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Research Article

Large-scale mitochondrial phylogeography in the halophilic fairy shrimp *Phallocryptus spinosa* (Milne-Edwards, 1840) (Branchiopoda: Anostraca)

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Abstract. In this study we analyzed patterns of sequence divergence in about 1kb of mitochondrial DNA coding for two genes (16S rRNA and Cytochrome Oxidase I, COI) in 15 populations and 61 individuals of the halophilic fairy shrimp *Phallocryptus spinosa* (Milne-Edwards, 1840). Populations were sampled in saline and hypersaline water bodies from Spain, France, Italy, Greece, Turkey, Ukraine, Iran, Uzbekistan, Cyprus, Algeria, Morocco and Botswana. Our genetic findings suggest complex phylogeographic relationships and pronounced genetic differentiation among populations. Multiple phylogenetic methods and nested clade analysis revealed the existence of four highly divergent maternal lineages with strong phylogeographic patterns and signatures of either allopatric fragmentation or restricted gene flow with isolation by distance. These results are further supported by the hierarchical analysis of molecular variance (AMOVA) and pairwise F_{ST} values, which indicate that most of detected genetic heterogeneity is apportioned among populations. Genetic relationships among haplotypes fit geographical hypotheses in most cases but one. Indeed, one haplotype is shared among French, Iranian and Uzbekistan populations. We hypothesize that this peculiar occurrence might be due to an avian-mediated long distance passive dispersal event.

Key words. Anostraca; *Phallocryptus spinosa*; halophilic crustaceans; dispersal; mitochondrial DNA; phylogeography.

Introduction

The fairy shrimp *Phallocryptus spinosa* (Milne-Edwards, 1840) inhabits brackish, saline and hypersaline astatic waters subjected to seasonal droughts. *P. spinosa* has a wide palearctic distribution. Populations have been reported from Spain, France, Italy, Cyprus, Turkey, Greece, Ukraine, Iran, Uzbekistan and Afghanistan (Rogers, 2003). The species is also known for few localities in North Africa (Morocco and Algeria) and from a single location south of the Sahara (Makgadikgadi Pans, Botswana) (Abatzopoulos et al., 1999 and references therein). The disjunct occurrence of the species most likely mirrors the

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scattered distribution of suitable habitats. It is, however, not yet entirely clear whether the description of the species distribution is accurate or rather reflects the general lack of knowledge on anostracan species richness at a regional scale and/or bad taxonomy (Abatzopoulos et al., 1999). Around the Mediterranean the species may coexist with the brine shrimp *Artemia*, while in Botswana it co-occurs with the fairy shrimp *Streptocephalus* (Rogers, 2003).

Like all other brine and fairy shrimps, *P. spinosa* is capable of producing encysted embryos (also termed resting/diapausing cysts) that can withstand prolonged droughts and high and low temperatures. This allowed anostracans to colonize extreme environments such as temporary rain pools and permanent hypersaline water bodies not accessible to bony fishes, which preyed them to extinction in other aquatic environments (Dumont and Negrea, 2002). The resting cysts should confer a high potential for long distance passive dispersal to these crustaceans. Indeed, cysts may attach to feathers of water birds and remain viable after passage through amphibian and bird digestive tracts (Bohonak and Whiteman, 1999; Charalambidou and Santamaria, 2002).

To date, anostracans have been studied molecularly to either resolve the placement of the lineage within branchiopods or to produce phylogenetic hypotheses at the family and genus levels (Hanner and Fugate, 1997; Remigio and Hebert, 2000; Spears and Abele, 2000; Weekers et al., 2002; Remigio et al., 2001, 2003; Ketmaier et al., 2003; Daniels et al., 2004). Conversely, studies at the intra-specific level are relatively rare. Yet, these organisms do represent a unique opportunity to test hypotheses on patterns of dispersal and genetic structuring in light of their peculiar ecology. The commercial importance of the brine shrimp *Artemia* has stimulated research aimed at elucidating its systematics and ecology. We therefore have a rather detailed knowledge on the life history, morphometrics, phylogeny and biogeography of this important genus (Abatzopoulos et al., 1986, 2002; Baxevanis et al., 2005, 2006; Beardmore and Abreu-Grobois, 1983; Browne, 1992; Browne and Hoopes, 1990; Mura et al., 2006; Eimanifar et al., 2006). Comparatively less is known on how genetic variation is partitioned within single *Artemia* species (Baxevanis et al., 2006). Data on freshwater taxa are relatively more abundant but mostly based on allozymes (Davies et al., 1997; Bohonak, 1998; Brendonck et al., 2000; Zarattini et al., 2001; Ketmaier et al., 2003; Meglécz and Thiéry, 2005).

We recently published the first mitochondrial DNA (mtDNA)-based phylogeographic study for a freshwater fairy shrimp (*Tanymastix stagnalis*; Ketmaier et al., 2005). For that study, we analyzed sequence polymorphisms in fragments of two mt genes, namely 16S rRNA (16S) and Cytochrome Oxidase I (COI). Our study revealed a complex phylogeographic pattern and pronounced genetic differentiation in the species. The other intra-specific genetic studies available on anostracans also detected low levels of gene flow. For instance, in the fairy shrimp *Branchipodopsis wolfi* gene flow among populations seems to be seriously reduced by distances of 2 km or even less (Brendonck et al., 2000). This pattern is not limited to anostracans but it apparently applies to all branchiopods (De Meester et al., 2002). These authors discussed the evident discrepancy between high theoretical potential for dispersal of these organisms and little ongoing gene flow by emphasizing the role of local adaptation and multiple independent founder events in reducing gene flow even at a local scale. The rapid population growth typical of these arthropods upon colonization would monopolize resources, hence the name of "Monopolization Hypothesis" proposed by the authors to explain the apparently paradoxical, yet widespread, pattern shown by a variety of pond-dwelling organisms (mainly cladocerans, rotifers and bryozoans). In addition, the presence of a large cyst bank would act as a sort of barrier against the settlement of invading genotypes.

Here we have used the 16S and COI genes to produce a large-scale phylogeographic study for *P. spinosa.* The present study has two main objectives. First, we wanted to determine the magnitude of genetic differentiation within a halophilic species with a widespread, yet disjunct distribution. To this end, we made an effort to cover most of the species known range with our sampling. We were able to obtain samples from Spain, France, almost all known Italian localities, Greece, Cyprus, Turkey, Ukraine (Crimea), Iran, Uzbekistan, Algeria, Morocco and Botswana for a total of 15 populations. We could not sample the type locality of the species (Hadjibe Salt Lake, Odessa, Ukraine). We collected, however, *P.spinosa* from the Atchi Lake, which is approximately 200 km east of the Hadjibe Salt Lake. Second, we wanted to test, within a rigorous phylogeographic framework, how and to what extent relationships among populations relate to their geographic distribution and whether the geographic distance among populations is the major force responsible for the pattern of genetic variation in the species.

Materials and methods

Sampling

For this study, we collected 15 populations of *P. spinosa*. Specimens were preserved in absolute ethanol. We analyzed from three to eight individuals for each population. Details on the sampling sites are given in figure 1.

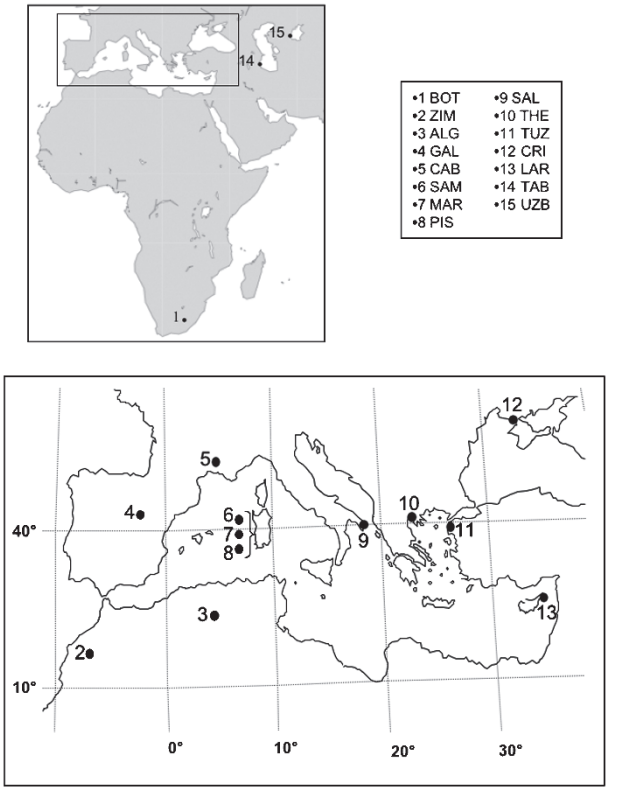

Figure 1. Schematic map showing the sampling localities of the *P.spinosa* populations included in the study. Abbreviations are population codes followed by sample sizes and geographical coordinates. 1-BOT (3; 20°15'7"S 26°01'28"E)= Makgadikgadi Pans, Botswana; 2-ZIM (3; 32°05'00"N 08°40'00"W)= Zima saltern, Morocco; 3-ALG (8; 35°39'00"N 02°50'00"E)= Boughzoul Lake, Algeria; 4-GAL (3; 40°58'12"N 01'30'00"W)= Gallocanta Lake, Spain; 5-CAB (4; 43°26'00"N 04°49'00"E)= Salin du Caban, France; 6-SAM (6; 40°02'08"N 08°24'32"E)= Sa Mesa Longa, Italy; 7-MAR (6; 39°52'46"N 08°26'21"E)= Mari Ermi, Italy; 8-PIS (5; 39°57'49"N 08°29'01"E)= Piscaredda, Italy; 9-SAL (5; 48°18'00"N 17°43'53"E)= Torre Colimena, Italy; 10-THE (3; 40°26'00"N 22°52'00"E)= Palioura saltmarsh, Greece; 11-TUZ (3; 40°07'57"N 25°57'19"E)= Lake Tuz, Gokceada Island, Turkey; 12-CRI (3; 46°08'00"N 33°54'00"E)= Atchi Lake, Ukraine; 13-LAR (3; 34°52'00"N 33°32'00"E)= Larnaka Lake, Cyprus; 14-TAB (3; 37°34'00"N 45°25°00"E)= Tabriz, Iran; 15-UZB (3; 44°18'00"N 58°59'00"E)= Cape Aktumsuk, Uzbekistan.

DNA sequencing

Genomic DNA was extracted from ethanol-preserved specimens by using the DNeasy kit from QIAGEN. PCR amplifications of a 413-bp (base pair) fragment of the 16S rRNA gene and of a 515-bp fragment of the Cytochrome Oxidase I (COI) gene were carried out as in Ketmaier et al. (2003; 2005). Sequences were determined with automated sequencers (Applied Biosystems 373A or AB3100) following the manufacturer's protocols. Strands were sequenced in both directions for each individual. Sequences were edited and aligned using Sequencher 3.1.1 (Gene Code Corporation, Ann Arbor, MI); alignments were also checked by eye. Sequences have been submitted to GenBank (Accession numbers EU236108-EU236141).

Phylogenetic and nested clade analyses

As an outgroup, we used the fairy shrimp *Streptocephalus dorothae* (retrieved from GenBank; Accession number AF209056 and AF209065 for 16S and COI genes, respectively) of the family Streptocephalidae. Remigio and Hebert (2000) and Spears and Abele (2000) suggested Streptocephalidae to be sister to Thamnocephalidae. To detect saturation in the data set, we plotted the absolute numbers of transitions (Ti) and transversions (Tv) against uncorrected-p distances. For the COI data set, these plots were done for all positions and for third codon positions separately. Aligned sequences (16S and COI separately and the two genes combined) were analyzed by maximum parsimony (MP; heuristic searches, ACCTRAN character-state optimization, 100 random stepwise additions, TBR branch-swapping algorithm) (Farris, 1970), maximum likelihood (ML; heuristic searches, 100 random stepwise additions, TBR branch swapping algorithm) (Felsenstein, 1981), Neighbor-Joining (NJ) (Saitou and Nei, 1987) and Bayesian methods (Rannala and Yang, 1996; Mau and Newton, 1997; Larget and Simon, 1999; Mau et al., 1999; Huelsenbeck, 2000). MP, ML and NJ analyses were performed using PAUP* 4.0β10 (Swofford, 2002); Bayesian analysis was carried out using MRBAYES 3.1 (Ronquist and Huelsenbeck, 2003). MP searches were run giving equal weight to all substitutions. We ran the ML analyses on PAUP* 4.0β10 after having determined the best model of DNA substitutions that fit our data using MODELTEST (Posada and Crandall, 1998). According to the results of this program, we ran all our ML analyses using the GTR + Γ model (combined data set, variable rates, shape parameter $\alpha = 0.455$; models and parameters selected for the two genes separately are not shown). NJ analyses were carried out on ML distances calculated with the same settings used for the ML analyses. For the Bayesian approach, we employed the same model of sequence evolution as in the ML searches allowing site-specific rate variation partitioned by gene and, for COI, by codon positions. MRBAYES was run for 2 million generations with a sampling frequency of 100 generations.

We ran one cold and three heated Markov chains. From the 20,000 trees found, we discarded the first 10 % ("burn-in") in order to include only trees for which convergence of the Markov chain had been reached. The remaining trees were used to construct a 50 % majority rule consensus tree using PAUP* 4.0β10. The robustness of the phylogenetic hypotheses was tested by bootstrap replicates (1,000 replicates for MP and NJ and 100 replicates for ML) (Felsenstein, 1985). For the Bayesian analysis, the posterior probabilities were estimated only for those generations sampled after the burn-in. Partitioned (by gene) Bremer Support (PBS) values (Bremer, 1988) were also calculated with TreeRotv.2 (Sorenson, 1999). Trees yielded by different phylogenetic methods as well as competing phylogenetic hypotheses were tested using the ML-based approximately unbiased tree selection test (AU; Shimodaira, 2002) implemented in the software package CONSEL (Shimodaira and Hasegawa, 2001). We always compared tree topologies simultaneously (Shimodaira and Hasegawa, 1999).

We also performed a network analysis to estimate gene genealogies using the TCS program (Clement et al., 2000), which implements the Templeton et al. (1992) statistical parsimony procedure. Input data were individual (16S + COI) mtDNA sequences. This program collapses sequences into haplotypes and produces a network linking different haplotypes only if they have a 95 % probability of being justified by the parsimony criterion. We used the Nested Clade Analysis (NCA) (Templeton, 1998) to test for association between genealogy and geography in the data set to infer population processes. We followed Templeton et al. (1992) and Crandall (1996) to construct the nested design. We then used the program GEODIS (Posada et al., 2000) to calculate distance measures and their statistical significance. The statistical distribution of distances was determined recalculating distances after 10,000 random permutations (i.e. clades against sampling locality). We finally complement NCA by computing an index for haplotypes and *n*-step clades ($n = 0, 1, 2, ..$) found in each sampling site, as suggested by Templeton (2001). The index is calculated by averaging the pairwise geographical distances between the geographical centers of haplotypes and clades found in each sampling site (geographical centers are provided in the GEODIS output). For a given sampling site, the index will increase if haplotypes/clades found in that site have geographically centers away from each other. This implies that that site hosts very divergent haplotypes, a phenomenon usually associated with secondary contacts of highly divergent lineages. Conversely, if the index decays with increasing the hierarchical level (i.e. from the haplotype to the 4th step clade) means that at that site haplotypes are all closely related and isolation by distance is the most likely process responsible for the observed pattern of haplotype occurrence.

We used ARLEQUIN 3.01 (Excoffier et al., 2005) to test levels of genetic diversity within and among the major lineages of *P.spinosa* identified by the phylogenetic and network searches by a hierarchical analysis of molecular variance (AMOVA; Excoffier et al., 1992). Significance of variance estimates was obtained using a randomization procedure (1000 permutations). We estimated Φ-statistics, which are haplotypic correlation measures analogous to F-statistics. We used the same package to calculate pairwise F_{ST} values for all pairs of *P. spinosa* populations; statistical significance of these values was assessed by 1000 permutations.

Results

Sequence variation

We sequenced 928 bp of mtDNA (413 bp for the 16S rRNA gene and 515 bp for the COI gene) for each of the 61 individuals of *P. spinosa* included in the study. We observed only few gaps in the final alignment; all gaps were in the 16S gene and were confined to the outgroup vs. ingroup comparisons. These indels were included in the MP analyses and counted as one single mutation each, regardless of size. Their removal or inclusion did not result in significant differences in tree topologies (not shown). Levels of sequence variability were much higher in COI than in 16S (variable sites 44.66 % for COI, 29.05 % for 16S; parsimony informative sites were 35.33 % and 17.43 % for the two genes). As expected, most of the variation resided in COI 3rd codon positions (83.72 % of sites were variable and of these 70.93 % were also parsimony informative). The combined data set had 37.71 % and 27.37 % of variable and parsimony informative sites. Sequences were generally A+T rich and anti-G biased, a pattern typical for a mitochondrial genome. A+T percentages ranged from 47.2 % in COI 1st codon positions to 72.1 % in COI 3rd codon positions; G percentages varied from 13.5 % in COI 3rd codon positions to 28.5 % in COI 1st codon positions. A χ^2 test of homogeneity of base frequencies among taxa revealed no significant differences, regardless of the data partition tested (both genes combined, each gene separately and COI 1st, 2nd, and 3rd codon positions separately). Visual inspection of saturation plots (not shown) revealed that saturation is not pronounced in the combined data set at the ingroup level.

Table 1. Haplotype frequencies of all individuals of *P.spinosa* sequenced for the study. For population codes see figure 1. Numbers in brackets are sample sizes.

	Populations														
Hapl.	BOT (3)	ZIM (3)	ALG (8)	GAL (3)	CAB (4)	SAM (6)	MAR (6)	PIS (5)	SAL (5)	THE (3)	TUZ (3)	CRI (3)	LAR (3)	TAB (3)	UZB (3)
A					3									1	3
B					1		1								
C							1								
D							1								
E							3								
F						2									
G						3									
H						1		5							
I									5						
J													3		
K										3					
L												3			
M											3				
N				3											
O			8												
P		3													
Q	3														

Haplotype frequencies

The 61 sequences obtained for the study identified a total of 17 unique haplotype for *P.spinosa*; these are listed in Table 1 (coded from A to Q) along with their frequencies in the 15 sampled populations. Haplotypes differed from one another by 1 to 214 substitutions; 12 out of the 15 analyzed populations had a single haplotype. CAB, SAM, and MAR had more than one with MAR scoring the highest number of haplotypes (4). Only in three circumstances, two or more populations share the same haplotype. Haplotypes B and H are in common between geographically close populations (CAB-MAR and SAM-PIS, respectively), whilst haplotype A has been found in CAB, TAB, and UZB, which are hundreds or thousands of kilometers apart from each other (see Fig. 1).

Phylogenetic analyses and genetic distances

Phylogenetic analyses were carried out on 16S and COI genes separately and on both genes combined. Tree topologies from individual genes were similar but not identical. When loci were combined, however, the number of equally most parsimonious trees in the MP searches was substantially reduced (35 for COI; 20 for 16S, and 2 for COI+16S) and more nodes were statistically supported independently from the phylogenetic method adopted. According to Brower et al. (1996) even though data sets are incongruent, the best estimate of relationships is still derived from the simultaneous interpretation of all the data. Because of these lines of reasoning, we present the results of the analyses based on the combined data set only.

Figure 2 shows the haplotype ML tree ($-\ln L = 3648.66$) obtained on the combined data set using the GTR + Γ model and summarizes the results of the other phylogenetic methods employed in the study. The ML tree is statistically indistinguishable from the unweighted MP, NJ and Bayesian searches with the AU test ($0.631 \leq P \leq 0.921$). MtDNA identifies four major clades (named I, II, III and IV in Fig. 2), which received maximum or nearly maximum statistical support in all phylogenetic searches.

Haplotypes O, P, and Q, which are restricted to North and South African populations, cluster together and are placed basal in the tree (cluster I). Clusters II and III comprise Spanish and Turkish/Crimean haplotypes, respectively. Cluster IV includes the highest number of haplotypes/populations. Relationships within this group are generally related to the geographical origins of samples. Haplotypes J and K (from Cyprus and continental Greece) are each other's closest relatives (but with no statistical support). The remaining part of the cluster includes haplotypes found in Sardinian and French populations with the remarkable exceptions of haplotype A, which has been found in the French (CAB), Iranian (TAB) and Uzbekistan populations (UZB) and of haplotype I, restricted to the Apulian population (SAL). Figure 2 also shows the results of the PBS analysis. It is evident that COI is the partition that contributes more to the overall support of the simultaneous analysis tree. This general pattern is reversed only in the case of clade I.

Given some unexpected results based on mere geographical considerations, we tested two alternative hypotheses against the unconstrained topology presented in Figure 2. First, we constrained haplotype A (shared among France, Iranian and Uzbekistan populations) to cluster together with haplotypes L and M

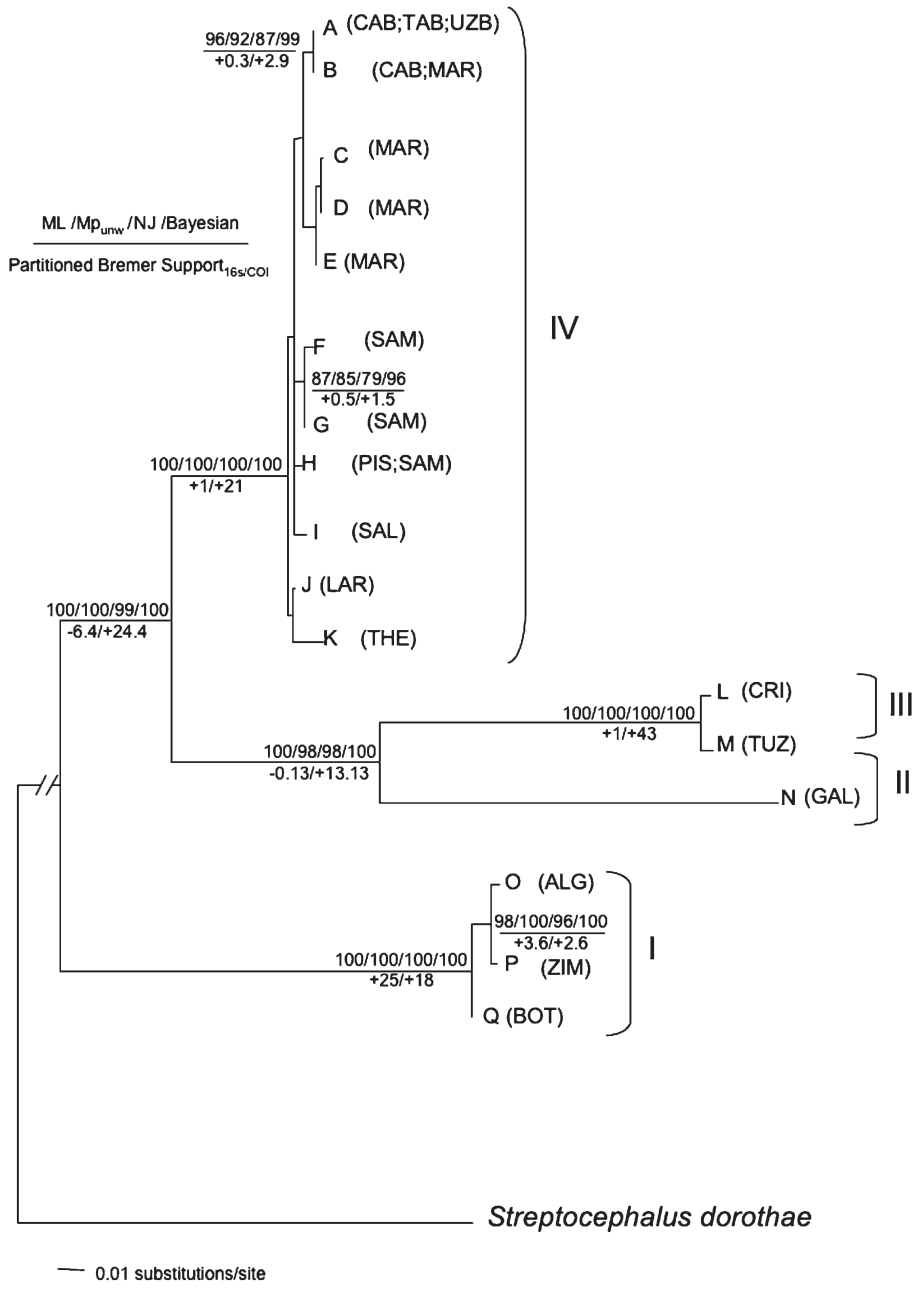

Figure 2. ML haplotype phylogram based on the GTR + Γ model (combined data set, variable rates, shape parameter $\alpha = 0.455$) as selected by MODELTEST. Numbers at nodes are the statistical supports for ML, MP, NJ and Bayesian searches (above branches) and Partitioned Bremer Support values for 16S and COI (below branches); only nodes with a statistical support $\geq 50\,\%$ are labeled. Clusters I, II, III, and IV are described in the text. Haplotype and population codes match those in Table 1 and figure 1. Branch lengths are proportional to the amount of genetic divergence within *P.spinosa* while the branch leading to the outgroup (*S. dorothae*) has been bracketed.

from Crimea and Turkey. Second, we forced Cypriot, Greek, Crimean, and Turkish haplotypes (J, K, L, and M) to form a monophyletic cluster. The AU test rejected both these alternative hypotheses ($P < 0.001$).

Table 2 shows the pairwise ML genetic distances and F_{ST} values among the populations included in the study. ML distance values range between 0.001 (SAM vs. PIS) and 0.483 *S. dorothae* vs. GAL. The mean ML genetic distances among the four major clades identified in the phylogenetic analyses range from 0.256 ± 0.008 to 0.389 ± 0.007 (cluster II vs. IV and III vs. IV). The latter value is only slightly lower than that between the ingroup and the outgroup (0.394 ± 0.002). At the intra-group level, genetic divergence is low within clusters I, III and III ($0.009 \pm 0.005, 0.007$, and 0.006 ± 0.005, respectively).

Table 2. Estimates of genetic differentiation based on the combined 16S and COI data set among the populations and species included in the study. Below the diagonal are shown the ML genetic distance values (settings to calculate the distances are the same as in the ML searches). Above the diagonal are reported the pairwise F_{ST} values as obtained using ARLEQUIN 3.01. Bold values indicate significance at the 0.05 level and below (significance assessed by 1,000 permutations).

Pop.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1-BOT	–	**1.000**	**1.000**	**1.000**	0.500	0.266	0.200	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**
2-ZIM	0.009	–	**1.000**	**1.000**	0.500	0.266	0.200	**1.000**	0.266	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**
3-ALG	0.011	0.005	–	**1.000**	**0.839**	**0.680**	**0.649**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**
4-GAL	0.333	0.346	0.349	–	0.500	0.266	0.200	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**
5-CAB	0.251	0.258	0.264	0.278	–	**0.365**	**0.299**	**0.781**	**0.781**	0.500	**0.641**	**0.641**	**0.707**	0.000	0.000
6-SAM	0.246	0.256	0.262	0.276	0.005	–	**0.233**	**0.526**	**0.603**	0.266	**0.445**	**0.445**	**0.518**	0.266	**0.518**
7-MAR	0.246	0.254	0.259	0.281	0.004	0.003	–	**0.568**	**0.568**	0.200	**0.400**	**0.400**	**0.478**	0.200	**0.478**
8-PIS	0.243	0.254	0.259	0.274	0.004	0.001	0.002	–	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**
9-SAL	0.248	0.258	0.264	0.286	0.008	0.005	0.006	0.004	–	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**
10-THE	0.264	0.275	0.281	0.307	0.017	0.015	0.017	0.014	0.017	–	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**
11-TUZ	0.386	0.398	0.397	0.287	0.250	0.250	0.251	0.248	0.258	0.273	–	**1.000**	**1.000**	**1.000**	**1.000**
12-CRI	0.378	0.390	0.390	0.284	0.255	0.255	0.256	0.252	0.263	0.278	0.007	–	**1.000**	**1.000**	**1.000**
13-LAR	0.241	0.251	0.257	0.281	0.006	0.005	0.006	0.004	0.008	0.012	0.249	0.254	–	**1.000**	**1.000**
14-TAB	0.250	0.258	0.264	0.278	0.000	0.005	0.004	0.004	0.008	0.017	0.250	0.255	0.006	–	0.000
15-UZB	0.251	0.258	0.264	0.278	0.000	0.005	0.004	0.004	0.008	0.017	0.250	0.255	0.006	0.000	–
S.dorothae	0.301	0.325	0.323	0.483	0.391	0.384	0.391	0.387	0.399	0.420	0.473	0.473	0.385	0.391	0.391

Table 3. Nested clade analysis of the geographical distribution of *P.spinosa* haplotypes. Only the five clades with significant GEODIS results are shown (see *P* value of the χ^2 test; third column). Clade names and nesting design are as in figure 3; the geographical distribution of clades is also shown. The last two columns show the sequence followed in the inference key (version 11 November 2005) and the resulting biological inference.

Clade	Distribution	*P*	Inference key sequence	Biological inference
3–2	CAB, MAR, TAB, UZB	0.0138	1,2 tip-interior status cannot be determined	Inconclusive outcome
3–5	BOT, ZIM, ALG	0.0060	1,19, no	Allopatric fragmentation
4–1	IV	0.0000	1,2,3,4, no	Restricted gene flow with isolation by distance
4–2	II-III	0.0360	1,19,20,2 tip-interior status cannot be determined	Inconclusive outcome
Total	I-II-III-IV	0.0000	1,2 tip-interior status cannot be determined	Inconclusive outcome

Nested Clade Analysis and AMOVA

Figure 3 illustrates the network as obtained with TCS (Clement et al., 2000) and the nesting design with four nested levels.

The pattern of relationships in the network is highly congruent with that depicted by the phylogenetic tree of figure 2. There are four major groups, which are not connected to each other under a 95 % statistical parsimony criterion and which correspond to the four clusters (I, II, III and IV) of figure 2. The sub-network in the left side of figure 3 perfectly matches cluster III in the ML tree of figure 2. This group includes by far the highest number of haplotypes (11 out of a total of 17). There is a clear structuring by geographic origin of samples in this part of the network, if we exclude the co-occurrence of haplotype A in French, Iranian and Uzbekistan locations. There are two main groups of Sardinian haplotypes (B, H, F, G and C, D, E respectively) with haplotypes C, D, and E being limited to a single population (MAR). Haplotypes from continental Greece and Cyprus (K and J) and from Apulia (I) are placed on independent branches, which are connected through many missing haplotypes to the remaining part of the network. The nesting procedure produced nine clades that could be tested for geographical association and of these five resulted in significant permutational contingency tests indicating non-random geographical distribution of haplotypes (Table 3).

The inferences derived from Templeton's keys suggested allopatric fragmentation for clade 3–5 and restricted gene flow with isolation by distance for clade 4–1. In three circumstances (3–2, 4–2, and for the total cladogram) the inference keys gave inconclusive outcomes because of the unresolved status of tip-interior position of the clades. We therefore carried out the analysis suggested by Templeton (2001); the results of this analysis are shown in figure 4.

The analysis was carried out only for those populations (MAR, SAM, and CAB) that showed genetic variation (if all haplotypes at a site are of the same type, the corresponding pairwise location distances is zero). In all cases as one moves from haplotypes to 4-step clades, the average pairwise distances go to zero. This suggests that haplotypes found in a single geographical site tend to be closely related evolutionarily. This also means that isolation

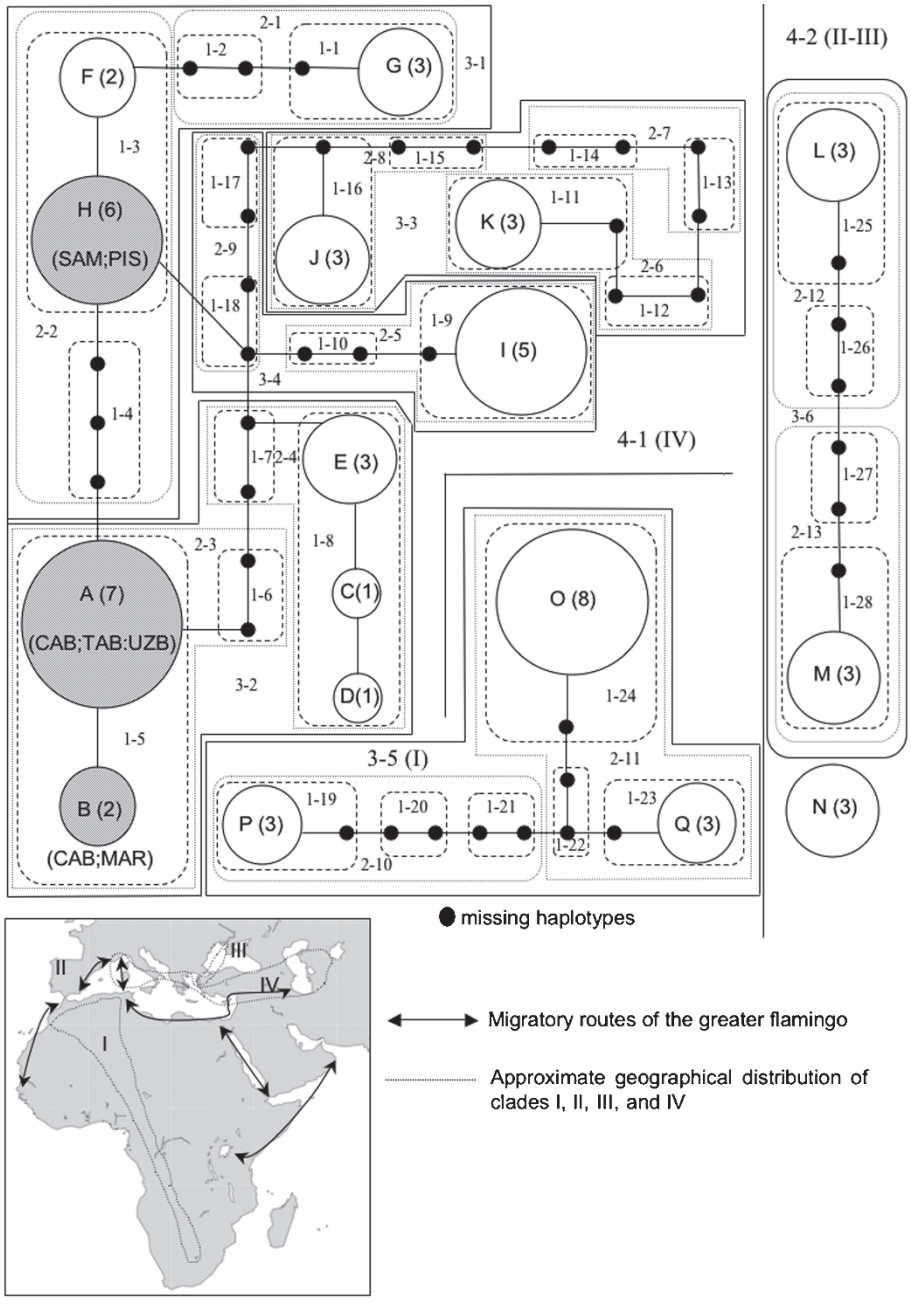

Figure 3. Haplotype networks derived from 928 bp of mtDNA (16S and COI). The relative size of the circles is proportional to the number of individuals carrying that particular haplotype; shadings identify haplotypes in common among more than one population. Letters identify haplotypes as in Table 1; numbers indicate how many individuals carried that particular haplotype. Black dots are missing haplotypes. The figure also shows the nesting used to infer the underlying population processes. Informative clades are designed as *x-y* where *x* represents the nesting level (from the haplotype level to the 4th level) and *y* is the number assigned to that particular clade. I, II, III, and IV correspond to clusters I, II, III and IV in figure 2. The approximate geographical distribution of these four clades is shown in the insert in the lower left corner. Double-arrows depict the migratory routes of the greater flamingo (re-drawn from http://www.unep-aewa.org).

by distance is the most appropriate process to explain the geographical distribution of haplotypes.

The hierarchical analysis of molecular variance (AMOVA) is reported in Table 4. This shows that most of the genetic heterogeneity is apportioned among populations within groups.

These results, along with those on pairwise F_{ST} analysis reported in Table 2, are indicative of a high level of genetic differentiation. The vast majority of the pairwise F_{ST} values are around 40 % or higher. F_{ST} is often 1.000, which indicates complete genetic differentiation between pairs of populations. About

Table 4. Results from the analysis of molecular variance (AMOVA). Populations have been pooled into the four groups (I, II, III, and IV) identified by both phylogenetic and nested clade analyses (see Figs. 2, 3).

Analysis structure	Source of variation	Sum of squares (d.f.)	Variance component	*P*	Fixation index	% of variation
By groups						
I, II, III, and IV	Among groups	4.59 (3)	Va = 0.046	0.005	$\Phi_{CT} = 0.089$	8.94
	Among populations	11.762 (10)	Vb = 0.326	<0.001	$\Phi_{SC} = 0.722$	63.32
	Within populations	4.583 (32)	Vc = 0.143	<0.001	$\Phi_{ST} = 0.722$	27.74

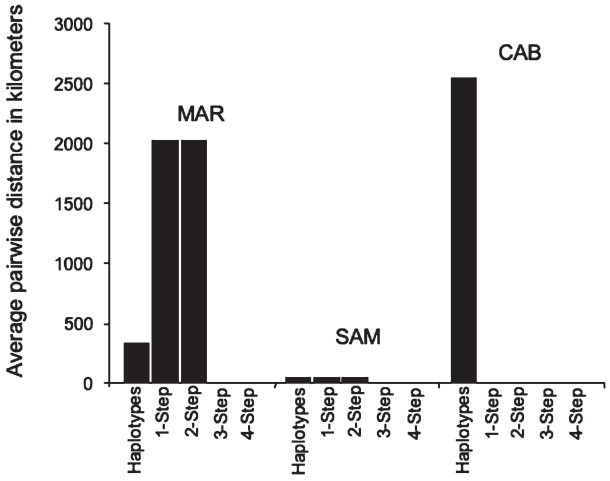

Figure 4. Average pairwise distances for haplotypes and clades (from the 1st to the 4th step clade level) on the three sampling sites that have more than one haplotype. The bars indicate the average pairwise geographical distance between the geographical centers of haplotypes and clades found at each location calculated from the corresponding coordinates given in the GEODIS output.

83 % of the F_{ST} values (87 out of 105) are significant at the 0.05 level.

Discussion

Molecular systematics

Our data revealed a remarkable degree of genetic divergence among the four clades into which the analyzed populations are grouped (see Figs. 2, 3), with sequence divergence values ranging from 25 % to 38 % for the combined data set (ranges of variation for COI and 16S alone were 17–29.2 % and 16.1–16.5 %, respectively). These values are exceptionally high and well within the range of variation reported for interspecific comparisons in anostracans. Remigio et al. (2001) reported an average sequence divergence of 11–22.4 % for 16S and percentages generally higher than 15 % for the COI gene among species of the Australian genus *Parartemia.* Baxevanis et al. (2006) found a range of 0.8–34.7 % and of 0–2.4 % for inter- and intraspecific comparisons in *Artemia.* These estimates are based on the nuclear ITS1 gene and therefore direct comparisons to our mitochondrial data are difficult to interpret. However, the overall pattern of genetic divergence recovered by Baxevanis et al. (2006) is in substantial agreement with previous mtDNA data (Gajardo et al., 2004). It is important to emphasize that here we are limiting our comparisons only to those studies centered on halophilic taxa. The rationale behind this decision relies on evidence reported in Hebert et al. (2002); these authors demonstrated a significant acceleration of mutation rates in halophilic branchiopods in comparison to their freshwater counterparts. Consequently, comparing sequence divergence values across ecologically different categories is not reliable, as it could lead to seriously biased conclusions.

Even taking into consideration halophilic taxa only and considering that our data are based exclusively on mitochondrial markers, the values of genetic divergence we obtained in this study strongly suggest the presence of multiple independent lineages in *P. spinosa.* These results, taken altogether, prompt the question of whether a re-evaluation of the species systematics is needed. This was not completely unexpected, given that anostracan specialists have traditionally had notable difficulties in finding the appropriate morphological characters on which grounding a reliable taxonomy. Anostracans seem to be affected by extensive convergent evolution, which makes taxonomically informative morphological characters difficult to pinpoint (Daniels et al., 2004; Remigio et al., 2003; Rogers, 2003; 2006). Following DeSalle et al. (2005) we can tentatively delineate at least two new taxa (corresponding to clades I and II) based on geographical and molecular grounds. The partially overlapping distribution of clades III and IV renders their delineation more problematic. Our sampling covers most of the known range of *P. spinosa* and the scattered distribution of the sampled populations accurately reflects the available knowledge on the distribution of the species (Rogers, 2003). We therefore consider as unlikely that the phylogeographic breaks we have identified would turn out as extremes of a more smooth clinal variation by simply increasing the number of studied populations, should such geographically intermediate populations remain to be discovered.

It is evident that our mitochondrial data need to be combined with additional sequencing of nuclear

genes, in order to detect any possible gene flow events among the maternal lineages we have described here. Nevertheless, given the large degree of mitochondrial divergence we have found, we suspect that the overall pattern would not change considerably after the inclusion of such nuclear markers. Even with these limitations, we do believe that this study could represent a valuable starting point for future efforts aimed to evaluate the systematics of the species in deeper details.

Phylogeography

Our phylogeographic analysis of mtDNA variation in *P.spinosa* suggests that historical processes are much more important than gene flow in this species. The large genetic distance among clade I and the others and its disjunct geographic distribution fit well with the allopatric fragmentation hypothesis yielded by the GEODIS analysis. The phylogeographic pattern of clade IV might be explained in terms of restricted gene flow and isolation by distance, these two processes having been the major forces that cast the observed pattern of genetic heterogeneity. The simultaneous occurrence of haplotype A in geographically remote populations is the most difficult result pertaining this clade to interpret. Theoretically, anostracans should be well adapted for long distance dispersal, due to the presence of diapausing cysts, which can withstand prolonged heat and drought stresses. The cysts are usually characterized by a complex ornamentation on their surface, with the presence of ridges, spines and bulges (Mura, 1992; Mura et al., 2002). The utility of this ornamentation as a reliable character for the taxonomy of the group has been largely debated (Mura, 2001 and references therein) and a general consensus on this point has still to be reached. This ornamentation is thought to enhance the hitchhiking ability of resting eggs by increasing their exposure to avian vectors. Passive transport is apparently the only means anostracans have to achieve long distance dispersal (Bohonak and Whiteman, 1999; Wissinger et al., 1999). Notwithstanding, current views on the effectiveness of passive transport are contradictory. Bohonak and Whiteman (1999) demonstrated that salamanders most probably disperse thousands of eggs of the fairy shrimp *Branchinecta coloradensis* across nearby ponds. Brendonck and Riddoch (1999) provided evidence for a wind-mediated dispersal of resting eggs of the African fairy shrimp *Branchipodopsis wolfi* only over short distances. Thus these authors cautioned against overestimating the importance of wind dispersal for population genetic processes. Green et al. (2005) had monitored movements of *Artemia* cysts dispersed by shorebirds along Portuguese and Spanish coasts, showing how birds can transported viable cysts over more than 1000 km. Conversely, Charalambidou and Santamaria (2002) stressed how probability of avian long distance dispersal of resting eggs drops dramatically at distances over 60–80 km in the spiny water flea *Bythotrephes longimanus*. In our case, the occurrence of the same haplotype over a wide geographical scale suggests that some (occasional?) long distance dispersal events must have taken place sometime in the past among the French, Iranian and Uzbekistan populations, the frequency of which is difficult to assess. However, it is worth noting that since we have analyzed only 10 individuals from these populations (nine of which carrying haplotype A) the probability to sample the same haplotype by pure random chance would have been negligible if the frequency of the haplotype itself was extremely low. Interestingly, the migratory routes of the greater flamingo, a species bound to shallow lagoons and salt lakes, almost perfectly overlap with the distribution of haplotype A. According to what published in the African Eurasian Waterbird Agreement (AEWA) website (http://www.unep-aewa.org) this species regularly migrates between Southern France and Tunisia and again from Tunisia to feeding and brooding sites around the Caspian Sea (Fig. 3). This migratory route apparently includes also Cyprus, where we have found a different haplotype (J), yet still belonging to the same clade IV as haplotype A. The fact that the greater flamingo is known for feeding on brine shrimps and their cysts (MacDonald, 1980), is a further support for the scenario proposed above. In addition, there are evidences suggesting that the larger the bird body sizes the longer the retention of cysts in the gut and, consequently, the distance over which they might be transported (Green et al., 2005).

The results of the AMOVA and pairwise F_{ST} analyses indicate that the statistically significant differences among populations account for most of the genetic heterogeneity in the data, this being in large part shaped by the geographical distance among populations, as attested by the visual inspection of the average pairwise geographical distances between the geographical centers of haplotypes and clades (Fig. 4). This leads us to conclude that *P. spinosa* is able to maintain genetic connectedness among populations only at a very local geographical scale, with the single notable exception of haplotype A. Our results are in agreement with the vast majority of population genetic studies conducted so far on anostracans, which have inevitably revealed a lack of ongoing gene flow among populations (see Introduction and the previous section on molecular systematics for appropriate references). These results point to the fact that the amount of movements of individuals (cysts)

and their subsequent reproductive success, although occurring to some extent, are not frequent enough to prevent local differentiation.

The overall pattern of genetic structuring of *P. spinosa* fits quite well into the Monopolization Hypothesis proposed by De Meester et al. (2002). Obviously, testing in details such a hypothesis is not feasible here, as it would have required a minute ecological study of each population to be coupled with our genetic analyses. Still, our results are in remarkable agreement with its general outline. They are also in line with previous findings on the water salt rotifer *Brachionus plicatilis* (Gómez et al., 2000; 2002; Suatoni et al., 2006) and therefore suggest that the Monopolization Hypothesis might be extended also to halophilic taxa inhabiting astatic waters.

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