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Original article

Genetic structure of bisexual and parthenogenetic populations of *Artemia* from Italian brackish–hypersaline waters

Structure génétique des populations bisexuelles et parthénogénétiques d'*Artemia* des eaux saumâtres à hypersalées en Italie

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Abstract

Eight populations of *Artemia* displaying different reproduction modalities (amphigonic vs. parthenogenetic) and ploidies (diploid vs. polyploid) have been collected from Italian salt-works or brackish coastal lagoons, and their genetic structure has been investigated using allozyme electrophoresis. The results obtained on genetic differentiation showed that the Tyrrhenian bisexual samples (*A. salina*) are genetically homogeneous even between populations located in relatively faraway sites (Sardinia–Latium), having a moderate gene flow assisted by migratory waterfowl. The parthenogenetic samples (*A. parthenogenetica*) are genetically well differentiated both from the bisexual ones and among them, even when characterised by the same ploidy. Each sample presents few genotypes (i.e. few or single clones have been found in each sampling site), well distinguished from all the others. Further results concern the levels of genetic variability recorded. The parthenogenetic populations have an extremely high level of heterozygosity, due to a remarkable number of loci showing fixed heterozygosity, up to 13 out of 18 analysed. The preliminary data presented here show that some strains of *A. parthenogenetica* have a number of biological features typical of species of hybrid origin: fixed heterozygosity at many loci, parthenogenetic reproduction and genome polyploidisation. While too preliminary to advance a new hypothesis on the origin of *A. parthenogenetica*, our data suggest that further studies need to be carried out aimed at the research of possible bisexual parental species of *A. parthenogenetica* suspected hybrid strains.

Résumé

L'électrophorèse multilocus a permis de caractériser génétiquement huit populations d'*Artemia* provenant aussi bien d'étangs saumâtres que de lagunes côtières italiennes. Ces populations sont caractérisées par des modes de reproduction différents (amphigonie à parthénogenèse) et par différents degrés de ploïdie (diploïdie à polyploïdie). Les échantillons bisexuels qui viennent de la mer Tyrrhénienne sont génétiquement homogènes même s'ils sont géographiquement lointains (Sardaigne-Latium), grâce à un faible flux génétique probablement dû à la dispersion par des oiseaux migrateurs. Les échantillons parthénogénétiques (*A. parthenogenetica*) sont génétiquement différents entre eux ou comparés aux bisexuels, qu'ils aient ou non le même degré de ploïdie. Chaque population est composée d'un nombre limité de génotypes nettement différents les uns des autres. Les populations parthénogénétiques ont des niveaux d'hétérozygotie extrêmement élevés puisqu'un grand pourcentage des locus étudiés montre une hétérozygotie fixe (jusqu'à 13 locus sur 18). Ces donnés préliminaires prouvent que certains clones d'*A. parthenogenetica* présentent plusieurs caractères biologiques typiques d'une espèce hybride : hétérozygotie fixée à plusieurs locus, reproduction par parthénogenèse et polyploïdisation du génome. Ces donnés sont cependant trop préliminaires pour avancer de nouvelles hypothèses concernant l'origine d'*A. parthenogenetica*, mais ils soulignent la nécessité d'identifier les espèces bisexuelles parentales des clones de *A. parthenogenetica* qui pourraient avoir une origine hybride.

Keywords:Artemia; Allozymes; Amphigony; Parthenogenesis; Heterozygosity

Mots clés : Artemia ; Allozymes ; Reproduction amphigonique ; Parthénogenèse ; Hétérozygotie

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1. Introduction

Brackish waters are habitats characterised by strong seasonal fluctuations of environmental parameters, mainly temperature and salinity. These make them particularly suitable environments to investigate the relationships between environmental challenge and biological features adapting local populations to these "extreme" habitats. Within crustaceans, the anostracans belonging to *Artemia* genus are particularly well adapted to hypersalted habitats, responding to environmental changes with many adaptive strategies, including parthenogenetic reproduction and/or genome polyploidisation. Consequently, brine shrimp is a good target species to address the above-mentioned ecological and evolutionary questions concerning the relationships between reproductive modalities, evolutionary history and environmental stress (Browne, 1992 and references herein).

Although distributed in all continents except Antarctica, brine shrimp habitat is restricted to hypersaline inland lakes and coastal salterns, often associated with commercial salt production (Browne and MacDonald, 1982). In the American continent, *Artemia* reproduces only by bisexual reproduction and three species have been described, *A. persimilis*, *A. franciscana* and *A. monica* (Bowen et al., 1978). In the Old World and Australia, *Artemia* consists of a number of siblings or morphologically similar species divided into sexual diploid and obligate parthenogenetic, although obligate parthenogenesis is the method of reproduction for approximately 70% of the populations (Browne and MacDonald, 1982). There are many bisexual species so far described and their number will increase together with the number of genetic investigations carried out on this complex, since it is characterised by a high number of sibling species (Browne, 1992; Triantaphyllidis et al., 1997; Abatzopoulos et al., 2002). In the Mediterranean region (southern Europe and North Africa), there is a single bisexual species, *A. salina*, previously called *A. tunisiana* (Bowen et al., 1978; Browne, 1983, 1988; Mura, 1990), as demonstrated by recent morphological and molecular studies (Triantaphyllidis et al., 1997). All the parthenogenetic species of the Old World are grouped collectively under the single binomen *A. parthenogenetica*, including both diploid and polyploid strains (Bowen and Sterling, 1978). This classification is, however, recognised as inadequate, because remarkable levels of genetic divergence have been observed between different strains using allozymes and molecular markers (Abreu-Grobois and Beardmore, 1982; Perez et al., 1994).

Recent works suggest that asexual polyploid clones evolved by autopolyploidisation from parthenogenetic diploid *Artemia*, branched in turn from a bisexual ancestor (possibly *A. urmiana* or *A. salina*) approximately 5.4 million years ago (Abreu-Grobois and Beardmore, 1982; Browne and Bowen, 1991; Browne 1992). According to this hypothesis, the high genetic variability typical of *A. parthenogenetica* would be due to the interaction between genetic variation (originating by mutation and recombination, the latter acting in diploid forms only) and environmental selection (Barigozzi, 1974; Browne, 1992; Zhang and King, 1992; Browne et al., 2002). As a consequence, the genetic divergence between cytotypes should be due to environment dependent selection rather than their phylogenetic history. All the strains included in the *A. parthenogenetica* complex would be, therefore, of monophyletic origin.

The present research is part of a national project aimed at analysing the biodiversity in brackish–hypersaline waters. Thus, we have examined the genetic structure of the widely studied branchiopods *A. salina* and *A. parthenogenetica* using allozyme electrophoresis. The aim was to produce an overall view of the genetics of a large part of the Italian populations. Although in the past single Italian populations have been studied by many authors (Abreu-Grobois and Beardmore, 1982; Zhang and King, 1992; Pilla and Beardmore, 1994; Badaracco et al., 1995; Triantaphyllidis et al., 1997), an inclusive outline is still lacking. Given the genetic complexity of the species, the availability of data collected at the same time on different populations could be important for further studies on the evolution and ecology of this crustacean.

2. Materials and methods

A total of 746 *Artemia* specimens were collected from seven sites in Italian brackish–hypersaline waters: four samples of bisexual *A. salina* from Tyrrhenian Sea (Tarquinia, S. Antioco, Cagliari, and Putzu Idu) and four samples of *A. parthenogenetica* from Adriatic Sea (Cervia, Comacchio, and Margherita di Savoia). The latter sample has both diploid and tetraploid strains, as already evidenced by Abreu-Grobois and Beardmore (1982) and confirmed by preliminary cytogenetic studies on karyotype (our unpublished data). The sampling locations are given in Table 1.

Standard horizontal starch gel electrophoresis (12%) was carried out on the whole organism. A total of 18 loci encoded by 15 enzymes were analysed as shown in Table 2. Allozymes were named numerically, according to their mobility relative to the most common allele (called *100*) in the reference population from Tarquinia. To be sure that no "alien" species were sampled instead of *A. salina* or *A. parthenogenetica*, a population of *A. franciscana* from Salt Lake City (USA) was used as reference material: 10 of the 12 loci scored showed fixed allelic differences with respect to the Italian samples (*Mdh-1*, *Mdh-2*, *Mdhp*, *Idh-2*, *Est*, *Pep-B*, *Ca*, *Mpi*, *Gpi*, *Pgm* vs. *Ldh*, *6Pgdh*).

Statistic analyses on allozyme data were performed using the computer package Biosys-1 (Swofford and Selander, 1989). Genotypic distributions at all loci were tested for conformance to Hardy–Weinberg expectations in bisexual populations using the exact probability test. Genetic variability was estimated in bisexual populations by the following parameters: (1) percentage of polymorphic loci, using the 0.99 (P_{99}) criterion; (2) overall number of alleles per locus (A); (3) observed mean heterozygosity per locus (H_o). Ob-

Table 1
Population codes with relative reproductive modality and ploidy, collecting sites and number of specimens genetically analysed

Species	Collecting sites	Abbreviation	Location	Reproductive modality	Ploidy	No. of specimens
A. salina	Tarquinia	TAR	Latium	Amphigonic	Diploid	156
"	S. Antioco	SAN	Sardinia	"	"	115
"	Cagliari	CAG	"	"	"	123
"	Putzu Idu	PUZ	"	"	"	95
A. parthenogenetica	Cervia	CER	Emilia Romagna	Parthenogenetic	Tetraploid	73
"	Comacchio	COM	"	"	"	112
"	Margherita di Savoia	MSD	Apulia		Diploid	67
"	Margherita di Savoia	MST	"	"	Tetraploid	5

Table 2
The enzymes scored, listed with their international code (EEC), encoding loci, electrophoretic migration conditions and staining references

Enzymes	EEC	Encoding loci	Migration	Buffer system[a]	V/cm	Ref.[b]
α-glycerophosphate dehydrogenase	1.1.1.8	*α-Gpdh*	+	2	8	d
Lactate dehydrogenase	1.1.1.28	*Ldh*	+	4	8	a
Malate dehydrogenase	1.1.1.37	*Mdh-1*	+	5	8	b
		Mdh-2	+			
NADP-Malate dehydrogenase	1.1.1.40	*Mdhp*	+	2	8	d
Isocitrate dehydrogenase	1.1.1.42	*Idh-1*	+	2–4	8	b
		Idh-2	+			
6-Phosphogluconate dehydrogenase	1.1.1.44	*6Pgdh*	+	5	8	b
Superoxide dismutase	1.15.1.1	*Sod*	+	2–4	8	c
Aspartate aminotransferase	2.6.1.1	*Aat-1*	+	1	8	b
		Aat-2	+			
Esterase	3.1.1.1	*Est*	+	1–3	8	d
Peptidase (Leu–Gly–Gly)	3.4.11	*Pep-B*	+	2	8	f
Aldolase	4.1.2.13	*Ald*	+	1	8	d
Carbonic anhydrase	4.2.1.1	*Ca*	+	3	8	e
Mannose-6-phosphate isomerase	5.3.1.8	*Mpi*	+	2	8	e
Glucose-6-phosphate isomerase	5.3.1.9	*Gpi*	–	3	8	c
Phosphoglucomutase	5.4.2.2	*Pgm*	+	4	8	a

+ = anodal.
– = cathodal.
[a] Buffer systems are the following: (1) discontinuous tris/citrate (Poulik, 1957); (2) continuous tris/citrate (Selander et al., 1971); (3) tris/versene/borate (Brewer and Sing, 1970); (4) tris/versene/maleate (Brewer and Sing, 1970); (5) phosphate/citrate (Harris and Hopkinson, 1976).
[b] Staining references: (a) Brewer and Sing (1970); (b) Shaw and Prasad (1970); (c) Selander et al. (1971); (d) Ayala et al. (1972); (e) Harris and Hopkinson (1976); (f) Richardson et al. (1986).

served mean heterozygosity has also been calculated for *A. parthenogenetica* samples. Genetic structuring within and between populations was calculated using the *F* statistics by Wright (1943, 1951). The statistical significance of F_{ST} was assessed using the computer software FSTAT (Goudet, 2001) and the method of Workman and Niswander (1970). Genetic divergence was estimated using distance indices by Nei (1972, D_{Nei}), further used to build up an unweighted pair group method with arithmetic mean (UPGMA) dendrogram showing the genetic relationships between studied populations.

The amount of gene flow between bisexual populations was estimated by means of the parameter *Nm*, where *m* is the fraction of immigrants in a population of effective size *N*, using the formula proposed by Wright (1943, 1951), modified by Crow and Aoki (1984): $Nm = [(1/F_{ST}) - 1]/4\alpha$ where F_{ST} is the standardised variance of allele frequencies (averaged over variable loci) and $\alpha = (n/n - 1)^2$, with *n* being the number of populations sampled.

3. Results

3.1. Genetic structure in amphigonic populations

Six of the 18 loci analysed (*Ldh*, *Mdhp*, *Aat-2*, *Ald*, *Est*, *Ca*) were found to be monomorphic for the same allele in all the samples tested. At six loci (*α-Gpdh*, *Mdh-1*, *Mdh-2*, *Idh-2*, *Pep-B*, *Gpi*), the frequency of the most common allele was higher than 0.90 in all the samples. The remaining six variable loci were found to be polymorphic (*Idh-1*, *Sod*, *Aat-1*) or highly polymorphic (*6Pgdh*, *Mpi*, *Pgm*) in all the four amphigonic populations with 2–6 alleles per locus (Table 3). Seven private alleles, i.e. alleles observed in a single population (Neel, 1973), have been found at five loci (*α-Gpdh*, *Mdh-1*, *Idh-2*, *6Pgdh*, *Pgm*). They are all at low percentages, generally close to 1%, except $Idh\text{-}2^{90}$ at 8.9% in S. Antioco and Pgm^{88} at 2.8% in Tarquinia. The populations from Tarquinia and S. Antioco are the only ones having private alleles. These results cannot be attributed to artificial

Table 3
Allele frequencies at the 12 loci found to be polymorphic in the studied populations of *A. salina*

Locus	Tarquinia	S. Antioco	Cagliari	Putzu Idu
α-Gpdh				
97	–	0.011	–	–
100	1.000	0.989	1.000	1.000
Mdh-1				
90	0.006	–	–	–
100	0.994	0.995	0.995	0.966
115	–	0.005	0.005	0.034
Mdh-2				
100	0.983	0.990	1.000	1.000
110	0.017	0.010	–	–
Idh-1				
92	0.224	0.232	0.382	–
100	0.776	0.768	0.618	1.000
Idh-2				
85	0.007	–	–	0.020
90	–	0.089	–	–
92	0.007	–	–	–
100	0.986	0.911	1.000	0.980
6Pgdh				
100	0.418	0.238	0.570	0.271
106	–	0.006	–	–
112	0.160	0.287	0.160	0.431
120	0.361	0.415	0.260	0.257
127	–	0.006	–	–
135	0.061	0.049	0.010	0.042
Sod				
95	–	0.020	0.008	–
100	0.937	0.760	0.629	0.711
107	0.062	0.220	0.363	0.289
Aat-1				
90	0.083	0.133	0.111	–
100	0.917	0.867	0.889	1.000
Pep-B				
94	0.063	0.014	–	0.020
100	0.937	0.986	1.000	0.980
Mpi				
75	0.008	0.006	0.056	–
82	0.038	0.032	–	–
88	0.195	0.083	0.247	0.250
93	0.148	0.141	0.056	0.303
97	0.140	0.173	0.167	0.079
100	0.470	0.564	0.475	0.368
Gpi				
95	0.010	0.030	–	–
100	0.980	0.963	1.000	0.981
108	0.010	0.007	–	0.019
Pgm				
88	0.028	–	–	–
95	0.233	0.080	0.150	0.265
100	0.712	0.375	0.539	0.596
105	0.028	0.545	0.311	0.140

sampling because a comparable number of specimens have been analysed for each sample (see Table 1).

No statistically significant deviations from Hardy–Weinberg expectations were seen in any of the studied samples.

Table 4
Parameters of genetic variability in the studied populations of *A. salina*

Samples	A	P_{99}	H_0
Tarquinia	39	0.61	0.135
S. Antioco	41	0.67	0.184
Cagliari	32	0.39	0.165
Putzu Idu	31	0.44	0.141

A = overall number of alleles.
P_{99} = percentage of polymorphic loci at 0.99 criterion.
H_0 = observed mean heterozygosity.

All amphigonic populations have similar levels of genetic variability, with a mean observed heterozygosity H_o ranging between 0.135 and 0.184, a total number of recorded alleles A between 31 and 41, and a percentage of polymorphic loci P_{99} from 0.39 to 0.67 (Table 4). Nei's genetic distances have been calculated to estimate the extent of genetic differentiation between the studied samples (Table 5). D_{Nei} ranged between 0.013 and 0.021, showing that populations are genetically closely related and that there is a scarce differentiation among them, as confirmed by the low value of mean F_{ST} (0.061) (Wright, 1943, 1951) and by the close values assumed by F_{IS} and F_{IT} at single loci (see Table 6). Although the studied populations are not geographically adjacent, being separated by the Tyrrhenian Sea, the low observed differentiation gives evidence of a moderate gene flow among them (Nm = 2.2).

3.2. Genetic structure in parthenogenetic populations

Genotypic frequencies based on observed phenotypes at the 18 scored loci (Table 7) were considered to analyse the genetic structure of parthenogenetic populations. All the three tetraploid samples have private alleles: Comacchio has eight private alleles, Cervia seven and M. Savoia (tetraploid) two. Private alleles have not been found in the diploid parthenogenetic strain from M. Savoia. The four populations have fixed different genotypes at many loci (Table 7). Even between Cervia and Comacchio, geographically neighbouring and both tetraploid, fixed differences have been recorded at 11 of the 18 scored loci (*α-Gpdh*, *Mdh-1*, *Idh-1*, *Idh-2*, *6Pgdh*, *Aat-1*, *Ald*, *Ca*, *Mpi*, *Gpi*, *Pgm*).

Each population presents few genotypes, well distinguished from those of the other populations. For example, Comacchio sample shows a single genotype for all the 18 scored loci in all the studied specimens, and at seven loci the allelic array is unique (*α-Gpdh*, *Idh-1*, *Aat-1*, *Ca*, *Mpi*, *Gpi*, *Pgm*), having never been observed in the other studied populations.

The values of observed heterozygosity for parthenogenetic populations are given in Fig. 1. The range of observed values is very wide. The tetraploid samples Cervia and tetraploid M. Savoia, have quite similar values of H_o (0.39 and 0.28, respectively), while Comacchio sample has a strikingly high level of heterozygosity, 0.72. The diploid sample from M. Savoia exhibits a lower level of heterozygosity, 0.15. The high genetic variability observed in tetraploid samples can be

Table 5
Matrix of Nei's (1972) genetic distance coefficients

	Population	1	2	3	4	5	6	7	8
1	Tarquinia	*							
2	S. Antioco	0.019	*						
3	Cagliari	0.014	0.013	*					
4	Putzu Idu	0.013	0.019	0.021	*				
5	Cervia	0.734	0.720	0.741	0.802	*			
6	Comacchio	0.540	0.577	0.557	0.599	0.266	*		
7	M. Savoia Dip	0.907	0.912	0.922	0.976	0.208	0.253	*	
8	M. Savoia Tet	0.791	0.793	0.813	0.836	0.223	0.249	0.098	*

Table 6
Wright's *F* statistics calculated for polymorphic loci of *A. salina*

Locus	F_{IS}	F_{IT}	F_{ST}	
α-Gpdh	–0.011	–0.003	0.009	NS
Mdh-1	–0.026	–0.011	0.014	NS
Mdh-2	–0.015	–0.007	0.008	NS
Idh-1	–0.035	0.081	0.112	*
Idh-2	0.148	0.187	0.045	NS
6Pgdh	0.068	0.115	0.051	*
Sod	–0.113	–0.037	0.068	*
Aat-1	–0.128	–0.089	0.034	NS
Pep-B	0.064	0.086	0.024	NS
Mpi	0.116	0.141	0.028	NS
Gpi	–0.025	–0.015	0.010	NS
Pgm	–0.089	0.018	0.098	*
Mean	0.006	0.067	0.061	NS

* $P < 0.01$.
NS, not significant ($P > 0.01$).

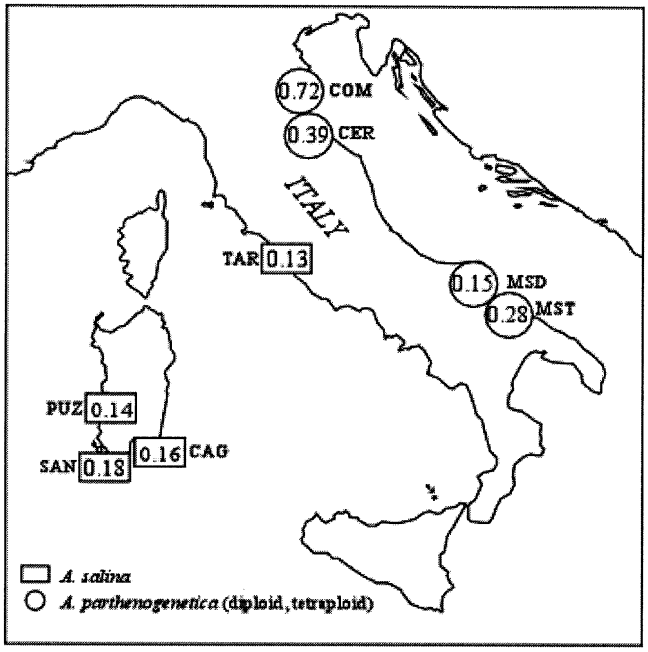

Fig. 1. Values of observed heterozygosity (H_0) for the studied populations of *A. salina* and *A. parthenogenetica*. Abbreviations as reported in Table 1.

due to the fixed heterozygosity found at several loci, for two or three alleles. Comacchio sample shows a fixed heterozygosity at 13 of the 18 studied loci, while Cervia and M. Savoia tetraploid strains have five loci with fixed heterozygosity. Cervia shows two or three alleles at heterozygous fixed loci and M. Savoia always shows two. Also, the diploid sample from M. Savoia has fixed heterozygosity at two loci (*6Pgdh*, *Pgm*), with two alleles each.

D_{Nei} values show a substantial genetic differentiation among the studied populations, ranging between 0.098 (observed between the two samples from M. Savoia, diploid and tetraploid) and 0.266 (between Comacchio and Cervia).

These results show that *A. parthenogenetica* is a heterogeneous group of clones characterised by a strong genetic differentiation, even within the same group of ploidy.

3.3. Genetic differentiation within and between A. salina *and* A. parthenogenetica

The dendrogram in Fig. 2 represents genetic relationships based on Nei's distances calculated among populations. High genetic distances were detected between bisexual and parthenogenetic populations (D_{Nei} from 0.540 to 0.976, Table 5) which join two main sub-clusters. This is in good agreement with the belonging to the studied samples of two well-differentiated species: *A. salina* and *A. parthenogenetica*.

The sub-cluster including *A. salina* bisexual samples shows a low genetic distance among them (D_{Nei} between 0.013 and 0.021), with a differentiation fully within the typical range of conspecific local populations (Hedgecock et al., 1982).

Within parthenogenetic populations, M. Savoia diploid and tetraploid samples show the highest affinity and are linked to the North Adriatic population from Cervia (mean distance 0.215). Comacchio sample is the most differentiated, being on a separate branch with respect to the other parthenogenetic populations, with an average distance of 0.256. This is also due to the high number of exclusive alleles (8) found in this population. It can be noted that the two nearby polyploid North Adriatic samples (Cervia and Comacchio) have a conspicuous genetic distance (0.266), mostly due to the high number of exclusive alleles found in each population, observed at 10 loci out of the 18 analysed.

Table 7
Genotypic frequencies based on observed phenotypes at the 18 loci analysed in *A. parthenogenetica* populations. Common alleles with *A. salina* evidenced in bold

Genotype	Cervia	Comacchio	M. Savoia dip	M. Savoia tet
α-Gpdh				
100/*110*	–	*1.000*	–	–
110/110	–	–	*1.000*	*1.000*
110/120	*1.000*	–	–	–
Ldh				
100/100	*1.000*	*1.000*	*1.000*	*1.000*
Mdh-1				
100/*110*	*0.086*	–	–	–
110/110	*0.914*	–	*1.000*	–
110/118	–	*1.000*	–	*1.000*
Mdh-2				
100/110	*0.065*	*1.000*	–	–
110/110	*0.935*	–	*1.000*	–
*105/**110***	–	–	–	*1.000*
Mdhp				
100/100	*1.000*	*1.000*	*1.000*	*1.000*
Idh-1				
92/92	*0.866*	–	–	–
92/*96*	*0.067*	–	–	–
92/*98*	–	*1.000*	–	–
96/96	*0.067*	–	*1.000*	–
*96/**100***	–	–	–	*1.000*
Idh-2				
96/96	–	–	*0.677*	–
96/104	–	*1.000*	*0.323*	–
100/104	*0.956*	–	–	–
104/104	*0.044*	–	–	*1.000*
6Pgdh				
100/106	–	*1.000*	*1.000*	*1.000*
106/112	*1.000*	–	–	–
Sod				
100/100	*1.000*	*1.000*	*1.000*	*1.000*
Aat-1				
*82/**90***	–	*1.000*	–	–
90/90	*1.000*	–	*1.000*	*1.000*
Aat-2				
115/115	*1.000*	*1.000*	*1.000*	*1.000*
Est				
100/100	*1.000*	*1.000*	*1.000*	*1.000*
Pep-B				
96/96	*0.643*	–	*1.000*	*1.000*
*96/**100***	*0.214*	*1.000*	–	–
100/100	*0.143*	–	–	–
Ald				
*92/**100***	–	*1.000*	*0.429*	–
100/100	–	–	*0.571*	*1.000*
100/*105*	*0.600*	–	–	–
105/105	*0.400*	–	–	–
Ca				
100/100	*1.000*	–	*1.000*	*1.000*
100/*110*	–	*1.000*	–	–
Mpi				
93/*103/105*	–	*1.000*	–	–
100/*103*	*1.000*	–	–	–
103/103	–	–	*1.000*	*1.000*
Gpi				
95/110	*1.000*	–	–	–
100/*110*	–	*1.000*	–	–
110/110	–	–	*1.000*	*1.000*
Pgm				
95/100	–	–	*0.015*	–
95/100/105	*1.000*	–	–	–
95/100/*115*	–	*1.000*	–	–
100/105	–	–	*0.985*	*1.000*

4. Discussion

The presented results add some data to the knowledge of the widely studied genetics of Italian *Artemia* populations. The main data obtained concern the degree of genetic differentiation between and within populations of *A. salina* and *A. parthenogenetica* and their levels of genetic variability.

The results obtained on genetic differentiation show that brine shrimp bisexual populations are genetically similar, with a moderate gene flow able to counteract genetic drift effect and to maintain genetic homogeneity between populations from far areas, i.e. Sardinia Island and Tyrrhenian Italian coasts. Maybe not all the assumptions needed to calculate gene flow rates are satisfied in *Artemia* populations. For example, the absence of selective forces is one assumption of the model, but it has been repeatedly suggested that selection could act on local populations of many *Artemia* species (Browne and Hoopes, 1990; Browne et al., 2002). However, the obtained rates of gene flow seem consistent with the effectiveness of the dispersal mechanisms of the species, that are usually carried out by the desiccation-resistant cysts and assisted by migratory waterfowl that can cover very long distances, allowing a gene flow able to counteract genetic drift effects ($Nm > 1$) even between populations located in relatively far away areas, such as Sardinia and Latium. Accordingly, previous electrophoretic studies carried out by Abreu-Grobois and Beardmore (1982) on Mediterranean populations of *A. salina* showed a range of genetic distances comparable to that reported here (D_{Nei} 0.01–0.02 against 0.01–0.06), with slightly higher values limited to very far away samples (Spain against Cyprus). The levels of genetic variability observed are close to those evidenced in other bisexual *Artemia* species. For example, the observed heterozygosity ranges from 0.135 to 0.184, while a range of 0.096–0.138 was recently found using allozymes by Abatzopoulos et al. (2002) in populations of *A. franciscana*, *A. urmiana*, *A. sinica* and *A. tibetiana*. The genetic differentiation between parthenogenetic populations is about one order of magnitude greater than among bisexual samples (D_{Nei} 0.098–0.266). These findings confirm that *A. parthenogenetica* is a cluster of very differentiated forms. However, previous electrophoretic studies evidenced strong differentiation mainly between forms characterised by different ploidies, while polyploid populations from the whole Mediterranean basin were shown as having similar genotypes (Abreu-Grobois and Beardmore, 1982). Other results obtained by Perez et al. (1994) comparing the mitochondrial DNA of diploid and tetraploid Spanish strains of *A. parthenogenetica*, showed them to be highly differentiated. While confirming the differentiation between strains with different ploidies, our results also highlight a noticeable differentiation even between polyploid neighbouring populations (Comacchio and Cervia; D_{Nei} 0.266), comparable to that observed between diploid/polyploid forms (M. Savoia diploid vs. Comacchio and Cervia; D_{Nei} 0.230). The estimates of genetic variability show high heterozygosity for the three tetraploid parthenogenetic populations, in particular, in the

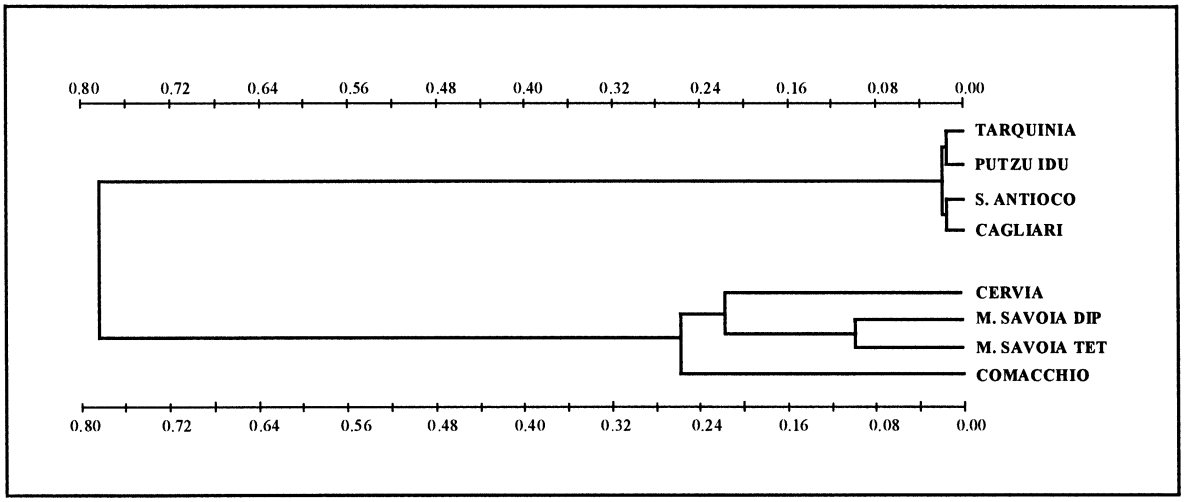

Fig. 2. Unweighted pair group method with arithmetic mean (UPGMA) dendrogram based on Nei's (1972) genetic distances.

tetraploid sample from Comacchio. This variability can be attributed to the fixation of heterozygous genotypes in all the studied individuals at many loci. Such a striking value (H_o = 0.72) is due to the fixation in heterozygosity of a relevant percentage of the studied loci, 13 out of the 18 analysed. Similar levels of fixed heterozygosity, supporting our explanation, were found, but not stressed, by Abreu-Grobois and Beardmore (1982) in tetraploid samples from Spain and France.

According to the usually accepted theories on evolutionary biology of brine shrimp, these data could be explained by the evolutionary history of *A. parthenogenetica*. The high heterozygosity evidenced here could be generated by mutation or recombination in diploid *Artemia*, while, in polyploid forms, only mutation could create variation (Barigozzi, 1974). Once this heterozygosity originates, it could be maintained by selective pressure due to environmental conditions (Zhang and King, 1992). Within this frame, mutations would account for the high heterozygosity recorded in polyploid forms, while different environmental conditions could be responsible for the selective fixation in heterozygosity of many loci and for the high genetic divergence observed between either diploid/polyploid or polyploid populations. Ecological knowledge supports this explanation, because it is well known that a range of environmental parameters, such as temperature, salinity, UV radiation and more, play a relevant role in moulding the genetic structure of *Artemia* local populations (Bowen et al., 1988; Browne and Hoopes, 1990; Lenz and Browne, 1991; Browne, 1992).

An alternative working hypothesis could be formulated. In fact, it is known that other unisexual invertebrate species, also living in rather extreme environments, originated by hybridisation (White, 1978; Nascetti and Bullini, 1980; Bullini and Nascetti, 1990; Bullini, 1994). There is growing evidence that hybridisation can generate clonal diversity (Butlin et al., 1998), since this process has been proved in some unisexual animal species (Avise et al., 1992; Bullini, 1994; Vrijenhoek, 1994). According to the "reticulate evolution" process (Darewsky, 1992), the random hybridisation between allopatrically differentiated bisexual taxa could give rise to new hybrid species, characterised by fixed heterozygosity at parental discriminating loci, non-amphigonic reproduction and frequent genome polyploidisation, as in the case of stick insects of the genus *Bacillus* (Bullini and Nascetti, 1990; Mantovani and Scali, 1992). Although quite rare, hybrid animal species usually have a remarkable ecological success, leading to parental competitive displacement or to the colonisation of new habitats, unsuitable for parental species, as proved for the snail, *Bulinus truncatus*, the grasshopper, *Warramaba virgo*, or the stick insects belonging to the genus *Clonopsis* (White, 1978; Nascetti and Bullini, 1980; Bullini and Nascetti, 1990; Bullini, 1994). Some strains of *A. parthenogenetica* (e.g. tetraploid Comacchio population) display many of these features, being characterised by a high number of fixed heterozygous loci, apomictic parthenogenetic reproduction and tetraploidy. Moreover, it has been experimentally demonstrated that *A. parthenogenetica* competitively displaces the sympatric sexual species, *A. salina* (Browne et al., 1988; Browne and Halanych, 1989). Considering that on an overall number of 44 alleles observed in *A. parthenogenetica*, 24 are common with *A. salina*, this species could be a good candidate for being one of parental bisexual species. Nevertheless, locus *Aat-2* is fixed for alternative alleles in the two species, so that a common ancestor (with a gene pool close to that of extant *A. salina*) as a parental species of *A. parthenogenetica* seems a more reasonable hypothesis.

In conclusion, if the hypothesis of the origin of some polyploid brine shrimps by hybridisation cannot be excluded, its demonstration needs further studies aimed at identifying the bisexual parental species of *A. parthenogenetica*.

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