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Fluvial basin history in the northeastern Mediterranean region underlies dispersal and speciation patterns in the genus *Dugesia* (Platyhelminthes, Tricladida, Dugesiidae)

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ABSTRACT

In this study we analyzed the phylogenetic relationships of eastern Mediterranean freshwater planarians of the genus *Dugesia*, estimated divergence times for the various clades, and correlated their phylogeographic patterns with geological and paleoclimatic events, in order to discover which evolutionary processes have shaped the present-day distribution of these animals. Specimens were collected from freshwater courses and lakes in continental and insular Greece. Genetic divergences and phylogenetic relationships were inferred by using the mitochondrial gene subunit I of cytochrome oxidase (COI) and the nuclear ribosomal internal transcribed spacer-1 (ITS-1) from 74 newly collected individuals from Greece. Divergence time estimates were obtained under a Bayesian framework, using the COI sequences. Two alternative geological dates for the isolation of Crete from the mainland were tested as calibration points. A clear phylogeographic pattern was present for *Dugesia* lineages in the Eastern Mediterranean. Morphological data, combined with information on genetic divergences, revealed that eight out of the nine known species were represented in the samples, while additional new, and still undescribed species were detected. Divergence time analyses suggested that *Dugesia* species became isolated in Crete after the first geological isolation of the island, and that their present distribution in the Eastern Mediterranean has been shaped mainly by vicariant events but also by dispersal. During the Messinian salinity crisis these freshwater planarians apparently were not able to cross the sea barrier between Crete and the mainland, while they probably did disperse between islands in the Aegean Sea. Their dependence on freshwater to survive suggests the presence of contiguous freshwater bodies in those regions. Our results also suggest a major extinction of freshwater planarians on the Peloponnese at the end of the Pliocene, while about 2 Mya ago, when the current Mediterranean climate was established, these Peloponnese populations probably began to disperse again. At the end of the Pliocene or during the Pleistocene, mainland populations of *Dugesia* colonized the western coast, including the Ionian Islands, which were then part of the continent.

1. Introduction

The Mediterranean Basin has a long and complex geological history and is therefore generally considered as an excellent laboratory region to study the effect of paleogeographic events on the evolutionary diversification of fauna and flora. This has resulted in a wealth of studies that focus on the biogeography and evolution of taxa in the western or in the eastern Mediterranean (e.g. de Jong, 1998; Veith et al., 2004; Lázaro et al., 2009; Lymberakis and Poulakakis, 2010).

Especially the northeastern Mediterranean region is well suited for phylogeographic studies to unravel the historical processes that underlie present-day species distributions and current levels of diversity and endemism (Sfenthourakis and Legakis, 2001). This area has been subjected to tectonism, volcanism and sea level changes since the Miocene (Dermitzakis, 1990; Perissoratis and Conispoliatis, 2003), resulting in a complex geological history. The major events in the geological history of the Aegean area are relatively well known. The Aegean archipelago started to form c. 16 million years ago (Mya), when the single landmass Ägäis started to fragment (Dermitzakis, 1990) as a consequence of the collision of the African/Arabian tectonic plates with the Eurasian plate

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(Krijgsman, 2002). The opening of the mid-Aegean trench (MAT) started at c. 12 Mya when the sea invaded the land from south to north, starting between Crete and Kasos–Karpathos; at c. 9 Mya the previously uniform landmass became divided into an eastern and a western Aegean sections (Dermitzakis and Papanikolaou, 1981). At about 10 Mya Crete was the first island to become separated from the mainland (Dermitzakis, 1990; Cosentino et al., 2007). Apart from tectonic fragmentation events, the Hellenic area also experienced several sea level changes, such as during the Messinian salinity crisis (MSC) (5.96–5.33 Mya; Krijgsman et al., 1999) and during the Pleistocene glaciations (2.58 Mya–11.7 kya; Perissoratis and Conispoliatis, 2003), thus leading to contact between previously isolated landmasses and ancient river drainage systems or to the severance of single landmasses and river basins (Maurakis et al., 2001).

In the past few years an increasing number of studies have carried out historical biogeographic analyses on a wide range of organisms in this region, such as snails (e.g. Parmarkelis et al., 2005; Kornilios et al., 2009), arthropods (e.g. Poulakakis and Sfenthourakis, 2008; Papadopoulou et al., 2009; Parmakelis et al., 2006), reptiles (e.g. Kasapidis et al., 2005; Poulakakis et al., 2003, 2005), frogs (Akın et al., 2010), and plants (Bittkau and Comes, 2005). Most of these studies conclude that the evolutionary diversification of organisms in the northeastern Mediterranean has been driven by vicariance induced by geological and marine barriers. In general, the three divergence patterns proposed by Lymberakis and Poulakakis (2010) can be recognized among the taxa in this region: (1) species already present before breakup into several component areas, (2) species that reached the area after the formation of the MAT (after c. 9 Mya), and (3) much more recent, human-mediated arrivals. Nevertheless, differences in the organisms' biology and ability to disperse can result in different responses to the geological history of the area and, therefore, to differences in current patterns of distribution (Douris et al., 2007).

In this study we used freshwater planarians of the genus *Dugesia* Girard, 1850 as a model to examine the effect of the paleogeography of the Hellenic region on the evolutionary diversification of its component fauna. For this purpose, the genus *Dugesia* is an ideal model group, in view of the fact that (1) the Mediterranean region is a hotspot of biodiversity, with over 20 species from a world total of about 75 species, (2) freshwater planarians do not possess larval dispersal stages and do not tolerate salt water and thus need contiguous freshwater bodies to survive and disperse (Ball and Fernando, 1969; Ball, 1975). A recent study on Mediterranean *Dugesia* species revealed a clear correspondence between phylogenetic relationships and paleogeography (Lázaro et al., 2009). Unfortunately, virtual absence of planarian fossils prevents absolute dating of divergence times and neither did paleogeographic information facilitate calibration of a molecular clock, thus impeding precise dating of the phylogeographic patterns. Further, that study concentrated on species in the western Mediterranean, in contrast to our present focus on the eastern Mediterranean region.

For the present study we sampled numerous *Dugesia* populations distributed across the northeastern Mediterranean region, comprising populations from Greek islands as well as the mainland (Fig. 1). We generated a calibrated phylogenetic tree for these populations, with the aim to examine the effects of geological processes, paleoclimatic events, and anthropogenic dispersal on the historical diversification and current distribution of these planarians in this region. Furthermore, we also set out to examine the correlation between molecular and morphological markers in species determination.

2. Materials and methods

2.1. Sample collection

Dugesia specimens were collected from the type localities of eight Greek species (de Vries, 1984, 1988) and from other localities on the mainland and some islands during the spring seasons of 2009 and 2010. For each locality some specimens were fixed and preserved in absolute ethanol for molecular analysis. Other animals were fixed with Steinmann's fluid (cf. Sluys, 1989) for morphological analyses and were, subsequently, preserved in 70% ethanol. For information on sampling localities, see Table 1 and Fig. 1.

2.2. Morphological analysis

Specimens that had been preserved for morphological analysis were cleared in clove oil and then embedded in paraffin wax, sectioned at intervals of 6 or 8 μm (depending on the size of the animals) and mounted on albumen-coated slides. Sections were stained in Mallory-Cason/Heidenhain (Humason, 1967; Romeis, 1989) and mounted in DPX. Reconstructions of the copulatory complex were obtained by using a camera lucida attached to a compound microscope. All material has been deposited in the collections of the Netherlands Center for Biodiversity Naturalis, Leiden, Netherlands.

2.3. Sequencing procedure

Total genomic DNA extraction was performed on two individuals fixed in absolute alcohol per sample locality, using the commercial reagent DNAzol (Molecular Research Center Inc., Cincinnati, OH) following the manufacturer's instructions.

Specific primers were used to amplify a fragment of the mitochondrial gene cytochrome c oxidase subunit I (COI) and the nuclear ribosomal internal transcribed spacer-1 (ITS-1) sequences. Sequences and annealing temperatures for each pair of primers are given in Table 2. Final PCR reaction volume for all molecules was 25 μl. To 1 μl of DNA sample to amplify we added: (1) 5 μl of Promega 5× Buffer, (2) 1 μl of dNTP (10 mM), (3) 0.5 μl of each primer (25 μM), (4) 2 μl of $MgCl_2$ (25 mM), (5) 0.15 μl of *Taq* polymerase (GoTaq® Flexi DNA Polymerase of Promega). Double-distilled and autoclaved water was added to obtain the final PCR volume. In order to obtain amplification of the sequences it was necessary in many cases to vary the annealing temperatures or the amount of $MgCl_2$ or DNA.

PCR products were purified before sequencing using the purification kit illustra™ (GFX™ PCR DNA and Gel Band of GE Healthcare) or by using a vacuum system (MultiScreen™$_{HTS}$ Vacuum Manifold of Millipore). Sequencing reactions were performed by using Big-Dye (3.1, Applied Biosystems) with the same primers used to amplify the fragment, except for the forward COI sequence that was obtained with a more internal primer (COIEF3), due to sequencing problems when using BarT. Reactions were run on an automated sequencer ABI Prism 3730 (Unitat de Genòmica of Serveis Científico-Tècnics of the Universitat de Barcelona). Obtained chromatograms were visually checked.

2.4. Sequence alignment and genetic divergence

An approximate 750 bp fragment of the mitochondrial gene COI and an approximately 700 bp fragment of ITS-1 were sequenced. Additionally, sequences of other *Dugesia* species available in GenBank were retrieved (Table 1). Alignments of the sequences were obtained with the online software MAFFT version 6 (Katoh and

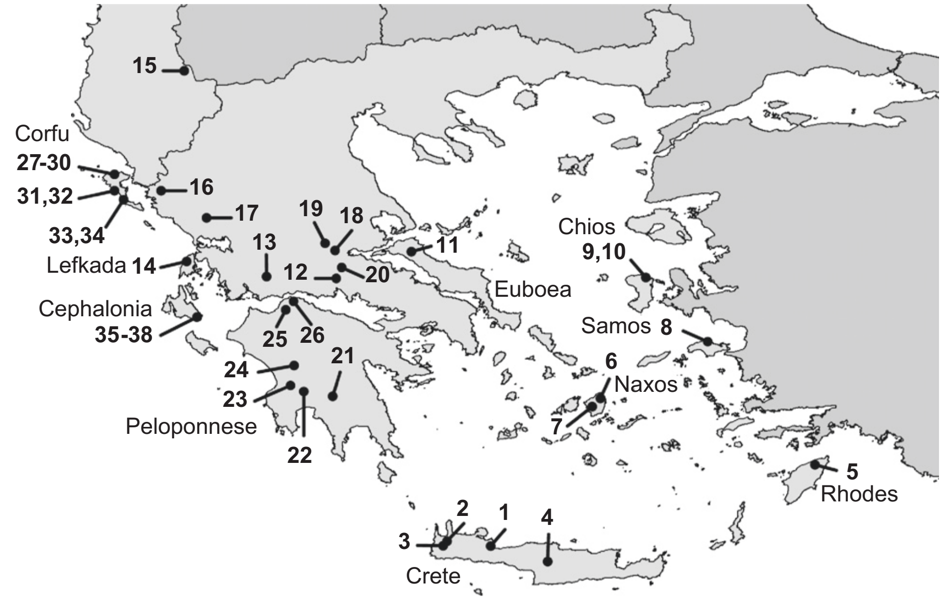

Fig. 1. *Dugesia* localities sampled in Greece; numbers correspond to the locality codes reported in Table 1. The Albanian population (15; cf. Lázaro et al., 2009) is also shown in the map.

Toh, 2008) and were manually edited with the software BioEdit (version 7.0 for PC) (Hall, 1999). Prior to analyses, the COI sequences were translated into amino acids showing no stop codons. Equivocal positions of ITS-1 alignment were removed with the software Gblocks (Talavera and Castresana, 2007), allowing half gap positions in the alignment. Genetic divergences among individuals were calculated with MEGA 5.0 computer package (Tamura et al., 2011) using the Kimura 2-parameters correction.

2.5. Phylogenetic analysis

Level of sequence saturation was analyzed by plotting observed transitions and transversions against the divergence for COI and ITS-1 under the TN93 nucleotide substitution pattern model with the program DAMBE (Xia and Xie, 2001). Three data sets were analyzed: ITS-1, COI, and an alignment with both molecules concatenated. Phylogenetic analyses were performed using two inference methods: Maximum Likelihood (ML) and Bayesian inference (BI). In all the likelihood and Bayesian analyses we set $GTR + I + \Gamma$ as evolutionary model, leaving the inference programs to estimate all the parameter values and hence the best model. In the analyses of the concatenated data set we set the parameters estimation as unlinked. ML analyses were performed with the program RaxML 7.0.0 (Stamatakis, 2006). 1000 replicates were calculated to obtain bootstrap supports. BI was conducted using the program MrBayes (v. 3.2: Ronquist et al., 2012). Given the high number of terminals we ran 1 cold and 4 heated chains for two runs to ensure a better sampling in the tree space. 1,000,000 generations were performed for each gene, saving a tree every 100 generations. The convergence of the topologies and model parameters of both runs was surveyed by checking that the standard deviation of the split frequencies reached a value below 0.01 (default burn-in = 25%). In order to infer the topology and the posterior probabilities we discarded the first 25% of trees for COI, ITS-1, and concatenated data sets in order to avoid inclusion of those trees obtained before likelihood values had stabilized, which were checked by plotting likelihoods against generations, and both runs had converged.

In a preliminary analysis, the genus *Schmidtea* Ball, 1974 was used as the outgroup (sister group of *Dugesia*; cf. Álvarez-Presas et al., 2008) to determine the root for the genus *Dugesia*. The results showed that *D. sicula* and *D. aethiopica* form a monophyletic clade that is the sister group of all other *Dugesia* species used in this study. Therefore, these two species were used to root all subsequent analyses.

2.6. Molecular clock calibration

In the absence of planarian fossils, only paleogeographic events of known age can be used to calibrate a molecular clock. However, in the case of planarians this is also not straightforward. Fortunately, the complex and well-known geological history of the eastern Mediterranean enables one to find such calibration points. In particular, the well-supported split in our phylogenetic trees between Cretan species and all other Greek species suggested this node as the best point to calibrate the phylogenetic tree. In order to assign a divergence date to this calibration point we considered two alternative scenarios, corresponding to the two times in its history that Crete became isolated. The first isolation of Crete took place c. 11–9 Mya (Dermitzakis, 1990), when it was separated from the mainland Ägäis. During the MSC, between 5.96 and 5.33 Mya, the Mediterranean dried out because of the closure of the Strait of Gibraltar (Hsü, 1972), reconnecting Crete to the mainland. Subsequently, the second Cretan isolation event occurred after the MSC, when the Mediterranean reflooded. In order to test, by using Bayes Factors (BFs), which of the two datings better explained our data we compared three temporal scenarios using a second calibration point, since a single calibration point does not provide a powerful test. As second calibration point we used the separation between eastern and western regions in the Aegean Sea (in our case corresponding to the Aegean islands, east, versus the rest, west), albeit that the clusters presumably correlated with that event have low support in our trees. For this splitting, we also considered two other possible datings. The first calibration point is the opening of the MAT (c. 12–9 Mya), as used in other studies (cf. Lymberakis and Poulakakis, 2010 and references therein). However, given the topology of the tree obtained, a scenario with Cretan lineages diverging at the end of the Messinian salinity crisis (5.3 Mya) and the east–west split occurring between 12 and 9 Mya was impossible because the east–west split occurs after

Table 1
Sampling localities of *Dugesia* populations used in this study (see also Fig. 1).

Locality code	Species	Sampling site	Coordinates	GenBank accession no.	
				COI	ITS-1
	Outgroups				
	D. aethiopica	Lake Tana, Ethiopia	Lázaro et al. (2009)	FJ646932 + FJ646976	FJ646889
	D. benazzii	R. Lernu, Sardinia, Italy	Lázaro et al. (2009)	FJ646933 + FJ646977	FJ646890
	D. etrusca	Tuscany, Italy	Lázaro et al. (2009)	FJ646939 + FJ646984	FJ646898
	D. gonocephala	Vijlen, Limburg, Netherlands	Lázaro et al. (2009)	FJ646941 + FJ646986	FJ646900
	D. hepta	R. S. Lucia, Sardinia, Italy	Lázaro et al. (2009)	FJ646943 + FJ646988	FJ646902
	D. ilvana	I. Elba, Tuscany, Italy	Lázaro et al. (2009)	FJ646944 + FJ646989	FJ646903
	D. sicula	S. Antioco, Sardinia, Italy	Lázaro et al. (2009)	FJ646947 + FJ646994	U84356
	Dugesia sp.	Vernár, Slovak Republic	48°55′21.06″N 20°18′34.45″E	KC007033	KC007104
				KC007017	KC007110
		Ludrová, Slovak Republic	49°1′46.18″N 19°19′49.07″E	KC007013	KC007114
		Prosiek, Slovak Republic	49°9′15.18″N 19°29′53.64″E	KC007030	KC007113
	Ingroups				
1	*D. cretica*	Georgioupoli, Crete, Greece	35°21′37.94″N 24°15′6.51″E	JN376141	KC007051
				KC006976	KC007050
2		Kakopetros, Crete, Greece	35°24′29.34″N 23°45′19.23″E	KC006974	KC007054
				KC006973	KC007053
3		Sasalos, Crete, Greece	35°24′9.86″N 23°42′42.39″E	KC006975	KC007052
				KC006977	KC007055
4	*Dugesia* sp.	Rouvas Gorge, Crete, Greece	35°9′48.66″N 24°54′34.71″E	KC007032	KC007102
				KC007012	KC007091
5	*D. elegans*	Petaloudes Valley, Rhodes, Greece	36°20′13.51″N 28°3′44.90″E	KC006985	KC007062
				KC006984	KC007063
6	*D. ariadnae*	Apollonas, Naxos, Greece	37°9′53.96″N 25°32′42.94″E	JN376142	KC007048
				KC006972	KC007049
7	*D. improvisa*	Melanes, Naxos, Greece	37°5′3.38″N 25°26′59.40″E	KC006987	KC007065
				KC006986	KC007064
8	*D. damoae*	Manolates, Samos, Greece	37°47′21.26″N 26°49′17.80″E	KC006979	KC007057
				KC006978	KC007056
9	*D. effusa*	Nagos, Chios, Greece	38°33′27.73″N 26°4′28.26″E	KC006983	KC007058
				KC006981	KC007061
10		Nagos, before the opening to the sea, Chios, Greece	38°33′34.73″N 26°4′56.86″E	KC006980	KC007060
				KC006982	KC007059
11	*Dugesia* sp.	Prokopi, Euboea, Greece	38°49′45.72″N 23°16′53.48″E	KC007026	KC007112
				KC007010	KC007089
12	*Dugesia* sp.	Eleonas – Gravia, Phocis, Greece	38°34′29.21″N 22°23′38.50″E	KC007018	KC007090
				KC007014	KC007101
13	*Dugesia* sp.	Varia, Aetolia-Acarnania, Greece	38°35′34.87″N 21°35′11.02″E	KC007011	KC007108
				KC007020	KC007092
14	*Dugesia* sp.	Vafkeri, Lefkada, Greece	38°43′31.41″N 20°39′46.59″E	KC007034	KC007088
				KC007009	KC007093
15	*Dugesia* sp.	Pogradec, Albania	~40°53′44.05″N 20°37′52.32″E	FJ646970 + FJ647015	FJ646930
16	*Dugesia* sp.	Filiates, Thesprotia, Greece	39°38′16.09″N 20°23′41.48″E	KC007028	KC007103
				KC007035	KC007107
17	*Dugesia* sp.	Potamia, Preveza, Greece	39°22′37.42″N 20°52′38.41″E	KC007037	KC007109
				KC007036	KC007105
18	*D. malickyi*	Gorgopotamos, Phthiotis, Greece	38°49′46.06″N 22°22′53.37″E	KC006990	KC007069
				KC006991	KC007066
19		Mexiates, Phthiotis, Greece	38°53′4.09″N 22°18′53.16″E	KC006988	KC007068
				KC006989	KC007067
20	*Dugesia* sp.	Polidrosos, Phocis, Greece	38°38′4.43″N 22°30′49.69″E	KC007022	KC007115
				KC007023	KC007094
21	*Dugesia* sp.	Tripi, Peloponnese, Greece	37°5′38.47″N 22°20′46.29″E	KC007025	KC007100
				KC007021	KC007106
22	*Dugesia* sp.	Agios Floros, Peloponnese, Greece	37°10′8.94″N 22°1′33.92″E	KC007029	KC007086
				KC007008	KC007087
23	*Dugesia* sp.	Dorio – Psari, Peloponnese, Greece	37°18′29.61″N 21°51′55.96″E	KC007024	KC007111
				KC007019	KC007099
24	*Dugesia* sp.	Theisoa – Andritsaina, Peloponnese, Greece	37°29′13.97″N 21°55′4.88″E	KC007031	KC007096
				KC007015	KC007098
25	*D. arcadia*	Chalandritsa, Peloponnese, Greece	38°6′31.85″N 21°47′13.73″E	KC006969	KC007044
				KC006971	KC007047

Table 1 (*continued*)

Locality code	Species	Sampling site	Coordinates	GenBank accession no.	
				COI	ITS-1
26		Sella, Peloponnese, Greece	38°17′3.02″N 21°52′45.80″E	JN376140 KC006970	KC007045 KC007046
27	*D. sagitta*	Roda, Corfu, Greece	39°47′23.94″N 19°47′29.46″E	KC007006 KC007003	KC007077 KC007074
28		Sfakera, Corfu, Greece	39°46′54.55″N 19°47′16.86″E	KC007002 KC006997	KC007081 KC007082
29		Kato vrisi spring, Klimatia, Corfu, Greece	39°44′30.48″N 19°46′49.20″E	KC007004 KC006996	KC007080 KC007075
30		Ano vrisi spring, Klimatia, Corfu, Greece	39°44′12.16″N 19°47′6.33″E	KC006999 KC007007	KC007083 KC007085
31	*D. parasagitta*	Ermones, Corfu, Greece	39°36′37.98″N 19°46′41.64″E	KC006995 KC006994	KC007072 KC007070
32		Ermones, slightly higher than 31, Corfu, Greece	39°36′41.93″N 19°47′1.40″E	KC006993 KC006992	KC007073 KC007071
33	*D. sagitta*	North of Vouniatades, Corfu, Greece	39°31′16.33″N 19°52′38.12″E	KC007000 KC007001	KC007076 KC007079
34		Benitses, Corfu, Greece	39°32′44.39″N 19°54′35.35″E	KC007005 KC006998	KC007078 KC007084
35	*D. aenigma*	Near Agia Eirini, Cephalonia, Greece	38°7′34.92″N 20°44′31.62″E	KC006968 KC006963	KC007040 KC007038
36		Digaleto, Cephalonia, Greece	38°10′46.99″N 20°40′46.80″E	KC006966 KC006967	KC007039 KC007042
37		Near Agia Eirini, Cephalonia, Greece	38°7′35.58″N 20°44′34.80″E	KC006965 KC006964	KC007043 KC007041
38	*Dugesia* sp.	Pastra, Cephalonia, Greece	38°6′4.38″N 20°45′4.14″E	KC007016 KC007027	KC007097 KC007095

Table 2
Forward (F) and reverse (R) primers used in amplification and sequencing.

Name	Sequence 5′–3′	Annealing temperature (°C)	Source
ITS-1			
9F (F)	GTAGGTGAACCTGCGGAAGG	45	Baguñà et al. (1999)
ITSR (R)	TGCGTTCAAATTGTCAATGATC	45	Baguñà et al. (1999)
COI			
BarT (F)	ATGACDGCSCATGGTTTAATAATGAT	43	Álvarez-Presas et al. (2011)
COIEF3 (F)	CCWCGTGCWAATAATTTRAG	48	This study
COIR (R)	CCWGTYARMCCHCCWAYAGTAAA	43	Lázaro et al. (2009)

the separation of Cretan species. Therefore, we also considered a second possible dating, i.e. that the final separation between the Aegean islands lineages and the rest of planarian lineages was a result of the refilling of the Mediterranean after the MSC. In this way, we generated three different temporal scenarios: (1) isolation of Crete at 10 Mya and east–west splitting during MAT opening between 12 and 9 Mya (scenario D1); (2) isolation of Crete at 10 Mya and east–west splitting at the end of MSC 5.3 Mya (scenario D2); (3) isolation of Crete and east–west splitting both occurring at 5.3 Mya (scenario D3). Once the best dating for the Crete separation was evaluated, we inferred a new dating tree using only that calibration point (D4), thus avoiding to fix a date for the separation between eastern and western lineages and to allow that dating to be deduced from the data itself.

We ran BEAST 1.6.1 software package (Drummond and Rambaut, 2007) in order to estimate clade divergence times for the fragment of COI, using relaxed molecular clock settings, following the uncorrelated relaxed lognormal clock. We applied the Yule or 'pure birth' prior process to the speciation model. The model of sequence evolution used was $GTR + I + \Gamma$, with runs of 12 million steps, sampling a tree every 1200 steps. Tracer vers. 1.5 (Rambaut and Drummond, 2007) was used to check convergence of parameters and to obtain mean and standard deviation (SD) of the substitution rates. We discarded 10% of the steps as burn-in. We assumed an age of 10 ± 1 Mya (mean of the normal prior distribution ± SD after relative 95% confidence intervals) for the first isolation of Crete, an age of 5.3 ± 0.3 Mya for the end of the MSC (both as the second isolation of Crete and for the splitting between east and west) and an age of 12–9 Mya for the opening of the MAT. Once we had the three calibrated trees, we applied a Bayesian model selection approach to decide which of the three temporal scenarios best fitted the data by running BF with Tracer and evaluating the results following Kass and Raftery (1995) criteria.

2.7. Biogeographic analyses

We used S-DIVA (Statistical Dispersal-Vicariance Analysis) implemented in RASP (Yu et al., 2010) in order to infer the biogeographic history of the Greek *Dugesia* lineages. This method facilitates statistical reconstruction of the ancestral distribution of species, taking into account phylogenetic uncertainty. We pruned the species tree, leaving one specimen per sampling locality, and excluding all species not present in the northeastern Mediterranean, with the exception of *Dugesia hepta*, which we used as outgroup in this analysis. S-DIVA was run using the trees sampled in a BEAST 1.6.1 analysis for COI and ITS-1. This analysis was performed with 50 million steps, sampling a tree every 10,000 steps. The condensed tree was obtained from the BEAST analysis using the TreeAnnotator 1.6.1 program with a 10% burn-in. We defined eight areas for biogeographic analysis: (A) Sardinia (outgroup); (B) Crete; (C) eastern Aegean islands; (D) Naxos; (E) Peloponnese; (F) Euboea; (G) Mainland; (H) Corfu; (I) Cephalonia. Although some of these areas could potentially be further divided, as mainland for example, we did not do it in order to avoid an excess of divisions.

3. Results

3.1. Taxonomic status

The taxonomic status of the animals from the various populations was determined through morphological analysis of histological sections. In this way we were able to assign the populations to eight out of the nine species known for Greece, viz. *Dugesia aenigma* de Vries, 1984, *D. arcadia* de Vries, 1988, *D. ariadnae* de Vries, 1984, *D. cretica* (Meixner, 1928), *D. damoae* de Vries, 1984, *D. elegans* de Vries, 1984, *D. malickyi* de Vries, 1984, and *D. sagitta* (Schmidt, 1861). Further, three new species were identified through both morphological and molecular markers; the new species names (*D. effusa*, *D. improvisa* and *D. parasagitta*) currently should be treated as *nomina nuda*. In addition, the molecular analysis suggests the presence of a few other genetic lineages, potentially new species (4, 11–17, 20–24 and 38). Unfortunately, we have been unable to ascertain the taxonomic status of these populations due to lack of (1) fixed material, (2) sexual specimens or (3) adequate histological sections. In a companion paper (Sluys et al., in preparation) we will examine and discuss more at length the species status of all populations examined in this study, in particular the new species, based on an integrative approach to taxonomy.

3.2. Sequence characteristics and divergence values

COI (706 bp) and ITS-1 (646 bp) sequences were analyzed for 74 new individuals from Greece and four from Slovakia. The saturation process plot shows that third codon position of the coding COI is not saturated. Therefore, final analyses included third codon positions. ITS-1 is also not saturated (Supplementary data Fig. 1).

Distance data between known species are given in Supplementary data Tables 1–4.

3.3. Phylogenetic and dating analyses

The concatenated tree (Fig. 2) and the tree derived from the COI gene (Supplementary data Fig. 2) have very similar topologies, although the first generally provides more resolved groups. In contrast, ITS-1 data only supports the split of the Cretan clade from the rest of the populations and also a few internal clades (Supplementary data Fig. 3). In particular, the Peloponnese clade is monophyletic and separated by a long branch from the rest, indicating the presence of a number of fixed substitutions in this group. Nevertheless, addition of ITS-1 data to those for the COI gene increases the resolution of the phylogenetic tree obtained from the latter gene. Although ML and BI trees inferred from the concatenated data set show some differences, the basic topology is the same for both.

Summarizing, all analyses reveal a clear correlation between the genetic lineages and their geographic distribution, albeit that ITS-1 provided a less resolved tree. The general picture emerging from these analyses shows a first divergence of the Cretan species, separated in all cases by a long branch from the remaining species, thus suggesting a relatively old event, as compared to the rest of splits. The next node corresponds to the separation of eastern and western MAT lineages. The eastern group, formed by populations from the eastern islands, includes two species (6 and 7) from Naxos (a priori a western island) and the only population studied from Euboea (11). The resolution within this group is poor, with the nodes receiving low support, likely indicating a radiation event that did not leave a clear signal in the molecules studied. In the western clade, the Peloponnese populations constitute a monophyletic group that is highly differentiated from the rest, presumably reflecting a relatively recent dispersal event. The mainland clade is only monophyletic in the dating analysis (Fig. 3); however, all analyses (Figs. 2 and 3, Supplementary data Fig. 2) show populations 15 (Ori Lake in Albania) and 16 (northwestern Greece) in a basal situation as well as two monophyletic clades constituted by the populations 18–20 and 12–14. These two monophyletic clades show a pattern of isolation by distance in the trees (geographically closer populations are more closely related in the trees; Figs. 2 and 3), a pattern to be expected in a case of dispersal, i.e. migration followed by genetic drift. Finally, mainland population 17 and all Ionian populations constitute a monophyletic group in all analyses, while within this group the populations from Corfu and from Cephalonia form two monophyletic clades.

Exceptions to these congruent results among methods concern the Euboea (11), Albanian (15), and Filiates (16) populations. In the COI tree (Supplementary data Fig. 2), the Euboea (11) population is positioned at the base of the western clade (including mainland and Peloponnese clades), whereas in the concatenated tree (Fig. 2) it is at the root of a monophyletic eastern clade (including eastern islands and Naxos species). However, BEAST Bayesian based tree (Fig. 3) positioned the Euboea (11) population within the eastern clade. Additional COI analyses without this Euboea population resulted in a COI tree (not shown) with a monophyletic eastern clade, but with similar node supports. Although the Albanian (15) and Filiates (16) populations always have a basal position with respect to the mainland clade, their relationships vary slightly and never receive high support, whereas the rest of mainland populations form two well-defined clusters.

The datings and substitution rates obtained with the three calibration scenarios (D1, D2, D3) are compared in Table 3, and the results of the BF comparison are presented in Table 4. The D2 model was best supported by the data, with the Bayes Factors providing substantial and strong support (Kass and Raftery, 1995 interpretation) for this scenario, as compared to scenarios D1 and D3, respectively. Scenario D1 received substantial support as compared to D3. Given the strong support for the calibration based on the separation of Crete at 10 Mya, and taking into account the low support for the two clusters defining our second calibration point, we performed a dating analysis using only the first calibration point at 10 Mya (Fig. 3; Table 3), in order to obtain an objective dating for the separation of the eastern and western lineages. This resulted in a value of 7.5 Mya, lying between the two values used in our previous calibration analyses (Table 3).

3.4. Biogeographic analyses

The topology obtained after pruning the tree was very similar to that obtained with the complete data set (Fig. 4, Supplementary data Table 5), with only some differences in nodes with low support

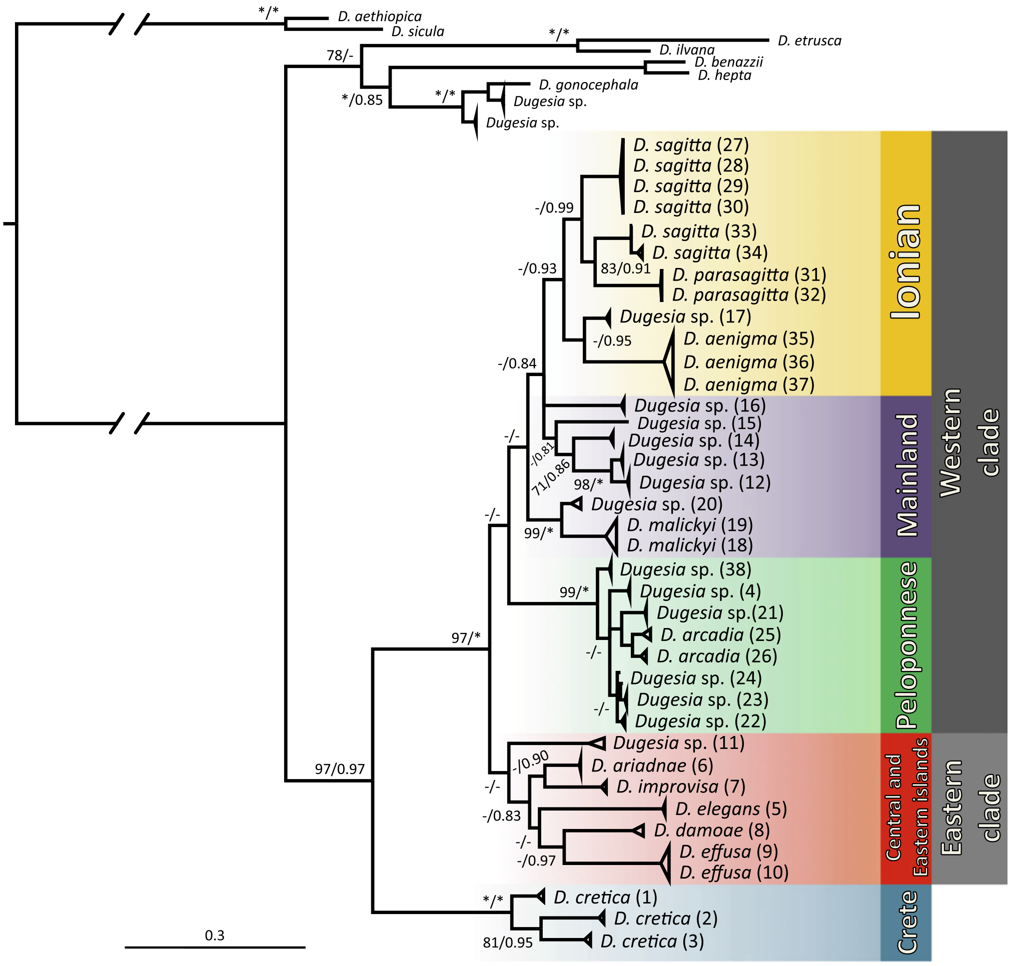

Fig. 2. Bayesian tree inferred from the concatenated data set (COI + ITS-1). Labels correspond to the species name (when known), and the numbers in parentheses refer to the locality codes reported in Table 1. Node numbers correspond to bootstrap (ML)/posterior probability (BI); values are only indicated when >50/>0.80, "*" indicates maximum support. The scale bar indicates substitutions per site.

in our phylogenetic and dating analyses. The specimens from Crete (4) and Cephalonia (38) that clustered together with the Peloponnese individuals were not included in the analysis because there is strong evidence that their distribution has been influenced by human transport. The results suggest seven vicariant events and two dispersals (indicated in green and blue, respectively, in Fig. 4). When we compare the dating studies and the biogeographic analyses (Figs. 3 and 4) it is clear that most of the results are in accordance with the geological history of the region. However, the hypothesized vicariant processes do not always precisely coincide with the presumed geological events. For example, in node 5 a dispersal event is inferred going from the mainland (G) to the western coast (HI, including present Corfu and Cephalonia), followed by a vicariant event (node 6) that splits region H (Corfu) from GI (Cephalonia plus a mainland population) and, subsequently, another vicariant event (node 61) that splits G (the mainland population) from I (Cephalonia). However, those hypothesized vicariant events occurred at a period long before the two islands were actually separated from the continent, implying that they were not the result of that geological event but most probably resulted from the isolation of several drainage basins in the Ionian region. Another complex situation is found in the eastern region, with S-DIVA deducing two vicariant events and one dispersal event. The first vicariant event separated the Euboea population (11) from the rest (node 21), after which dispersal took place from the east (C) to Naxos (D), followed by a vicariant event (nodes 22 and 23). Finally, there are three vicariances coinciding with geological breakages: the separation of Crete from the mainland (node 1), the separation of eastern and western Aegean lineages (node 2), and the separation of Peloponnese populations from the mainland (node 3).

4. Discussion

4.1. Differentiation among genetic lineages, speciation and systematic implications

The phylogenetic trees and also the genetic distances (Supplementary data Table 1), show that the species described previously on morphological grounds coincide with well-defined genetic lineages. The COI distances between species vary between 2.8% and 9.6%. These values are slightly lower than those found between *Dugesia* species in the western Mediterranean (Lázaro et al., 2009), excepting *D. benazzii* and *D. hepta* (the latter two species presumably representing a case of recent speciation). This situation suggests a younger diversification process in the eastern region (as also seen in the dating analyses, Fig. 3), which corresponds well with the fact that when Crete became isolated for the first time (c. 10 Mya) and the MAT began to form (c. 12 Mya), the western part of the Mediterranean had practically reached its present configuration (Rosenbaum and Lister, 2004a,b; Schettino and Turco, 2006).

In general, levels of intraspecific divergence (Table 1) fall within the range found for other planarian species (Lázaro et al., 2009,

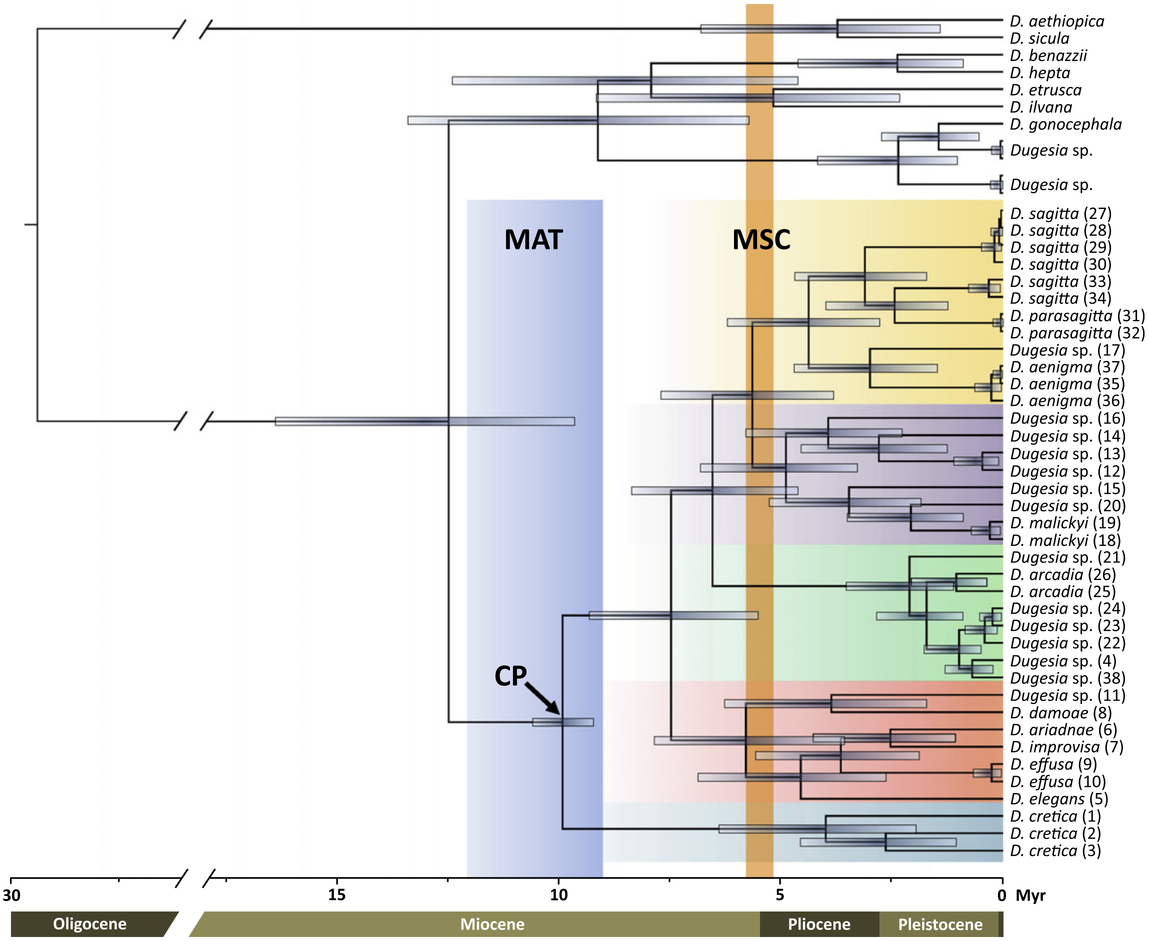

Fig. 3. Divergence times between Greek lineages of *Dugesia* inferred from COI by Bayesian analysis using a relaxed molecular clock and fixing the calibration point (CP) at 11–9 Mya (scenario D4). Bars at nodes represent the 95% highest posterior density (HPD) credibility interval. Vertical color bars indicate the periods of opening of the mid-Aegean trench (MAT; blue) and Messinian salinity crisis (MSC; orange).

Table 3
Inferred mean dates and highest posterior density (HPD) confidence interval for three scenarios using two calibration points: (1) the isolation of Crete from the Greek mainland (CP1: CR-GR) and (2) the split of the east and west Aegean (CP2: WMAT-EMAT); bottom row (D4) presents the calibration inferred from the data, using only the early isolation of Crete (c. 11–9 Mya) (for further explanation, see Material and Methods). Abbreviations: CR (Crete), EMAT (central and eastern islands), GR (All Greek populations without Crete), ION (Ionian), MNL (mainland), PEL (Peloponnese), and WMAT (Peloponnese, Mainland, and Ionian).

	Calibration point (CR-GR)	Node dating (MYA) [95% HPD]				Mean rate[b]
		WMAT-EMAT	PEL-(MNL + ION)	MNL-ION	Naxos-EMAT[a]	
D1	CP1: 11–9 MYA	Fixed	8.1	6.7	4.4	0.015
	CP2: 12–9 MYA		[9.5–6.2]	[8.5–4.8]	[6.5–2.3]	[0.001–0.021]
D2	CP1: 11–9 MYA	Fixed	4.9	4.3	2.7	0.022
	CP2: 5.5–5 MYA		[5.4–4.1]	[4.3–2.3]	[4.3–1.6]	[0.015–0.029]
D3	CP1: 5.5–5 MYA	Fixed	4.4	3.6	2.3	0.028
	CP2: 5.5–5 MYA		[5.2–3.3]	[4.7–2.6]	[3.5–1.2]	[0.002–0.041]
D4	11–9 MYA	7.5	6.6	5.7	3.7	0.017
	[Mean: 10; SD: 0.3]	[9.3–5.5]	[8.4–4.6]	[7.7–3.8]	[5.6–1.9]	[0.011–0.024]

[a] eastern islands excluding Naxos.
[b] Number of substitutions per site divided by tree length.

2011). The populations from Corfu (i.e. *D. sagitta* and *D. parasagitta*) are structured in three differentiated clades, corresponding with their geographical distribution (northern, central and southern part of the island; Figs. 1–4), with distances between the clades reaching 4.7% (Supplementary data Table 2), a value slightly higher than the maximum found between populations of the same species in the western Mediterranean (Lázaro et al., 2009). Despite the similar genetic distances between the three clades, only the central group presents morphological differences with respect to the other two, thus allowing the delimitation of a new species (*D. parasagitta*). For *D. cretica* the distances between populations also reach high values (5.7%), whereas study of their internal anatomy shows all of these animals to be identical.

On the other hand, there are some genetic lineages that do not correspond to any known species. Morphological analysis of some of these populations revealed cases in which defining characters exist for some genetic clades, whereas in other cases the opposite was the case, i.e. that morphological differences appear in genetically closely related populations. This complex situation calls for a deeper analysis, both from a morphological and a molecular point

Table 4
Bayes Factors results for the comparison of the three temporal scenarios. Probability of the three models with standard error and log 10 Bayes factors.

Scenario	ln P (model|data)	S.E.	CP1: 11–9 MYA CP2: 12–9 MYA D1	CP1: 11–9 MYA CP2: 5.5–5 MYA D2	CP1: 5.5–5 MYA CP2: 5.5–5 MYA D3
CP1: 11–9 MYA CP2: 12–9 MYA	−5336.426	±0.224	–	−0.74	0.811
CP1: 11–9 MYA CP2: 5.5–5 MYA	−5334.721	±0.218	0.74	–	1.551
CP1: 5.5–5 MYA CP2: 5.5–5 MYA	−5338.292	±0.221	−0.811	−1.551	–

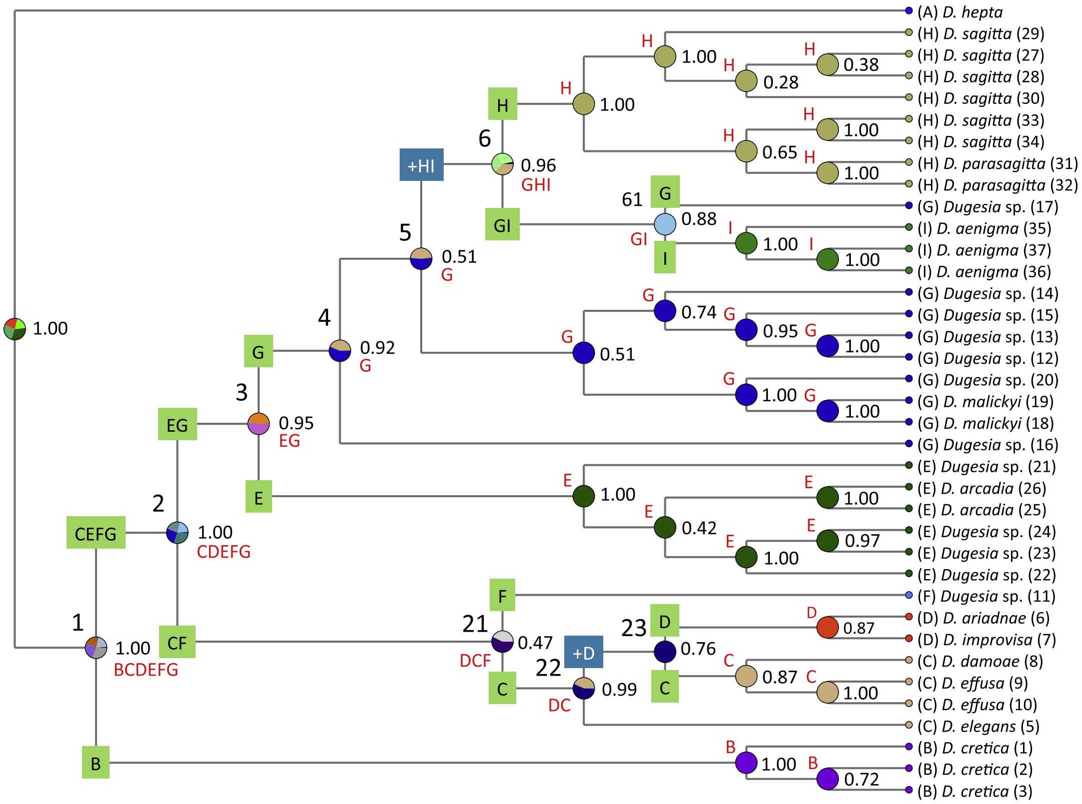

Fig. 4. Cladogram showing the results of the S-DIVA analysis. The node charts show the relative probabilities of alternative ancestral distribution ranges (see Supplementary data Table 5 for the exact values), red letters at the nodes indicate the area with highest probability. Vicariant and dispersal events inferred by the program are highlighted in green and blue, respectively. Numbers shown over some nodes are used to identify them in the text. Posterior probabilities of nodes are shown at their right side. The areas used in this analysis are: (A) Sardinia (outgroup); (B) Crete; (C) eastern Aegean islands; (D) Naxos; (E) Peloponnese; (F) Euboea; (G) Mainland; (H) Corfu; (I) Cephalonia.

of view. In a companion paper we will provide a more in-depth analysis of these cases by taxonomically integrating morphological and molecular data.

4.2. Phylogenetic congruence with geological and climatic history

Although the phylogenetic pattern is congruent with the geographical distribution of the lineages, it remains to be examined whether the timings for the splittings found in the dating analyses coincide with the geological and climatic history of the region. For this we have used the paleogeographic isolation of Crete from the continent as a calibration point for our divergence time analyses. This island became isolated twice, and there has been some contention on which dating is the most adequate to do this calibration. For many terrestrial animals it has been demonstrated that they used the exposed land surface to migrate from the continent to the island during the MSC (Lymberakis and Poulakakis, 2010 and references therein). Even seawater sensitive animals, such as amphibians and freshwater crabs, migrated during the Lago-Mare phase of the MSC (Akın et al., 2010; Jesse et al., 2011). However, unlike freshwater planarians these organisms are able to survive outside of freshwater and therefore their presumed dispersal still provides no firm evidence that the land bridge between Crete and the Peloponnese contained contiguous freshwater bodies facilitating dispersal of the planarians. Nevertheless, we considered the possibility that the Lago-Mare may have offered *Dugesia* an opportunity for dispersal, and hence we calibrated the splitting of the Cretan lineage at the two known moments that this island became isolated in order to compare both scenarios. The results show that our data give stronger support to the 11–9 Mya calibration (Table 4), pointing to a situation where planarians probably did not disperse between Crete and the mainland during the MSC. Either, there was no contact among freshwater bodies between Crete and the continent or planarians did not take the opportunity to disperse.

In the calibration based only on the earlier isolation of Crete at 11–9 Mya (Fig. 3; Table 3), the evolutionary rate (1.7% per site per lineage per million years) is in agreement with that found in other groups of organisms (e.g. Papadopoulou et al., 2010; Allegrucci et al., 2011) and also with what is considered a universal rate for mitochondrial DNA (Brown et al., 1979). In this scenario, the divergence between western (including the Peloponnese, mainland, and Ionian clades) and eastern (central and western islands clade) Aegean populations occurred between the end of the MAT opening and the beginning of the MSC, thus leaving open the possibility that it was a late consequence of the opening of the MAT. The situation that after the end of the MAT, other climatic and geographic events probably resulted in renewed contact of eastern and western lineages at some point in time (see below) may explain the fact that the support for this splitting is not high, while its dating does not exactly fit the 12–9 Mya period. Furthermore, the divergence times in this scenario, together with the biogeographic analysis, suggest that the common ancestor of the two Naxos species (*D. ariadnae* and *D. improvisa*), which are closely related to the eastern lineages, colonized this island from the eastern Aegean region during the Lago-Mare phase of the MSC (5.5–5.33 Mya). In that case, the freshwater systems on Naxos must have been in contact at one moment with the western Aegean systems during the MSC, perhaps flowing into common freshwater or brackish lakes. Recent human introduction seems a less parsimonious alternative hypothesis, since the two sister species then must have speciated in the east and, subsequently, have been transported on two occasions to the island, given that their speciation is much older than human activity. Additionally, the radiation suggested by the lack of resolution found in this region may have resulted from the cessation of contact between landmasses, due to rapid reflooding of the Mediterranean after the MSC, resulting in vicariant speciation on islands during the latest Messinian (5.33 Mya), although this could not be evaluated in S-DIVA since it was defined as a single distribution area. In a similar way, the Messinian has been postulated as the time of diversification of Mediterranean cyprinids (Bianco, 1990). These freshwater fishes would have dispersed across the basin during the Lago-Mare stage and underwent a fast speciation as a consequence of the return of the basin to marine conditions. This may be reflected in the deep polytomies found in some molecular analyses (Durand et al., 2003; Ketmaier et al., 2004; Tsigenopoulos et al., 2003). Although not all authors agree that the Messinian would have resulted in diversification around the whole Mediterranean basin (Perea et al., 2010), it seems clear that the Lago-Mare stage has acted at a local scale, especially in the eastern Mediterranean (Durand et al., 1999; Ketmaier et al., 2004). The individuals from Euboea (11) are also part of the eastern group in most analyses, if this situation is confirmed, this population and the eastern islands species share a common ancestor. Furthermore, the S-DIVA analysis infers with a higher probability an ancestral area for this clade comprising the eastern islands (C), Naxos (D) and Euboea (F) or CF (node 21 in Fig. 4, Supplementary data Table 5). Taking into account the geological history of the Aegean, this implies that the ancestors of this clade shared the same area in the east, and that the occurrence of species of this clade in the middle Aegean and in the west is due to dispersal. In that case, the Euboea individuals may result from dispersal by some eastern populations, followed by a vicariant event (as estimated by S-DIVA). This situation is also congruent with the findings of Durand et al. (1999) for the cyprinids. In that study a population from Euboea was found to be closely related to species from the rivers in eastern Greece. Our finding reinforces their hypothesis that freshwater habitants in Euboea would have evolved from eastern populations, which arrived as a result of contact between freshwater bodies in the northern Aegean Sea during a decrease in salinity in interglacial seven at about 200,000 years ago (Bianco, 1990).

The Peloponnese clade splits in a vicariant event (node 3 in Fig. 4) from the rest of the continental species at an earlier period (8.5–4.6 Mya) than the geological isolation of the Peloponnese (4–3 Mya), the latter event proposed as an explanation for the evolution of endemic species and lineages on this peninsula (Ursenbacher et al., 2008; Jesse et al., 2011). Presumably, the split between the Peloponnese clade and the other mainland and Ionian lineages was due to the severance of freshwater drainages before the peninsula was actually formed. In fact, the long branch separating this clade from all other groups, both in the COI and ITS-1 trees, and the low variability within it, point to the occurrence of a bottleneck event within the Peloponnese lineage (genetic drift during the bottleneck would have fixed mutations in the DNA that otherwise could have become lost). Although our data do not allow us to statistically test this demographic event, the dating tree shows that *Dugesia* populations on the Peloponnese did not diversify in a period between c. 7 and c. 2 Mya. This last point in time coincides with the beginning of the Pleistocene, a period characterized by an increase in humidity in the Mediterranean area (Haywood et al., 2000), which may have promoted the diversification of *Dugesia* on the Peloponnese peninsula through colonization of newly established freshwater environments.

On the mainland, the dispersal of freshwater planarians seems to have followed a north to south direction along both sides of the Pindus mountain range (east and west, Fig. 3 and Supplementary data Fig. 4). Again, although our data does not allow statistical testing of this dispersal hypothesis, while it could neither be seen in the biogeographic analyses (since we defined all mainland as a single distribution area to avoid an excess of regions), this result is congruent with that for freshwater fishes (Durand et al., 1999). The planarian dispersals are dated at the end of the Pliocene or during the Pleistocene, a little before the datings proposed for the fishes (middle and end of the Pleistocene for western and eastern Pindus lineages, respectively). However, the fishes' datings were based on a rate calculated for other organisms and were used very cautiously by Durand et al. (1999).

These two latter hypotheses, i.e. (1) loss of diversity on the Peloponnese with a recent recovery and (2) a possible north–south recolonization pattern on the mainland, need more detailed population studies in order to test the occurrence of bottleneck or dispersal events. However, the currently available dated tree induced us to erect a bold hypothesis, viz. a freshwater crisis before or during the MSC on the southern part of the Balkan Peninsula that resulted in the disappearance of most planarian populations in that region. When the climate became more suitable, surviving *Dugesia* populations on the Peloponnese would have dispersed through the entire Peloponnese peninsula, resulting in the radiation that is apparent from the phylogenetic trees. Moreover, populations situated in the north of the Balkan Peninsula could move southwards, colonizing Greece along both sides of the Pindus mountain range (Supplementary data Fig. 4).

Finally, the Ionian clade comprises *D. aenigma* from Cephalonia, *D. sagitta* and *D. parasagitta* from Corfu, and population 17 from the mainland in a basal position. This group reflects the presence of a third mainland lineage that, after the biogeographic analysis, presumably dispersed from the mainland to the west coast (node 5 in Fig. 4). Later, it experienced at least two vicariant events: (1) the splitting of Corfu lineages from the rest and (2) the splitting of population 17 from Cephalonia lineages (Fig. 3, Fig. 4). These two vicariant events (at about 4 and 1.7 Mya, respectively; Fig. 3) as well as the diversification of the three Corfu lineages (at about 1.7 Mya, Fig. 3) predate the isolation of Corfu and Cephalonia from the mainland at c. 9000 cal. yr BP. This suggests that during the last glaciation the river drainage basins inhabited by these lineages were not in contact, although Corfu was joined with the mainland through a large coastal plain traversed by many rivers (Van Andel and Shackleton, 1982). Hence, the vicariant events estimated are most probably due to the severance of those drainages.

Interestingly, the rate of substitution obtained for *Dugesia* in this study differs considerably from the only other molecular calibration for triclads available from the literature: *Schmidtea mediterranea* (Dugesiidae), with a 0.27% substitution per lineage and Mya for COI (Lázaro et al., 2011). The different rates of diversification observed for *Dugesia* and *Schmidtea* (the latter genus with only four species and a restricted area of distribution) may also explain the observed differences in molecular substitution rates.

4.3. Impact of human activities on planarian distribution

An unexpected result is that population four from Crete and population 38 from Cephalonia fall within the Peloponnese clade. According to the ingroup genetic distance between the Crete and Cephalonia populations and the Peloponnese specimens (COI: 0.8–2.5%; ITS-1: 0–1.1%) and the rather recent divergence times (0.96 Mya), postdating the last contact between these landmasses, it does not seem likely that they spread by their own means from the Peloponnese to these two islands. It has been suggested that biochore dispersal is of no importance in the dispersal of freshwater triclads (Reynoldson, 1966). However, all evidence in this case points to humans as a vector of their dispersal, a possibility already mentioned by de Vries (1985). The genetic similarity between Cretan and Cephalonian populations suggests that they originated from the same source population or from two genetically and geographically close populations. Despite this case, the total effect of anthropochorous transport on the current distribution of the planarians seems to be limited in this section of the Mediterranean region.

4.4. Fluvial basin history underlies planarian dispersal and speciation patterns

Fluvial basins may act as ‘‘ecological islands’’ for exclusively freshwater organisms, even on islands, in the same way as mountain peaks, landslides, or puddles (Heads, 2011). This is also evident in Mediterranean *Dugesia*. For example, the three genetic lineages found in Corfu diverged on the continent in the absence of marine barriers. This illustrates how the lack of contact between freshwater drainages for these organisms is as important in their diversification processes as island formation is for many other organisms. The Peloponnese lineage also represents a case of divergence before a well-known geographical barrier appears, viz. the opening of the Gulf of Corinth. This extreme dependence of planarians on contiguous freshwater bodies for their dispersal makes them an ideal group of organisms (1) to examine the effect of the geological history of freshwater drainages on their evolutionary diversification, and (2) to elucidate geological events, such as presence of land bridges and fluvial basins, which at times may be difficult to ascertain from geological data. In some respects this also applies to freshwater fishes, but planarians have the added advantage that they are able to live in smaller watercourses or even in temporary ones, thus enabling the extension of such historical inferences to a more fine-grained geographic scale. For example, the present study suggests that planarians were able to disperse between eastern and central islands in the Aegean Sea during the MSC, whereas there was likely no full contact between freshwater courses on the continent and Crete during that period.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.11.010.

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