



Dugesia hepta and *Dugesia benazzii* (Platyhelminthes: Tricladida): two sympatric species with occasional sex?

Daniel Dols-Serrate¹ · Laia Leria¹ · Juan Pablo Aguilar¹ · Giacinta Angela Stocchino² · Marta Riutort¹

Received: 19 December 2019 / Accepted: 17 April 2020 / Published online: 29 May 2020
© Gesellschaft für Biologische Systematik 2020

Abstract

Dugesia hepta Pala, Casu & Vacca, 1981 and *Dugesia benazzii* Lepori, 1951 are two freshwater planarian species from the islands of Corsica and Sardinia. *Dugesia hepta* is endemic of Sardinia and distributed in four northern hydrographic basins where it co-occurs with *D. benazzii*, which has a wider Tyrrhenian distribution. Although these species have been broadly studied—especially *D. benazzii*—as regards to their variety of reproductive patterns as well as for their karyological diversity, little is known about them from a molecular phylogenetic perspective. For the first time, we present a molecular phylogenetic tree of the two species and their populations based on two molecular markers—one mitochondrial, *Cox1*, and one nuclear, *Dunuc12*. Our results not only confirm that both species are molecularly distinct but also show that *D. benazzii*'s Corsican and Sardinian populations could belong to separate species. Furthermore, we present the first demonstration of a natural hybridization between different species in the genus *Dugesia* on the basis of molecular data.

Keywords *Dugesia* · Hybridization · Hybrid speciation · Molecular phylogeny · Haplotype network

Introduction

Tricladida (Lang 1884)—most commonly known as planarians—have been the subject to an in-depth research in a wide-ranging spectrum of scientific fields, such as regeneration, pattern formation, genomics, and transcriptomics (Abril et al. 2010; Newmark and Alvarado 2002; Reddien and Alvarado 2004; Robb et al. 2007, 2015) as well as on diversity and phylogeographical analyses (Álvarez-Presas and Riutort 2014; Leria et al. 2018; Leria et al. 2020; Sluys et al. 2013; Solà et al. 2013). However, historically, the Tricladida have been a challenging group for taxonomists and systematists due

to the unsuspected complexity for classifying their specimens, which relies, ironically, in their morphological simplicity. Within any genus, most planarian species share a common external morphology; hence, the diagnostic characters are mainly found in their most complex anatomical system, the copulatory apparatus. Nonetheless, the existence of fully fissiparous species (Stocchino and Manconi 2013) as well as the fact that freshwater planarians are known for being able to resorb their own reproductive organs during starvation periods (Berninger 1911; Newmark and Alvarado 2002; Schultz 1904) can sometimes render the morphological approach useless.

A good example of this situation is the longstanding freshwater flatworm genus *Dugesia*. This genus inhabits the Afrotropical, Palearctic, Oriental, and Australasian biogeographic regions and comprises an approximate number of 90 described species (Harrath et al. 2019; Leria et al. 2020; Stocchino et al. 2017). In this genus, three main types of life cycle related to their reproductive strategy can be found: sexual, asexual (fissiparous), and facultative, which seasonally alternate between the former modes (Stocchino and Manconi 2013). Asexual reproduction in this genus occurs by transverse fission of the architomic type (i.e. cellular differentiation, organ formation, and body reorganization occur in the new individual after the fission process). Due to the existence

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13127-020-00438-z>) contains supplementary material, which is available to authorized users.

✉ Marta Riutort
mriutort@ub.edu

¹ Departament de Genètica, Microbiologia i Estadística, Facultat de Biologia and Institut de Recerca de la Biodiversitat (IRBio), Universitat de Barcelona, Av. Diagonal 643, 08028 Barcelona, Catalonia, Spain

² Dipartimento di Medicina Veterinaria, University of Sassari, Via Vienna 2, I-07100 Sassari, Italy

of the aforementioned fissiparous-reproducing populations that do not develop the copulatory organs, it has not been unusual to group together several different species into one unique species or species group. For instance, *Dugesia gonocephala* s.l. (Dugès 1830) was a hodgepodge where all the sexual, asexual, and nonconclusive forms of European *Dugesia* were put together (Benazzi 1955; Benazzi and Banchetti 1972; Benazzi and Benazzi-Lentati 1976; Benazzi and Deri 1980; De Vries 1984, 1986; Sluys and De Jong 1984). The incorporation of molecular data not only helped to identify fissiparous individuals but also helped to delimit new species and to clarify the phylogenetic relationships within the genus (Álvarez-Presas and Riutort 2014; Lázaro et al. 2009; Leria et al. 2019; Leria et al. 2020; Riutort et al. 2012; Sluys et al. 2013).

Two species once belonging to the *D. gonocephala* s.l. complex, *Dugesia benazzii* Lepori 1951 and *Dugesia hepta* Pala et al. 1981, are the main focus of the present study. The former inhabits the islands of Corsica, Sardinia, and Capraia (Benazzi and Benazzi-Lentati 1976; De Vries 1985), whereas the latter is endemic of Sardinia restricted to four fluvial basins in the northern region, where both species occur in sympatry. *Dugesia hepta* differs from other Sardinian *Dugesia* species in its haploid chromosomal number ($n = 7$), which is unique in the Western Palearctic region (Stocchino et al. 2005), while in contrast, *D. benazzii* presents the most common haploid chromosomal number among *Dugesia* species, $n = 8$ (Fig. 1). Moreover, *D. benazzii* is known for comprising diploid ($2n = 16$), triploid ($2n = 3x = 24$), tetraploid ($2n = 4x = 32$), hexaploid ($2n = 6x = 48$), and aneuploid (with a mean chromosomal number of 32) populations (Lepori 1951; Pala et al. 1982, 1999; Stocchino 2018). Concurrently, *D. benazzii* characteristically presents sexual and mixed (coexistence of sexual and fissiparous individuals) natural populations, whereas for *D. hepta*, there are no reports on asexual and/or polyploid individuals (Stocchino and Manconi 2013). At first, both species were considered to be identical and indistinguishable except for their karyotypes (Pala et al. 1981), yet in a later study (Stocchino et al. 2005), differential morphological features were described in the copulatory apparatus and in the external morphology. Molecularly, *D. benazzii* and *D. hepta* have had a meager presence in the current molecular phylogenetic era,

being represented in all cases only by a few individuals (Lázaro et al. 2009; Solà et al. 2013). From those studies, we learnt that they are sister groups and closely related to the *Dugesia* species from the Western Palearctic Region.

These two sister species externally nearly identical and biogeographically sympatric pose an interesting case study. Since no thorough molecular study with a broad taxon sampling centered on these species have been carried out before, and adding to the fact that *D. hepta* is restricted to only four fluvial basins where co-occurs with *D. benazzii* individuals, one may wonder whether *D. hepta* could have had multiple origins or if it is really a monophyletic species. In the first scenario, *D. hepta* would be the result of a recurrent chromosomal disorder from *D. benazzii* specimens and, thus, should not be considered as a species per se. In the second scenario, we could face a possible case of biogeographical sympatric speciation due to a chromosomal rearrangement, with a concomitant parallel dispersion over the same fluvial basins. Speciation due to chromosomal rearrangements has been proposed to take place in other planarian genera (Benazzi 1982; Leria et al. 2018) as well as in several turbellarian groups (Curini-Galletti et al. 1985; Galleni and Puccinelli 1986). On the other hand, regardless of the taxonomical status of *D. hepta*, given its external morphological similarity to *D. benazzii* and their spatial distribution, one could wonder whether they are able to intercross and if they do it naturally. In fact, there are reports on an aneuploid ($2n = ?x = 32$) population of *D. benazzii*—reproducing by gynogenesis and referred to as the “biotype G” (Benazzi 1949)—located in Rio Bunnari (where the two species co-occur) that was considered to be a stabilized natural hybrid population (Pala et al. 1982) based on karyological data, yet further research rejected that hypothesis (Benazzi-Lentati and Benazzi 1985).

Hence, *D. hepta* and *D. benazzii* pose a case in which first, sympatric speciation may have occurred as the consequence of a chromosomal reorganization, and in the second place, posterior hybridization may have occurred. In the case of plants, approximately 25% of flowering species are considered to be involved in natural hybridization and introgression (Mallet 2007), and the prevalence of this processes has been demonstrated to facilitate speciation and adaptive radiation (Mallet 2007; Pennisi 2016). In animals, hybridization is less frequent, affecting only 10% of species (Arnold et al. 2008; Mallet et al. 2016). Moreover, the existence of this reticulate evolutionary process leads to difficulties in phylogenetic inferences and species delimitation studies, challenging for instance the concept of species grounded on the evolutionary independence of lineages. Hence, studying the patterns and processes of reticulate evolution is not only important to understand evolutionary processes generating new species but may also help to resolve the relationship among closely related taxa affected by them.

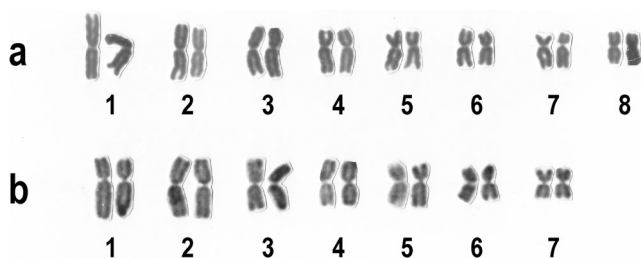


Fig. 1 Karyogram with chromosome complements arranged in pairs of *Dugesia benazzii* (a) and *Dugesia hepta* (b)

In the present study, we aim to answer the following questions using molecular tools: (i) is *D. hepta* a monophyletic distinct species from *D. benazzii* or the result of a recurrent chromosomal disorder from the last species? (ii) Do these two species (or taxonomic entities) hybridize? To accomplish our goals, we have performed a broad sampling of both species from Sardinia; we make use of karyological data to identify some individuals and molecular data to infer the phylogeny of the Sardinian populations of *D. hepta* and *D. benazzii* as well as to reconstruct a haplotype network to assess the existence of hybridism. The results obtained show that *D. hepta* and *D. benazzii* are indeed two different species. Interestingly, we also uncover a complex genetic scenario for their relationships compatible with the existence of ancestral and recent hybridization events between them.

Material and methods

Sampling

Dugesia benazzii and *Dugesia hepta* specimens were sampled from 32 localities distributed on the islands of Corsica and Sardinia (Fig. 2; Table S1) between 1997 and 2010. For each locality, some specimens were fixed and preserved in absolute ethanol for molecular analysis. Others were kept alive and taken to the laboratory to obtain karyotypes.

Assignment of individuals to species

Diagnostic differences between the two species rely on their karyotypes and differences in the copulatory apparatus. Aguilar (2011) found the external morphological differences proposed by Stocchino et al. (2005) to be misleading due to the fact that, in some populations, these differences were not always clearly recognizable but, in contrast, it was envisaged that *D. hepta* and *D. benazzii* differed in 6 divergent sites in their *Internal Transcribed Spacer-1 (ITS-1)* sequences of the ribosomal cluster (Aguilar 2011) (Table 1). A situation that may allow the assignment of individuals from sympatric populations without a tedious work of karyotyping and/or obtaining histological sections from all the individuals. In the present work in the first place, we checked whether this correlation was univocally true by karyotyping 31 individuals from different populations to assign them to a species based on their chromosomal number. Once the karyological assignment was established, *ITS-1* sequences were obtained. Our newly obtained data corroborated Aguilar's (2011) hypothesis. Thereafter, *ITS-1* sequences were obtained to assign individuals to species.

Karyotyping

Karyotypes were obtained for 31 animals from four populations where *D. hepta* and *D. benazzii* were known to coexist (Table S1) in order to assign them to species and corroborate the *ITS-1* criterion. Chromosome metaphasic plates were obtained by the squashing method. Regenerative blastemas of caudal fragments were treated with a solution of colchicine (0.3%) for 4 h then transferred onto glass slides and treated with a solution of acetic acid (5%) for 5 min. Subsequently, they were stained with acetic orcein for 2 h and squashed using a small coverslip (cf. Stocchino et al. 2019).

The criterion to assign the individuals to species based on karyological data was the following: sexual specimens were assigned to *D. hepta* when the karyotype was $2n = 14$ and any other case with a chromosomal number equal or higher than $2n = 16$ was considered a *D. benazzii* individual. This criterion was used because *D. hepta* is only described from diploid sexual populations, while *D. benazzii* is known to present diploid, triploid, tetraploid, hexaploid, and also different types of aneuploids biotypes (Lepori 1951; Pala et al. 1982). We compared our karyological results with our molecular phylogenetic results to ascertain the usefulness of the *ITS-1* criterion as well as the correct species assignment.

DNA extraction, quantification, and sequence amplification

Total genomic DNA extraction was performed for 161 individuals using DNAzol Reagent (Molecular Research Center Inc., Cincinnati, OH) and Wizard Genomics DNA Purification Kit (Promega Corporation) following the manufacturer's instructions. DNA quantification was performed for each sample using a spectrophotometer NanoDrop™ 1000 (Thermo Fisher Scientific Inc.) using 2 µl per sample.

Specific primers were used to amplify a fragment of the mitochondrial gene *Cytochrome c oxidase I (Cox1)*, the *ITS-1*, and the *transmembrane p24 trafficking protein 9 (TMED9)*; referred in the present study as *Dunuc12*). Sequences and annealing temperatures of each pair of primers are given in Table 2. Final PCR reaction volume for all markers was 25 µl, consisting of (1) 5 µl of Promega 5× Green GoTaq Flexi Buffer, (2) 2 µl of MgCl₂ (25 mM), (3) 1 µl of dNTP (0.5 mM), (4) 0.5 µl of each primer (either 10 or 25 µM), (5) 0.15 µl of *Taq* polymerase (5 u/µl) (GoTaq Flexi DNA Polymerase of Promega), and (6) 1 µl of genomic DNA sample (50 ng/µl). Autoclaved Milli-Q water was added to obtain the final PCR volume. It was necessary in many cases to vary the annealing temperatures or the amount of MgCl₂ and/or DNA in order to achieve sequence amplification. The resulting PCR products were visualized in a 1% agarose gel in order to verify the correct amplification of the different molecular markers.

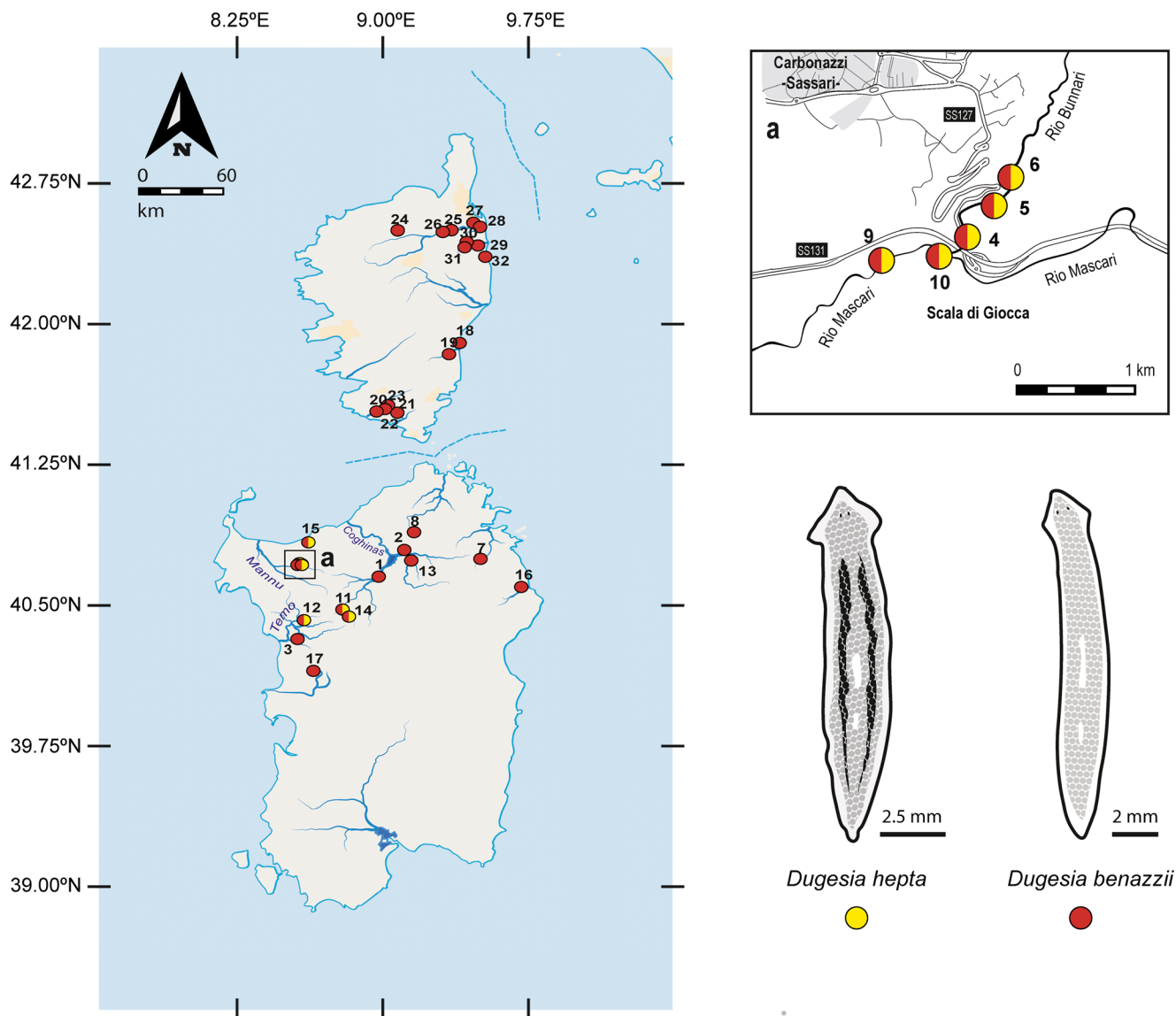


Fig. 2 Sampling localities used for this study and geographical distribution of *D. hepta* and *D. benazzii*. Localities are numbered according to Supporting information Table S1. Red-colored circles

indicate the presence of *D. benazzii* species, whereas yellow-colored circles indicate *D. hepta*'s presence. **a** Zoom in of Scala di Giocca

Viable-checked PCR products were purified before sequencing using a vacuum system (MultiScreen™ HTS Vacuum Manifold of Millipore) or a digestion with exonuclease I (0.2 u/μl; Tebu-Bio) and shrimp alkaline

Table 1 Polymorphic diagnostic site positions of *D. benazzii* and *D. hepta* in the *ITS-1* alignment and GenBank accession numbers for the type haplotypes

Species	Alignment position						Acc. number
	12	24	49	223	372	458	
<i>D. benazzii</i>	C	–	G	C	A	C	MN162692
<i>D. hepta</i>	T	T	A	T	G	T	MN162693

phosphatase (0.2 u/μl; SAP, Sigma-Aldrich) (1:2 ExoSAP per PCR product at 37 °C for 15 min plus an additional phase at 80 °C for 15 min). Sequencing reactions were performed using Big Dye (3.1, Applied Biosystems) and ran in an automated sequencer ABI Prism 3730 (Unitat de Genòmica dels Serveis Científic-Tècnics de la Universitat de Barcelona) or at Macrogen Inc. (Amsterdam). The same primers used to amplify were used for sequencing both strands. Chromatograms were visually checked for quality with Geneious R8 (Biomatters, <http://www.geneious.com/> last visited June 2019) and then contig and consensus sequences were obtained.

Since some individuals presented double-band patterns in their *Dunuc12* chromatograms, we decided to clone their PCR products to inspect their origin; we also cloned some animals

Table 2 Sets of primers used in this study

Gene	Primers	Sequence (5'-3')	Ann. Temp. ^a	Source
<i>Dunuc12</i>	Jon_12F	GATTACGAAAGCTATTTATAATT	52	Present study
	Snow_12R	CATGCACAAGATTACAAAG	52	Present study
	Elo_12F	AAGCTATTTATAATTCAGCG	54	Present study
	Hell_12R	AAAGAAATTGCTGCTAAAG	54	Present study
	Dunuc12_1F	CTCGTATCTCTGAATCTAGCCTC	55	Leria et al. 2019
	Dunuc12_1R	G TTCATACA ACTCATTCTTC	55	Leria et al. 2019
<i>Cox1</i>	SamCF	GCTAATAATTTGAGTTTTTG	51	Present study
	TarlyCR	CATTTTAAAACAACATTACC	51	Present study
	COIF	CCNGD TTTGGDATDRTWTCWCA	49	Lázaro et al. 2009
	COIR	CCWGTYARMCCHCCWAYAGTAAA	49	Lázaro et al. 2009
<i>ITS-1</i>	ITS-9F	GTAGGTGAACCTGCGGAAGG	45	Baguña et al. 1999
	ITSR	TGCGTTCAAATTGTCAATGATC	45	Baguña et al. 1999

^a Annealing temperature (C°)

that presented a mito-nuclear discordance or other peculiar molecular features (see results), and animals not presenting such features as a control. In total, 17 individuals (4 controls and the rest presenting double bands, mito-nuclear discordances, or other peculiarities) were cloned using a TOPO@TA Cloning Kit (Thermo Fisher Scientific Inc.) following the manufacturer's instructions. Approximately fifteen to thirty colonies from each individual were sequenced using the T3 and T7 primers (included in the kit).

Phylogenetic analysis

Alignments of the sequences were performed with the online software MAFFT v.7 (Katoh and Standley 2013) and posteriorly revised in BioEdit v.7.2.5 (Hall 1999). Prior to analyses, *Cox1* and *Dunuc12* sequences were translated into amino acids to verify that there were no stop codons within coding regions. Three alignments were obtained: (1) *ITS-1*, used to perform the species assignment; (2) *Cox1* (dataset I) and (3) *Dunuc12* (dataset II), which were used to perform the subsequent phylogenetic analyses. *Cox1* and *Dunuc12* sequences of four specimens belonging to three other *Dugesia* species were used as outgroup for the phylogenetic inferences (Supporting information Table S1).

Levels of sequence saturation were assessed by means of the Xia et al. (2003) test implemented in the software DAMBE (Xia and Xie 2001). The best substitution model was selected with jModelTest (Posada 2008) based on the Akaike information criterion (AIC). In both cases the best fitting model resulted in a HKY + G. Phylogenetic analyses were performed using two inference methods: Maximum Likelihood (ML) and Bayesian inference (BI). ML analyses were performed with RaxML 7.0.3 (Stamatakis 2006), applying a GTR + G substitution model—owing to the absence of

the HKY model in the aforementioned RaxML version. A total of 5000 replicates were calculated to obtain bootstrap supports (bs) conducting a rapid bootstrap analysis, and the ML search was performed starting from a random tree. Furthermore, we applied an optimization of both branches and model parameters on bootstrapped trees. BI analyses were conducted using MrBayes v.3.2 (Ronquist et al. 2012) and applying a HKY + G substitution model. Prior to run the analyses, nexus files were generated with MEGA6 (Tamura et al. 2013). We ran one cold and three heated chains for two parallel runs. Both topological and model parametrization convergence were surveyed by checking that the standard deviation of the split frequencies reached a value below 0.01. A total of 10,000,000 generations were performed for each gene, saving a tree every 5000 generations. We applied the default burn-in—set at 25%—in order to avoid the inclusion of trees obtained before likelihood values had stabilized to infer the topology and the posterior probabilities (pp). Obtained trees were visualized with FigTree v.1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/> last visited June 2019).

Haplotype networks

In order to construct the haplotype networks, we generated two additional alignments only comprising specimens from Sardinia: (1) comprising haplotypes from the *Cox1* sequences (dataset III) and (2) consisting of cloned *Dunuc12* sequences from 17 individuals (dataset IV). In the latter, we included Sardinian individuals that presented chromatograms either with double-bands or patterns indicating possible heterozygosity for indels, which had aroused our interest (individuals MR0092-05, MR0092-11, MR0025-02, MR0091-01, MR0092-04, and MR0030-06). We also included individuals belonging to the COI mixed clade (see results) that did not

present polymorphic bands (MR0088-02 and MR0092-03) as well as two individuals whose karyotype was known yet formed part of the mixed clade (MR0353-01 and MR0352-01). Furthermore, individuals MR0022-04, MR0022-05, and MR0172-01 were included as they showed signs of having mito-nuclear discordance. In addition, MR0022-04 and MR0172-01 had double-band patterns too. Finally, four individuals previously identified as *D. hepta* (MR0354-01 and MR0355-01) and *D. benazzii* (MR0368-01 and MR0370-01) based on karyological data were also analyzed as controls.

As for the *CoxI* haplotype network (dataset III), all individuals with polymorphic positions in dataset I were excluded from the alignment. Sequence ends were trimmed using BioEdit v.7.2.5 to avoid overestimating the number of underlying haplotypes due to the terminal missing data. DnaSP v.5 (Librado and Rozas 2009) was used to determine and assign the haplotypes for each individual analyzed and Network v.5.0.0.0 (Fluxus Technology Ltd.) was used to construct the haplotype networks. We used a default *epsilon* value set at zero.

Genetic diversity

Levels of nucleotide and haplotype diversity were calculated using DnaSP v.5. for each *CoxI* haplogroup. We also calculated a *CoxI* distance matrix using Kimura 2P evolutionary model for all the individuals included in the study.

Figures edition

The maps in figures were created with Q-GIS v.3.2.2 (<https://qgis.org/es/site/> last visited June 2019). Figures 2, 3, 4, 5, and 6 were edited with Illustrator CC v.22.0.1 (<https://www.adobe.com/products/illustrator.html> last visited June 2019).

Results

ITS-1 as diagnostic marker

The karyological analysis of the 31 individuals from localities 4, 5, 7, 9, and 14 revealed that nine of them matched to *D. benazzii* and 22 of them to *D. hepta*. *ITS-1* sequences could be recovered from only 23 of the aforementioned individuals. In 19 cases, the six species-specific sites proposed by Aguilar (2011) revealed the individuals to belong to *D. hepta* and four cases to *D. benazzii*, coinciding with the expected for the karyologically identified species. These results confirm the *ITS-1*'s validity as a marker for molecular diagnosis of the two species. Henceforth, we refer to it as the *ITS-1* criterion in the present manuscript.

We attained an overall number of 146 *ITS-1* sequences; none presented double peaks in the chromatograms. The

Fig. 3 Bayesian Inference tree of dataset I (*CoxI*). Node values are displayed qualitatively using squares for posterior probability (pp) and circles for bootstrap support (bs) values. Used colors indicate fully supported (black), significantly supported (gray), and nonsupported (white) nodes. Locality numbers from Table S1 are highlighted in black hexagons. Sampling localities are displayed in the map as follows: in Sardinia—grouped according to hydrographical distribution, and in Corsica—grouped into northern and southern geographical regions as showed in the tree

resulting alignment had a length of 496 bp—base pairs. The six species-specific sites were highlighted as key elements for the species diagnose. An overall number of 91 individuals were identified as *D. benazzii*, 55 as *D. hepta*, and 15 remained unidentified since our attempts to amplify and sequence their respective *ITS-1* were unsuccessful (*Dugesia* sp. in the Supporting information Table S1).

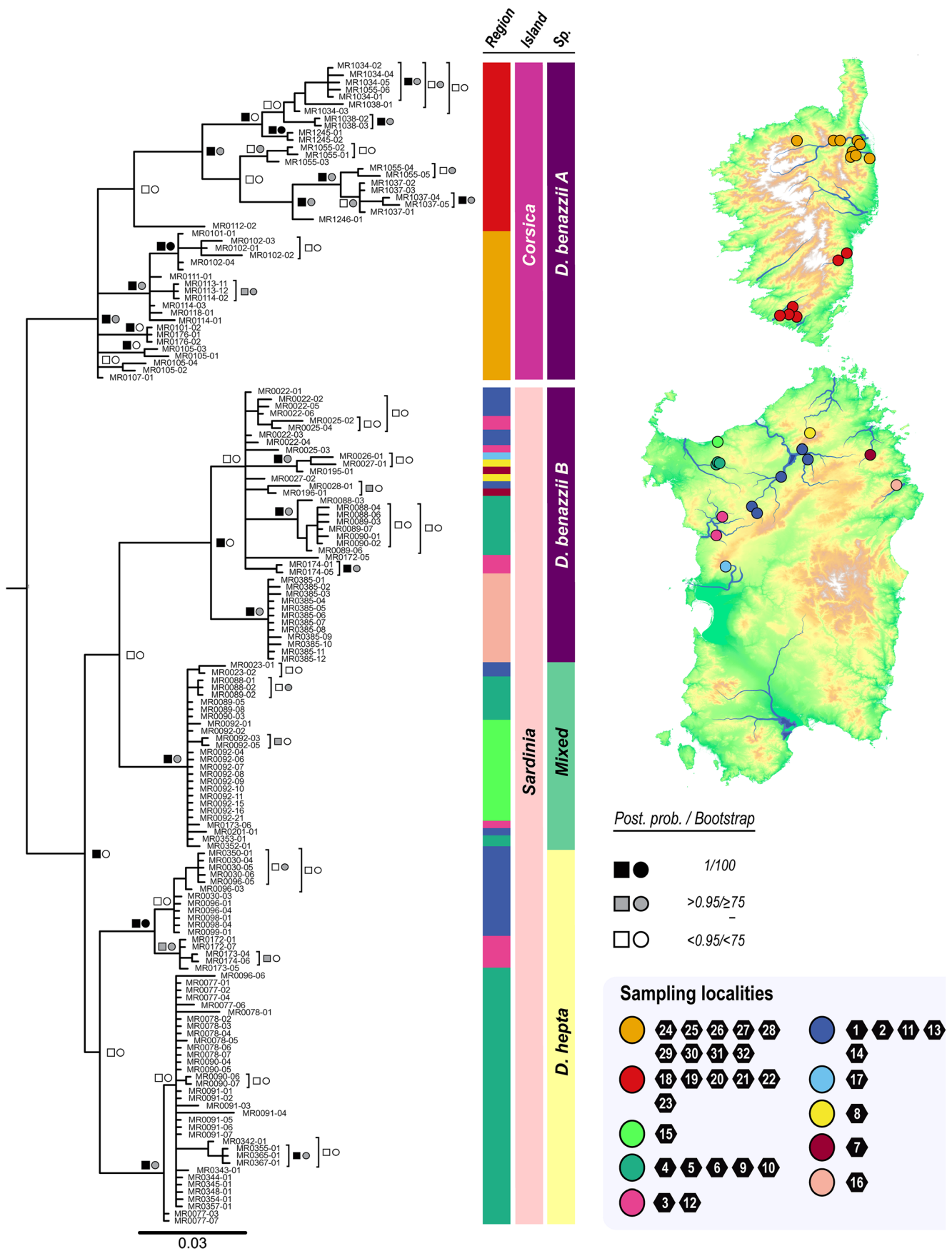
Dataset characteristics

We set four alignments to be analyzed either to estimate phylogenies (dataset I and II) or to construct the haplotype networks (dataset III and IV). Dataset I was comprised of 163 *CoxI* sequences (706 bp, 44 from Corsica, 115 from Sardinia, and 4 outgroup sequences) and dataset II was comprised of 102 *Dunuc12* sequences (642 bp total, 81 exonic bp, 2 from Corsica, 96 from Sardinia, and 4 outgroup sequences). Dataset III was constructed with nonpolymorphic *CoxI* sequences (685 bp, 53 sequences representing the 43.1% of the original Sardinian alignment)—from which 19 haplotypes were identified (Supporting information Table S2). Lastly, dataset IV consisted of cloned *Dunuc12* sequences (530 bp, 55 exonic bp, 259 sequences) from 17 individuals, from which a total of 176 haplotypes were recovered (Supporting information Table S3) with a mean of 10.0 ± 4.83 different haplotypes per individual. All new sequences have been deposited in GenBank (accession numbers available in Supporting information Tables). The tests revealed no significant saturation signals from any of the alignments.

Phylogenetic analyses

The phylogenetic inferences carried out by means of both Bayesian Inference (BI) and maximum likelihood (ML) yielded no topological incongruities.

Based on the mitochondrial marker (dataset I), the BI tree showed four major clades (Fig. 3): (1) *D. benazzii* specimens from Corsica (*D. benazzii* A), (2) Sardinian *D. benazzii* specimens (*D. benazzii* B), (3) *D. hepta* individuals belonging to Sardinia (*D. hepta*), and (4) an unexpected, apparently mixed clade comprised of 13 individuals identified via *ITS-1* as *D. benazzii*, 5 individuals identified as *D. hepta*, and 8 *Dugesia* sp. individuals. Most of the clades were well-



supported as indicated by posterior probability ($pp \geq 0.95$) and *bootstrap* ($bs \geq 75$) values—with the exception of the *D. hepta* clade, yet the resolution within clades was scarce. *Dugesia benazzii* did not result in a monophyletic group but a paraphyletic one, being the Corsican clade (A), the sister group to a clade constituted by *D. benazzii* from Sardinia (B), *D. hepta*, and the mixed clade. However, the phylogenetic relationship among these last three clades was unclear due to low support values.

The topology yielded by the nuclear marker (dataset II; Fig. 4) differed from the one obtained with *Cox1*. First of all, the populations of *D. benazzii* from Corsica and Sardinia clustered as a monophyletic clade. Likewise,

the main clades—namely one for *D. benazzii* and one for *D. hepta*—were unambiguously supported as indicated by *pp* and *bs* values, albeit the resolution within the groups was again poor. Secondly, there were no traces of the aforementioned mixed clade. Instead, the individuals belonging to the mitochondrial mixed clade were integrated within either the *D. hepta* or the *D. benazzii* group, in concordance with the *ITS-1* criterion. However, two individuals (MR0022-04 and MR0022-05) showed signs of mito-nuclear discordance given that their *Dunuc12* sequences belonged to the *D. hepta* clade—contrary to the *ITS-1* identification—while the

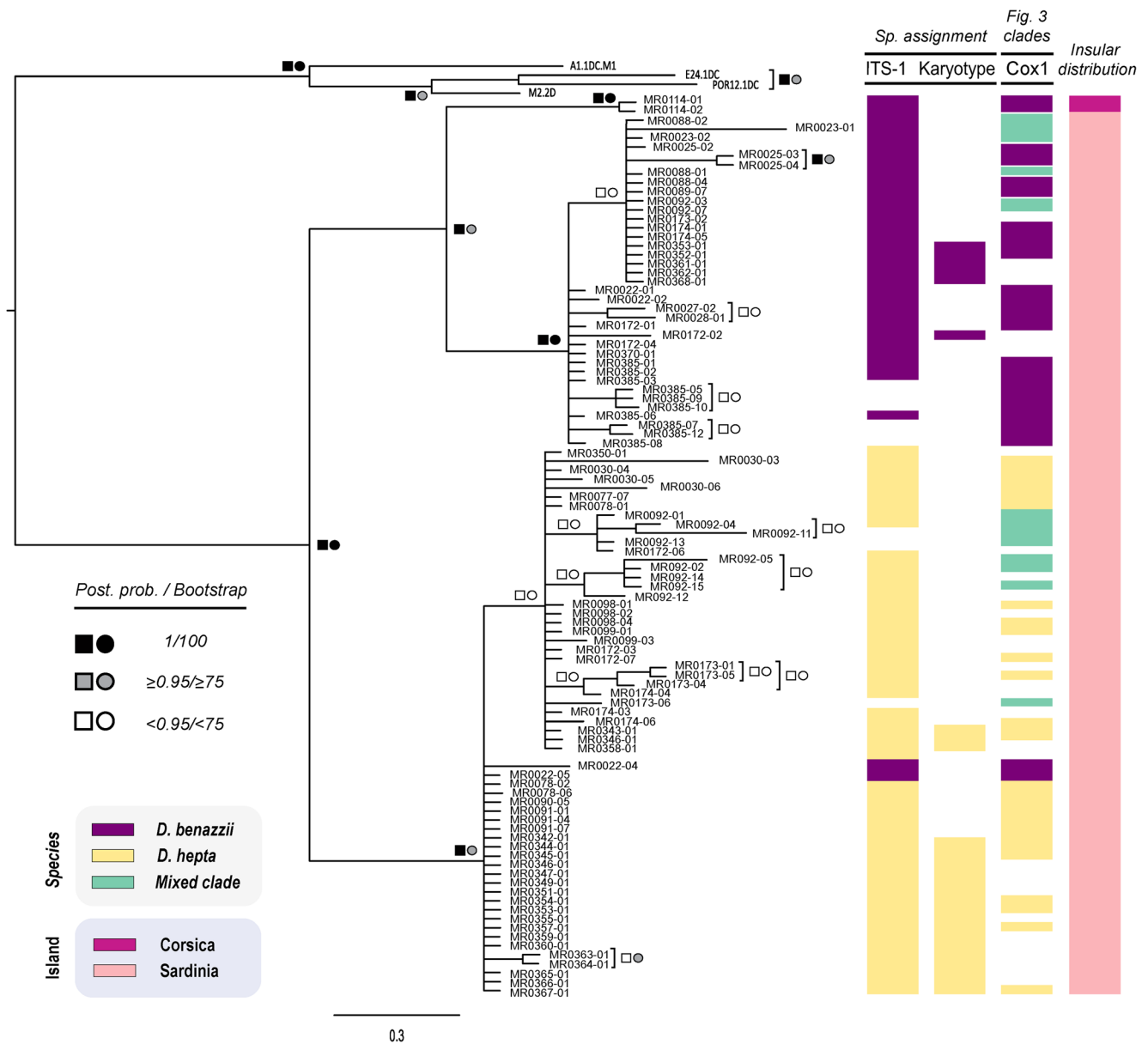
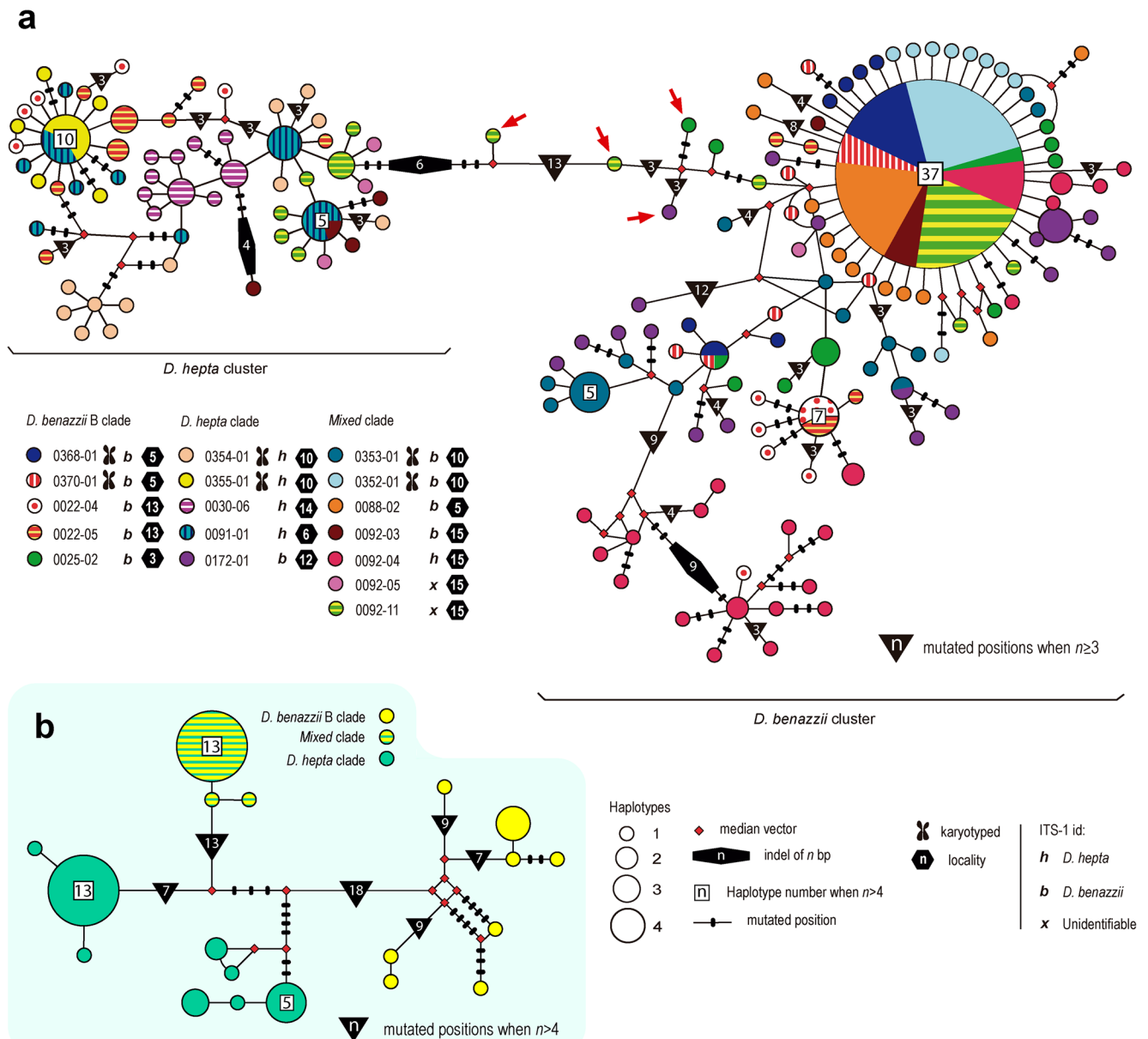


Fig. 4 Bayesian inference tree of dataset II (*Dunuc12*). Node support values are displayed as in Fig. 3. Bar diagrams indicate the species assignment of samples based on the *ITS-1* criterion and karyology. It is

also showed the phylogenetic position in dataset I's tree and the insular distribution (fuchsia—Corsica and light pink—Sardinia)



threshold number. Insertions and deletions are represented with an elongated hexagon indicating numerically the length of the *indel*. **a** For each cloned individual, information regarding *ITS-1* species identification, sampling locality, and availability of karyotype is given. Recombinant haplotypes are highlighted with red arrows

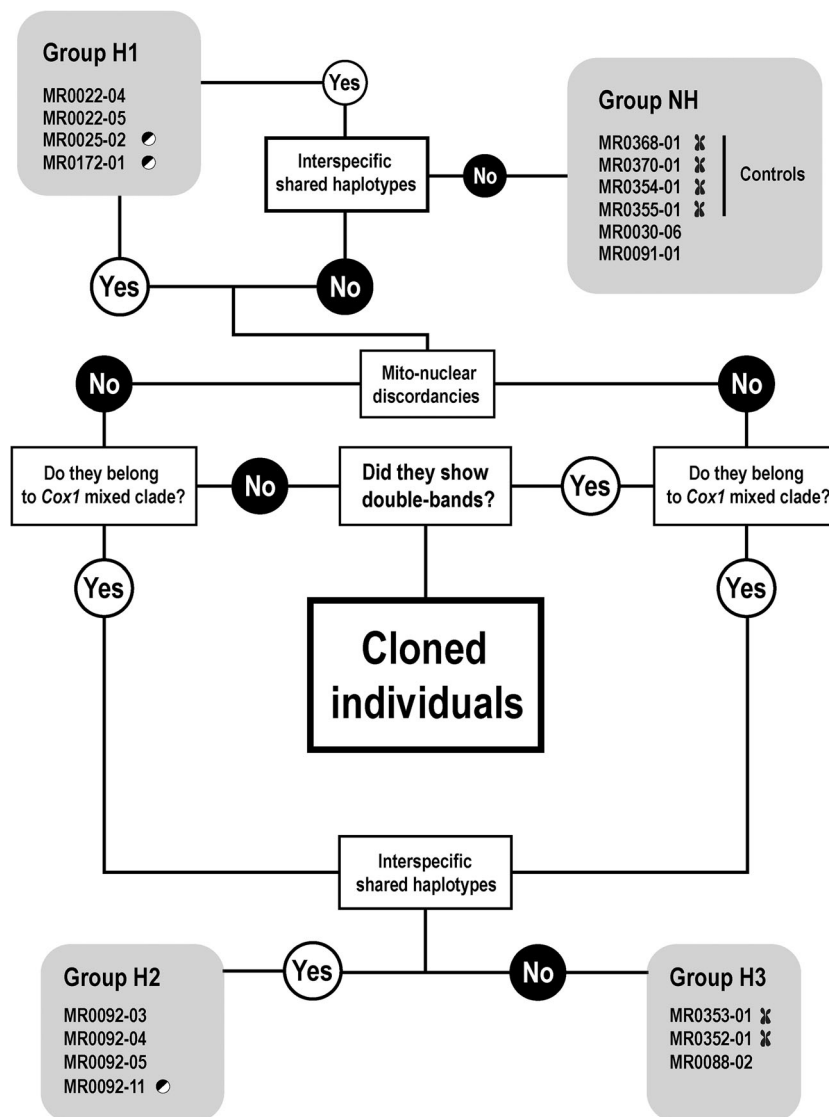
as *D. benazzii* (MR0368-01 and MR0370-01—from *D. benazzii* B clade) had their haplotypes in the other cluster, thus indicating that each cluster could be matched to a different species. Although some of the haplotypes sequenced may have been artificially generated by errors of the polymerase, the high differentiation between the two species' clusters ensures that their differentiation is real so that we can assign haplotypes from individuals as belonging to one species or the other depending on the cluster they belong to. Out of all the individuals assigned to the species *D. hepta* based solely on the *ITS-1* criterion, MR0030-06 and MR0091-01 had all

mitochondrial sequences placed them unequivocally within the *D. benazzii* B clade.

Haplotype networks

As regards to the *Dumuc12* (dataset IV) cloned haplotype network (Fig. 5a), we found two distinct haplotype clusters separated by at least 24 substitutions and a 6-nucleotide indel. The individuals karyologically identified as *D. hepta* (MR0354-01 and MR0355-01) had all their haplotypes assigned to one cluster, while those karyologically identified

Fig. 6 Summarization of dataset IV groups and their characteristics. Individuals whose karyotype is established are highlighted with bivalent chromosome symbol. Black and white split-up circles indicate individuals with recombinant sequences



their haplotypes unequivocally assigned to the *D. hepta* cluster, in accordance also with their mitochondrial data. In contrast, MR0092-04 had all its haplotypes associated to the *D. benazzii* cluster despite being part of the mitochondrial mixed clade. On the other hand, the individuals assigned to *D. benazzii* based on the *ITS-1* criterion showed more disparate results. Out of the specimens from mitochondrial *D. benazzii* B clade, MR0022-04 and MR0022-05 presented haplotypes in both clusters, while MR0025-02 had all its haplotypes in the *D. benazzii* cluster. Individual MR0172-01 from mitochondrial *D. hepta* clade presented only *benazzii* haplotypes, coinciding with its *ITS-1* assignment. As regards to the individuals belonging to the mitochondrial mixed clade, individual MR0088-02 had all its haplotypes associated to the *benazzii* cluster, while MR0092-03 showed haplotypes in both clusters. At the same time, karyotyped individuals MR0353-01 and MR0352-01 showed only *benazzii* haplotypes. Lastly, individuals MR0092-05 and MR0092-11 that

were not possible to identify based on *ITS-1* had haplotypes assigned to both clusters. It should be pointed out that inter-cluster recombinant haplotype variants were found in three individuals—MR0025-02, MR0092-11, and MR0172-01. In summary (Fig. 6), the clonal analyses of the nuclear gene of different “anomalous” individuals have resulted in the finding of four groups of individuals: some that are “pure” *D. hepta* or *D. benazzii* and probably presented double bands in their sequences due to their heterozygosity (Fig. 6, group NH); a second group that presents mitochondrial haplotypes either from *hepta* or *benazzii*, but in whose nucleus we can find haplotypes from both species or some recombinant (group H1); a third group presenting the mixed clade mitochondrial haplotypes and also presenting in the nucleus haplotypes from both species (group H2); and finally a group presenting the mixed clade mitochondrial haplotype but only *benazzii* nuclear haplotypes (group H3).

As for the *Cox1* haplotype network (Fig. 5b), the same three Sardinian clades observed in the tree (Fig. 3) were recovered. Curiously enough, in this case, the mixed clade derives from within the *hepta* cluster, separated by 13 substitutions, instead of being closer to the *benazzii* cluster as shown in the phylogenetic tree (a relationship that receives a low support, Fig. 3). The *benazzii* clade is separated from the *hepta* clade by 18 substitutions. Hence, the three groups are well separated, but in fact, the internal differentiation within the *hepta* and the *benazzii* clades is also quite high.

Genetic diversity

The *Cox1* distances estimated with Kimura 2P are shown in Table 3.

We also calculated nucleotide (π) and haplotype diversity (H_D) within each of the three *Cox1* haplogroups (Table 4). The values of H_D were high for the *hepta* and *benazzii* haplogroups in contrast to a low value for the mixed group. For π again, *hepta* and *benazzii* presented higher values than the mixed clade; however its values were also moderately low.

Discussion

Outstanding intraindividual haplotype diversity: artifact or commonality?

We cloned the PCR products of the nuclear gene *Dunuc12* to unveil the haplotypes of the putative heterozygous individuals of *D. hepta* and *D. benazzii*. Surprisingly, we found a mean of 10 different haplotypes per individual. Some of these haplotypes may have resulted from polymerase errors during the PCR step before the cloning procedure; however, our results exceed whatever expectations of errors caused by the malfunction of the polymerase. Even though the recovered number of haplotypes was surprisingly high, similar results have been previously found in *D. japonica* (Nishimura et al. 2015) and in a closely related group of species—*D. subtentaculata*, *D. aurea*, *D. corbata*, and *D. vilafarrei* (Leria et al. 2019; . Leria and coworkers demonstrated in said study the presence of mosaicism due to the accumulation of mutations in planarian stem cells during homeostatic and fission processes,

Table 4 Estimations of nucleotide (π) and haplotype diversity (H_D) for the haplotype groups in dataset III (*Cox1*)

Group	Sequences	Haplotype num.	H_D	π
<i>Hepta</i>	$n = 27$	8	0.738 ± 0.005	0.0131
<i>Benazzii</i>	$n = 11$	8	0.891 ± 0.008	0.0178
<i>Mixed</i>	$n = 15$	3	0.257 ± 0.020	0.0006

resulting in multiple closely related haplotypes in sexual animals (star-like patterns) and in multiple distantly related haplotypes in fissiparous animals (divergent pattern). Thus, we have reasons to believe that this might be characteristic of *Dugesia* species—perhaps even of other dugesid genera—especially when asexuality is involved as a reproductive strategy in the species.

Dugesia benazzii species status

The clues provided by the phylogenetic *Cox1* tree obtained in this study (Fig. 3) point out that *D. benazzii* could constitute more than one species. We base this suggestion on the paraphyletic arrangement of the *D. benazzii* clades in the tree and especially on the high genetic differentiation among them. The genetic differentiation between the populations from Corsica and Sardinia for COI has a mean value of $5.8 \pm 0.8\%$, far superior to the ones found between populations within each of the islands (Table 3). Moreover, the values found between islands concur with some of the interspecific genetic distance values for *Dugesia* species from the Western and Eastern Mediterranean (Lázaro et al. 2009; Solà et al. 2013) that vary between 2.8% for closely related species in the Aegean region and 11% for some species on the Western region. On the other hand, the phylogenetic tree obtained from the nuclear marker (*Dunuc12*, Fig. 4) showed a monophyletic *D. benazzii* clade, yet the two individuals from Corsica appeared again to be highly differentiated from the Sardinian populations. Nonetheless, the fact that only two sequences for the aforementioned nuclear marker of Corsican individuals were used in this study gives us little information regarding genetic diversity of the populations of the Corsican *D. benazzii*. On the other hand, populations of *D. benazzii* from both islands are identical regarding the *ITS-1*, which will

Table 3 Distance matrix of mean K2P distances (%) with standard deviations for the *Cox1* marker. Number of sequences is given inside the parentheses

	<i>D. benazzii</i> A	<i>D. benazzii</i> B	Mixed clade	<i>D. hepta</i>
<i>D. benazzii</i> A (44)	2.9 ± 1.4			
<i>D. benazzii</i> B (39)	5.8 ± 0.8	1.6 ± 0.9		
Mixed clade (26)	5.2 ± 0.8	4.2 ± 0.4	0.1 ± 0.1	
<i>D. hepta</i> (52)	4.9 ± 0.7	4.0 ± 0.5	3.2 ± 0.1	1.3 ± 1.2

support the monophyly of *D. benazzii* but not its division in more than one species, unless this is a very recent event and ribosomal clusters are still being kept similar by concerted evolution. As for the morphology, the original description of the species given by Lepori (1951) did not establish any remarkable differences between Corsican and Sardinian *D. benazzii* populations regarding the copulatory apparatus, but it did point out some minor dissimilarities that he deemed insufficient neither to consider the Corsican and Sardinian populations as distinct geographical subspecies. However, considering that modern descriptions take into account more characters than in the past, the possibility to find valid differences supporting a specific differentiation cannot be ruled out. To this purpose, a new detailed morphological study has been already undertaken on populations of *D. benazzii* from the two islands that will be the subject of a companion paper.

Hence, there is incongruence between the mitochondrial history and the nuclear and morphological accounts. In some cases, a potentially high degree of genetic variation may only be reflected by recondite morphological traits (according to Kucera and Darling 2002) that are not evident at first sight. Sibling species often have minor morphological differences that are only noticed once species are recognized for other reasons—such as karyological data or molecular evidences. The species that fit this profile are known as pseudo-cryptic (Knowlton 1993), and this may be the case for Corsican and Sardinian *D. benazzii* populations. Nonetheless, speciation is a continuum. Theoretically, the further we stray from the starting point, the clearer and more evident should be the differences between descendant lineages, but in the first stages of speciation, there can be divisiveness among sources of evidence—i.e. genetic data versus morphological data, nuclear versus mitochondrial DNA—because changes do not accumulate uniformly and at a fixed rate. This interval of speciation is known as the “gray zone” (De Queiroz 2007) and could explain why we find differences regarding the *CoxI* sequences between Corsican and Sardinian *D. benazzii* populations but not in the *ITS-1* or in their morphology.

These results point to the need for a revision of the taxonomic status of *D. benazzii*, based on more data ranging from an increase of the number of nuclear markers and the use of molecular methods for species delimitation to a morphological and karyological revision of the individuals. A similar situation has been resolved in a close relative, *D. subtentaculata*, by the concurrent use of all these lines of evidence in an integrative way, resulting in the description of three new species that are morphologically cryptic with *D. subtentaculata* (Leria et al. 2020).

Species status and origin of *D. hepta*

Dugesia hepta is a monophyletic species beyond questioning as ascertained by both phylogenetic trees. We sustained a

reasonable doubt regarding its taxonomical status owing to (1) the atypical chromosomal number ($n = 7$) and (2) their geographical distribution—restricted to four fluvial basins and in co-occurrence with *D. benazzii* individuals. However, our results rule out the possibility that *D. hepta* could be an aberrant chromosomal form of *D. benazzii* in which case we might expect to find *D. hepta* as a polyphyletic ensemble appearing in the phylogenetic trees independently from different *D. benazzii* clades. Our phylogenetic trees do not support that hypothetical scenario but rather show that *D. hepta* and *D. benazzii* are two different species that shared a common ancestor.

It is not clear from our results whether *D. hepta* is sister to only the Sardinian *D. benazzii* or to an older lineage that gave rise to the *D. benazzii* group from Sardinia (group B) and Corsica (group A). Nuclear data seem to indicate that *D. hepta* could be in fact the sister group of *D. benazzii* A and B (Fig. 4), yet such relationship is questioned when the mitochondrial data is considered. However, discordances in nodal support depending on the inference method used to build the tree based on *CoxI* data (Fig. 3) let open the possibility that *D. hepta* could be the sister group of *D. benazzii* A and B. It is worth noticing that although both species present similar values of diversity for their *CoxI* sequences (Tables 3 and 4), they differ in how this genetic variation is geographically distributed. The Sardinian populations of *D. benazzii* from the sampled localities showed no remarkable signs of geospatial structure or isolation, with the exception of the samples from Monte Albo (Table S1; Fig. 3 locality 16) that are appreciably genetically isolated from the rest of *D. benazzii* B populations. In contrast, the populations of *D. hepta* appeared to be more structured with no apparent admixture of individuals from different fluvial basins (Fig. 3). A plausible explanation for the differences in mitochondrial structuration degree could reside within the reproductive strategy of each species. *Dugesia hepta* is exclusively sexual, while *D. benazzii* is strategically more flexible having both sexual and sexual-fissiparous mixed populations, which could be advantageous towards rapidly colonizing new fluvial basins, as it has been shown for other *Dugesia* species (Lázaro and Riutort 2013; Leria et al. 2020). This also could explain why *D. benazzii* has a broader geographic distribution. A speculative scenario will be that, *D. hepta*'s ancestral populations may had undergone through a constraint in numbers due to direct competition with other species—possibly *D. benazzii* itself who could have colonized Sardinia from Corsica and displaced *D. hepta*. However, similar results could be expected if recent bottlenecks caused by abiotic phenomena—such as the desiccation of the brooks and springs where they can usually be found—affect *D. hepta* population's survival more than those of *D. benazzii* due to its exclusive sexual way of reproduction. There was no apparent correlation between the genetic lineages and their geographic distribution for the

nuclear gene, for any of the species. This can be a result of the gene analyzed being highly conserved and hence lacking information for recent dispersal events.

Whether *D. hepta* is sister to *D. benazzii* from Sardinia or to the lineage that gave rise to *D. benazzii* group from Sardinia and Corsica, the speciation event may have been related to a chromosomal rearrangement. Bearing in mind that *D. hepta*'s chromosomal number ($n = 7$) is uncommon within the whole Western Palearctic Region and that most species of *Dugesia* commonly share the $n = 8$ chromosomal number, we suggest that the ancestor that gave rise to the lineages leading to the current *D. hepta* and *D. benazzii* species might have shared the same chromosomal number, $n = 8$. Therefore, *D. hepta* could pose a case of speciation due to a chromosomal rearrangement. In most animal and plant groups, there are differences regarding the chromosomal number among closely related species (King 1993). Nonetheless, not all of the changes that may operate on the chromosomes are implicated in speciation phenomena (King 1987) but only those that have potential to diminish the biological efficiency of the hybrids, which are known as negative heterotic (Forsdyke 2004; King 1987, 1992; Rieseberg 2001), or that even impede their viability. Both cases, at shorter or longer term, give rise to reproductive isolation among populations and therefore are likely to cause speciation (White 1978). Chromosomal rearrangement speciation cases have gained presence over time (Coates and Shaw 1984; Kawakami et al. 2011; Talavera et al. 2013). There are other cases in freshwater planarians where a chromosomal rearrangement is suspicious of being the speciation cause, within the dugesiid genus *Schmidtea*. *Schmidtea nova* and *S. lugubris* are two sibling species with haploid chromosomal numbers of $n = 3$ and $n = 4$, respectively. Within the genus, $n = 4$ is the plesiomorphic karyological state. *Schmidtea nova* would have originated from a common ancestor through a Robertsonian translocation plus a pericentric inversion resulting in its three basic chromosomes that would have rapidly isolated reproductively the descendent lineages (Benazzi and Puccinelli 1973; Leria et al. 2018). In *D. hepta*, we also have a reduction of the chromosomal number as well as changes in the chromosomal structure—being the most remarkable a large submetacentric chromosome 1—within a predominantly metacentric set—that could be the by-product of a nonreciprocal translocation that led to the loss or the assimilation of the eighth chromosome. We can conclude that these two species exhibit a great karyological plasticity regarding ploidy and chromosomal composition as it has been previously proposed for other planarian groups (Leria et al. 2018; Leria et al. 2020; Ribas 1990) in comparison with their conservative morphology, and this plasticity may in some cases be related to speciation events. However, does the chromosomal difference between *D. hepta* and *D. benazzii* really impede their intercrossing?

Dangerous liaisons: a complex relationship between *D. hepta* and *D. benazzii*

Pala et al. (1982) had proposed that *D. hepta* and *D. benazzii* might be able to intercross to explain the presence of individuals reproducing gynogenetically bearing a variable number of chromosomes (being the most frequent number 32 but never eutetraploid) in “Scala di Giocca” locality (Rio Bunnari), although it was posteriorly refuted by Benazzi-Lentati and Benazzi (1985) based on karyometric analyses. To try to elucidate whether hybrids exist, we have planned our analyses to detect a classical basic case of hybridization, the detection of nuclear haplotypes from both parent species in the putative hybrids, together with the mitochondrial haplotypes from only one of them. Our results, however, show a much more complex and interesting situation that should be deeply looked into in further researches. We have found evidence that prove the existence of at least three types of “anomalous” individuals collected in the four hydrographic basins in which the two species co-occur (H1 to H3, Fig. 6) that most probably could be the result of hybridizations. Even though it would be tempting to jump into hasty conclusions, we cannot unerringly relate Pala's et al. individuals to our hybrids. The situation is certainly much more complex than we could expect.

H1 individuals could be the result of a recent hybridization. Those individuals have *Cox1* sequences either belonging to the Sardinian *D. benazzii* group—as well as their *ITS-1*—or to *D. hepta* group yet the *Dunuc12* nuclear marker presents haplotypes from both species or presumptive recombinant alleles. These individuals could be the result of a recent hybridization in which *D. benazzii* or *D. hepta* will have acted as a mother so that the hybrids have one or the other mitochondrial DNA. In the nucleus, we will in this case expect to find haplotypes from both parents, which is the case for individuals MR0022-04 and M0022-05, while in the other two individuals, we only find *benazzii* haplotypes but some presumptive recombinants. This latter case could be a consequence of the hepta *Dunuc12* haplotypes not having been PCR-amplified as efficiently as *benazzii* *Dunuc12* haplotypes (so a methodological artifact) or else that hybrid individuals have been able to backcross with *D. benazzii* parental species resulting in the loss of the hepta nuclear haplotypes.

For the *ITS-1* sequences (showing *D. benazzii* origin in the four H1 individuals), either a similar situation is found (lack of amplification or backcross to parental species) or else the concerted evolution processes that regularly homogenize the multiple copies of the ribosomal clusters (Dover 1982; Hillis and Dixon 1991) may have resulted in the original *D. benazzii* cluster having overruled the *D. hepta* cluster. Subsequently, the validity of the *ITS-1* criterion as a highly reliable method to identify the species that Aguilar (2011) originally proposed is questioned. It will work for the parental species, but it will

certainly fail to determine hybrids unless it is cloned. Since a hybrid will have the genomes of both parental species, theoretically one could be amplifying the *ITS-1* of any of the two parental genomes.

The H2 group individuals also present nuclear *Dnuc12* haplotypes from both species (or a recombinant), hence likely being of hybrid origin. However, what makes these hybrids special is that they bear the mixed clade *Cox1* haplotypes that appear as a monophyletic clade in the *Cox1* tree, completely independent from the *D. hepta* and *D. benazzii* clades. Therefore, we have individuals bearing in their nucleus haplotypes coming from both species, while their mitochondrial genome seems to have differentiated from both parentals, showing a closer relationship to the hepta haplogroup from which most probably derived (Fig. 5b). Moreover, to make the picture more complex, within this mixed clade we also find the group H3 presenting the mixed clade *Cox1* haplotypes but only *benazzii* nuclear sequences.

Many of the members in the mixed clade (H2-H3 individuals) belong to the same river where Pala et al. (1982) found and described the putative stabilized 32-aneuploid hybrids (Bunnari). Yet the 32-aneuploid can also be found in Rio Silis (locality 15) where a karyological study carried out on the *D. gonocephala* s.l. planarian populations by Vacca et al. (1988) discovered another anomalous karyotype of constantly 22 chromosomes with low frequency (described in 10 individuals out of the 95 studied). Vacca et al. (1988) were unable to neither reconstruct the idiogram nor to establish a solid ploidy due to the differences in size and shape of chromosomes of the same plates and also between different chromosome plates. Furthermore, all individuals had copulatory apparatus and, under laboratory conditions, they were able to lay cocoons, but these were always sterile.

This 22 chromosomal number could result from the sum of 14 + 8 chromosomes, which would be possible if a diploid *D. hepta* gamete (most likely an oocyte) and a haploid *D. benazzii* gamete (most probably a sperm) joined, which could point to these animals to be the putative H2 or H3 hybrids. The fact that in the *Cox1* network (Fig. 5b), the mixed clade *Cox1* haplotypes derive from the *D. hepta* haplotypes would give further support to this latter possibility. Thus, we find two karyotypes in Rio Silis that could be a match to our H2-H3 hybrids. Unfortunately, we only have karyological information from two individuals of the mixed clade, and they were found not to bear a diploid set of chromosomes (neither 14 nor 16 chromosomes), but the exact number was not registered.

Molecular evidence could point to a hybrid lineage that originated through the cross of a *D. hepta* oocyte with a *D. benazzii* sperm and that now seems to be stabilized with its individuals reproducing by their own (H2 individuals) so that no mitochondrial genomes from any of both parental species is newly introduced in this lineage. A possibility would be

that the hybrid populations could carry on reproducing by fission, a reproductive strategy frequently used in *Dugesia* when they become triploids (Stocchino and Manconi 2013) and, on time, evolve their own mitochondrial lineage by accumulating changes, as we observe in this case. That they use this type of reproduction will be supported by the low nucleotide and haplotype diversity found within this group, especially as compared with those found for the hepta and *benazzii* haplogroups (Table 4), expected for clonal individuals. This hypothesis would explain why the hybrids bear nuclear haplotypes of the two parental haplogroups but would not explain the existence of a recombinant haplotype (individual MR0092-11), neither the individuals of the H3 group. However, if the hybrids are able to intercross these latter cases would be explained, but this hypothesis has the problem of how the two different karyotype compositions can combine to produce viable gametes in the hybrids. A possibility would be that these animals use a similar strategy to that observed in triploid ex-fissiparous lineages of *D. ryukyuensis* (Chinone et al. 2014). In a lineage from this species before spermatogenesis begins, the spermatogonia eliminate a whole set of chromosomes, hence in a triploid hybrid between *D. hepta* and *D. benazzii*, two sets of the same species could remain in a certain proportion of cases and pass a regular meiosis. In the female oogenesis in *D. ryukyuensis*, the three sets of chromosomes are retained until the metaphase I occurs. During the meiosis, two chromosome sets pair and the third remains alone. Thus, there is a certain probability that either the two sets of chromosomes from the same species pair or that those homologous chromosomes from the two parental species pair. This process would provide some haploid genetically equilibrated (bearing one copy of each gene) oocytes and also some diploid oocytes, either bearing two sets of chromosomes coming both from one species or even recombinants between the two species' chromosomes. This situation would clearly render these animals mostly sterile (explaining for instance the observations of Vacca et al. 1988) since the probability of getting two gametes with an equilibrated set of chromosomes each to mate and give offspring would be low. Nonetheless, even if this happened with a low frequency, it would be enough to explain the presence of a recombinant haplotype among so many sequenced and especially that some individuals may have only nuclear haplotypes from one of the parent species. Thus, in this hypothesis, the hybrids may mostly reproduce by fission but could be able to mate and produce some fertile offspring from time to time. In any case, both hypotheses point to a probable case of speciation by hybridization since the hybrids would have stabilized and have established populations reproducing on their own.

There is also a third possibility that these mixed clade hybrids can cross with the parental species. Almost all the individuals in the mixed clade belong to localities where both species coexist (localities 5, 6, 10, 12, 14 and 15; Supporting

information Table S1; Fig. 2), thus giving the hybrids that have produced some genetically viable gamete the opportunity to backcross. When the hybrids act as females, the mitochondrial lineage is retained to evolve independently, while the nuclear genome is continuously being introgressed by parental species chromosomes. A situation of this type is known from the vertebrate genus *Rana* (Blankenhorn, 1977). We wonder if such reproductive strategy may explain the *D. hepta*, *D. benazzii*, and putative hybrids conundrum and if environmental and/or competition factors may explain the existence of the hybrids and the co-occurrence of the three lineages.

However, similar results can be expected under incomplete lineage sorting (ILS) when a radiation takes place as it has been demonstrated in other cases (Suh et al. 2015). What we consider to be the *hepta* and *benazzii* exclusive haplotypes for *Dunuc12* and *ITS-1* would have been population alleles in a polymorphic ancestor. Stochastically, the *benazzii* variant could have gone lost in the *D. hepta* lineage and preserved as a polymorphic state in *D. benazzii* populations. Thus, the mitochondrial mixed clade would be a distinct *D. benazzii* clade—C—that would have diverged from the other Sardinian *D. benazzii*. We could expect to find in said clade homozygous *Dunuc12* individuals for the *D. benazzii* variant—even for the *D. hepta* variant, though we found none—as well as heterozygous individuals that would be our “hybrids.” This could also justify why there are individuals whose karyotype is *benazzii*-like and homozygous for the *benazzii* haplotypes within the mixed clade. However, this hypothesis fails to explain why there are individuals bearing anomalous karyotypes and showing infertility as found in previous studies. On the other hand, from an ecological point of view, three differentiated lineages, probably depending on the same resources, co-occurring in the same localities seem a more difficult scenario to explain than two separated lineages co-occurring with their hybrids.

Conclusions

We present for the first time molecular evidence of the species status for *D. hepta* as a sister group and not derived from *D. benazzii*. In addition, we have found that *D. benazzii* individuals from Corsica may in fact be a different species. At the same time, we have uncovered an unexpected and complex situation in those rivers from Sardinia where the two species, *D. benazzii* and *D. hepta*, co-occur. *Dugesia benazzii* was thought to be a complex species presenting different ploidies and even aneuploids and with sexual and asexual reproduction, while *D. hepta* is exclusively diploid and sexual. Our results show that some aneuploids may in fact be the result of crossings between both species, which represents the first

demonstration of planarian hybridism in natural conditions on the base of molecular data, and what is more relevant is that they may even have become a new species. But the complexity of the mitochondrial and nuclear haplotype combinations found makes present information not enough to solve the riddle on how these hybrids may have originated and how they reproduce (if they do) and point to the need of a thorough study. An extensive sampling in the rivers where they co-occur, followed by a study at the genomic level of karyotyped individuals, so that the reproductive behavior, morphology, karyotype, and genomic information is known from each individual as well as the performance of interbreeding tests might render an interesting view on how this complex situation has been generated and is evolving.

Acknowledgments We kindly thank Prof. Maria Pala and Dr. Eva Lázaro for providing specimens of *D. hepta* and *D. benazzii*. We are also grateful to Dr. Marta Álvarez-Presas for her always-welcomed advice and guiding throughout the development of the analytical part of this study, and to all of our laboratory members for their help.

Data availability statement The sequences generated and analyzed during the current study are available in the GenBank repository (<https://www.ncbi.nlm.nih.gov/genbank/>), under accession numbers MN162692, MN162693 (ITS-1 haplotypes), MN442429 to MN442516 (*Dunuc12*), MN442517 to MN442535 (COI cloned haplotypes), MN525628 to MN525771 (COI), and MN565083 to MN565258 (*Dunuc12* cloned haplotypes).

Funding information This research was supported by the Ministerio de Economía y Competitividad, Spain (project CGL2015-63527-P).

References

- Abril, J. F., Cebrià, F., Rodríguez-Esteban, G., Horn, T., Fraguas, S., Calvo, B., Bartscherer, K., & Saló, E. (2010). Smed454 dataset: unravelling the transcriptome of *Schmidtea mediterranea*. *BMC Genomics*, 11(1), 731. <https://doi.org/10.1186/1471-2164-11-731>.
- Aguilar, J.P. (2011) Genética Evolutiva de *Dugesia benazzii* y *Dugesia hepta* de las islas de Córcega y Cerdeña. Master thesis, Universitat de Barcelona. Research Gate. <https://doi.org/10.13140/RG.2.2.23010.27849>.
- Álvarez-Presas, M., & Riutort, M. (2014). Planarian (Platyhelminthes, Tricladida) diversity and molecular markers: a new view of an old group. *Diversity*, 6, 323–338. <https://doi.org/10.3390/d6020323>.
- Arnold, M. L., Sapir, Y., & Martin, N. H. (2008). Review. Genetic exchange and the origin of adaptations: prokaryotes to primates. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 363(1505), 2813–2820. <https://doi.org/10.1098/rstb.2008.0021>.
- Baguña, J., Carranza, S., Pala, M., Ribera, C., Giribet, G., Amedo, M. A., Ribas M., Riutort M. (1999). From morphology and karyology to molecules. New methods for taxonomical identification of asexual populations of freshwater planarians. A tribute to Professor Mario

- Benazzi. *Italian Journal of Zoology*, 66, 207–214. <https://doi.org/10.1080/11250009909356258>.
- Benazzi, M. (1949). Problemi di differenziazione specifica e razziale nei Tricladidi. *Bollettino della Società Italiana di Biologia Sperimentale*, 25, 666.
- Benazzi, M. (1955). Appunti sulla distribuzione dei Tricladidi in Italia. *Bollettino di Zoologia*, 22(2), 149–164. <https://doi.org/10.1080/11250005509439195>.
- Benazzi, M. (1982). Speciation events as evidenced in Turbellaria. In C. Barigozzi (Ed.), *Mechanisms of speciation* (pp. 307–344). New York: Alan R. Liss.
- Benazzi, M., & Banchetti, R. (1972). Descrizione di *Dugesia biblica*, nuova microspecie del “gruppo *Dugesia gonocephala*” trovata nel fiume Giordano (Israele). *Atti della Società Toscana di Scienze Naturali*, 79, 83–91. http://www.stsn.it/AttiB1972/Benazzi_Banchetti_M.pdf. Accessed 20 February 2020.
- Benazzi, M., & Benazzi-Lentati, G. (1976). Platyhelminthes. In B. John (Ed.), *Animal cytogenetics* (pp. 1–182). Berlin-Suttgart: Gebrüder Borntraeger.
- Benazzi, M., & Deri, P. (1980). Histo-cytological study of fissiparous planarian testicles (Tricladida, Paludicola). *Italian Journal of Zoology*, 14(3), 151–163. <https://doi.org/10.1080/00269786.1980.10736353>.
- Benazzi, M., & Puccinelli, I. (1973). A Robertsonian translocation in the fresh-water triclad *Dugesia lugubris*: karyometric analysis and evolutionary inferences. *Chromosoma*, 198, 193–198. <https://fdocuments.in/document/a-robertsonian-translocation-in-the-fresh-water-triclad-dugesia-lugubris.html>. Accessed 20 February 2020.
- Benazzi-Lentati, G., & Benazzi, M. (1985). On the origin and the true nature of the tetraploid asynaptic planarian called Benazzi's G biotype. *Caryologia*, 38(3–4), 269–279. <https://doi.org/10.1080/00087114.1985.10797750>.
- Berninger, J. (1911). Über die Einwirkung des Hungers auf Planarien. *Zoologische Jahrbücher, Abteilung für allgemeine Zoologie und Physiologie der Tiere*, 30, 181–216.
- Chinone, A., Nodono, H., & Matsumoto, M. (2014). Triploid planarian reproduces truly bisexually with euploid gametes produced through a different meiotic system between sex. *Chromosoma*, 123(3), 265–272. <https://doi.org/10.1007/s00412-013-0449-2>.
- Coates, D. J., & Shaw, D. D. (1984). The chromosomal component of reproductive isolation in the grasshopper *Caledia captiva* III. Chiasma distribution patterns in a new chromosomal taxon. *Heredity*, 53, 85–100. <https://doi.org/10.1038/hdy.1984.65>.
- Curini-Galletti, M. C., Puccinelli, I., & Martens, P. M. (1985). Meccanismi di evoluzione del cariotipo nei monocelididi (Turbellaria, Proseriata). *Atti della Società Toscana di Scienze Naturali (Serie B)*, 92, 291–297. <http://www.vliz.be/imisdocs/publications/276721.pdf>. Accessed 20 February 2020.
- De Queiroz, K. (2007). Species concepts and species delimitation. *Systematic Biology*, 56(6), 879–886. <https://doi.org/10.1080/10635150701701083>.
- De Vries, E. J. (1984). On the species of the *Dugesia gonocephala* group (Platyhelminthes, Turbellaria, Tricladida) from Greece. *Bijdragen tot de Dierkunde*, 54(1), 101–126. <http://cat.inist.fr/?aModele=afficheN&csid=9712729>.
- De Vries, E. J. (1985). The biogeography of the genus *Dugesia* (Turbellaria, Tricladida, Paludicola) in the Mediterranean region. *Journal of Biogeography*, 12(6), 509–518. <https://doi.org/10.2307/2844906>.
- De Vries, E. J. (1986). On the karyology of *Dugesia gonocephala* s.l. (Turbellaria, Tricladida) from Montpellier, France. *Hydrobiologia*, 132(1), 251–256. <https://doi.org/10.1007/BF00046257>.
- Dover, G. (1982). Molecular drive: a cohesive mode of species evolution. *Nature*, 299(5879), 111–117. <https://doi.org/10.1038/299111a0>.
- Dugès, A. (1830). Aperçu de quelques observations nouvelles sur les Planaires et plusieurs genres voisins. *Annales des Sciences Naturelles*, 21, 72–91. <http://gallica.bnf.fr/ark:/12148/bpt6k55070078/f4.image.r>. Accessed 20 February 2020.
- Forsdyke, D. R. (2004). Chromosomal speciation: a reply. *Journal of Theoretical Biology*, 230(2), 189–196. <https://doi.org/10.1016/j.jtbi.2004.04.020>.
- Galleni, L., & Puccinelli, I. (1986). Chromosomal evolution in marine triclads and polyclads (Turbellaria). *Hydrobiologia*, 132, 239–242. <https://doi.org/10.1007/BF00046255>.
- Hall, T. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*. <https://doi.org/citeulike-article-id:691774>.
- Harrath, A. H., Sluys, R., Mansour, L., Lekeufack Folefack, G., Aldahmash, W., Alwasel, S., Solà, E., & Riutort, M. (2019). Molecular and morphological identification of two new African species of *Dugesia* (Platyhelminthes, Tricladida, Dugesidae) from Cameroon. *Journal of Natural History*, 53(5–6), 253–271. <https://doi.org/10.1080/00222933.2019.1577508>.
- Hillis, D. M., & Dixon, M. T. (1991). Ribosomal DNA: molecular evolution and phylogenetic inference. *The Quarterly Review of Biology*, 66(4), 411–453. <https://doi.org/10.1086/417338>.
- Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, 30(4), 772–780. <https://doi.org/10.1093/molbev/mst010>.
- Kawakami, T., Butlin, R. K., & Cooper, S. J. B. (2011). Chromosomal speciation revisited: Modes of diversification in Australian morabine grasshoppers (*Vandiemenella viatica* species group). *Insects*, 2(1), 49–61. <https://doi.org/10.3390/insects2010049>.
- King, M. (1987). Chromosomal rearrangements, speciation and the theoretical approach. *Heredity*, 59 (Pt 1)(1), 1–6. <https://doi.org/10.1038/hdy.1987.90>.
- King, M. (1992). A dual level model for speciation by multiple pericentric inversions. *Most*, 68(May 1991), 437–440. <https://doi.org/10.1038/hdy.1992.63>.
- King, M. (1993). *Species evolution: the role of chromosome change*. Cambridge: Cambridge University Press.
- Knowlton, N. (1993). Sibling species in the sea. *Annual Review of Ecology and Systematics*, 24, 189–216. <https://doi.org/10.2307/2097177>.
- Kucera, M., & Darling, K. F. (2002). Cryptic species of planktonic foraminifera: their effect on palaeoceanographic reconstructions. *Philosophical transactions. Series A, Mathematical, physical, and engineering sciences*, 360, 695–718. <https://doi.org/10.1098/rsta.2001.0962>.
- Lang, A. (1884). *Die polycladen (seeplanarien) des colfes von Neapel und der angrenzenden meeresabschnitte*. Leipzig: W. Engelmann. <http://www.biodiversitylibrary.org/bibliography/10545#summary>. Accessed 20 February 2020.
- Lázaro, E., & Riutort, M. (2013). *Dugesia sicula* (Platyhelminthes, Tricladida): the colonizing success of an asexual Planarian. *BMC Evolutionary Biology*, 13(1), 268. <https://doi.org/10.1186/1471-2148-13-268>.
- Lázaro, E., Sluys, R., Pala, M., Stocchino, G. A., Bagaña, J., & Riutort, M. (2009). Molecular barcoding and phylogeography of sexual and asexual freshwater planarians of the genus *Dugesia* in the Western Mediterranean (Platyhelminthes, Tricladida, Dugesidae). *Molecular Phylogenetics and Evolution*, 52(3), 835–845. <https://doi.org/10.1016/j.ympev.2009.04.022>.
- Lepori, N. G. (1951). Sulle caratteristiche morfologiche e sulla posizione sistematica della planaria di Sardegna e Corsica già ascritta a *Dugesia (Euplanaria) gonocephala* (Dugès). *Atti della Società Toscana di Scienze Naturali*, 58, 28–47.
- Leria, L., Sluys, R., & Riutort, M. (2018). Diversification and biogeographic history of the Western Palearctic freshwater flatworm genus

- Schmidtea* (Tricladida: Dugesiidae), with a redescription of *Schmidtea nova*. *Journal of Zoological Systematics and Evolutionary Research*, 56(3), 335–351. <https://doi.org/10.1111/jzs.12214>.
- Leria, L., Vila-Farré, M., Solà, E., & Riutort, M. (2019). Outstanding intraindividual genetic diversity in fissiparous planarians (*Dugesia*, Platyhelminthes) with facultative sex. *BMC Evolutionary Biology*, 19, 1–19. <https://doi.org/10.1186/s12862-019-1440-1>.
- Leria, L., Vila-Farré, M., Álvarez-Presas, M., Sánchez-Gracia, A., Rozas, J., Sluys, R., & Riutort, M. (2020). Cryptic species delineation in freshwater planarians of the genus *Dugesia* (Platyhelminthes, Tricladida): Extreme intraindividual genetic diversity, morphological stasis, and karyological variability. *Molecular Phylogenetics and Evolution*, 143(May), 106496. <https://doi.org/10.1016/j.ympev.2019.05.010>.
- Librado, P., & Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25(11), 1451–1452. <https://doi.org/10.1093/bioinformatics/btp187>.
- Mallet, J. (2007). Hybrid speciation. *Nature*, 446, 279–283. <https://doi.org/10.1038/nature05706>.
- Mallet, J., Besansky, N., & Hahn, M. W. (2016). How reticulated are species? *BioEssays*, 38(2), 140–149. <https://doi.org/10.1002/bies.201500149>.
- Newmark, P. A., & Alvarado, A. S. (2002). Not your father's planarian: a classic model enters the era of functioning genomics. *Nature Reviews Genetics*, 3(3), 210–219. <https://doi.org/10.1038/nrg759>.
- Nishimura, O., Hosoda, K., Kawaguchi, E., Yazawa, S., Hayashi, T., Inoue, T., Umesono, Y., & Agata, K. (2015). Unusually large number of mutations in asexually reproducing clonal planarian *Dugesia japonica*. *PLoS One*, 10(11), 1–23. <https://doi.org/10.1371/journal.pone.0143525>.
- Pala, M., Casu, S., & Vacca, R. A. (1981). *Dugesia hepta*, nuova specie di planaria di acqua dolce di Sardegna appartenente alla superspecie *Dugesia gonocephala* (Dugès). *Bollettino della Società Sarda di Scienze Naturali*, 20, 97–107. <https://core.ac.uk/reader/11689490>. Accessed 20 February 2020.
- Pala, M., Casu, S., & Lepori, N. G. (1982). Stabilized natural interspecific hybrid population of the fresh water planarians *Dugesia gonocephala* s. l. (Turbellaria, Tricladida). *Caryologia*, 35(2), 247–256. <https://doi.org/10.1080/00087114.1982.10796929>.
- Pala, M., Casu, S., & Stocchino, G. A. (1999). Karyology and karyotype analysis of diploid freshwater planarian populations of the *Dugesia gonocephala* group (Platyhelminthes, Tricladida) found in Sardinia. *Hydrobiologia*, 392(2), 113–119. <https://doi.org/10.1023/A:1003534507632>.
- Pennisi, E. (2016). Shaking up the tree of life. *Science*, 354(6314), 817–821. <https://doi.org/10.7748/ns.16.21.24.s37>.
- Posada, D. (2008). jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution*, 25(7), 1253–1256. <https://doi.org/10.1093/molbev/msn083>.
- Reddien, P. W., & Alvarado, A. S. (2004). Fundamentals of planarian regeneration. *Annual Review of Cell and Developmental Biology*, 20, 725–757. <https://doi.org/10.1146/annurev.cellbio.20.010403.095114>.
- Ribas, M. (1990). *Cariologia. Sistemàtica i biogeografia de les planàries d'aigües dolces als Països Catalans*: Universitat de Barcelona.
- Rieseberg, L. H. (2001). Chromosomal rearrangements and speciation. *Trends in Ecology and Evolution*, 16(7), 351–358. [https://doi.org/10.1016/S0169-5347\(01\)02187-5](https://doi.org/10.1016/S0169-5347(01)02187-5).
- Riutort, M., Álvarez-Presas, M., Lázaro, E., Solà, E., & Paps, J. (2012). Evolutionary history of the Tricladida and the Platyhelminthes: an up-to-date phylogenetic and systematic account. *International Journal of Developmental Biology*, 56(1–3), 5–17. <https://doi.org/10.1387/ijdb.113441mr>.
- Robb, S. M. C., Ross, E., & Alvarado, A. S. (2007). SmedGD: the *Schmidtea mediterranea* genome database. *Nucleic Acids Research*, 36, 599–606. <https://doi.org/10.1093/nar/gkm684>.
- Robb, S. M. C., Gotting, K., Ross, E., & Alvarado, A. S. (2015). SmedGD 2.0: the *Schmidtea mediterranea* genome database. *Genesis*, 53(8), 535–546. <https://doi.org/10.1002/dvg.22872>.
- Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., et al. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, 61(3), 539–542. <https://doi.org/10.1093/sysbio/sys029>.
- Schultz, E. (1904). Über Reduktionen. I. Über Hungerserscheinungen bei *Planaria lactea*. *Arch. Entw. Org.*, 18, 555–557.
- Sluys, R., & De Jong, H. (1984). Chromosome morphological studies of *Dugesia gonocephala* s.l. (Platyhelminthes, Tricladida). *Caryologia*, 37(1–2), 9–20. <https://doi.org/10.1080/00087114.1984.10797683>.
- Sluys, R., Solà, E., Gritzalis, K., Vila-Farré, M., Mateos, E., & Riutort, M. (2013). Integrative delineation of species of Mediterranean freshwater planarians (Platyhelminthes: Tricladida: Dugesiidae). *Zoological Journal of the Linnean Society*, 169(3), 523–547. <https://doi.org/10.1111/zoj.12077>.
- Solà, E., Sluys, R., Gritzalis, K., & Riutort, M. (2013). Fluvial basin history in the northeastern Mediterranean region underlies dispersal and speciation patterns in the genus *Dugesia* (Platyhelminthes, Tricladida, Dugesiidae). *Molecular Phylogenetics and Evolution*, 66(3), 877–888. <https://doi.org/10.1016/j.ympev.2012.11.010>.
- Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*, 22(21), 2688–2690. <https://doi.org/10.1093/bioinformatics/btl446>.
- Stocchino, G. A. (2018). 80 years of research on planarians (Platyhelminthes, Tricladida) from Sardinia, Italy: an annotated checklist. *Zootaxa*, 4532(4), 539–552. <https://doi.org/10.11646/zootaxa.4532.4.5>.
- Stocchino, G. A., & Manconi, R. (2013). Overview of life cycles in model species of the genus *Dugesia* (Platyhelminthes: Tricladida). *Italian Journal of Zoology*, 803(3), 319–328. <https://doi.org/10.1080/11250003.2013.822025>.
- Stocchino, G. A., Corso, G., Manconi, R., Casu, S., & Pala, M. (2005). Endemic freshwater planarians of Sardinia: redescription of *Dugesia hepta* (Platyhelminthes, Tricladida) with a comparison of the Mediterranean species of the genus. *Journal of Natural History*, 39(22), 1947–1960. <https://doi.org/10.1080/00222930500060025>.
- Stocchino, G. A., Sluys, R., Riutort, M., Solà, E., & Manconi, R. (2017). Freshwater planarian diversity (Platyhelminthes: Tricladida: Dugesiidae) in Madagascar: new species, cryptic species, with a redefinition of character states. *Zoological Journal of the Linnean Society*, 181, 727–756. <https://doi.org/10.1093/zoolinlean/zlx017>.
- Stocchino, G. A., Sluys, R., Harrath, A. H., Mansour, L., & Manconi, R. (2019). The invasive alien freshwater flatworm *Girardia tigrina* (Girard, 1850) (Platyhelminthes, Tricladida) in Western Europe: new insights into its morphology, karyology and reproductive biology. *Contributions to Zoology*, 88, 1–21. <https://doi.org/10.1163/18759866-20191406>.
- Suh, A., Smeds, L., & Ellegren, H. (2015). The dynamics of incomplete lineage sorting across the ancient adaptive radiation of neoavian birds. *PLoS Biology*, 13(8), 1–18. <https://doi.org/10.1371/journal.pbio.1002224>.
- Talavera, G., Lukhtanov, V. A., Rieppel, L., Pierce, N. E., & Vila, R. (2013). In the shadow of phylogenetic uncertainty: the recent diversification of *Lysandra* butterflies through chromosomal change. *Molecular Phylogenetics and Evolution*, 69(3), 469–478. <https://doi.org/10.1016/j.ympev.2013.08.004>.
- Tamura, K., Stecher, G., Peterson, D., Filipiński, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0.

- Molecular Biology and Evolution*, 30(12), 2725–2729. <https://doi.org/10.1093/molbev/mst197>.
- Vacca, R. A., Casu, S., & Pala, M. (1988). Popolamento planariologico dei fiumi del nord Sardegna: 1. I cariotipi delle planarie d'acqua dolce del gruppo *Dugesia gonocephala* (Turbellaria, Tricladida) presenti nel fiume Silis (Sassari). *Bollettino della Società Sarda di Scienze Naturali*, 26, 131–147. <https://www.researchgate.net/publication/277857627>. Accessed 20 February 2020.
- White, M. J. D. (1978). *Modes of speciation*. San Francisco: W. H. Freeman and Co..
- Xia, X., & Xie, Z. (2001). DAMBE: software package for data analysis in molecular biology and evolution. *Journal of Heredity*, 92(4), 371–373. <https://doi.org/10.1093/jhered/92.4.371>.
- Xia, X., Xie, Z., Salemi, M., Chen, L., & Wang, Y. (2003). An index of substitution saturation and its application. *Molecular Phylogenetics and Evolution*, 26(1), 1–7. [https://doi.org/10.1016/S1055-7903\(02\)00326-3](https://doi.org/10.1016/S1055-7903(02)00326-3).

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.