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Spatially explicit genetic structure in the freshwater sponge *Ephydatia fluviatilis* (Linnaeus, 1759) within the framework of the monopolisation hypothesis

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ABSTRACT

An apparent paradox is known for crustaceans, rotifers and bryozoans living in inland small water bodies: a potential for wide distribution due to the presence of resting stages is coupled with marked genetic differences between nearby water bodies, with enclave distributions masking clear phylogeographic patterns. According to the monopolisation hypothesis, this is due to the accumulation of resting stages, monopolising each water body. Freshwater sponges could represent a useful system to assess the generality of the monopolisation hypothesis: these organisms i) live in the same habitats as crustaceans, rotifers and bryozoans, ii) produce resting stages that can accumulate, and iii) have indeed a wide distribution. Currently, no studies on spatially explicit genetic differentiation on freshwater sponges are available. The aim of the present study is to provide additional empirical evidence in support of the generality of the scenario for small aquatic animals with resting stages by analysing genetic diversity at different spatial scales for an additional model system, the freshwater sponge Ephydatia fluviatilis *(Linnaeus, 1759). We expected that system genetic variability would follow enclave distributions, no clear phylogeographical patterns would be present, and nearby unconnected water bodies would show markedly different populations for this new model too. We analysed the ribosomal internal transcribed spacer regions 5.8S-ITS2-28S, the D3 domain of 28S subunit, the mitochondrial Cytochrome c Oxidase I (COI) and ten specific microsatellite markers of nine Italian and one Hungarian populations. Mitochondrial and nuclear sequences showed no or very low genetic polymorphism, whereas high levels of differentiation among populations and a significant polymorphism were observed using microsatellites. Microsatellite loci also showed a high proportion of private alleles for each population and an overall correlation between geographic and genetic distances among populations. All the expectations from the monopolisation hypothesis seemingly were confirmed for the analysed sponge.*

Key words: Ephydatia fluviatilis*, monopolisation hypothesis, microsatellites, 5.8-ITS2-28S, Cytochrome c Oxidase I.*

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INTRODUCTION

Inland still water habitats host a peculiar set of small animals that are able to withstand desiccation through resting stages (Caceres, 1997; Wharton, 2002). These water bodies, such as small lakes, ponds, swamps and lagoons, desiccate occasionally or regularly and the small animals living there adopted different strategies to withstand the lack of water (Caceres, 1997). The resting stages, surviving desiccation for long time, may act as efficient propagules for dispersal (Artois *et al.,* 2011). Various crustaceans (De Gelas and De Meester, 2005; Muñoz and Pacios, 2010), rotifers (Mills *et al.,* 2007; Fontaneto *et al.,* 2008) and bryozoans (Hoare *et al.,* 2001; Massard and Gelmer, 2008) are indeed known to have species with wide distributions, but often with unclear phylogeographic patterns at the local scale, such as enclave distributions and strong genetic differences between nearby water bodies (De Meester *et al.,* 2002; Urban and De Meester, 2009).

The monopolisation hypothesis provides an explanation for the discrepancy between wide distribution and genetic differences among nearby water bodies in small animals living in inland small water bodies (De Meester *et al.,* 2002). On the one hand, the paradox can be reconciled by the small size and dispersal ability that allow small aquatic animals to attain widespread and even cosmopolitan distribution (Artois *et al.,* 2011). On the other hand, it can be reconciled by the parthenogenetic reproduction and the possibility to accumulate resting stages that *monopolise* the water body, producing the striking genetic differences between water bodies at the local scale (Urban and De Meester, 2009). The generality of this scenario has been proposed and studied using rotifers, crustaceans and bryozoans (De Meester *et al.,* 2002). The aim of the present paper is to provide additional empirical evidence in support of the generality of the scenario for small aquatic animals with resting stages by analysing genetic diversity at different spatial scales for an additional

model system: the freshwater sponge *Ephydatia (E.) fluviatilis* (Linnaeus, 1759).

Freshwater sponges could represent a useful system to analyse the generality of the monopolisation hypothesis, due to their similarities with rotifers, crustaceans and bryozoans. They live in inland water habitats that are prone to desiccation (Manconi and Pronzato, 2008), and have asexual reproduction (Maldonado and Riesgo, 2008) producing gemmules. Such asexually produced resting stages can act both as propagules for passive dispersal (Bilton *et al.,* 2001) and as a numerical buffer against new invaders by accumulating in the water body (Gaino *et al.,* 2003). Moreover, several freshwater sponges are known to have the potential for cosmopolitan distribution (Manconi and Pronzato, 2008). To our knowledge, no studies on the phylogeography and local genetic structure of sponges are available. The present manuscript fills this gap and discusses the results within the framework of the monopolisation hypothesis. In order to have a reliable picture of this animal's genetic variability, we analyse nuclear and mitochondrial markers and the allelic size variation of a set of ten microsatellite loci in 301 samples from ten populations: nine from Central Italy and one from lake Balaton, Hungary.

On the basis of current knowledge on small crustaceans, rotifers and bryozoans in inland waters, our main hypotheses for the genetic structure of the sponge are: i) low genetic variability is expected in each water body; ii) genetic differences between ponds are expected to be strong and not related to geographic distances at the local scale in Central Italy; iii) comparing Italy and Hungary, a high degree of exchange and genetic overlap between these populations is expected. Moreover, low overall variability in mitochondrial sequences is expected in sponges (Duran *et al.,* 2004; Dailianis *et al.,* 2011). Thus, the main scenario expected would encompass a lack of clear spatial patterns, but a strong local genetic structure with differences between nearby water bodies equal to or higher than those between distant water bodies.

METHODS

The model system

The freshwater sponge *E. fluviatilis*, family Spongillidae (Fig. 1), is the most common and widespread freshwater sponge in the world, mostly inhabiting temperate regions (Lopp *et al.,* 2007). It is a gonochoric species and performs both sexual and asexual reproduction, depending on environmental conditions (Gaino *et al.,* 2003, 2011).

Two hundred and sixty-seven samples of *E. fluviatilis* were collected in different areas of Umbria region in Central Italy from June 2006 to June 2008, by overturning rocks or drawing out reed stalks and scraping sponge tissues from the surface (Tab. 1). Thirty-four samples of *E. fluviatilis* collected in lake Balaton, Hungary, were generously provided by Dr. Müller Tamàs of Szent Istvan University, Godollo (Tab. 1). Samples were stored in sterile tubes with 70% ethanol at -20°C. Specimens were ob-

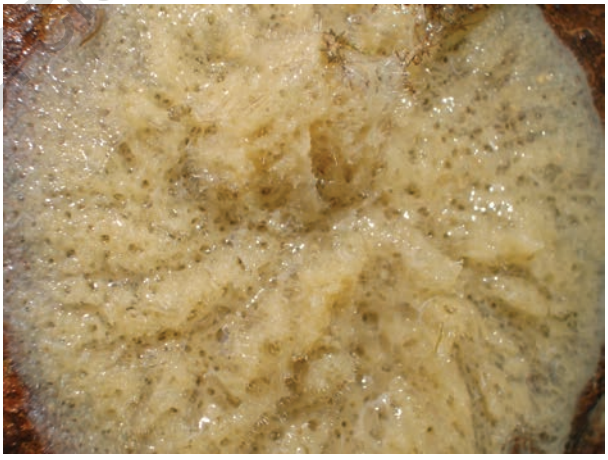

Fig. 1. A sample of the freshwater sponge *E. fluviatilis.*

Tab. 1. Sampling sites, populations, coordinates and sample numbers of *E. fluviatilis* collected in this research.

Sampling site	Population	Coordinates		Sample size number
		°N	°W	
Chiani stream	Chiani	42.42	12.06	31
Fersinone river	Fersinone	42.55	12.14	99
Lake Balaton (Hungary)	Balaton	46.50	17.44	34
Menotre river	Menotre	42.58	12.46	30
Piediluco lake	Piediluco	42.32	12.45	17
Tevere river (Attigliano)	Tevere AT	42.30	12.16	11
Tevere river (Città di Castello)	Tevere CC	43.27	12.14	26
Timia stream	Timia	42.59	12.35	8
Topino river	Topino	43.00	12.06	20
Trasimeno lake	Trasimeno	43.08	12.05	25

N, north; W, west; Tevere AT, Tevere (Attigliano); Tevere CC, Tevere (Città di Castello).

served under a stereomicroscope and cleaned, removing all other organisms hosted by the sponge, algae and superficial films.

DNA extraction and amplification

DNA was extracted from 10 mg tissues samples following a modified protocol (Lucentini *et al.,* 2006b) and using the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA). Extracted DNA was then stored at -20°C and qualitatively and quantitatively checked by means of a spectrophotometric assay at 260 nm and 280 nm wavelengths and of electrophoretic runs in agarose gel in presence of MassRuler™ DNA Ladder Mix (Fermentas, Hanover, MD, USA).

First, using a method based on the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach (Gigliarelli *et al.,* 2008), we confirmed the taxonomic identification of all the samples, in order to exclude apparent genetic variability belonging to a closely related and very similar species – *E. mülleri* (Lieberkühn, 1856). Then, only for samples unambiguously confirmed as *E. fluviatilis*, we proceeded with the genetic characterisation of rDNA, mtDNA and microsatellites.

We analysed the ribosomal internal transcribed spacer regions 5.8S-ITS2-28S, the D3 domain of 28S subunit, the mitochondrial Cytochrome c Oxidase subunit I (COI) and ten specific microsatellite markers. The 5.8S-ITS2-28S and D3/28S fragments were amplified as reported in Gigliarelli *et al*. (2008). For COI, we amplified two overlapping regions: one was the traditional Folmer region, amplified following Gigliarelli *et al*. (2008); the other was the I3-M11 partition (Erpenbeck *et al.,* 2006), obtained thanks to specifically designed primers. These were designed from the alignment of three complete poriferan COI sequences available from GenBank: *E. mülleri* NC010202, *Lubomirskia baicalensis* (Pallas, 1773) NC013760, and *Tethia actinia* (de Laubenfels, 1950) NC006991 (Lavrov *et al.,* 2008, Lavrov, 2010). The new primers, COI3FOR (5'- TAATATGAGAGCGCCCGGTA-3') and COI3REV (5'-ACAGCCCCCATTGATAGAACAT-3'), amplified a 651 bp segment that overlapped approximately for 200 bp with Folmer's 3' partition. For each fragment, amplicons (25 µL) were loaded and electrophoresed in 2% agarose gel together with the Gene Ruler 100 bp Plus DNA ladder. Bands were excised from gel and purified through Wizard SV Gel and PCR Clean-Up System (Promega) following manifacturer's instructions. Purified samples were labelled in both directions, purified through sepharose columns and sequenced through an Abi Prism 310 sequencer (Applera, Foster City, CA, USA). Chromatograms were analysed with Sequencing Analysis 5.2 (Applera) and sequences aligned with Mega 5.0 (Tamura *et al.,* 2011).

Ten microsatellite loci were analysed: Efl3, Efl4, Efl5, Efl7, Efl9, Efl10, Efl12, Efl14, Efl17 and Efl20 (Gigliarelli *et al.,* 2010). Loci were amplified using a 25 µL mix containing 2.5 µL 10X Reaction-Buffer (Euroclone S.p.A., Pero, Italy), 1 µL 5mM primer forward (Applera), 1 µL 5mM primer reverse (Applera), 1 µL 2.5mM dNTPs, 1.25 µL 50mM MgCl2, 0.06 µL 5u/ µL EuroTaq (Euroclone), 25ng DNA template and sdH2O up to the final volume. The amplification programme consisted in a first denaturation step of 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at primer s annealing temperature (Ta: 61°C or 63°C), 45 s at 72°C, and a longer final extension of 40 min at 72°C, in order to uniform polyadenilation of fragments. Amplicons were run in automatic sequencer Abi Prism 310 with GeneScan-500 LIZ Size Standard as dimensional marker (both from Applera), and obtained data were analysed through GeneMapper 5.0 (Applera). In order to evaluate and minimise genotyping errors, and to characterise allelic dropout or misprinting, all the experiments were replicated as suggested for non destructive samples (Hoffman and Amos, 2005). The experiments were also analysed, with Bonferroni's correction, through Microchecker 2.2.3 (Van Oosterhout *et al.,* 2004) by estimating null allele frequencies for each locus and comparing the obtained values with those calculated with the methods reported by Chakraborty *et al*. (1992) and Brookfield (1996). Microsatellite loci are informative when they are independent one another and do not produce redundant information: linkage disequilibrium (LD) was tested for each pair of loci within each population using Fisher's exact tests (Raymond and Rousset, 1995), with unbiased P values derived by a Markov chain method with the expectation that no such linkage should be found. The significance value for multiple significance tests was set using the sequential Bonferroni procedure (Rice, 1989). The P value of the exact test could be affected by sample size; thus, its effect was evaluated on the proportion of significant LD P values by drawing 80 random samples, 8 for each population, from the entire specimens set. Random samples were drawn without replacement and the proportion of significant LD P values was compared with those obtained for each population. LD analyses were conducted by means of Arlequin 3.5 (Excoffier and Lischer, 2010).

Hypothesis testing and statistical analyses

According to the monopolisation hypothesis, the set of scenarios we wanted to test regarded comparisons of genetic diversity within and between water bodies. Thus, for the three sets of sequence data, COI, 5.8S-ITS2-28S and D3/28S, we produced a matrix of uncorrected pairwise genetic distances among all individuals and obtained the average values between populations. For the microsatellite data, we focused only on differences among populations and, merging the full dataset of all 10 mi-

crosatellite loci, we obtained a matrix of Fst values among the 10 populations. Fst values were calculated using Arlequin 3.5 (Excoffier and Lischer, 2010) on the basis of presence/absence of alleles; Fst values among populations range from 0 – indicating complete similarity – to 1 – indicating complete dissimilarity.

In order to test our first hypothesis – that there is low genetic diversity within each water body, in contrast to high genetic diversity between water bodies – we performed an analysis of molecular variance, AMOVA, partitioning genetic variance within *vs* between water bodies. We performed the analysis for all the genetic data, either sequence data or microsatellite. We expectat to have the largest partition of genetic variance within each water body and not between them. AMOVA was performed using Arlequin 3.5 (Excoffier and Lischer, 2010) with 1000 permutations to assess the significance of the results. To provide further evidence on the independence among populations in different water bodies, we tested geographical grouping of populations through Spatial Analysis of MOlecular Variance (SAMOVA 1.0) (Dupanloup *et al.,* 2002). This method is based on a simulated annealing procedure that aims at maximising the proportion of total genetic variance due to differences among groups of populations in a spatially explicit scenario. The geographical grouping of populations was performed for the microsatellite data following the procedure based on the hierarchical analysis of ρ_{ST} and maximising the total genetic variance proportion between the minimum (two) and the maximum (nine) number of groups in the dataset (ρ_{CT}), as described by Lucentini *et al.* (2006a).

To test the hypothesis that genetic distances between water bodies are not related to geographic ones, we performed Mantel tests between matrices of genetic and geographic distances between water bodies (Borcard *et al.,* 2011). As a metric of genetic distances, we used Fst values from microsatellite polymorphism. To assess the effect of different spatial scales, we first performed the analyses by considering only the water bodies from Central Italy (within 110 km) and then by including also the sample from lake Balaton, Hungary (more than 600 km far away from the Italian samples). Mantel tests were performed in R 2.15.0 (R Development Core Team, 2012) and package *vegan* 2.0-3 (Oksanen *et al.,* 2012). Changes in correlation values between the analyses excluding and including lake Balaton and the degree of overlap on the genetic metrics of this population and the Italian ones are able to show if and how gene flow act over long distances.

In order to control for the reliability of our microsatellite dataset for the description of genetic variability in each population, we plotted the cumulative curves of the occurrence of alleles in each population. If the sample size for the analysis was appropriate, we expected to see a beginning of saturation; alternatively, if the number of analysed individuals was too low in comparison to the observed genetic diversity, the cumulative curves should still have been in the maximum growing part. An additional test for the reliability of the dataset was performed by calculating the expected number of alleles per population, given the observed distribution of the occurrence of the alleles. We used Chao estimates from incidence data, based on the proportion of alleles present only once or twice in the population (Colwell and Coddington, 1994) to calculate the expected number of alleles for each population, to be compared to the observed number. Expecting loci to be as variable as microsatellites meant having many unique alleles; thus, Chao estimates would surely be higher than the observed number. Nevertheless, we expected that the observed diversity should fall within the 95% confidence interval of the potential diversity, estimated through Chao. Cumulative curves and Chao estimates with their standard errors and confidence intervals were obtained in R, package *vegan*.

RESULTS

All the 301 samples were confirmed as *E. fluviatilis*, and no evidence of *E. mulleri* was found. Genetic variability among the 301 samples was confirmed to be low: both COI and D3/28S were monomorphic across all the dataset, and the alignments revealed no variable positions. Therefore, these loci were not included in the subsequent analyses. 5.8S-ITS2-28S showed a unique mutation in position 90 in the alignment, concerning the insertion of a thymine. This mutation was present in both homozygous (inserted/inserted and not inserted/not inserted) and in the heterozygous (not inserted/inserted) configuration. Both alleles (inserted and not inserted) were present in all the analysed populations (Tab. 2), thus only their relative proportion was variable between water bodies. Such proportion is not informative to establish genetic connectivity between water bodies. Also, this marker was not included in statistical analyses.

The microsatellite analysis revealed genetic polymor-

Tab. 2. Percentage of 5.8-ITS2-5.8S genotypes in the ten populations.

Population	3T/3T (%)	4T/4T (%)	3T/4T (%)
Balaton	0.00	0.00	100.0
Chiani	21.1	52.6	26.3
Fersinone	1.5	41.0	57.5
Menotre	0.00	100.0	0.0
Piediluco	0.00	81.2	18.8
Tevere AT	66.7	0.00	33.3
Tevere CC	10.0	40.0	50.0
Timia	0.00	50.0	50.0
Topino	9.00	45.5	45.5
Trasimeno	20.0	20.0	60.0

Tevere AT, Tevere (Attigliano); Tevere CC, Tevere (Città di Castello).

phism. Overall, 174 alleles were registered across all loci: the highest number of alleles (n=31) belonging to Efl5, and the lowest value (n=8) to Efl7. No evidence for scoring error due to stuttering or for large allele dropout was found (confidence interval=95%) neither significant linkage disequilibria. All microsatellite loci exhibited polymorphism among the 10 sponge populations with allelic richness per population ranging from 1 (Tevere Attigliano) to 8 (Timia), with an average of 4.74 across loci and populations (Supplementary Table). The mean number of alleles across loci ranged from 4.9 (Trasimeno and Timia populations) to 8.5 (Balaton). Private alleles were present in all the populations (Supplementary Table); the number of private alleles per population ranged from 1 (Chiani, locus Efl5) to 20 (Balaton, all loci with the exception of Efl7 and Efl17). There were private alleles in all loci except for Efl7. The number of private alleles per locus ranged from 2 (Efl10) to 9 (Efl5) (Supplementary Table).

The dataset on microsatellites can be considered reliable in itself (no linkage disequilibria), and also for a quantitative comparative analysis, as no evidence of undersampling was detected: the number of observed alleles for all the populations fell within the 95% confidence interval of Chao estimates, except for Tevere, where the observed number – 83 – is just below the minimum value of the confidence interval – 85.9 – (Tab. 3). Also, the cumulative curves showed that the number of alleles potentially not found in each population is not too high, given that all the curves are found in the part where the rate of increase is diminishing and approaching the asymptotic values (Supplementary Figure).

The AMOVA on microsatellite data performed for the entire group of populations showed a 20% of variance between individuals within populations, 15% among populations, and 65% among individuals. When using an additional hierarchy, *i.e.* dividing the Italian populations from the Hungarian one in lake Balaton, similar values were shown: 19% among individuals within populations, 12% among populations within groups, 8% between groups (Italy *vs* Hungary) and 61% due to variance among individuals (Tab. 4A and B).

Estimates of Fst (Tab. 5) were significantly different from zero (95% confidence interval) for all the pairwise population comparisons. The Mantel test between genetic and geographic distances was not significant when analysing only the nine populations from Central Italy (r=-0.04, P=0.53). The same test provided evidence of a significant and positive correlation when including lake Balaton (r=0.54, P=0.03).

The SAMOVA analysis assuming different numbers of groups of populations underlined that genetic differentiation among groups was slightly higher in the case of seven groups (ρCT=0.41, Tab. 6), but values were relatively similar also for six and eight groups. The only population that always resulted separated from the others is Balaton. On the contrary, two groups of populations seemed to be often present: Fersinone, Menotre and Topino on the one hand, and Tevere CC, Timia and Trasimeno on the other.

Tab. 3. Number of observed alleles in the microsatellite dataset, Chao estimates, and 95% confidence interval of the Chao estimates for each population.

Population	Observed	Chao	CI
Balaton	94	105.5	92.8-118.2
Chiani	88	96.6	86.3-106.9
Fersinone	88	109.1	88.0-130.3
Menotre	77	90.4	75.7-105.1
Piediluco	82	100.8	82.0-119.6
Tevere CC	83	140.8	85.9-195.7
Tevere AT	60	68.1	58.1-78.1
Timia	51	65.3	48.9-81.6
Topino	75	92.0	73.0-111.0
Trasimeno	45	59.5	41.4-77.5

CI, confidence interval; Tevere CC, Tevere (Città di Castello); Tevere AT, Tevere (Attigliano).

CONCLUSIONS

This work is the first research testing the population genetics within a spatially explicit framework in a freshwater sponge. The main results of our survey of genetic diversity in *E. fluviatilis* are: i) a very low genetic diversity is present in mtDNA and rDNA sequence data; ii) microsatellite polymorphism is present, identifying private alleles in all populations; iii) a geographic structure in the distribution of the microsatellite polymorphism is visible only at the largest scale we analysed.

Low variability in mtDNA and rDNA

The low variability in mtDNA and rDNA is a common feature in marine sponges (Pöppe *et al.,* 2010; Vargas *et al.,* 2012), and we here confirm the same scenario also for freshwater sponges. The analysed loci, COI, ITS2 and 28S are usually highly variable in other animals, and commonly used to study variability within and among populations and phylogeographic patterns (*e.g.* Stat *et al.,* 2011; Lucentini *et al.,* 2011a, 2011b). On the other hand, mitochondrial DNA sequences appear to be conserved in sponges and anthozoa, so that they do not provide adequate information to resolve intraspecific relationships (Shearer *et al.,* 2002; Wörheide *et al.,* 2002). Indeed, all the COI sequences we obtained from the 301 individuals show no mutation; they were all identical and, thus, did not allow any further investigation. Even the I3-M11 partition of COI (*Erpenbeck fragment*), which was analysed to overcome this issue, did not reveal any variability, although Erpenbeck *et al*. (2006) suggested it as signifi-

cantly more variable than the classical Folmer region, and Xavier *et al*. (2010) employed it to infer genetic variability in the poecilosclerid sponge *Phorbas fictitius* (Bowerbank, 1866). Hence, rDNA and mtDNA cannot be used to answer our main hypotheses within the framework of the monopolisation hypothesis (De Meester *et al.,* 2002).

Microsatellite polymorphism

The microsatellite loci proved to be reliable and useful data: a large number of private alleles for each population was present, as expected from the framework of the monopolisation hypothesis (De Meester *et al.,* 2002) based on analyses on rotifers, crustaceans and bryozoans. Nevertheless, the large number of private alleles in our study could be due to the confounding factor of a too large genetic variability, coupled with a limited within-population sample size. We can exclude this alternative explanation, as the observed number was within the 95% confidence interval of the Chao estimates for each population.

Another potential confounding factor, masking and/or

Tab. 4. Results for AMOVA analysis performed considering all the populations togheter (A) or divided into two groups (Italy *vs* Balaton) (B).

A

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among populations	9	235.4	0.57 Va	15.00
Among individual within populations	189	755.9	0.76 Vb	20.00
Within individuals	199	492.0	2.47 Vc	65.00
Total	397	1482. 3	3.80	
Fixation indices		P value (1023 permutations)		
F_{is}	0.235	0.00		
F_{it}	0.35	0.00		
F_{st}	0.15	0.00		

B

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among groups	1	65.2	0.31 Va	7.81
Among populations within groups	8	170.2	0.47 Vb	11.84
Among individuals within populations	189	754.8	0.76 Vc	18.91
Within individuals	199	492.0	2.47 Vd	61.44
Total	397	1.48	4.02	
Fixation indices		P value (1023 permutations)		
F_{is}	0.235	0.00		
F_{it}	0.386	0.00		
F_{sc}	0.13	0.00		
F_{ct}	0.07	0.10		

df, degree of freedom; Va, variance component a; Vb, variance component b; Vc, variance component c; Vd, variance component d.

Tab. 5. F_{st} per pair of populations using paiwise differences as distance methods (below the diagonal) and matrix of Slatkin linearised F_{st}s as $t/M=F_{st}/(1-F_{st})$ (above the diagonal).

$F_{st}/(1-F_{st})F_{st}$	Piediluco	Trasimeno	Fersinone	Topino	Tevere CC	Timia	Tevere AT	Chiani	Menotre	Balaton
Piediluco	-	0.08	0.22	0.19	0.17	0.15	0.16	0.16	0.20	0.22
Trasimeno	0.07	-	0.11	0.12	0.09	0.09	0.11	0.16	0.12	0.17
Fersinone	0.18	0.10	-	0.10	0.13	0.14	0.17	0.25	0.07	0.28
Topino	0.16	0.11	0.09	-	0.09	0.07	0.16	0.22	0.06	0.20
Tevere CC	0.14	0.08	0.12	0.08	-	0.06	0.20	0.25	0.12	0.19
Timia	0.13	0.08	0.12	0.06	0.05	-	0.17	0.27	0.12	0.22
Tevere AT	0.14	0.10	0.14	0.14	0.17	0.14	-	0.30	0.19	0.13
Chiani	0.14	0.14	0.20	0.18	0.20	0.21	0.16	-	0.20	0.29
Menotre	0.17	0.11	0.07	0.06	0.11	0.11	0.11	0.17	- 0.28	
Balaton	0.18	0.14	0.22	0.17	0.16	0.18	0.23	0.22	0.22	-

Tevere CC, Tevere (Città di Castello); Tevere AT, Tevere (Attigliano).

inflating the proportion of rare private alleles, can be due to the unbalanced proportion of heterozygotes: among all loci, F_{is} was significantly higher then zero and the average Ho per populations were lower than He (Supplementary Table), indicating a certain level of heterozygote deficiency. Furthermore, many loci did not result in a Hardy-Weinberg equilibrium, as highlighted from significance P values. Such heterozygote deficiency could be the result of technical and/or biological factors, such as the presence of non-amplifying alleles (*i.e.* null alleles) (Callen *et al.,* 1993) and the sessile nature of sponges.

Among technical factors, null alleles were detected in six loci in five populations. However, incorrect scoring of bands is unlikely, as the use of an automated sequencer with an internal standard allowed us a proper estimation of alleles' size with high resolution. High frequency of

Tab. 6. Results of SAMOVA analyses established trough 1000 permutations. ρSC defines the distribution of molecular variance among populations within groups; ρCT defines the same distribution among groups.

Groups	Group composition	F_{sc}	F_{ct}	ρSC	ρCT
2	(1) Balaton (2) Piediluco, Trasimeno, Fersinone, Topino, Timia, Tevere AT, Tevere CC, Chiani, Menotre	0.13	0.08	0.49***	0.30*
3	(1) Balaton (2) Chiani (3) Piediluco, Trasimeno, Fersinone, Topino, Timia, Tevere AT, Tevere CC, Menotre	0.11	0.09	0.40***	0.35**
4	(1) Balaton (2) Chiani (3) Piediluco (4) Trasimeno, Fersinone, Topino, Timia, Tevere AT, Tevere CC, Menotre	0.10	0.10	0.35***	0.38***
5	(1) Balaton (2) Chiani (3) Piediluco (4) Tevere AT (5) Trasimeno, Fersinone, Topino, Timia, Menotre, Tevere CC	0.09	0.10	0.31***	0.39*
6	(1) Balaton (2) Chiani (3) Piediluco (4) Tevere AT (5) Fersinone, Topino, Menotre (6) Trasimeno, Timia, Tevere CC	0.07	0.10	0.24***	0.40***
7	(1) Balaton (2) Chiani (3) Piediluco (4) Tevere AT (5) Trasimeno (6) Tevere CC, Timia (7) Fersinone, Topino, Menotre	0.07	0.11	0.23***	0.41***
8	(1) Balaton (2) Chiani (3) Piediluco (4) Tevere AT (5) Trasimeno (6) Topino (7) Tevere CC, Timia (8) Fersinone, Menotre	0.06	0.10	0.21***	0.40***
9	(1) Balaton (2) Chiani (3) Piediluco (4) Tevere AT (5) Trasimeno (6) Topino (7) Menotre (8) Fersinone (9) Tevere CC, Timia	0.10	0.08	0.31***	0.37**

****, P<0.01; **, P<0.05; *, P>0.05; Tevere CC, Tevere (Città di Castello); Tevere AT, Tevere (Attigliano).*

null alleles has been reported for several species of mollusks (Hedgecock *et al.,* 2004; Panova *et al.,* 2008; Wang *et al.,* 2010) and nematodes (Grillo *et al.,* 2007; Redman *et al.,* 2008) as a result of polymorphism in primer annealing sites. It is likely that a similar situation could occur also in sponges, contributing to heterozygote deficiency.

As for the biological factor, sponges are sedentary and once they have colonised a solid surface, they spent all their lives anchored to that substratum (Hooper, 2000). Ciliated larvae can contribute to a modest dispersal (Wielspütz and Saller, 1988) that likely leads to non-random mating in a certain area and to subpopulation structuring with a consequent Wahlund effect. Moreover, fragments of sponge may be passively moved over short distances by insects, especially on their larval cases (Corallini and Gaino*,* 2003). In addition, *E. fluviatilis* is prone to adopt mainly asexual reproduction trough gemmule formation (Gaino *et al.,* 2003). Therefore, many individuals are possibly clones generated from the same sponge, and genetically identical. Genetic variability could depend mainly from casual genetic drift instead of recombination events. Thus, a low level of excess of homozygotes across all loci and populations can be expected in sponges, and the high number of private alleles is not due to technical bias, but is a proper biological feature of the analysed sponge.

Spatially explicit genetic structure

Differences in the allelic composition in the microsatellite dataset among populations in Central Italy were not related to geographic distances. This means that at the analysed spatial scale – within about 100 km – no limiting barriers seem to be present for dispersal in sponges: part of the variability is due to private alleles, but the shared variability between populations is not related to geographic distances. When including lake Balaton in the analysis, a significant correlation between geographic and genetic distances could be found, showing that this population is significantly different from the Italian ones. Also, SAMOVA always divided the population in lake Balaton from all the Italian ones. Given the small number of analysed populations (only ten) we cannot infer whether the differences between Italy and Hungary can be ascribed to geographic distances, or simply to ecological differences. Still, even if the process driving such differences cannot be understood at the moment, the pattern is clear.

Such correlation between geographic and genetic distances is known also for the organisms on which the monopolisation hypothesis was based. Indeed, in both rotifers and crustaceans, a spatial correlation is present (De Gelas and De Meester, 2005; Mills *et al.,* 2007; Campillo *et al.,* 2011). Thus, sponges seem to be very similar to crustaceans and rotifers in the spatial structure of their genetic composition, and may be incorporated in future studies testing the assumptions of the monopolisation hypothesis.

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REFERENCES

Artois T, Fontaneto D, Hummon WD, McInnes SJ, Todaro MA, Sørensen MV, Zullini A, 2011. Ubiquity of microscopic animals? Evidence from the morphological approach in species identification, p. 244-283. In: D. Fontaneto (ed.), Biogeography of microscopic organisms, is everything small everywhere? Cambridge University Press, Cambridge.

Bilton DT, Freeland JR, Okamura B, 2001. Dispersal in freshwater invertebrates. Annu. Rev. Ecol. Syst. 32:159-181.

Borcard D, Gillet F, Legendre P, 2011. Numerical Ecology with R. Use R! Springer, New York: 306 pp.

Brookfield JFY, 1996. A simple new method for estimating null allele frequency from heterozygote deficiency. Mol. Ecol. 5:4534-4555.

Caceres CE, 1997. Dormancy in invertebrates. Invertebr. Biol. 116:371-383.

Callen DF, Thompson AD, Shen Y, Phillips HA, Richards RI, Mulley JC, Sutherland GR, 1993. Incidence and origin of "null" alleles in the (AC)n microsatellite markers. Am. J. Hum. Genet. 52:922-927.

Campillo S, Serra M, Carmona MJ, Gómez A, 2011. Widespread secondary contact and new glacial refugia in the halophilic rotifer *Brachionus plicatilis* in the Iberian Peninsula. PLoS ONE 6:e20986.

Chakraborty R, Kamboh MI, Nwankwo M, Ferrell RE, 1992. Caucasian genes in American blacks: new data. Am. J. Hum. Genet. 50:145-155.

Colwell RK, Coddington JA, 1994. Estimating terrestrial biodiversity through extrapolation. Philos. T.R. Soc. B 345:101-118.

Corallini C, Gaino E, 2003. The caddisfly *Ceraclea fulva* and the freshwater sponge *Ephydatia fluviatilis*: a successful relationship. Tissue Cell 35:1-7.

Dailianis T, Tsigenopoulos CS, Dounas C, Voultsiadou E, 2011. Genetic diversity of the imperilled bath sponge *Spongia officinalis* Linnaeus, 1759 across the Mediterranean Sea: patterns of population differentiation and implications for taxonomy and conservation. Mol. Ecol. 20:3757-3772.

De Gelas K, De Meester L, 2005. Phylogeography of *Daphnia magna* in Europe. Mol. Ecol. 14:753-764.

De Meester L, Gomez A, Okamura B, Schwenck K, 2002. The Monopolization Hypothesis and the dispersal-gene flow paradox in aquatic organisms. Acta Oecol. 23:121-135.

Dupanloup I, Schneider S, Excoffier L, 2002. A simulated annealing approach to define the genetic structure of populations. Mol. Ecol. 11:257-281.

Duran S, Pascual M, Turon X, 2004. Low levels of genetic variation in mtDNA sequences over the western Mediterranean and Atlantic range of the sponge *Crambe crambe* (Poeciloscleride). Mar. Biol. 144:31-35.

Erpenbeck D, Hooper JNA, Wörheide G, 2006. CO1 phylogenies in diploblasts and the 'Barcoding of Life' - are we sequencing a suboptimal partition? Mol. Ecol. Notes 6:550-553.

Excoffier L, Lischer HEL, 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol. Ecol. Resour. 10:564-567.

Fontaneto D, Barraclough TG, Chen K, Ricci C, Herniou EA, 2008. Molecular evidence for broad-scale distributions in bdelloid rotifers: everything is not everywhere but most things are very widespread. Mol. Ecol. 17:3136-3146.

Gaino E, Rebora M, Corallini C, Lancioni T, 2003. The life-cycle of the sponge *Ephydatia fluviatilis* (L.) living on the reed *Phragmites australis* in an artificially regulated lake. Hydrobiologia 495:127-142.

Gaino E, Scoccia F, Piersanti S, Rebora M, Bellucci LG, Ludovisi A, 2011. Spicule records of *Ephydatia fluviatilis* as a proxy for hydrological and environmental changes in the shallow Lake Trasimeno (Umbria, Italy). Hydrobiologia 679:139-153.

Gigliarelli L, Lucentini L, Palomba A, Puletti ME, Lanfaloni L, Bruford MW, Panara F, 2010. Isolation of microsatellite markers in the freshwater sponge *Ephydatia fluviatilis* (Porifera: Spongillidae) and cross-priming testing within the Demospongiae class. Mol. Ecol. Resour. 10:576-579.

Gigliarelli L, Lucentini L, Palomba A, Sgaravizzi G, Lancioni H, Lanfaloni L, Willenz P, Gaino E, Panara F, 2008. Applications of PCR-RFLPs for differentiating two freshwater sponges: *Ephydatia fluviatilis* and *Ephydatia mülleri*. Hydrobiologia 605:265-269.

Grillo V, Jackson F, Cabaret J, Gilleard JS, 2007. Population genetic analysis of the ovine parasitic nematode *Teladorsagia circumcincta* and evidence for a cryptic species. Int. J. Parasitol. 37:435-447.

Hedgecock D, Li G, Hubert S, Bucklin K, Ribes V, 2004. Widespread null alleles and poor cross-species amplification of microsatellite DNA loci cloned from the Pacific oyster, *Crassostrea gigas*. J. Shellfish Res. 2:379-385.

Hoare K, Goldson AJ, Giannasi N, Hughes RN, 2001. Molecular phylogeography of the cosmopolitan bryozoan *Celleporella hyalina*: cryptic speciation? Mol. Phylogenet. Evol. 18:488-492.

Hoffman J, Amos W, 2005. Microsatellite genotyping errors: detection approaches common sources and consequences for paternal exclusion. Mol. Ecol. 14:599-612.

Hooper JNA, 2000. 'Sponguide'. Guide to sponge collection and identification. Queensland Museum ed., Brisbane. Available from: http://www.qmuseum.qld.gov.au/organisation/sections/SessileMarineInvertebrates/index.asp

Lavrov DV, 2010. Rapid proliferation of repetitive palindromic elements in mtDNA of the endemic baikalian Sponge *Lubomirskia baicalensis*. Mol. Biol. Evol. 27:757-760.

Lavrov DV, Wanga X, Kelly M, 2008. Reconstructing ordinal relationships in the Demospongiae using mitochondrial genomic data. Mol. Phylogenet. Evol. 49:111-124.

Lopp A, Reintamm T, Vallmann K, Päri M, Mikli V, Richelle-Maurer E, Kelve M, 2007. Molecular identification, characterization and distribution of freshwater sponges (Porifera: Spongillidae) in Estonia. Fund. Appl. Limnol. 168:93-103.

Lucentini L, Palomba A, Gigliarelli L, Lancioni H, Natali M, Panara F. 2006a. Microsatellite polymorphism in Italian populations of northern pike (*Esox lucius* L.). Fish. Res. 80:251-262.

Lucentini L, Palomba A, Lancioni H, Natali M, Panara F. 2006b. A nondestructive, rapid, reliable and inexpensive method to sample, store and extract high-quality DNA from fish body mucus and buccal cells. Mol. Ecol Notes 6:257-260.

Lucentini L, Puletti ME, Ricciolini C, Gigliarelli L, Fontaneto D, Lanfaloni L, Bilò F, Natali M, Panara F, 2011a. Molecular and phenotipic evidence of a new species of genus *Esox* (Esocidae, Esociformes, Actinopterygii): the Southern Pike, *Esox flaviae*. PLoS ONE 6:e25218.

Lucentini L, Rebora M, Puletti ME, Gigliarelli L, Fontaneto D, Gaino E, Panara F 2011b. Geographical and seasonal evidence of cryptic diversity in the *Baetis rhodani* complex (Ephemeroptera, Baetidae) revealed by means of DNA taxonomy. Hydrobiologia 673:215-228.

Maldonado M, Riesgo A, 2008. Reproduction in the Phylum Porifera: a synoptic overview. Treballs de la Societat Catalana de Biologia 59:29-49.

Manconi R, Pronzato R, 2008. Global diversity of sponges (Porifera: Spongillina) in freshwater. Hydrobiologia 595:27-33.

Massard JA, Galmer G, 2008. Global diversity of bryozoans (Bryozoa or Ectoprocta) in freshwater. Hydrobiologia 595:93-99.

Mills S, Lunt DH, Gomez A, 2007. Global isolation by distance despite strong regional phylogeography in a small metazoan. BMC Evol. Biol. 7:225.

Muñoz J, Pacios F, 2010. Global biodiversity and geographical distribution of diapausing aquatic invertebrates: the case of the cosmopolitan brine shrimp, *Artemia* (Branchiopoda, Anostraca). Crustaceana 83:465-444.

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Wagner H, 2012. Vegan: Community Ecology Package. R package version 2.0-3. R Foundation for Statistical Computing ed., Vienna, Austria. Available from: http://CRAN.R-project.org/package=vegan

Panova M, Mäkinen T, Fokin M, André C, Johannesson K, 2008. Microsatellite cross-species amplification in the genus *Littorina* and detection of null alleles in *Littorina saxatilis*. J. Mollus. Stud. 74:111-117.

Pöppe J, Sutcliffe P, Hooper JNA, Wörheide G, Erpenbeck, D, 2010. CO I barcoding reveals new clades and radiation patterns of indo-pacific sponges of the family Irciniidae (Demospongiae: Dictyoceratida). PLoS ONE 5:e9950.

R Development Core Team 2012. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing ed., Vienna, Austria. Available from: http://www.R-project.org

Raymond M, Rousset F, 1995. Genepop (version 1.2), population genetics software for exact tests and ecumenicism. J. Hered. 86:248-249.

Redman E, Grillo V, Saunders G, Packard E, Jackson F, Berriman M, Gilleard JS, 2008. Genetics of mating and sex determination in the parasitic nematode *Haemonchus contortus*. Genetics 180:1877-1887.

Rice WR, 1989. Analyzing tables of statistical tests. Evolution 43:223-225.

Shearer TL, Van Oppen MJH, Romano SL, Wörheide G, 2002. Slow mitochondrial DNA sequence evolution in the Anthozoa (Cnidaria). Mol. Ecol. 11:2475-2487.

Stat M, Bird CE, Pochon X, Chasqui L, Chauka LJ, Concepcion GT, Logan D, Takabayashi M, Toonen RJ, Gates RD, 2011.

Variation in *Symbiodinium* ITS2 sequence assemblages among coral colonies. PLoS ONE 6:e15854.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S, 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28:2731-2739.

Urban MC, De Meester L, 2009. Community monopolization: local adaptation enhances priority effects in an evolving metacommunity. P. Roy. Soc. Lond. B Bio. 276:4129-4138.

Van Oosterhout C, Hutchinson WF, Willis DPM, Shipley P, 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol. Ecol. Notes 4:535-538.

Vargas S, Schuster A, Sacher K, Büttner G, Schätzle S, Läuchli B, Hall K, Hooper JNA, Erpenbeck D, Wörheide G, 2012. Barcoding sponges: an overview based on comprehensive sampling. PLoS ONE 7:e39345.

Wang, Y, Wang A, Guo X, 2010. Development and characterization of polymorphic microsatellite markers for the northern quahog *Mercenaria mercenaria* (Linnaeus, 1758). J. Shellfish Res. 29:77-82.

Wharton DA, 2002. Life at the limits. Organisms in extreme environments. Cambridge University Press, Cambridge.

Wielspütz C, Saller U, 1988. The metamorphosis of the parenchymula-larva of *Ephydatia fluviatilis* (Porifera, Spongillidae). Zoomorphology 109:173-177.

Wörheide G, Hooper JNA, Degnan BM, 2002. Phylogeography of western Pacific *Leucetta 'chagosensis'* (Porifera: Calcarea) from ribosomal DNA sequences: implications for population history and conservation of the Great Barrier Reef World Heritage Area (Australia). Mol. Ecol. 11:1753-1768.

Xavier JR, van Soest RWM, Breeuwer JAJ, Martins AMF, Menken SBJ, 2010. Phylogeography, genetic diversity and structure of the poecilosclerid sponge *Phorbas fictitius* at oceanic islands. Contrib. Zool. 79:119-129.