Molecular Phylogenetics and Evolution 66 (2013) 877–888

Contents lists available at SciVerse ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

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

Eduard Solà [a], Ronald Sluys [b], Konstantinos Gritzalis [c], Marta Riutort [a,*]

[a] Departament de Genètica, Facultat de Biologia and Institut de Recerca de la Biodiversitat (IRBio), Universitat de Barcelona, Barcelona, Catalonia, Spain
[b] Naturalis Biodiversity Center, Leiden and Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, The Netherlands
[c] Hellenic Centre for Marine Research, Institute of Inland Waters, Anavyssos, Greece

ARTICLE INFO

Article history:
Received 2 May 2012
Revised 8 November 2012
Accepted 10 November 2012
Available online 24 November 2012

Keywords:
Aegean
Biogeography
COI
Divergence times
Freshwater planarians
ITS-1
Molecular clock
Phylogeography

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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* Corresponding author. Address: Departament de Genètica, Facultat de Biologia and Institut de Recerca de la Biodiversitat (IRBio), Universitat de Barcelona, Av. Diagonal 643, 08028 Barcelona, Catalonia, Spain. Fax: +34 934 034 420.
E-mail address: mriutort@ub.edu (M. Riutort).

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http://dx.doi.org/10.1016/j.ympev.2012.11.010

(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