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

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26 Data availability statement

27 The sequences generated and analysed during the current study are available in the GenBank
28 repository (https://www.ncbi.nlm.nih.gov/genbank/), under accession numbers MN162692,
29 MN162693(ITS-1 haplotypes), MN442429 to MN442516 (Dunuc12), MN442517 to MN442535
30 (COI cloned haplotypes), MN525628 to MN525771 (COI), and MN565083 to MN565258
31 (Dunuc12 cloned haplotypes).

32 Abstract

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

45 Keywords: *Dugesia*, hybridization, hybrid speciation, molecular phylogeny, haplotype network

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63 Introduction

64 Tricladida (Lang 1884) –most commonly known as planarians- have been the subject to an in-
65 depth research in a wide-ranging spectrum of scientific fields, such as regeneration, pattern
66 formation, genomics, and transcriptomics (Abril et al. 2010; Newmark and Alvarado 2002;
67 Reddien and Alvarado 2004; Robb et al. 2007, 2015) as well as on diversity and
68 phylogeographical analyses (Álvarez-Presas and Riutort 2014; Leria et al. 2018; Leria et al. 2020;
69 Sluys et al. 2013; Solà et al. 2013). However, historically, the Tricladida have been a challenging
70 group for taxonomists and systematists due to the unsuspected complexity for classifying their
71 specimens which relies, ironically, in their morphological simplicity. Within any genus most
72 planarian species share a common external morphology, hence the diagnostic characters are
73 mainly found in their most complex anatomical system, the copulatory apparatus. Nonetheless,
74 the existence of fully fissiparous species (Stocchino and Manconi 2013) as well as the fact that
75 freshwater planarians are known for being able to resorb their own reproductive organs during
76 starvation periods (Berninger 1911; Newmark and Alvarado 2002; Schultz 1904) can sometimes
77 render the morphological approach useless.

78 A good example of this situation is the longstanding freshwater flatworm genus *Dugesia.* This
79 genus inhabits the Afrotropical, Palearctic, Oriental, and Australasian biogeographic regions and
80 comprises an approximate number of 90 described species (Harrath et al. 2019; Leria et al. 2020;
81 Stocchino et al. 2017). In this genus three main types of life cycle related to their reproductive
82 strategy can be found: sexual, asexual (fissiparous) and facultative, which seasonally alternate
83 between the former modes (Stocchino and Manconi 2013). Asexual reproduction in this genus
84 occurs by transverse fission of the architomic type (i.e. cellular differentiation, organ formation
85 and body reorganization occur in the new individual after the fission process). Due to the
86 existence of the aforementioned fissiparous reproducing populations, that do not develop the
87 copulatory organs, it has not been unusual to group together several different species into one
88 unique species or species group. For instance, the *Dugesia gonocephala* s.l. (Dugès, 1830) was a
89 hodgepodge where all the sexual, asexual, and non-conclusive forms of European *Dugesia* were
90 put together (Benazzi 1955; Benazzi and Banchetti 1972; Benazzi and Benazzii-Lentati 1976;
91 Benazzi and Deri 1980; De Vries 1984, 1986; Sluys and De Jong 1984). The incorporation of
92 molecular data not only helped to identify fissiparous individuals but also to delimit new species
93 and to clarify the phylogenetic relationships within the genus (Álvarez-Presas and Riutort 2014;
94 Lázaro et al. 2009; Leria et al. 2019; Leria et al. 2020; Riutort et al. 2012; Sluys et al. 2013).

95 Two species once belonging to the *D. gonocephala* s.l. complex, *Dugesia benazzii* Lepori, 1951
96 and *Dugesia hepta* Pala, Casu & Vacca, 1981 are the main focus of the present study. The former
97 inhabits the islands of Corsica, Sardinia and Capraia (Benazzi and Benazzii-Lentati 1976; De

98 Vries 1985) whereas the latter is endemic of Sardinia restricted to four fluvial basins in the
99 northern region, where both species occur in sympatry. *Dugesia hepta* differs from other
100 Sardinian *Dugesia* species in its haploid chromosomal number (n = 7), which is unique in the
101 Western Palearctic region (Stocchino et al. 2005), while in contrast *D. benazzii* presents the most
102 common haploid chromosomal number among *Dugesia* species, n = 8 (Fig. 1). Moreover, *D.*
103 *benazzii* is known for comprising diploid (2n = 16), triploid (2n = 3x = 24), tetraploid (2n = 4x =
104 32), hexaploid (2n = 6x = 48) and aneuploid (with a mean chromosomal number of 32)
105 populations (Lepori 1951; Pala et al. 1982, 1999; Stocchino 2018). Concurrently, *D. benazzii*
106 characteristically presents sexual and mixed (coexistence of sexual and fissiparous individuals)
107 natural populations whereas for *D. hepta* there are no reports on asexual and/or polyploid
108 individuals (Stocchino and Manconi 2013). At first, both species were considered to be identical
109 and indistinguishable except for their karyotypes (Pala et al. 1981), yet in a later study (Stocchino
110 et al. 2005) differential morphological features were described in the copulatory apparatus and in
111 the external morphology. Molecularly, *D. benazzii* and *D. hepta* have had a meager presence in
112 the current molecular phylogenetic era, being represented in all cases only by a few individuals
113 (Lázaro et al. 2009; Solà et al. 2013). From those studies we learnt that they are sister groups and
114 closely related to the *Dugesia* species from the Western Palearctic Region.

115 These two sister species externally morphologically nearly identical, and biogeographically
116 sympatric pose an interesting case study. Since no thorough molecular study with a broad taxon
117 sampling centered on these species have been carried out before and adding to the fact that *D.*
118 *hepta* is restricted to only four fluvial basins where co-occurs with *D. benazzii* individuals, one
119 may wonder whether *D. hepta* could have had multiple origins or if it is really a monophyletic
120 species. In the first scenario, *D. hepta* would be the result of a recurrent chromosomal disorder
121 from *D. benazzii* specimens and, thus, should not be considered as a species *per se*. In the second
122 scenario, we could face a possible case of biogeographical sympatric speciation due to a
123 chromosomal rearrangement, with a concomitant parallel dispersion over the same fluvial basins.
124 Speciation due to chromosomal rearrangements has been proposed to take place in other planarian
125 genera (Benazzi 1982; Leria et al. 2018) as well as in several turbellarian groups (Curini-Galletti
126 et al. 1985; Galleni and Puccinelli 1986). On the other hand, regardless of the taxonomical status
127 of *D. hepta*, given its external morphological similarity to *D. benazzii* and their spatial
128 distribution, one could wonder whether they are able to intercross and if they do it naturally. In
129 fact, there are reports on an aneuploid (2n = ?x = 32) population of *D. benazzii* – reproducing by
130 gynogenesis and referred to as the 'biotype G' (Benazzi 1949)- located in Rio Bunnari (where the
131 two species co-occur) that was considered to be a stabilized natural hybrid population (Pala et al.
132 1982) based on karyological data, yet further research rejected that hypothesis (Benazzi-Lentati
133 and Benazzi 1985).

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

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

156

157 **Material and methods**

158 Sampling

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

163 Assignment of individuals to species

164 Diagnostic differences between the two species rely on their karyotypes and differences in the
165 copulatory apparatus. Aguilar (2011) found the external morphological differences proposed by
166 Stocchino et al. (2005) to be misleading due to the fact that in some populations these differences

167 were not always clearly recognizable but in contrast it was envisaged that *D. hepta* and *D. benazzii*
168 differed in 6 divergent sites in their *Internal Transcribed Spacer-1* (*ITS-1*) sequences of the
169 ribosomal cluster (Aguilar, 2011) (Table 1). A situation that may allow the assignment of
170 individuals from sympatric populations without a tedious work of karyotyping and/or obtaining
171 histological sections from all the individuals. In the present work in the first place, we checked
172 whether this correlation was univocally true by karyotyping 31 individuals from different
173 populations to assign them to a species based on their chromosomal number. Once the
174 karyological assignation was established, *ITS-1* sequences were obtained. Our newly obtained
175 data corroborated Aguilar's (2011) hypothesis. Thereafter, *ITS-1* sequences were obtained to
176 assign individuals to species.

177 Karyotyping

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

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

193 DNA extraction, quantification and sequence amplification

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

199 Specific primers were used to amplify a fragment of the mitochondrial gene *Cytochrome c oxidase*
200 *I* (*Cox1*), the *ITS-1* and the *transmembrane p24 trafficking protein 9* (*TMED9*; referred in the

201 present study as *Dunuc12*). Sequences and annealing temperatures of each pair of primers are
202 given in Table 2. Final PCR reaction volume for all markers was 25 µl, consisting of: (1) 5µl of
203 Promega 5x Green GoTaq Flexi Buffer, (2) 2 µl of $MgCl_2$ (25 mM), (3) 1 µl of dNTP (0.5 mM),
204 (4) 0.5 µl of each primer (either 10 or 25 µM), (5) 0.15 µl of *Taq* polymerase (5 u/µl) (GoTaq
205 Flexi DNA Polymerase of Promega), (6) 1 µl of genomic DNA sample (50 ng/ µl). Autoclaved
206 miliQ water was added to obtain the final PCR volume. It was necessary in many cases to vary
207 the annealing temperatures or the amount of $MgCl_2$ and/or DNA in order to achieve sequence
208 amplification. The resulting PCR products were visualized in a 1% agarose gel in order to verify
209 the correct amplification of the different molecular markers.

210 Viable-checked PCR products were purified before sequencing using a vacuum system
211 (MultiScreen™$_{HTS}$ Vacuum Manifold of Millipore) or a digestion with exonuclease I (0.2 u/µl;
212 Tebu-Bio) and shrimp alkaline phosphatase (0.2 u/µl; SAP, Sigma-Aldrich) (1:2 ExoSAP per
213 PCR product at 37°C for 15 min plus an additional phase at 80°C for 15 min). Sequencing
214 reactions were performed using Big Dye (3.1, Applied Biosystems) and ran in an automated
215 sequencer ABI Prism 3730 (Unitat de Genòmica dels Serveis Científico-Tècnics de la Universitat
216 de Barcelona) or at Macrogen Inc. (Amsterdam). The same primers used to amplify were used for
217 sequencing both strands. Chromatograms were visually checked for quality with Geneious R8
218 (Biomatters, http://www.geneious.com/ last visited June 2019) and then contig and consensus
219 sequences were obtained.

220 Since some individuals presented double-band patterns in their *Dunuc12* chromatograms, we
221 decided to clone their PCR products to inspect their origin; we also cloned some animals that
222 presented a mito-nuclear discordance or other peculiar molecular features (see results), and
223 animals not presenting such features as a control. In total 17 individuals (4 controls and the rest
224 presenting double bands, mito-nuclear discordances or other peculiarities) were cloned using a
225 TOPO® TA Cloning Kit (Thermo Fisher Scientific Inc.) following the manufacturer's
226 instructions. Approximately fifteen to thirty colonies from each individual were sequenced using
227 the T3 and T7 primers (included in the kit).

228 Phylogenetic analysis

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

236 information Table S1).

237 Levels of sequence saturation were assessed by means of the Xia et al. (2003) test implemented
238 in the software DAMBE (Xia and Xie 2001). The best substitution model was selected with
239 jModelTest (Posada 2008) based on the Akaike information criterion (AIC). In both cases the best
240 fitting model resulted in a HKY+G. Phylogenetic analyses were performed using two inference
241 methods: Maximum Likelihood (ML) and Bayesian inference (BI). ML analyses were performed
242 with RaxML 7.0.3 (Stamatakis 2006), applying a GTR+G substitution model -owing to the
243 absence of the HKY model in the aforementioned RaxML version. 5,000 replicates were
244 calculated to obtain bootstrap supports (bs) conducting a rapid bootstrap analysis and the ML
245 search was performed starting from a random tree. Furthermore, we applied an optimization of
246 both branches and model parameters on bootstrapped trees. BI analyses were conducted using
247 MrBayes v.3.2 (Ronquist et al. 2012) and applying a HKY+G substitution model. Prior to run the
248 analyses, nexus files were generated with MEGA6 (Tamura et al. 2013). We ran one cold and
249 three heated chains for two parallel runs. Both topological and model parametrization
250 convergence were surveyed by checking that the standard deviation of the split frequencies
251 reached a value below 0,01. 10,000,000 generations were performed for each gene, saving a tree
252 every 5,000 generations. We applied the default burn-in –set at 25%- in order to avoid the
253 inclusion of trees obtained before likelihood values had stabilized to infer the topology and the
254 posterior probabilities (pp). Obtained trees were visualized with FigTree v.1.4.2
255 (http://tree.bio.ed.ac.uk/software/figtree/ last visited June 2019).

256 Haplotype networks

257 In order to construct the haplotype networks, we generated two additional alignments only
258 comprising specimens from Sardinia: 1) consisting of cloned *Dunuc12* sequences from 17
259 individuals (dataset III), and 2) comprising haplotypes from the *Cox1* sequences (dataset IV). In
260 the former we included Sardinian individuals that presented chromatograms either with double-
261 bands or patterns indicating possible heterozygosity for indels which had aroused our interest
262 (individuals MR0092-05, MR0092-11, MR0025-02, MR0091-01, MR0092-04 and MR0030-06).
263 We also included individuals belonging to the COI mixed clade (see results) that did not present
264 polymorphic bands (MR0088-02 and MR0092-03) as well as two individuals whose karyotype
265 was known yet formed part of the mixed clade (MR0353-01 and MR0352-01). Furthermore,
266 individuals MR0022-04, MR0022-05 and MR0172-01 were included as they showed signs of
267 having mito-nuclear discordance. In addition, MR0022-04 and MR0172-01 had double-band
268 patterns too. Finally, four individuals previously identified as *D. hepta* (MR0354-01 and
269 MR0355-01) and *D. benazzii* (MR0368-01 and MR0370-01) based on karyological data were also
270 analyzed as controls.

271 As for the *Cox1* haplotype network (dataset IV), all individuals with polymorphic positions in
272 dataset I were excluded from the alignment. Sequences ends were trimmed using BioEdit v.7.2.5
273 to avoid overestimating the number of underlying haplotypes due to the terminal missing data.
274 DnaSP v.5 (Librado and Rozas 2009) was used to determine and assign the haplotypes for each
275 individual analyzed and Network v.5.0.0.0 (Fluxus Technology Ltd.) was used to construct the
276 haplotype networks. We used a default *epsilon* value set at zero.

277 Genetic diversity

278 Levels of nucleotide and haplotype diversity were calculated using DnaSP v.5. for each *Cox1*
279 haplogroup. We also calculated a *Cox1* distance matrix using Kimura 2P evolutionary model for
280 all the individuals included in the study.

281 Figures edition

282 The maps in figures were created with Q-GIS v.3.2.2 (https://qgis.org/es/site/ last visited June
283 2019). Figures 2 to 6 were edited with Illustrator CC v.22.0.1
284 (https://www.adobe.com/products/illustrator.html last visited June 2019).

285

286 **Results**

287 *ITS-1* as diagnostic marker

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

295 We attained an overall number of 146 *ITS-1* sequences; none presented double peaks in the
296 chromatograms. The resulting alignment had a length of 496 bp -base pairs. The six species-
297 specific sites were highlighted as key elements for the species diagnose. An overall number of 91
298 individuals were identified as *D. benazzii,* 55 as *D. hepta* and 15 remained unidentified since our
299 attempts to amplify and sequence their respective *ITS-1* were unsuccessful (*Dugesia* sp. in the
300 Supporting information Table S1).

301 Dataset characteristics

302 We set four alignments to be analyzed either to estimate phylogenies (dataset I and II) or to
303 construct the haplotype networks (dataset III and IV). Dataset I was comprised of 163 *Cox1*
304 sequences (706 bp, 44 from Corsica, 115 from Sardinia and 4 outgroup sequences) and dataset II
305 was comprised of 102 *Dunuc12* sequences (642 bp total, 81 exonic bp, 2 from Corsica, 96 from
306 Sardinia and 4 outgroup sequences). Dataset III was constructed with non-polymorphic *Cox1*
307 sequences (685 bp, 53 sequences representing the 43.1% of the original Sardinian alignment) -
308 from which 19 haplotypes were identified (Supporting information Table S2). Lastly, dataset IV
309 consisted of cloned *Dunuc12* sequences (530 bp, 55 exonic bp, 259 sequences) from 17
310 individuals, from which a total of 176 haplotypes were recovered (Supporting information Table
311 S3) with a mean of 10.0 ± 4.83 different haplotypes per individual. All new sequences have been
312 deposited in GenBank (accession numbers available in supporting information Table S1). The
313 tests revealed no significant saturation signals from any of the alignments.

314 Phylogenetic analyses

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

317 Based on the mitochondrial marker (dataset I), the BI tree showed four major clades (Fig. 3): (1)
318 *D. benazzii* specimens from Corsica (*D. benazzii* A), (2) Sardinian *D. benazzii* specimens (*D.*
319 *benazzii* B), (3) *D. hepta* individuals belonging to Sardinia (*D. hepta*) and (4) an unexpected,
320 apparently mixed clade comprised of 13 individuals identified via *ITS-1* as *D. benazzii*, 5
321 individuals identified as *D. hepta* and 8 *Dugesia* sp. individuals. Most of the clades were well-
322 supported as indicated by posterior probability (pp; ≥0,95) and *bootstrap* (bs; ≥75) values -with
323 the exception of the *D. hepta* clade, yet the resolution within clades was scarce. *Dugesia benazzii*
324 did not result in a monophyletic group but a paraphyletic one, being the Corsican clade (A) the
325 sister group to a clade constituted by *D. benazzii* from Sardinia (B), *D. hepta* and the mixed clade.
326 However, the phylogenetic relationship among these last three clades was unclear due to low
327 support values.

328 The topology yielded by the nuclear marker (dataset II; Fig. 4) differed from the one obtained
329 with *Cox1*. First of all, the populations of *D. benazzii* from Corsica and Sardinia clustered as a
330 monophyletic clade. Likewise, the main clades -namely one for *D. benazzii* and one for *D. hepta*-
331 were unambiguously supported as indicated by pp and bs values, albeit the resolution within the
332 groups was again poor. Secondly, there were no traces of the aforementioned mixed clade.
333 Instead, the individuals belonging to the mitochondrial mixed clade were integrated within either
334 the *D. hepta* or the *D. benazzii* group, in concordance with the *ITS-1* criterion. However, two
335 individuals (MR0022-04 and MR0022-05) showed signs of mito-nuclear discordance given that
336 their *Dunuc12* sequences belonged to the *D. hepta* clade -contrary to the *ITS-1* identification-

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

338 Haplotype networks

339 As regards to the *Dunuc12* (dataset IV) cloned haplotype network (Fig. 5a), we found two distinct
340 haplotype clusters separated by at least 24 substitutions and a 6-nucleotide indel. The individuals
341 karyologically identified as *D. hepta* (MR0354-01 and MR0355-01) had all their haplotypes
342 assigned to one cluster, while those karyologically identified as *D. benazzii* (MR0368-01 and
343 MR0370-01 – from *D. benazzii* B clade) had their haplotypes in the other cluster, thus indicating
344 that each cluster could be matched to a different species. Although some of the haplotypes
345 sequenced may have been artificially generated by errors of the polymerase, the high
346 differentiation between the two species' clusters ensures that their differentiation is real, so that
347 we can assign haplotypes from individuals as belonging to one species or the other depending on
348 the cluster they belong to. Out of all the individuals assigned to the species *D. hepta* based solely
349 on the *ITS-1* criterion, MR0030-06 and MR0091-01 had all their haplotypes unequivocally
350 assigned to the *D. hepta* cluster, in accordance also with their mitochondrial data. In contrast,
351 MR0092-04 had all its haplotypes associated to the *D. benazzii* cluster despite being part of the
352 mitochondrial mixed clade. On the other hand, the individuals assigned to *D. benazzii* based on
353 the *ITS-1* criterion showed more disparate results. Out of the specimens from mitochondrial *D.*
354 *benazzii* B clade, MR0022-04 and MR0022-05 presented haplotypes in both clusters whilst
355 MR0025-02 had all its haplotypes in the *D. benazzii* cluster. Individual MR0172-01 from
356 mitochondrial *D. hepta* clade presented only *benazzii* haplotypes, coinciding with its *ITS-1*
357 assignment. As regards to the individuals belonging to the mitochondrial mixed clade, individual
358 MR0088-02 had all its haplotypes associated to the *benazzii* cluster while MR0092-03 showed
359 haplotypes in both clusters. At the same time, karyotyped individuals MR0353-01 and MR0352-
360 01 showed only *benazzii* haplotypes. Lastly, individuals MR0092-05 and MR0092-11 that were
361 not possible to identify based on *ITS-1* had haplotypes assigned to both clusters. It should be
362 pointed out that intercluster recombinant haplotype variants were found in three individuals –
363 MR0025-02, MR0092-11 and MR0172-01. In summary (Fig. 6), the clonal analyses of the nuclear
364 gene of different "anomalous" individuals have resulted in the finding of four groups of
365 individuals. Some that are "pure" *D. hepta* or *D. benazzii*, and probably presented double bands
366 in their sequences due to their heterozygosity (Fig. 6, group NH). A second group that presents
367 mitochondrial haplotypes either from *hepta* or *benazzii* but in whose nucleus we can find
368 haplotypes from both species or some recombinant (group H1). A third group presenting the
369 mixed clade mitochondrial haplotypes and also presenting in the nucleus haplotypes from both
370 species (group H2), and finally a group presenting the mixed clade mitochondrial haplotype but
371 only *benazzii* nuclear haplotypes (group H3).

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

379 Genetic diversity

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

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

385

386 DISCUSSION

387 **Outstanding intraindividual haplotype diversity: artefact or commonality?**

388 We cloned the PCR products of the nuclear gene *Dunuc12* to unveil the haplotypes of the
389 putatively heterozygous individuals of *D. hepta* and *D. benazzii*. Surprisingly, we found a mean
390 of 10 different haplotypes per individual. Some of these haplotypes may have resulted from
391 polymerase errors during the PCR step before the cloning procedure; however, our results exceed
392 whatever expectations of errors caused by the malfunction of the polymerase. Even though the
393 recovered number of haplotypes was surprisingly high, similar results have been previously found
394 in *D. japonica* (Nishimura et al. 2015) and in a closely related group of species -*D. subtentaculata*,
395 *D. aurea*, *D. corbata* and *D. vilafarrei*- (Leria et al. 2020). Leria and coworkers demonstrated in
396 said study the presence of mosaicism due to the accumulation of mutations in planarian stem cells
397 during homeostatic and fission processes, resulting in multiple closely related haplotypes in
398 sexual animals (star-like patterns) and in multiple distantly related haplotypes in fissiparous
399 animals (divergent pattern). Thus, we have reasons to believe that this might be characteristic of
400 *Dugesia* species -perhaps even of other dugesid genera-, especially when asexuality is involved
401 as a reproductive strategy in the species.

402 ***Dugesia benazzii*** **species status**

403 The clues provided by the phylogenetic *Cox1* tree obtained in this study (Fig. 3) point out that *D.*

404 *benazzii* could constitute more than one species. We base this suggestion on the paraphyletic
405 arrangement of the *D. benazzii* clades in the tree and especially on the high genetic differentiation
406 among them. The genetic differentiation between the populations from Corsica and Sardinia for
407 COI has a mean value of 5.8 ± 0.8%, far superior to the ones found between populations within
408 each of the islands (Table 3). Moreover, the values found between islands concur with some of
409 the interspecific genetic distance values for *Dugesia* species from the Western and Eastern
410 Mediterranean (Lázaro et al. 2009; Solà et al. 2013) that vary between 2.8% for closely related
411 species in the Aegean region, and 11% for some species on the Western region. On the other hand,
412 the phylogenetic tree obtained from the nuclear marker (*Dunuc12*, Fig. 4) showed a monophyletic
413 *D. benazzii* clade, yet the two individuals from Corsica appeared again to be highly differentiated
414 from the Sardinian populations. Nonetheless, the fact that only two sequences for the
415 aforementioned nuclear marker of Corsican individuals were used in this study give us little
416 information regarding genetic diversity of the populations of the Corsican *D. benazzii*. On the
417 other hand, populations of *D. benazzii* from both islands are identical regarding the *ITS-1*, which
418 will support the monophyly of *D. benazzii*, but not its division in more than one species unless
419 this is a very recent event and ribosomal clusters are still being kept similar by concerted
420 evolution. As for the morphology, the original description of the species given by Lepori (1951)
421 did not establish any remarkable differences between Corsican and Sardinian *D. benazzii*
422 populations regarding the copulatory apparatus, but it did point out some minor dissimilarities
423 that he deemed insufficient neither to consider the Corsican and Sardinian populations as distinct
424 geographical subspecies. However, considering that modern descriptions take into account more
425 characters than in the past, the possibility to find valid differences supporting a specific
426 differentiation cannot be ruled out. To this purpose, a new detailed morphological study has been
427 already undertaken on populations of *D. benazzii* from the two islands that will be the subject of
428 a companion paper.

429 Hence, there is incongruence between the mitochondrial history and the nuclear and
430 morphological accounts. In some cases, a potentially high degree of genetic variation may only
431 be reflected by recondite morphological traits (according to Kucera & Darling 2002) that are not
432 evident at first sight. Sibling species often have minor morphological differences that are only
433 noticed once species are recognized for other reasons –such as karyological data or molecular
434 evidences. The species that fit this profile are known as pseudo-cryptic (Knowlton 1993), and this
435 may be the case for Corsican and Sardinian *D. benazzii* populations. Nonetheless, speciation is a
436 continuum. Theoretically, the further we stray from the starting point the clearer and more evident
437 should be the differences between descendant lineages but in the first stages of speciation there
438 can be divisiveness among sources of evidence -i.e. genetic data *versus* morphological data,
439 nuclear *versus* mitochondrial DNA- because changes do not accumulate uniformly and at a fixed

440 rate. This interval of speciation is known as the 'gray zone' (De Queiroz 2007) and could explain
441 why we find differences regarding the *Cox1* sequences between Corsican and Sardinian *D.*
442 *benazzii* populations but not in the *ITS-1* or in their morphology.

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

449 **Species status and origin of *D. hepta***

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

459 It is not clear from our results whether *D. hepta* is sister to only the Sardinian *D. benazzii* or to an
460 older lineage that gave rise to the *D. benazzii* group from Sardinia (group B) and Corsica (group
461 A). Nuclear data seem to indicate that *D. hepta* could be in fact the sister group of *D. benazzii* A
462 and B (Fig. 4), yet such relationship is questioned when the mitochondrial data is considered.
463 However, discordances in nodal support depending on the inference method used to build the tree
464 based on *Cox1* data (Fig. 3) let open the possibility that *D. hepta* could be the sister group of *D.*
465 *benazzii* A and B. It is worth noticing that although both species present similar values of diversity
466 for their *Cox1* sequences (Tables 3 and 4) they differ in how this genetic variation is
467 geographically distributed. The Sardinian populations of *D. benazzii* from the sampled localities
468 showed no remarkable signs of geospatial structure or isolation, with the exception of the samples
469 from Monte Albo (Table S1, Fig. 3 locality 16) that are appreciably genetically isolated from the
470 rest of *D. benazzii* B populations. In contrast, the populations of *D. hepta* appeared to be more
471 structured with no apparent admixture of individuals from different fluvial basins (Fig. 3). A
472 plausible explanation for the differences in mitochondrial structuration degree could reside within
473 the reproductive strategy of each species. *Dugesia hepta* is exclusively sexual while *D. benazzii*
474 is strategically more flexible having both sexual and sexual-fissiparous mixed populations which

475 could be advantageous towards rapidly colonizing new fluvial basins, as it has been shown for
476 other *Dugesia* species (Lázaro and Riutort 2013; Leria et al. 2019). This also could explain why
477 *D. benazzii* has a broader geographic distribution. A speculative scenario will be that, *D. hepta*'s
478 ancestral populations may had undergone through a constrain in numbers due to direct
479 competition with other species -possibly *D. benazzii* itself who could have colonized Sardinia
480 from Corsica and displaced *D. hepta*-. However, similar results could be expected if recent
481 bottlenecks caused by abiotic phenomena -such as the desiccation of the brooks and springs where
482 they can usually be found- affect *D. hepta* population's survival more than those of *D. benazzii*
483 due to its exclusive sexual way of reproduction. There was no apparent correlation between the
484 genetic lineages and their geographic distribution for the nuclear gene, for any of the species. This
485 can be a result of the gene analyzed being highly conserved and hence lacking information for
486 recent dispersal events.

487 Whether *D. hepta* is sister to *D. benazzii* from Sardinia or to the lineage that gave rise to *D.*
488 *benazzii* group from Sardinia and Corsica, the speciation event may have been related to a
489 chromosomal rearrangement. Bearing in mind that *D. hepta*'s chromosomal number (n = 7) is
490 uncommon within the whole Western Palearctic Region, and that most species of *Dugesia*
491 commonly share the n = 8 chromosomal number, we suggest that the ancestor that gave rise to
492 the lineages leading to the current *D. hepta* and *D. benazzii* species might have shared the same
493 chromosomal number, n = 8. Therefore, *D. hepta* could pose a case of speciation due to a
494 chromosomal rearrangement. In most animal and plant groups there are differences regarding the
495 chromosomal number among closely related species (King 1993). Nonetheless, not all of the
496 changes that may operate on the chromosomes are implicated in speciation phenomena (King
497 1987), but only those that have potential to diminish the biological efficiency of the hybrids,
498 which are known as negative heterotic (Forsdyke 2004; King 1987, 1992; Rieseberg 2001), or
499 that even impede their viability. Both cases, at shorter or longer term, give rise to reproductive
500 isolation among populations and therefore, are likely to cause speciation (White 1978).
501 Chromosomal rearrangement speciation cases have gained presence over time (Coates and Shaw
502 1984; Kawakami et al. 2011; Talavera et al. 2013). There are other cases in freshwater planarians
503 where a chromosomal rearrangement is suspicious of being the speciation cause, within the
504 dugesiid genus *Schmidtea*. *Schmidtea nova* and *S. lugubris* are two sibling species with haploid
505 chromosomal numbers of n = 3 and n = 4 respectively. Within the genus, n = 4 is the
506 plesiomorphic karyological state. *Schmidtea nova* would have originated from a common ancestor
507 through a Robertsonian translocation plus a pericentric inversion resulting in its three basic
508 chromosomes that would have rapidly isolated reproductively the descendent lineages (Benazzi
509 and Puccinelli 1973; Leria et al. 2018). In *D. hepta* we also have a reduction of the chromosomal
510 number as well as changes in the chromosomal structure -being the most remarkable a large

511 submetacentric chromosome 1 -within a predominantly metacentric set- that could be the by-
512 product of a non-reciprocal translocation that led to the loss or the assimilation of the eighth
513 chromosome. We can conclude that these two species exhibit a great karyological plasticity
514 regarding ploidy and chromosomal composition as it has been previously proposed for other
515 planarian groups (Leria et al. 2018; Leria et al. 2020; Ribas 1990), in comparison to their
516 conservative morphology, and this plasticity may in some cases be related to speciation events.
517 However, does the chromosomal difference between *D. hepta* and *D. benazzii* really impede their
518 intercrossing?

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

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

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

546 For the *ITS-1* sequences (showing *D. benazzii* origin in the four H1 individuals), either a similar
547 situation is found (lack of amplification or backcross to parental species) or else the concerted
548 evolution processes that regularly homogenize the multiple copies of the ribosomal clusters
549 (Dover 1982; Hillis and Dixon 1991) may have resulted in the original *D. benazzii* cluster having
550 overruled the *D. hepta* cluster. Subsequently, the validity of the *ITS-1* criterion as a highly reliable
551 method to identify the species that Aguilar (2011) originally proposed is questioned. It will work
552 for the parental species, but it will certainly fail to determine hybrids unless it is cloned. Since a
553 hybrid will have the genomes of both parental species, theoretically one could be amplifying the
554 *ITS-1* of any of the two parental genomes.

555 The H2 group individuals also present nuclear *Dunuc12* haplotypes from both species (or a
556 recombinant), hence likely being of hybrid origin. However, what makes these hybrids special is
557 that they bear the mixed clade *Cox1* haplotypes that appear as a monophyletic clade in the *Cox1*
558 tree, completely independent from the *D. hepta* and *D. benazzii* clades. Therefore, we have
559 individuals bearing in their nucleus haplotypes coming from both species, while their
560 mitochondrial genome seems to have differentiated from both parentals, showing a closer
561 relationship to the hepta haplogroup from which most probably derived (Fig. 5b). Moreover, to
562 make the picture more complex, within this mixed clade we also find the group H3 presenting the
563 mixed clade *Cox1* haplotypes but only benazzii nuclear sequences.

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

574 This 22 chromosomal number could result from the sum of 14+8 chromosomes, which would be
575 possible if a diploid *D. hepta* gamete (most likely an oocyte) and a haploid *D. benazzii* gamete
576 (most probably a sperm) joined, which could point to these animals to be the putative H2 or H3
577 hybrids. The fact that in the *Cox1* network (Fig. 5b) the mixed clade *Cox1* haplotypes derive from
578 the *D. hepta* haplotypes would give further support to this latter possibility. Thus, we find two
579 karyotypes in Rio Silis that could be a match to our H2-H3 hybrids. Unfortunately, we only have
580 karyological information from two individuals of the mixed clade and they were found not to bear

581 a diploid set of chromosomes (neither 14 nor 16 chromosomes), but the exact number was not
582 registered.

583 Molecular evidence could point to a hybrid lineage that originated through the cross of a *D. hepta*
584 oocyte with a *D. benazzii* sperm, and that now seems to be stabilized with its individuals
585 reproducing by their own (H2 individuals), so that no mitochondrial genomes from any of both
586 parental species is newly introduced in this lineage. A possibility would be that the hybrid
587 populations could carry on reproducing by fission, a reproductive strategy frequently used in
588 *Dugesia* when they become triploids (Stocchino and Manconi 2013) and, on time, evolve their
589 own mitochondrial lineage by accumulating changes, as we observe in this case. That they use
590 this type of reproduction will be supported by the low nucleotide and haplotype diversity found
591 within this group, especially as compared to the found for the hepta and benazzii haplogroups
592 (Table 4), expected for clonal individuals. This hypothesis would explain why the hybrids bear
593 nuclear haplotypes of the two parental haplogroups but would not explain the existence of a
594 recombinant haplotype (individual MR0092-11), neither the individuals of the H3 group.
595 Howbeit, if the hybrids are able to intercross these latter cases would be explained, but this
596 hypothesis has the problem of how the two different karyotype compositions can combine to
597 produce viable gametes in the hybrids. A possibility would be that these animals use a similar
598 strategy to that observed in triploid ex-fissiparous lineages of *D. ryukyuensis* (Chinone et al.
599 2014). In a lineage from this species before spermatogenesis begins the spermatogonia eliminate
600 a whole set of chromosomes, hence in a triploid hybrid between *D. hepta* and *D. benazzii* two sets
601 of the same species could remain in a certain proportion of cases and pass a regular meiosis. In
602 the female oogenesis in *D. ryukyuensis* the three sets of chromosomes are retained until the
603 metaphase I occurs. During the meiosis two chromosome sets pair and the third remains alone.
604 Thus, there is a certain probability that either the two sets of chromosomes from the same species
605 pair or that those homologous chromosomes from the two parental species pair. This process
606 would provide some haploid genetically equilibrated (bearing one copy of each gene) oocytes and
607 also some diploid oocytes, either bearing two sets of chromosomes coming both from one species
608 or even recombinants between the two species' chromosomes. This situation would clearly render
609 these animals mostly sterile (explaining for instance the observations of Vacca et al. 1988) since
610 the probability of getting two gametes with an equilibrated set of chromosomes each to mate and
611 give offspring would be low. Nonetheless, even if this happened with a low frequency it would
612 be enough to explain the presence of a recombinant haplotype among so many sequenced, and
613 especially that some individuals may have only nuclear haplotypes from one of the parent species.
614 Thus, in this hypothesis the hybrids may mostly reproduce by fission but could be able to mate
615 and produce some fertile offspring from time to time. In any case, both hypotheses point to a
616 probable case of speciation by hybridization, since the hybrids would have stabilized and have

617 stablished populations reproducing on their own.

618 There is also a third possibility: that these mixed clade hybrids can cross with the parental species.
619 Almost all the individuals in the mixed clade belong to localities where both species coexist
620 (localities 5, 6, 10, 12, 14 and 15; Supporting information Table S1; Fig. 2), thus giving the
621 hybrids that have produced some genetically viable gamete the opportunity to backcross. When
622 the hybrids act as females, the mitochondrial lineage is retained to evolve independently while
623 the nuclear genome is continuously being introgressed by parental species chromosomes. A
624 situation of this type is known from the vertebrate genus *Rana* (Blankenhorn, 1977). We wonder
625 if such reproductive strategy may explain the *D. hepta*, *D. benazzii* and putative hybrids
626 conundrum, and if environmental and/or competition factors may explain the existence of the
627 hybrids and the co-occurrence of the three lineages.

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

643 **Conclusions**

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

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

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897 Figure captions

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

900 **Fig. 2** Sampling localities used for this study and geographical distribution of *D. hepta* and *D.*
901 *benazzii*. Localities are numbered according to Supporting information Table S1. Red-colored
902 circles indicate the presence of *D. benazzii* species whereas yellow-colored circles indicate *D.*
903 *hepta*'s presence. (a) zoom in of Scala di Giocca.

904 **Fig. 3** Bayesian Inference tree of dataset I (*Cox1*). Node values are displayed qualitatively using
905 squares for posterior probability (pp) and circles for bootstrap support (bs) values. Used colors
906 indicate fully supported (black), significantly supported (gray) and non-supported (white) nodes.
907 Locality numbers from Table S1 are highlighted in black hexagons. Sampling localities are
908 displayed in the map as follows: in Sardinia - grouped according to hydrographical distribution
909 and in Corsica – grouped into northern and southern geographical regions as showed in the tree.

910 **Fig. 4** Bayesian inference tree of dataset II (*Dunuc12*). Node support values are displayed as in
911 Fig. 3. Bar diagrams indicate the species assignation of samples based on the *ITS-1* criterion and
912 karyology. It is also showed the phylogenetic position in dataset I's tree and the insular
913 distribution (fuchsia – Corsica and light pink – Sardinia).

914 **Fig. 5** Haplotype networks for datasets III (a – *Dunuc12*) and IV (b – *Cox1*). Haplotypes are
915 depicted as individual circles which are proportional to their abundancy (number of sequences),
916 highlighted in a white square. Mutations are either depicted with black bars or black triangles
917 when the number of mutations between linked haplotypes is equal to or exceeds a certain
918 threshold number. Insertions and deletions are represented with an elongated hexagon indicating
919 numerically the length of the *indel.* (a) For each cloned individual, information regarding *ITS-1*
920 species identification, sampling locality and availability of karyotype is given. Recombinant
921 haplotypes are highlighted with red arrows.

922 **Fig. 6** Summarization of dataset III groups and their characteristics. Individuals whose karyotype
923 is stablished are highlighted with bivalent chromosome symbol. Black and white split-up circles
924 indicate individuals with recombinant sequences.

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

Species\Alignment position	**12**	**24**	**49**	**223**	**372**	**458**	***Acc. number***
D. benazzii	C	-	G	C	A	C	MN162692
D. hepta	T	T	A	T	G	T	MN162693

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930

931 **Table 2.** Sets of primers used in this study.

Gene	Primers	Sequence (5’-3’)	Ann. Temp. [a]	Source
Dunuc12	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	GTTCATACAACTCATTCTTC	55	Leria et al. 2019
Cox1	SamCF	GCTAATAATTTGAGTTTTTG	51	Present study
	TarlyCR	CATTTTAAAACAACATTACC	51	Present study
	COIF	CCNGGDTTTGGDATDRTWTCWCA	49	Lázaro et al. 2009
	COIR	CCWGTYARMCCHCCWAYAGTAAA	49	Lázaro et al. 2009
ITS-1	ITS-9F	GTAGGTGAACCTGCGGAAGG	45	Baguñà et al. 1999
	ITSR	TGCGTTCAAATTGTCAATGATC	45	Baguñà et al. 1999

932 **[a]**: *Annealing temperature (C°)*

933

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

		D. benazzii A	***D. benazzii B***	***Mixed clade***	***D. hepta***
D. benazzii A	**(44)**	2.9 ± 1.4			
D. benazzii 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	
D. hepta	**(52)**	4.9 ± 0.7	4.0 ± 0.5	3.2 ± 0.1	1.3 ± 1.2

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938 **Table 4.** Estimations of nucleotide (π) and haplotype diversity (H_D) for the haplotype groups in
939 dataset IV (*Cox1*).

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

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