





RESEARCH ARTICLE

Three dispersal routes out of Africa: A puzzling biogeographical history in freshwater planarians

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Abstract

Aim: Freshwater planarians may have a wide geographical range despite their assumed low vagility. Found across four continents, *Dugesia* may have either an ancient origin on a large palaeo landmass, followed by colonisation in different regions before continental fragmentation, or a more recent origin and subsequent transoceanic dispersal. We seek to resolve between these two hypotheses.

Location: Africa, Eurasia and Australasia.

Taxon: Genus *Dugesia* (Platyhelminthes: Tricladida: Dugesiidae).

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Methods: We used data from the sequencing of six gene fragments and comprehensive taxonomic sampling of *Dugesia* from across its distribution range to reconstruct the phylogeny of this genus using maximum likelihood and bayesian inference methods. We conducted two phylogenetic dating analyses using Platyhelminthes fossils and palaeogeological events. Basing on the time-calibrated molecular phylogenetic framework we evaluated the contribution of vicariance and dispersal to the biogeographical evolution of *Dugesia*. By reconstructing the ancestral areas and present-day potential distribution using BioGeoBEARS and niche modelling, we elucidated the biogeographical history of the genus.

Results: The present-day distribution of *Dugesia* is a result of different vicariance and dispersal events. However, we also found evidence of transoceanic dispersal. Consistent with previous hypotheses, *Dugesia* dates to the Upper Jurassic in the Afro-Malagasy Gondwana region. We unveiled a novel biogeographical scenario for the genus, involving multiple events of colonisation in Eurasia from continental Africa via at least three dispersal routes.

Main conclusions: *Dugesia* is an ancient genus having reached its present distribution through a complex history of dispersal and vicariant events following its origin in southern Gondwana. Despite the low vagility of *Dugesia*, we found evidence of their overseas dispersal.

KEYWORDS

Gondwana, Madagascar, Mesozoic, Platyhelminthes, Tricladida, vicariance

1 | INTRODUCTION

Historical biogeography seeks to explore the relative roles of vicariance, dispersal and/or extinction in the formation of contemporary species distributions (e.g. Bourguignon et al., 2018; Sanmartín & Ronquist, 2004). Such studies are centred on the availability of well-resolved and statistically supported phylogenies as well as the presence of fossils that hint at ancient distributions and allow for the time calibration of the phylogenies. In the case of soft-bodied invertebrates, there are two primary limitations. First, the fossil record of these organisms is usually very poor and sparse (Hipsley & Müller, 2014), and second, they often possess few synapomorphic characteristics to reconstruct a resolved phylogeny (e.g. in the case of Tricladida; Sluys et al., 1998). Consequently, there have been limited studies on soft-bodied invertebrate groups, and there is a generally poor understanding of their phylogenetic origin. In recent years, the inclusion of molecular data to infer phylogenetic relationships and to place these groups in a temporal and spatial framework (e.g. Scarpa et al., 2015; Solà et al., 2013) has promoted research on soft-bodied invertebrates.

One of these soft-bodied invertebrate groups is the free-living freshwater planarian genus *Dugesia*. Its large distribution range remains an intriguing issue despite five decades of biogeographical speculations (Ball, 1974; Kawakatsu, 1968; Sluys et al., 1998). Indeed, *Dugesia* species inhabit a wide array of freshwater bodies in Africa, Europe, the Middle East, South Asia, Far East and Australasia (Figure 1). The widespread and disjunct distribution of a group of

organisms may be explained by vicariant and/or dispersal events. As in the case of most organisms with low dispersal ability, the presence of *Dugesia* across different continental land masses and islands has traditionally been attributed to vicariance. This hypothesis is based on the assumption that the dispersal of freshwater planarians is limited by their low vagility (Ball, 1974), due to the lack of protection against water loss and direct mode of development. While the planariids *Hymenella retenuova* and, probably, *Polycelis nigra* are known to form cocoons that offer some degree of resistance to desiccation (Ball, 1974; Vila-Farré et al., 2011), this phenomenon has not been recorded as yet in any known *Dugesia* species. Sexually reproducing *Dugesia* species lay spherical cocoons—capsules containing fertilised eggs and yolk cells from which several individuals (reduced versions of mature *Dugesia*) hatch. Cocoons in Dugesiidae are mostly stalked, cemented and usually left attached by an endplate to the under surfaces of stones, fallen leaves or other objects (Sluys & Riutort, 2018), rendering their biochore dispersal difficult, if not impossible (Ball, 1974).

Among the different hypotheses put forth to explain the geographical distribution of *Dugesia*, the most recently published one, based on morphological characters, places the origin of the genus in a Gondwanan scenario (Sluys et al., 1998). According to this hypothesis, the dispersal of *Dugesia* ancestors in Eurasia from those in Gondwana is a result of either (1) vicariance via rafting on the Indian subcontinent following its split from Madagascar (c. 88 Ma) until its collision with Asia (c. 55 to 20 Ma) or (2) wide dispersal throughout Africa, until the collision of the Arabian plate with the Eurasian plate, that bridged the

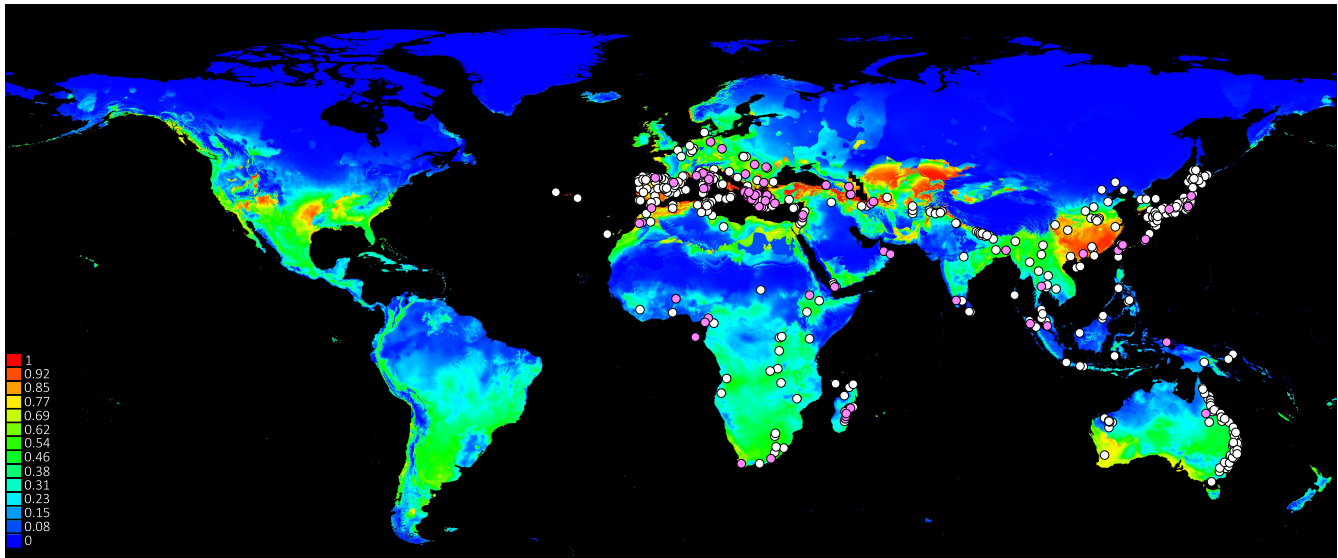


FIGURE 1 Potential distribution of the genus *Dugesia* inferred using niche modelling analysis based on localities retrieved from scientific literature (white dots) and on localities collected for the present work's molecular analyses (pink dots). Colour scale indicates predicted probability for habitat suitability (red: high probability of suitable conditions; blue: low predicted probability of suitable conditions). Equirectangular projection. Complete list of localities and their references is provided in Table S8

two continents (c. 20Ma). Nonetheless, given the lack of both morphological characteristics and fossil data, this hypothesis remains untested. Now, the use of molecular techniques may shed light on which of the existing hypotheses, or any alternative, is more plausible.

The use of molecular tools and discovery of new fossils have demonstrated that many organisms inhabiting different regions derived from the fragmentation of the Gondwana supercontinent are not the product of vicariant events but of posterior dispersal to their present distributions. This has been proven for angiosperm and vertebrate taxa (e.g. Friedman et al., 2013; Phillips et al., 2010; Samonds et al., 2013; Sanmartín & Ronquist, 2004). Interestingly, most of the vertebrate representatives in Madagascar have dispersed overseas—mainly from Africa to the island during the Cenozoic (Yoder & Nowak, 2006). Meanwhile, the impacts of Gondwana fragmentation on other groups, including centipedes (Muriénne et al., 2010), harvestmen (Giribet et al., 2012) and velvet worms (Giribet et al., 2018; Muriénne et al., 2013), have been pointed out. Furthermore, both vicariant and dispersal events have been proposed to have played a role in the diversification of groups inhabiting the former Gondwanan landmasses, such as galaxiid fishes (BurrIDGE et al., 2012) and stoneflies (McCulloch et al., 2016).

To this end, the present study used molecular phylogenetic data and biogeographical analyses to reconstruct the biogeographical history of *Dugesia* species. Based on the Gondwana origin hypothesis, we expect that the Afrotropical species of the genus are older than the Eurasian lineages. Moreover, either continental African or Malagasy lineages are more closely related to the present-day Eurasian groups, depending on which of the competing hypotheses better reflects the dispersal route followed by planarians (dispersal through the Arabian Peninsula or rafting on the India subcontinent).

Briefly, to attain these objectives (1) we collected *Dugesia* specimens across its global distribution range, including biogeographically important regions, such as Madagascar, India, Africa and the Arabian

Peninsula, aiming to (2) determine the phylogenetic relationships of *Dugesia* species, (3) infer the age of the genus and of its subsequent diversification events, (4) put forward hypotheses regarding their putative dispersal routes to the present-day distribution range, and (5) attribute the historical palaeogeographical events that have more likely shaped the diversification and phylogeny of this group.

Interestingly, *Dugesia* originated in the Afro-Malagasy region of Gondwana during the Upper Jurassic. Unexpectedly, however, we propose a new biogeographical scenario for the arrival of *Dugesia* to Eurasia, involving three independent dispersal events from Africa to Asia, to the Middle East and eastern Europe, and to western Europe. Our findings question the notion that the overseas dispersal of freshwater flatworms is impossible.

2 | MATERIALS AND METHODS

2.1 | *Dugesia* samples

To achieve the broadest sampling of *Dugesia* throughout its known distribution range (Figure 1), we sought a collaborative effort among scientists working in different regions of the distribution range of the genus and searched for *Dugesia* specimens either in known localities from the literature or by exploring suitable habitats. Most samples were fixed in absolute ethanol immediately after the collection for DNA extraction and sequencing. We did not examine the internal morphology of the individuals sampled due to technical and time constraints; thus, most specimens were identified to the genus level. In a few cases, the species of the material received had previously been identified (e.g. Harrath et al., 2019; Stocchino et al., 2017). Details of the localities and collectors are provided in Appendix S1.

2.2 | DNA extraction, polymerase chain reaction (PCR) and sequencing

Total genomic DNA was extracted using either DNAzol® (Molecular Research Center Inc. Cincinnati, OH) or Wizard® Genomic DNA Purification Kit (Promega), following the manufacturer's instructions. The following gene fragments were PCR amplified: (1) the mitochondrial gene cytochrome c oxidase subunit I (COI) as well as the nuclear ribosomal cluster (2) 18S ribosomal gene (18S), (3) 28S ribosomal gene (28S) and (4) ribosomal internal transcribed spacer-1 (*ITS-1*). Moreover, two novel nuclear markers (Leria et al., 2020) were used—an anonymous nuclear marker called (5) *Dunuc3* and (6) a disulphide isomerase called *Dunuc5*. A third marker, *Dunuc2*, was tested, but due to the difficulty in amplification, it was not included in the final analysis. Markers (5) and (6) include intronic and exonic regions (see Extended Methods in Supporting Information, Table S1). For primer sequences, PCR conditions and sequencing strategy, see Table S2 and Extended Methods in Supporting Information.

2.3 | Phylogenetic analyses

2.3.1 | Alignment

Nuclear and mitochondrial genes were aligned using MAFFT version 7 (Kato & Standley, 2013) and Geneious 10.2.3 (<https://www.geneious.com>; Kearse et al., 2012), respectively. The putative existence of sequence saturation was evaluated (see Extended Methods in Supporting Information).

Four datasets were prepared for different analyses (Tables S3–S5). Dataset I included 18S, 28S, *Dunuc3* and *COI* sequences of *Dugesia* representatives, with *Recurva* and *Schmidtea* (*Dugesiidae*) as outgroups, to root the *Dugesia* phylogeny (Sluys et al., 2013). Dataset II included *COI*, 18S, 28S, *ITS-1*, *Dunuc3* and *Dunuc5* sequences analysed concatenated or independently (no outgroup included) to construct the phylogenetic trees and perform dating analyses. Dataset III included 18S sequences of *Catenuclida* and *Rhabditophora* (including *Tricladida*) representatives, with a chaetognath species as the outgroup, to run dating analysis. Finally, dataset IV was obtained by pruning dataset II to include only one representative per biogeographical region and at least one representative per clade, thus avoiding overrepresentation of certain areas per lineage, to run biogeographical analyses. Species of *Recurva* and *Schmidtea* were used as outgroup representatives.

2.3.2 | Phylogenetic inference

Tree inference was performed using the Maximum Likelihood (ML) criterion with RaxML 8.2.8 (Stamatakis, 2014) and Bayesian Inference (BI) using MrBayes 3.2.4 (Ronquist et al., 2012) and/or

BEAST 1.8.4 (Drummond et al., 2012). RaxML and MrBayes analyses were run with partitioned concatenated gene alignments, while independent gene alignments were used for BEAST analysis. We applied the same evolutionary model for all genes in all phylogenetic analyses: GTR+I+G. For BEAST analysis, the input file was prepared using BEAUti 1.8.4. For model selection and program parametrisations, see Extended Methods in Supporting Information.

2.4 | Dating analyses

Dataset files were prepared using BEAUti 1.8.4 and run in BEAST 1.8.4 implemented on the online server CIPRES Science Gateway (Miller et al., 2010). Two different calibration approaches were used. In the first approach, performed with dataset III, we used published parasitic Platyhelminthes' fossil records (De Baets et al., 2015; De Baets et al., 2021; Dentzien-Dias et al., 2013; Upeniece, 2011) along with a secondary calibration point (dos Reis et al., 2015). Fossil calibrations were set under a lognormal distribution. Substitution model was GTR, and Yule speciation was selected.

In the second calibration approach, using dataset II, two calibration points were selected based on geological events a priori, having putatively impacted the diversification of the genus: (1) the split of Madagascar from Africa around 160–130 Ma (Ali & Aitchison, 2008; Rabinowitz & Woods, 2006; Schettino & Scotese, 2005) to calibrate the different scenarios at two alternative nodes, because two Madagascar clades appeared in the phylogeny (Figure S1: *MDG1* and *MDG2*) and (2) the formation of the mid-Aegean trench (MAT) between 12 and 9 Ma (Dermitzakis & Papanikolaou, 1981) to calibrate the split between insular and mainland species from both sides of the Aegean region. Five dating analyses were performed (Table 1). The parameters set were as follows: GTR+I+G as the substitution model; uncorrelated relaxed clock type; birth–death model as the speciation model. See Extended Methods in Supporting Information for a more detailed account on calibration strategies and dating analyses.

2.5 | Ancestral range estimation

RASP 4beta (Yu et al., 2015) was used to run BioGeoBEARS (Matzke, 2013) for statistically inferring the ancestral geographical ranges of the *Dugesia* clades using dataset IV. SDEC model (Lagrange; Ree & Smith, 2008) was implemented alone and taking into account the founder effect (parameter *j*). BEAST 1.8.4 was used to obtain the condensed tree (Extended Methods). We defined eight major geographical areas: (A) western Europe, (B) Asia, (C) Africa, (D) Arabian Peninsula, (E) India, (F) Australasia, (G) Madagascar and (H) eastern Europe. Different time layers were set according to the landmass's connectivity along time (Table S6) on the basis of different data sources (Table S7; Extended Methods).



TABLE 1 Calibration results of the five scenarios tested based on dataset II. Values in the table indicate time in million years ago; values in brackets are the 95% confidence intervals for the estimated ages of the nodes; the calibration points used in each scenario are highlighted in boldface

	MDG1-rest of <i>Dugesia</i>	MDG2-<i>AFR1</i>	(MDG2 + <i>AFR1</i>) - (<i>AFR2</i> + Eurasia)	<i>AFR2</i>-Eurasia	Europe-Asia	Europe and Middle East diversification	'Aegean' clade^a
Calibration 1	100.3 [131.8-73]	81.1 [105.2-58.5]	90.6 [115.6-68]	74.3 [94.1-55.7]	63.4 [80.7-47.5]	24.7 [31.7-18.8]	Mean: 9.5 Stdev: 0.6
Calibration 2	Mean: 145 Stdev: 6	102.3 [125-81.2]	113.7 [134.8-93]	91.9 [109.9-74.3]	77.3 [92.7-61.8]	29.4 [35.9-23.4]	Mean: 9.5 Stdev: 0.6
Calibration 3	Mean: 145 Stdev: 6	111.8 [137-85.87]	124.6 [148-99]	101.6 [123.5-80.1]	86 [105.9-66.7]	33.1 [42.3-24.7]	12.8 [16.8-9.4]
Calibration 4	155.7 [184.7-134.2]	Mean: 145 Stdev: 6	143.8 [158.7-130.4]	113.4 [130.7-96.3]	93.8 [110.3-77.7]	34.1 [41.4-27.4]	Mean: 9.5 Stdev: 0.6
Calibration 5	170.1 [222-140]	Mean: 145 Stdev: 6	154.6 [186.7-133.9]	126.5 [156.7-102.6]	107.1 [134.1-84.8]	41 [55.4-30.5]	16.1 [21.7-11.4]

^aIncludes representatives from other Mediterranean regions.

2.6 | Ecological niche modelling

To test whether the present-day distribution of *Dugesia* is consistent with its potential distribution, we prepared a datafile listing the coordinates of 856 *Dugesia* localities (Figure 1; Table S8) from all across its distribution range, including data from scientific literature and the present work.

The formatted coordinates file was used as the input for Maxent 3.4.4 (Elith et al., 2011; Phillips et al., 2017). The following four independent climatic variables were selected as described previously (Lázaro & Riutort, 2013): isothermality, mean temperature of the wettest quarter, mean temperature of the driest quarter and precipitation seasonality. The following settings were used: 25% random test percentage and 100 bootstrap replicates. The resulting model was evaluated based on the area under the curve (AUC) for both training and testing datasets and visualised using the cloglog output format.

3 | RESULTS

3.1 | *Dugesia* individuals analysed, sequences, and alignments

The sampling strategy employed was successful for most countries, except Mongolia, where only Planariidae specimens were found. The sampled specimens, together with the already available sequences, provided *Dugesia* representatives from 106 localities across 30 countries (Appendix S1; Figure 1), covering the entire distribution range of the genus.

We obtained 56 new sequences of *COI*, 62 of *ITS-1*, 87 of *18S*, 89 of *28S*, 103 of *Dunuc3*, and 73 of *Dunuc5*, and 32 of *Dunuc2* (not included in the present analysis due to its low representation but deposited in GenBank). From GenBank, we downloaded 53 *Dugesia* *COI* sequences, 48 *ITS-1* sequences, 23 *18S* sequences, 17 *28S* sequences, 4 *Dunuc3* sequences and 2 *Dunuc5* sequences. The alignments of molecular markers used in the present study did not show significant substitution saturation (Table S9). Dataset I contained sequences of 115 individuals, covering 4444 base pairs (bp). Dataset II contained sequences of 112 specimens, covering 5501 bp from six genes. Information on the alignment characteristics is shown in Table S3, and details of the individuals included in each dataset are provided in Tables S4 and S5. Table S10 lists all the sequences newly obtained or downloaded from GenBank for each individual.

3.2 | Phylogeny of *Dugesia*

3.2.1 | Outgroup rooting

The resulting tree based on dataset I (Figure S1) revealed a clade including species from Madagascar (*MDG1*) as sister to the remainder of *Dugesia* with the maximum Bayesian support. Thus, *MDG1* was

used as root in the analyses including exclusively *Dugesia* species, avoiding the use of distantly related outgroups.

3.2.2 | Phylogenetic relationships within *Dugesia*

The ML and BI tree topologies obtained based on dataset II and rooted based on the MDG1 clade are shown in Figures S2 and S3. The analyses recovered six highly supported clades. The first clade (AFR1) included specimens from Benin, South Africa, Ethiopia, Yemen and Chios Island in Greece. The second clade (MDG2) included specimens from Madagascar and Oman, with the latter being well-nested amongst the Malagasy species. The third clade included specimens from Cameroon and São Tomé (AFR2). The fourth clade included Asian specimens (ASIA). The ASIA clade was sister to the European and Middle East clade, which was split in two highly supported clades including species from the east (EEURME) and the west (WEUR) of Europe. The relationships among all these major groups were well supported (BS = 100, PP > 0.96), with a single exception. The clustering of MDG2 and AFR1 was moderately supported in BEAST analysis (PP = 0.92; Figure S3), while ML and BI analyses placed MDG2 as sister to the AFR2 plus Eurasian clade, albeit with low support (BS = 39, PP = 0.83; Figure S2). However, the two MDG clades never constituted a monophyletic group or appeared as sisters.

3.3 | Node age estimation

The divergence times obtained based on dataset III are indicated in Figure S4 and Table S11. The mean value for the split of the *Dugesia* lineage from its sister group (i.e. *Schmidtea* and *Recurva*) is estimated to be 135.9 Ma, and the estimated age of the initial diversification of the genus is 97.74 Ma. Considering that the parasitic Neodermata fossil ages were used as the minimum values to date the corresponding splits, the resulting age ranges should also be considered the minimums.

The divergence times obtained based on dataset II for seven representative splitting into five calibration scenarios are shown in Table 1 and the corresponding trees are presented in Figures 2 and Figures S5–S8. In these dating analyses, the 95% highest posterior density (HPD) range was narrower than that in the first calibration. Among the five calibration scenarios, although divergence dates varied, all placed the node dates within similar time periods. Among the single-point calibrations, in two cases where MAT was not used as a calibration point (calibrations 3 and 5), the age of the node corresponding to this event was dated in range within or only slightly beyond the values of the geological date of the MAT opening (c. 12–9 Ma). In the calibration where the Madagascar split was not used as a calibration point (calibration 1), both nodes that may be related to this event were moderately younger than the known geological date of the split of Madagascar from Africa (160–130 Ma). In the two two-point calibrations (calibrations 2 and 4), the age of each node was close and their ranges overlap. Thus, placing the

split of Madagascar from the African continent as the calibration point for the first (MDG1) or second (MDG2) split of Madagascar species does not produce markedly different (from a geological time perspective) divergence estimates (Table 1). Therefore, in the Section 4, we consider the values obtained from the calibration 4 trees when discussing the internal nodes of *Dugesia*.

The dating analysis performed based on dataset II after removing the third codon position of *COI* and intron of *Dunuc5* (Extended Methods) resulted in a tree (Figure S9) with the same topology of the relevant clades and node ages within the equivalent range as the trees obtained with calibration 4 based on the complete dataset II (Figure 2). This result corroborates that putative saturation in the most variable markers did not affect tree topology or dating inferences in the present analysis.

3.4 | Biogeography of *Dugesia*

Statistical model comparison with BioGeoBEARS revealed that the DEC+j model, including founder event dispersal, showed a significantly improved fit (DEC+j LnL = -75.5, AIC = 157 vs DEC LnL = -93.53, AIC = 191.1). The results of biogeographical analysis are shown in Figure 3 and Table S12. The usage of the alternative matrix of connectivity did not result in any significant difference. DEC+j model results showed most of the nodes present in the consensus tree with a well-supported estimated ancestral range reconstruction. At node 1, the range points for the ancestor of all present *Dugesia* were estimated to be in Africa, Madagascar or a landmass including both regions (accounting for 82.44% of range estimation). Subsequently, populations from the MDG1 clade have likely diversified in Madagascar (node 2). Node 3 indicated a similar result to node 1. Although Africa is the more probable ancestral region, Madagascar or a landmass including both areas may also be the ancestral regions (adding to an 82.59%). Ancestors of the AFR1 and MDG2 (nodes 7 and 6) clades were distributed on the respective landmasses, and their common ancestor (node 4) was most probably in Africa, Madagascar or a landmass including both areas (96.78%). The ancestors of the AFR2 + Eurasia clade (node 5) showed a high probability of being distributed in Africa (79.59%) whilst Europe and other combinations accounted for the rest. The ancestors of the Eurasian clade (node 9) were estimated to have been distributed in Asia or Europe at an approximately equal probability. Finally, the ancestors of the Asian and European clades (nodes 10 and 11) were estimated to have been distributed in the respective regions.

3.5 | Potential distribution

The Maxent model produced AUC values exceeding 0.85 for both training and testing datasets (0.872 ± 0.006 and 0.866 ± 0.010 , respectively), indicating that the model showed a high predictive power. The maximum range of potential distribution spanned the temperate zone of Eurasia and Africa, notably avoiding the desert regions (Figure 1).

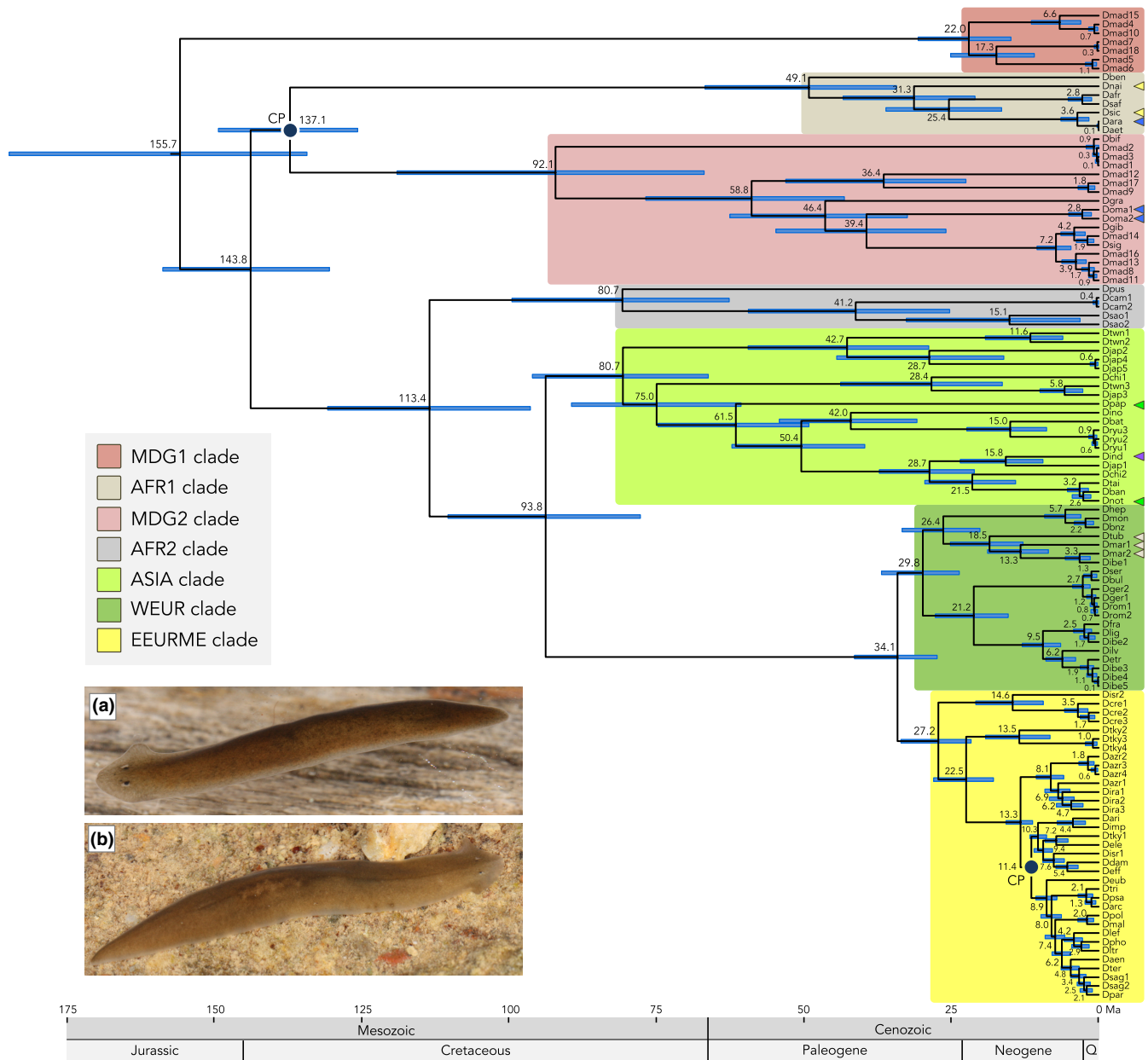


FIGURE 2 Calibrated phylogenetic tree of *Dugesia* obtained using BEAST with dataset II. Blue circles indicate the calibrated nodes (CP): the split of Madagascar from Africa approximately 160–130 Ma and the formation of the mid-Aegean trench (MAT) approximately 12–9 Ma (Table 1). Coloured triangles beside some terminals indicate either geographical outliers or specific distributions (i.e. *Dind*; India) and correspond to colours in Figure 3. Legend, the main clades are labelled according to their geographical distribution: MDG, Madagascar; AFR, Africa; ASIA, Asia; WEUR, western Europe; EEURME, eastern Europe and the Middle East. Blue bars represent 95% confidence intervals for the estimated ages of the nodes. (a) Specimen of *Dugesia cretica* and (b) specimen of *Dugesia sicula*

Moreover, wide unexplored geographical areas in Africa, Asia and Australasia may harbour *Dugesia* representatives. Finally, the Americas present vast geographical expanses suitable for *Dugesia*.

4 | DISCUSSION

In the present study, we illustrated the first well-resolved phylogeny of the genus *Dugesia* including representatives from across its known range of distribution, shedding light on the complex history for this ancient genus. Our findings indicate that the current distribution of

Dugesia has been shaped by not only vicariance due to Gondwana fragmentation but also speciation within continents. To explain the results in detail, we hypothesise multiple colonisation events of Eurasia from continental Africa via at least three different dispersal routes and at least one putative event of overseas dispersal.

4.1 | Calibration considerations

Both dating approaches employed have limitations. In the first calibration approach, one limitation was the lack of Tricladida fossils



to estimate the age of clades at the order level. The second limitation was a general lack of certainty regarding the placement of the Neodermata fossils in the phylogeny (Lee, 1999). The use of a single gene (i.e. 18S), in addition to the aforementioned drawbacks, may have contributed to the wide 95% HPD ranges obtained. In contrast, confidence intervals obtained with the second calibration approach were much narrower, thanks perhaps to the broader specimen and gene representation. In the second set of calibration analyses, as in the case of any geological calibration, we cannot entirely be certain regarding the events impacting the diversification of specific clades. Nonetheless, in the three single-point calibrations, the dating estimates obtained for the node of the non-used calibration point fell within the range of published dates of those events (MAT opening = c. 12–9 Ma and Madagascar split from Africa = 160–130 Ma), supporting the validity of our findings, irrespective of the limitations. Moreover, the two independent calibration approaches placed the origin of the genus *Dugesia* and some of its divergences within a similar range of ages, spanning from the Upper Jurassic to the Early Cretaceous, lending further support to the obtained dating estimates.

4.2 | Biogeographical origin of the genus *Dugesia*: Out of Gondwana

Different lines of evidence derived from our analyses support the hypothesis that the centre of origin of the genus *Dugesia* was located in southern Gondwana—the region that gave rise to the Afro-Malagasy landmasses. First, in the phylogeny, the African and Madagascar lineages of *Dugesia* represent genetically highly diversified groups, based both on the number of lineages found and the genetic differentiation amongst them (Figure S2). The Eurasian clade of *Dugesia* comprises samples from a broad geographical region, stretching from the Iberian Peninsula to the Japanese Archipelago, and constitutes a robust monophyletic group well-nested within the Afro-Malagasy *Dugesia* diversification (Figure S2). Second, putting this evolutionary history in a temporal frame (Figure 2), the first *Dugesia* divergence occurred at the Jurassic–Cretaceous boundary or during the Early Cretaceous—a period during which Gondwana started undergoing fragmentation (Ali & Aitchison, 2008; Powell et al., 1988; Schettino & Scotese, 2005; Seton et al., 2012). Third, our biogeographical analyses support Afro-Malagasy landmasses as the areas of origin of *Dugesia* (Figure 3; Table S12). Overall, our results regarding the origin of the genus *Dugesia* are partially consistent with previous biogeographical hypotheses (Ball, 1974; Sluys et al., 1998). These hypotheses were based on the highest present-day diversity of Dugesidae in the continents of the southern hemisphere, together with the intercontinental distribution of many of its genera, which may be attributed to vicariant events associated with Gondwana fragmentation. According to these hypotheses, Gondwana fragmentation shaped the origin of *Dugesia* in Africa. Meanwhile, the absence of *Dugesia* in the Americas or other regions like New Zealand, despite the presence of suitable habitats (Figure 1), suggests that the genus

either did not occupy these landmasses when contiguous with Africa or that the genus went extinct from the region.

4.3 | Improbable journey of *Dugesia* to Madagascar

The non-monophyletic status of the Malagasy species of *Dugesia* suggests that the ancestors of the two Madagascar lineages were already present on the island when it separated from Africa during the Mesozoic (c. 130 Ma), or that they dispersed overseas to Madagascar afterwards, or that both events occurred in sequence. The overseas colonisation of Madagascar has been attributed for most of the extant vertebrate lineages on the island (Crottini et al., 2012; Samonds et al., 2012). Such dispersal was favoured by periodic oceanic paleo-currents, which likely allowed rafting from Africa to Madagascar in the Early Cenozoic (Ali & Huber, 2010). However, podocnemid and testudinid turtles and tenrecs probably diversified on Madagascar prior to its isolation (Crottini et al., 2012). Our results indicate that the latter may also have been the case for *Dugesia*. First, the ancestral origin of *Dugesia* in Madagascar, supported by the great molecular and morphological diversity within both lineages, suggests a long time of speciation after their arrival to the area (Stocchino et al., 2017), discarding the recent colonisation hypothesis. Second, the journey of an adult or a cocoon from the mainland to a freshwater body on an island via 400 kilometres of drifting in the ocean (Ali & Huber, 2010) is considered improbable, although not impossible, due to the sensitiveness to desiccation and salt water of freshwater planarians (Ball, 1974). The coast of Mozambique Channel has not received the flow of rivers with abundant discharge, which may eventually generate freshwater plumes in the sea, thus increasing the probability of the overseas colonisation of freshwater fauna. Interestingly, some geological hypotheses (Scotese, 2016; Wells, 2003 and references there in) indicate that the west coast of Madagascar separated from Africa, leading to the formation of the Somalia–Madagascar Gulf during the mid-Jurassic (c. 170 Ma); since this incursion was formed by a shallow sea, it would have allowed the contact between Africa and Madagascar around 150 Ma, until the definitive split of the latter around 130 Ma. This complex palaeogeological history, if confirmed, can better explain our phylogenetic results. According to our hypothesis (Figure 3), after originating and diversifying in the eastern part of Gondwana, *Dugesia*, gave rise to two lineages that radiated within the Madagascar region through two consecutive splitting events of the island.

4.4 | *Dugesia* dispersal within Africa

After its origin in Gondwana, *Dugesia* must have dispersed across Africa, arriving to the western coast. Within the AFR1 clade, there were individuals belonging to populations distributed from the Gulf of Guinea to South Africa and Ethiopia. In this group, we also detected two species inhabiting the European coasts of the Mediterranean Basin: *Dugesia sicula* and *Dugesia naidis*. The presence of *D. sicula*

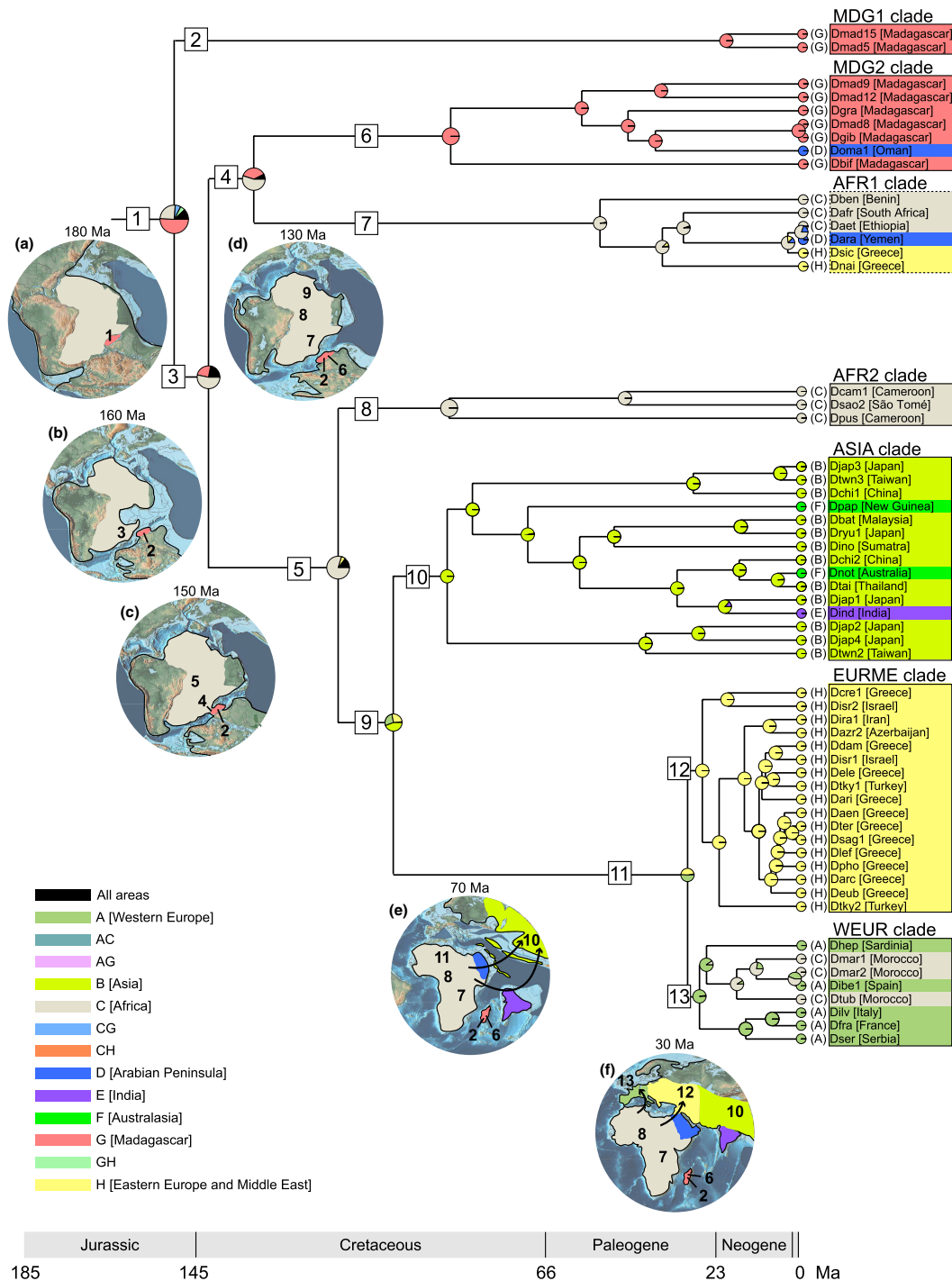


FIGURE 3 *Dugesia* ancestral ranges estimated with BioGeoBEARS using the DEC+*j* model (*Schmidtea* and *Recurva* were used as the outgroups). Pies at nodes indicate the probability of the ancestral distribution areas per clade (values are provided in Table S12). Legend: geographical areas. Geographical reconstructions (a–f) are based on PALEOMAP (Scotese, 2016). (a) Africa and Madagascar constitute a continuous terrain still within Gondwana supercontinent, 1 indicates the approximate situation of the ancestor of *Dugesia*; (b) first split of Madagascar from Africa, lineage 2 stays in Madagascar, 3 in continental Africa; (c) African clade 3 diversifies and disperses throughout Africa (lineage 5), including a region of new contact of the continent with Madagascar (lineage 4); (d) second breakage of Madagascar from Africa isolates lineage 6 in Madagascar while lineage 7 stays in Africa (node 4); in Africa, lineage 5 diversifies and gives rise to lineages 8 and 9 (node 5); (e) North Africa ancestor 9 diversifies, descendant lineage 10 moves to Asia, the other (11) stays in Africa; (f) ancestor 11 diversifies in North Africa, one lineage disperses to West Europe through terrestrial connections in the mid-Mediterranean area (13) and another to East Europe through Arabia (12). The last geographical reconstruction corresponds to the present Eurasian and African outline to better illustrate the putative dispersal routes of *Dugesia* into Eurasia, although at the time of dispersal some continental areas did not correspond to the current situation. See Section 4 for a more detailed account of events and hypotheses

throughout the Mediterranean Basin may be attributed to recent anthropogenic activities (Lázaro & Riutort, 2013). This may also be the case for *D. naiadis*. Furthermore, the close genetic relationship between *Dugesia aethiopica* (Ethiopia) and *Dugesia arabica* (Yemen) may imply anthropogenic interventions in their distribution. Likewise, anthropogenic translocation may also be involved in the finding of specimens from the Arabian Peninsula (*Doma1* and *Doma2*) well-nested within the Madagascar clade (*MDG2*). Of note, in most of these cases, the species implicated multiply via fission (the animals perform a binary fission and posteriorly regenerate the missing parts)—a reproductive strategy that has been proposed to favour the colonisation ability (Lázaro & Riutort, 2013; Leria et al., 2019).

The volcanic origin of the São Tomé Island (*AFR2*) indicates that animals must have arrived here through transoceanic dispersal. In the course of history, these islands have accumulated hundreds of endemic species, including crabs, shrews, burrowing reptiles and amphibians (Daniels et al., 2015; Daniels & Klaus, 2018; Jones, 1994), which do not typically cross saltwater barriers and are absent from most oceanic islands (Darwin, 1859; Heim de Balsac & Hutterer, 1982; Vitt & Caldwell, 2013). Contrary to the Mozambique Channel, the Atlantic coast of Africa has a rich network of large, fast-flowing rivers. The Congo, Ogooué and Niger rivers create extensive freshwater plumes in the Atlantic Ocean (Heim de Balsac & Hutterer, 1982; Measey et al., 2007) and have been hypothesised to explain the presence of certain frog species on the São Tomé and Príncipe islands (Bell et al., 2015; Measey et al., 2007). The presence of land (Sluys et al., 2017) and freshwater flatworms on São Tomé (*Dsao1* and *Dsao2*) might be explained by the same hypothesis. However, the low vagility of freshwater flatworms supports the recent anthropogenic translocation of already diversified lineages. Overall, our findings question the dogma of the impossibility of the overseas dispersal of freshwater flatworms.

4.5 | Multiple dispersal routes of *Dugesia* from Africa to Eurasia

Different hypotheses have been posed regarding the arrival of *Dugesia* to Eurasia. Ball (1974) proposed that the genus likely dispersed in Eurasia when the Tethys Sea closed, probably through a route from Africa to the Middle East and then to Europe and Asia. Sluys et al. (1998) proposed a similar dispersal route to Eurasia through the Middle East following the impact of the Arabian plate, around 23–16 Ma (Robertson, 2000), implying a relatively recent expansion in Eurasia. The same authors also suggested a different path of the introduction of the genus in Asia following the collision of India with the continent (Aitchison et al., 2007; Ali & Aitchison, 2008; van Hinsbergen et al., 2012), which will theoretically place the Madagascar species as sister to the Eurasian ones.

Surprisingly, our results do not support any of these hypotheses. First, the sister clade of Eurasian *Dugesia* comprised representatives from Cameroon and São Tomé (*AFR2*), rather than Madagascar. Second, assuming that the most recent common ancestor of Eurasian

species was in India, one would expect to find the older lineages of the Eurasian clade there or in surrounding regions, which is not the case.

Alternatively, based on our results, the Eurasian lineages shared the immediate common ancestor with Sub-Saharan African lineages, and our ancestral range estimation placed the ancestor of the *AFR2*–Eurasian clade in Africa with high probability (Figure 3, node 5). These results indicated a possible route through Arabia, although it was not supported by the results of our dating analysis. The collision between the Arabian and Eurasian plates occurred around 20 Ma (Robertson, 2000)—much later than the splitting of the *AFR2* and Eurasian lineages (node 5, 113.43 Ma; 130.72–96.34 Ma 95% HPD) and the diversification for the Eurasian clade (93.77 Ma; 110.3–77.7 Ma 95% HPD) in the dated trees. Thus, *Dugesia* likely arrived in Eurasia through a different path from the one passing through the Arabian Peninsula.

Notably, there were symmetric relationships between the European plus Middle East clade and the Asian clade (Figure 3, node 9), and the genetic diversification of the Asian lineages was older than that of the European and Middle Eastern ones. According to the previous hypotheses of planarian dispersion, after arriving to Eurasia riding on India (Sluys et al., 1998), some lineages migrated westward to arrive in the Middle East and then Europe. Our results do not show any Asiatic or Middle East representatives as sister to the European–Middle East clade as will be expected if animals dispersed from Asia to Europe. On the contrary, the Middle East representatives included in the analyses (i.e. Azerbaijan, Turkey, Israel and Iran) were well-nested within the eastern European clade. Therefore, the European–Middle Eastern *Dugesia* probably used a different route from the Asian *Dugesia* to disperse out of Africa. Some alternative geological hypotheses may explain the evidence derived from our results, implying three different paths, which are explained in the following sections.

4.6 | India as a ferry to Asia with stops in Africa

Based on the fossils of animals found in different combinations in America, Europe, Africa and India, it has been proposed that India may have not been a completely isolated continent moving northwards through the Indian Ocean (Briggs, 2003; Chatterjee et al., 2017; Kapur et al., 2018; Kapur & Khosla, 2016). Instead, it was likely still in some contact with Africa and Madagascar even as it began to contact Eurasia. This would have allowed the interchange of fauna between Africa and Eurasia during the Late Cretaceous–Early Paleogene. According to this alternative hypothesis, between 94 and 65 Ma, fauna from Africa likely entered India and partially replaced the original Indian–Madagascar endemics. Then, around 65 Ma, India, upon contact with Eurasia, allowed subsequent interchange of fauna. Most of this evidence is based on the records of vagile vertebrates, but also some amphibians depending on freshwater and presenting low vagility indicate the existence of such connections among landmasses following Gondwana fragmentation



(Duellman & Trueb, 1994). Nonetheless, there remain contradictory signals between biological and geological clues referring to the connection between India and Africa (Ali & Aitchison, 2008; Kapur & Khosla, 2016).

However, the above-mentioned hypothetical connections among Africa, India and Eurasia may have offered to the *Dugesia* representatives dwelling in the central and/or north-eastern parts of Africa, the opportunity to disperse to Eurasia aboard the moving Indian plate. This can further explain the beginning of diversification of the Asian group during the Late Cretaceous (93–66 Ma; Figures 2 and 3, node 10), probably while riding the Indian plate. Once India collided with Asia, multiple lineages present in the region dispersed in different directions through Asia, giving rise to the current distribution pattern of diverse ancient lineages scattered across Asia rather than forming geographically structured groups of clades.

4.7 | Asian *Dugesia*

Although many of the samples obtained from Asia were initially assigned to *Dugesia japonica*, it is evident from our results that they comprise more than one species and that a thorough study of the Asian species may reveal much greater species diversity (Chen et al., 2015; Song et al., 2020; Wang et al., 2021). Moreover, our results revealed some outliers in the Asian group: specimens from Papua New Guinea (*Dpap*) and Australia (*Dnot*). Both Papua New Guinea and Australia are on the eastern side of the Wallace Line, which marks a biogeographical limit traditionally considered a biological barrier between the Southeast Asian and Australasian fauna (Mayr, 1944). Moreover, *Dugesia* found in Australia (*Dnot*) was genetically very close to representatives from Bangladesh (*Dban*) and Thailand (*Dtai*), suggesting anthropogenic transportation and geographical expansion in Australia (Grant, 2017; Figure 1), similar to the case of *D. sicula* in the Mediterranean Basin (Lázaro & Riutort, 2013).

4.8 | Arrival of *Dugesia* to Europe in two steps

According to our previous hypothesis, *Dugesia* likely diversified within Africa. A part of this *Dugesia* diversity might have moved to Asia through the moving Indian plate. Meanwhile, the *Dugesia* species that remained in the north of Africa might have had the opportunity to move to Europe through two different routes, which will explain the two well-differentiated European groups. First, during the Eocene (55–33 Ma), there were continuous terrestrial connections between the African and the European plate in the western European area (*WEUR* clade) when the Tethys sea was closing (Stampfli & Hochard, 2009). Second, the *Dugesia* species from the eastern area (*EEURME* clade) might have passed through the Arabian Peninsula and the landmass that was destined to become the Aegean region. This hypothesis is supported by the topology and dating of the two clades.

Following the two dispersal events from Africa to Europe, most diversity in northern Africa may have disappeared because of the desertification of the region around 3 Ma (Foley et al., 2003). The desertification of northern Africa may also explain the long stem branch for the European clade. Meanwhile, the European continent did not form a continuous landmass until around 30 Ma (Meulenkamp & Sissingh, 2003), which may explain the lack of dispersion through this continent from both sides in older times.

5 | CONCLUSIONS

The present study puts forth the first biogeographical hypotheses drawn for the *Dugesia* genus as a whole based on molecular data. Our results confirm that *Dugesia* is an ancient genus that most likely originated in the eastern part of Gondwana at the Jurassic–Cretaceous boundary. Moreover, following its origin, the genus has gone through an extraordinary diversification over time and space, reaching most of its present-day suitable distribution range through both vicariant and dispersal events. The first diversification processes occurring within *Dugesia* putatively involved two consecutive vicariant–dispersal events during the split of Madagascar from Africa. Subsequently, and contrary to the previously proposed biogeographical hypotheses based on distribution data, *Dugesia* colonised Eurasia via three possible independent dispersal routes. Surprisingly, *Dugesia* might have travelled through the ocean to reach the oceanic island of São Tomé, thus questioning the assumed impossibility of animals with low vagility, such as freshwater planarians, to disperse overseas. Overall, although vicariant processes have certainly shaped part of the evolutionary history of *Dugesia*, the higher dispersal capability of these organisms than previously thought might have played a key role in driving the diversification and distribution of this genus, which, together with adaptation and chance, has given rise to an extremely intricate biogeographical history.

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CONFLICT OF INTEREST

Authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All sequences have been deposited in GenBank. The alignments are available in DRYAD database link: <https://doi.org/10.5061/dryad.mw6m905xb>

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BIOSKETCHES

Eduard Solà obtained his PhD in 2014 after defending his thesis mainly focused on systematics and phylogeography of the genus *Dugesia*. One of the thesis' chapters has been reviewed and its data expanded to publish the present paper. Marta Riutort was the supervisor of his PhD and is leader of the planarian diversity and evolution research team at Universitat de Barcelona (Research team web page: www.ub.edu/geisan).

Author contributions: Eduard Solà and Marta Riutort did the initial study design. Eduard Solà, Laia Leria, Giacinta Angela Stocchino, Reza Bagherzadeh, Michael Balke, Savel R. Daniels, Abdel Halim Harrath, Tsung Fei Khang, Duangduen Krailas,



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