1.772	2.157	0.012	2.600	2.501	0.216	0.206	0.182	0.216	0.232	0.188	0.091	0.087			
EGYn	1.892	1.621	1.579	1.921	0.190	2.017	2.074	0.335	0.351	0.338	0.319	0.319	0.333	0.115	0.110	0.183		
MAR	1.704	1.717	1.792	1.965	0.210	2.175	2.168	0.120	0.111	0.097	0.091	0.124	0.088	0.247	0.244	0.197	0.352	

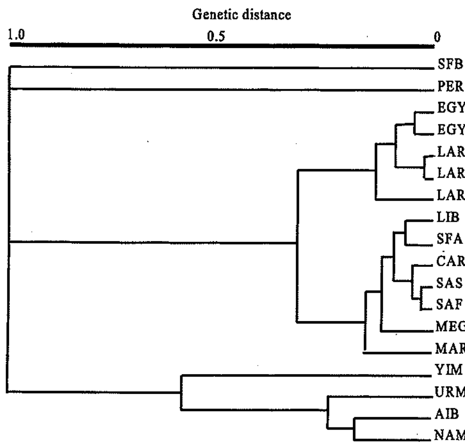

Fig. 6 *Artemia.* Dendrogram based on Nei's genetic distance after AFLP analysis

orientation of the electron beam and can be used as a reliable taxonomic character.

The fact that many scientists are using the binomen *Artemia salina* or *A. tunisiana* to refer to the same population causes misidentification and confusion. Our data, which show striking morphological similarities between the LYM population and those of the broader Mediterranean basin sufficiently prove that *tunisiana* and *salina* are synonyms for the same taxon. According to the International Commission of Zoological Nomenclature *salina* has priority and *tunisiana* becomes a synonym (The Code, Principle of Priority, Article 23; see also Belk and Brtek 1995).

In conclusion, allozyme polymorphisms, as well as amplified DNA fragment length polymorphisms, revealed that the bisexual Mediterranean populations belong to the same species. The males of the Mediterranean populations are lacking a spine-like outgrowth at the base of their penis contrary to the representatives of the species *Artemia franciscana*, *A. persimilis*, *A. sinica*, *A. urmiana* and *Artemia* sp. from Kazakhstan which have this character. The fact that the extinct population from Lymington presents morphological similarities with the present Mediterranean populations suggests that they belong to the same species. The binomen *A. tunisiana* is a synonym and following the Principle of Priority (the Code, Article 23) it should not be used any longer.

Acknowledgements We acknowledge the help of Dr. R. Huys and the Natural History Museum of London (UK) for kindly providing the Lymington specimens for this study. Professor D. Belk is acknowledged for his constructive comments on the manuscript. M. Vallejo is acknowledged for sending us the fixed specimens from San Pedro del Pinatar, Murcia, Spain. We thank S. Van Hulle for her technical assistance with the photographs. GVT is a scholar of the "Alexander S. Onassis" Public Benefit Foundation and the "Empirikion Foundation" (Greece). This paper is part of an international interdisciplinary study on *Artemia* populations coordinated by the Laboratory of Aquaculture and *Artemia* Reference Center, University of Ghent, Belgium.

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