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REVIEW

Molecular systematics, population genetics and phylogeography on planarians (Platyhelminths: Turbellaria): A brief review of molecular markers

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Abstract Due to their characteristics, such as powerful regeneration capability, high susceptibility to xenobiotics, wide distribution and weak dispersion ability, planarians are used as ideal test animals in several research fields, and planarianology has become a more and more active subject. This paper reviews the research progress of planarians from the aspects of molecular phylogeny, molecular taxonomy, population genetics and phylogeography, and also summarizes the corresponding molecular markers applied. Moreover, the future direction and tendency in this field is briefly analyzed and prospected.

Key words Planarian, molecular marker, molecular phylogeny, molecular taxonomy, population genetics, phylogeography.

1 Introduction

Planarians, known in scientific literature as Platyhelminthes, are a phylum of relatively simple bilaterian, unsegmented, soft-bodied invertebrate animals. They are numerous, diverse and globally distributed, occurring in marine, limnetic and terrestrial environments. Considering their unique biological characteristics, planarians are used as ideal test animals in several research fields, and planarianology has become a more and more active subject. First of all, planarians possess the amazing ability of regeneration and have been considered being "immortal under the edge of knife" (Randolph, 1897). Therefore, they have been traditionally used as an ideal model system for regeneration and developmental biology (Mogan, 1898; Reddien & Alvarado, 2004; Elliott & Alvarado, 2013). *Schmidtea mediterranea* is often used in the European and American countries while *Dugesia japonica* is usually used in the Asian countries as the model organism to carry out researches on regeneration, development, tissue homeostasis and stem cells (Wagner *et al*, 2011; Tasaki *et al*, 2016; Reddien, 2018; Levin *et al*., 2019). Secondly, planarians can grow or de-grow depending on the nutritional status. When food is scarce, planarians can endure starvation for several months by digesting and absorbing their own reproductive organs and present de-growth. Thus planarians are also a good model for studying cell autophagy (Newmark & Alavarado, 2002). Thirdly, due to their high susceptibility to xenobiotics, ease of acquisition and low maintenance cost, freshwater planarians are ideal test animals in toxicological research. More and more literature has verified that they can serve not only as alternative models for chemical toxicity screenings in laboratories but also as potential bioindicators for the quality of freshwater environments (Horvat *et al*., 2005; Wu & Li, 2018; Zhang *et al*., 2017, 2018, 2019). Last but not the least, with the development of molecular marker technology and next generation sequencing methodology, planarians, which are widely distributed and have low dispersion capacity, gradually become an ideal model for the study of population genetic and phylogeography patterns (Álvarez-Presas & Riutort, 2014). In view of their biological importance in so many fields, a robust phylogenetic scheme for the planarians is required for both taxonomic and comparative purposes. In this review, researches on the

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molecular systematics, population genetic structure and phylogeography of planarians and the corresponding molecular markers applied (Table 1) are summarized.

Table 1. The molecular markers applied in researches on planarians.

Molecular Marker	Type	Usage	Reference
Microsatellite loci	Nuclear DNA	Population genetics	Pongratz *et al*., 2002
18S rDNA	Nuclear DNA	Molecular phylogeny	Baguñà *et al*., 2001; Carranza *et al*., 1998; Álvarez-Presas *et al*., 2008; Yang *et al*., 2018
28S rDNA	Nuclear DNA	Molecular phylogeny	Álvarez-Presas *et al*., 2008
ITS-1	Nuclear DNA	Molecular taxonomy; Phylogeography	Baguñà *et al*., 1999; Lázaro *et al*., 2009; Solà *et al*., 2013; Álvarez-Presas *et al*., 2011, 2012, 2014
ITS-2	Nuclear DNA	Phylogeography	Riesgo *et al*., 2017
N13	Nuclear DNA	Phylogeography	Lázaro *et al*., 2011
COI	Mitochondrial DNA	Molecular phylogeny; Molecular taxonomy; Population genetics; Phylogeography	Baguñà *et al*., 2001; Álvarez-Presas *et al*., 2008, 2011, 2012, 2014; Pongratz *et al*., 2003; Lázaro *et al*., 2009, 2011; Lázaro & Riutort, 2013; Solà *et al*., 2013
CYB	Mitochondrial DNA	Phylogeography	Lázaro *et al*., 2011
Transcriptome	RNA	Molecular phylogeny	Egger & Noreña, 2015
18S rRNA	Nuclear RNA	Molecular phylogeny	Carranza *et al*., 1998
16S rRNA	Mitochondrial RNA	Phylogeography	Riesgo *et al*., 2017

2 Molecular systematics of planarians

Molecular systematics refers to the study of the evolution of biological macromolecules (proteins, nucleic acids, *etc*.) to clarify the genealogical relationships among various groups of organisms. With the application of molecular biological technology in the study of systematics, molecular systematics has rapidly developed into a hot research field, and the corresponding techniques and analysis methods have also been widely used in the study of planarian systematics.

2.1 Molecular phylogeny

An independent method to test the current phylogeny of Turbellaria (Platyhelminthes) and to overcome some of the present difficulties associated with assessing morphological homology is to employ molecular systematics. Molecular data obtained from nuclear ribosomal DNA, mitochondrial genes and even mitogenome or transcriptome data over an important new source of informative characters were used for inferring phylogenetic relationships at several levels (Field *et al*., 1988; Riutort *et al*., 1993; Carranza *et al*., 1997, 1998; Baguñà *et al.*, 2001; Álvarez-Presas *et al*., 2008; Egger*et al*., 2015; Solà *et al*., 2015).

The interrelationships of Platyhelminthes are poorly resolved despite decades of morphological and molecular phylogenetic studies (Laumer & Giribet, 2014). Egger *et al.* (2015) sequenced transcriptomes from 18 flatworms and 5 other metazoan groups. Together with other published data they reconstructed the phylogenetic tree of flatworms, and the results suggested that Platyhelminthes is a monophyletic group while Turbellaria within it is paraphyletic. Coincidently, almost at the same time Laumer and his coworkers (2015) drew the similar phylogentic tree and further confirmed the results of Egger's group. In order to reassess the taxonomic status and the phylogenetic position of Proseriata and Tricladida within Platyhelminthes, Baguñà *et al.* (2001) constructed a molecular phylogenetic tree of Platyhelminthes based on *18S rDNA*. The results showed that the Tricladida is a monophyletic group being not related to the Proseriata, but sister group of Prolecithophora. Therefore, validity of the taxon Seriata (Tricladida + Proseriata) is rejected, and the suborder Tricladida was promoted to order category with infra-orders within it being promoted to suborders. Carranza *et al.* (1998) analyzed the phylogeny and classification of the Tricladida with additional independent molecular data from complete sequences of *18S rDNA* and *18S rRNA*. The results showed that Maricola is monophyletic and is the sister group to the rest of Tricladida (Paludicola + Terricola). The Paludicola are paraphyletic since the Terricola and one paludicolan family, the Dugesiidae, share a more recent common ancestor than the dugesiids with other paludicolans (dendrocoelids and planariids). Therefore, the authors suggested replacing the Paludicola and Terricola with a new clade, the Continenticola, which comprises the families Dugesiidae, Planariidae, Dendrocoelidae and the Terricola. After Carranza *et al.* (1998) established the monophyly of Maricola, a serise of phylogenetic studies on the triclads consistently reveal the Maricola as a monophyletic taxon (Charbagi-Barbirou *et al.*, 2011; Harrath *et al.*, 2016; Yang *et al.*, 2018). Also, Baguñà *et al.* (2001) using *COI* sequences gave further support to the sister group relationship of Terricola and Dugesiidae, as well as the paraphyly of Paludicola. However, neither *18S rDNA* nor *COI* based trees have been able to resolve the relationships among species of Terricola and Dugesiidae, particularly the monophyly of Terricola. Thus, Álvarez-Presas *et al.* (2008) presented new molecular data including sequences of nuclear genes (*18S rDNA*, *28S rDNA*) and a mitochondrial gene (*COI*) of a wider sample of Dugesiidae and Terricola species. In the end, they concluded that Terricola has a monophyletic origin, and the terrestrial planarian is evolved from the freshwater planarian and followed by a likely return of some species to freshwater habitats.

2.2 Molecular taxonomy

A general characteristic of planarians is their simplicity in morphology, which has, from the beginning, made the planarian taxonomists' work difficult and tedious. Firstly, because they are all similar in appearance, histological sections of individuals must be made to identify species by observing the anatomical characteristics of their reproductive structures, which is time-consuming. Secondly, the lack of a taxonomic index and loss of type specimens of many species made the work more difficult (Leal-Zanchet & Carbayo, 2000). In addition, there are many asexual populations in planarians and they reproduce asexually without developing the reproductive system or the copulatory apparatus. Therefore, the lack of both external and internal taxonomic features makes traditional taxonomy even more powerless. Molecular taxonomy based on genetic tools brings a new opportunity for planarian identification. The use of *ITS-1* in the 1990s demonstrated that this sequence could help identify asexual populations in a DNA barcoding style shortly before this methodology was formally proposed. In the study of Baguñà *et al.* (1999), they used *ITS-1* of rDNA to study 20 populations in western Mediterranean, sexual and asexual, of the genus *Dugesia*. All asexual populations clearly clustered with individuals from a diploid identified species, which allows them to be unambiguously assigned to a known sexual species. In 2002, Tautz *et al.* advocated the establishment of an identification system based on the analysis of sequence diversity in small segments of DNA. A fragment of the *COI* sequence was proposed as a universal marker in what has since been called the DNA barcoding system for all animal life (Hebert *et al.*, 2003; Hebert & Gregory, 2005). Lázaro *et al.* (2009) extended the analysis based on *ITS-1* sequences (Baguñà *et al.*, 1999) and developed a DNA barcoding method, based on *COI* and *ITS-1* sequences, which allows the assignment of the fissiparous asexual population of the genus *Dugesia* in the western Mediterranean to sexual species. Solà *et al.* (2013) identified three new species through both morphological and molecular markers. Overall, molecular data, especially mitochondrial genes, are highly effective as DNA barcoding tools to identify species, even from fragments, eggs or immature individuals. Thus this methodology is a breakthrough that could greatly speed up the description of planarian species and allow for the rapid identification of individuals, thus facilitating taxonomic work.

3 Population genetics and phylogeography of planarians

With the development of molecular marker technique and the next generation sequencing methodologies (NGS), planarians with wide distribution and low dispersal ability has become an ideal model on population genetics and phylogeographic pattern studies (Álvarez-Presas & Riutort, 2014). Population genetics mainly studies the genetic evolution of a population, including selection, gene flow, mutation and genetic drift. Population genetic structure is the main content of studies on population genetics, which refers to the distribution of genetic variation within and between populations. The study of population genetic structure is helpful to clarify the relationship between biological variation and environment under natural conditions and the mechanism of speciation. In addition, it can also deepen the understanding of biological evolution process, thus providing an important scientific basis for the protection of species. Phylogeography is a branch subject that studies the evolutionary process and historical reasons for the formation of the current distribution pattern of different populations within or among species. In the study of phylogeography, the genetic diversity of populations is often measured by the nucleotide diversity and haplotype diversity of different geographical populations or whole populations.

3.1 Population genetics of planarians

Dispersal of individuals is an important factor that can influence genetic differentiation between populations. Genetic structure within, and gene flow between populations have been studied in many different freshwater species (Pongratz *et al.*, 2002). The evolutionary significance of gene flow can be seen from different viewpoints: on the one hand, restricted gene flow may allow local adaptation and micro-evolutionary changes that increase fitness of local populations, while on the other, gene flow can be necessary to maintain genetic variation when population size is small (Slatkin *et al.*, 1985; Slatkin, 1987). The extent to which genetic variation and differentiation among populations can be observed, strongly depends on the markers used. Microsatellite DNA, also known as simple sequence repeats (SSR), is the most revealing and widely used nuclear DNA marker available so far for inferring population structure and dynamics, even though it has important and unavoidable limitations (Zhang & Hewitt, 2003). SSR has been developed in planarians and has been widely used in population genetic structure, intraspecies genetic distance, reproduction and other aspects (Ramachandran *et al.*, 1997; Pongratz *et al.*, 2002; Bruvo *et al.*, 2004). Ramachandran *et al.* (1997) used library enrichment method to screen and isolate eight microsatellite loci with rich polymorphism from *Schmidtea polychroa*. The genus of *Schmidtea*, together with *Recurva*, forms the sister group of *Dugesia*, to which *D. japonica* belongs. This is the first report on the screening and separation of microsatellite markers from planarians, laying a foundation for the subsequent study on population structure and mating system.

Genetic differentiation occurs when there is restricted gene flow between populations. Several studies of different taxa reported stronger genetic differentiation of populations for species with limited dispersal abilities, and a negative relationship was found between dispersal ability and genetic differentiation (Bohonak, 1999). *Schmidtea polychroa* is a freshwater planarian with a Europe-wide distribution. It is a simultaneous hermaphrodite with sexual and parthenogenetic biotypes (Weinzierl *et al.*, 1999; Michiels *et al.*, 2001). Pongratz *et al.* (2002) used three highly polymorphic microsatellite markers for analyzing small-scale and large-scale genetic structure within one, and between four natural lake populations of *S. polychroa*. Genetic differentiation could already be observed between samples collected at least 13 m apart, but not between neighbouring samples, and was most pronounced between samples from different lakes. These results showed that individual dispersal of *S. polychroa* was limited, but that there was gene flow between subpopulations from the same lake. The next year, also Pongratz's group studied the phylogenetic relationships of sexual and parthenogenetic *S. polychroa* collected at 38 sites, a phylogenetic relationship between 36 populations based on partial sequence of mitochondrial gene *COI* revealed that haplotypes were often similar over large geographic distances. Combining these results with those based on nuclear markers, the data suggest that repeated hybridization between sexuals and parthenogenetic lineages in mixed populations maintains high levels of genetic diversity in parthenogens (Pongratz *et al.*, 2003).

3.2 Phylogeography on planarians

Due to their relatively high abundance and broad distribution, planarians are often selected as ideal organisms for phylogeographic study. At present, the phylogeography has been widely used to explore the distribution pattern, migration and dispersion of planarians. Although relevant reports haven't been seen in China, studies in some European and American countries and regions have revealed the genealogy differentiation pattern of different geographical populations and the possible source of the genealogy differentiation (Pongratz *et al.*, 2003; Lázaro *et al.*, 2009, 2011; Álvarez-presas *et al.*, 2011, 2012; Lázaro & Riutort, 2013). The Mediterranean basin, located between Asia, Europe and Africa, has become a hotspot of biodiversity in the world and played an important role in the study of biodiversity and lineages due to its complex and long geological history (Myers *et al.*, 2000). Although *Schmidtea mediterranea* is a model species for regeneration and development research, little is known about its evolutionary history and demographics. *S. mediterranea* is found in scattered localities on a few islands and in coastal areas of the western Mediterranean. Lázaro *et al.* (2011) analyzed the genetic variability and demographic parameters on 11 populations from across its distribution area based on mitochondrial *COI*, *CYB* and a nuclear intron *N13*. The results indicated that *S. mediterranea* have formed an obvious pedigree pattern in the whole distribution area, namely the west, middle and southeast branches, and there is no haplotype shared among the branches. Therefore, the hypothesis that the present distribution pattern of this species was formed by ancient geological events was verified. It also illustrates the antiquity of the Mediterranean planarian species. In contrast with the restricted distribution of *S. mediterranea*, *Dugesia sicula* is the only cosmopolitan species of its genus within the Mediterranean area. Most populations of it are asexual populations with fissiparous reproduction. Lázaro & Riutort (2013) estimated haplotype and nucleotide diversity of *COI* on 58 *D. sicula* populations across the whole Mediterranean coast and found that the distribution of haplotypes is not geographically structured. Based on the phylogeographic pattern, the authors inferred the origin and the disperse route of *D. sicula* in the past and also predicted the population expansion in the future. The northeastern Mediterranean region has been subjected to tectonism, volcanism and sea level changes since the Miocene, resulting in a complex geological history (Perissoratis & Conispoliatis, 2003). This area is well suited for phylogeographic studies to unravel the historical processes that underlie present-day species distributions and current levels of diversity and endemism (Sfenthourakis & Legakis, 2001). Based on two molecular markers *COI* and *ITS-1*, Solà *et al.* (2013) analyzed the phylogenetic relationships of eastern Mediterranean freshwater planarians of the genus *Dugesia*, estimated divergence times for the various clades, and correlated their phylogeographic patterns with geological and paleoclimatic events. They found that a clear phylogeographic pattern was present for *Dugesia* lineages in the Eastern Mediterranean, and *Dugesia* species present distribution in the Eastern Mediterranean had been shaped mainly by vicariant events but also by dispersal.

In addition, phylogeography can discuss the genetic diversity and genealogical relationship of species from the perspective of genetics, so as to provide reference for their protection. Apart from the Mediterranean basin, the Brazilian Atlantic Forest (AF) is another one of the world's most important biodiversity hotspots (Myers *et al*., 2000) and, at the same time, one of the most threatened biomes on the planet. The distribution of most land planarians requires the presence and good condition of wet forests, suggesting that they may be good indicators of forests health and also an excellent model for phylogeographic studies (Sluys, 1999; Carbayo *et al*., 2002; Sunnucks *et al*., 2006; Fonseca *et al*., 2009; Álvarez-Presas *et al*., 2011). With the use of *COI* and *ITS-1*, Álvarez-Presas *et al.* (2011) carried out a population genetics and comparative phylogeographic study on two terrestrial planarian species *Cephaloflexa bergi* and *Geoplana goetschi* in a national park located within the Southern Atlantic Forest (SAF). The results found that both species harbour high levels of genetic variability without exhibiting the molecular footprint of recent colonization or population expansions, suggesting a long-term stability scenario. This study provides new useful information for the origin and pattern formation of biodiversity in this region, and also scientific guidance for the formation of biodiversity protection policies in this region. Also based on *COI* and *ITS-1*, Álvarez-Presas & Riutort (2014) subsequently performed another comprehensive phylogeographic study using the low-dispersal land planarian *C. bergi* in 13 conservation areas within SAF region. They found that most sampled localities harbour high levels of genetic diversity, with lineages sharing common ancestors that predate the Pleistocene. In addition, they also demonstrated that land planarians are an advantageous biological model for making phylogeographic and, particularly, fine-scale evolutionary inferences, and propose appropriate conservation policies. The availability of molecular data and up-to-date methodologies to analyze them, has transformed planarians from being mostly of interest to a few taxonomists and researchers investigating their regenerative capacity to a cutting-edge model on phylogeographic and demographic studies. This is so also because their features, such as their low dispersal ability, make them ideal for providing insight into past events and for inferring how biodiversity is generated, the influence of geological, climatic events, and the history of populations found in forests and rivers.

4 Summary and outlook

Planarians is an excellent model for the study of many scientific problems in biology, and planarianology has become an active research field. Many scientists have conducted in-depth studies on different biological problems in different research directions and levels. In this review, researches on the molecular systematics, population genetic structure and phylogeography of planarians are reviewed. The first part of this article summarizes the molecular phylogeny and molecular taxonomy of planarians. Molecular phylogenetic studies have shown that "Turbellaria" is a paraphyletic group. Molecular markers applied in planarian phylogeny and taxonomy reveals a great deal of diversity and makes a great contribution to the construction of better classification and the increase of taxonomic richness. The second section of this review addresses the population genetics and phylogeography of planarians. Many specialists surveyed detailed population genetic structure of planarians by using molecular marker technique. And, in the study of the phylogeography of planarians, they found that phylogeography can study the evolution of biogeographical patterns of species and the genealogy patterns of population genes; it can also compare phylogenetic biogeography and reveal hotspots areas of biological protection from the genetic level.

At present, the phylogenetic relationships within higher categories such as class and order of planarians have been relatively clear. The next work will focus on the exploration of phylogenetic relationships of different families and subfamilies within orders, especially Tricladida. However, the above phylogenetic problems should be discussed on the basis of species classification, and therefore species identification based on morphological, molecular and geographical distribution information is still necessary. With the increasing serious environmental pollution, the global warming and water drying up, and the accelerating tourism development, the population genetic structure, phylogeography and conservation of planarians should be strengthened in the future, especially in our China. In the current era of omics, NGS-based RNA sequencing (RNAseq) and restriction site associated DNA sequencing (RADseq) are expected to help develop new markers to achieve a greater understanding of the group evolutionary history and to have a more accurate taxonomy.

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References

Álvarez-Presas, M., Baguñà, J., Riutort, M. 2008. Molecular phylogeny of land and freshwater planarians (Tricladida, Platyhelminthes): From freshwater to land and back. *Molecular Phylogenetics and Evolution*, 47(2): 555–568.

Álvarez-Presas, M., Carbayo, F., Rozas, J., Riutort, M. 2011. Land planarians (Platyhelminthes) as a model organism for fine-scale phylogeographic studies: understanding patterns of biodiversity in the Brazilian Atlantic Forest hotspot. *Journal of Evolutionary Biology*, 24(4): 887–896.

Álvarez-Presas, M., Mateos, E., Vila-Farré, M., Sluys, R., Riutort, M. 2012. Evidence for the persistence of the land planarian species *Microplana terrestris* (Müller, 1774) (Platyhelminthes, Tricladida) in microrefugia during the Last Glacial Maximum in the northern section of the Iberian Peninsula. *Molecular Phylogenetics and Evolution*, 64(3): 491–499.

Álvarez-Presas, M., Riutort, M. 2014. Planarian (Platyhelminthes, Tricladida) diversity and molecular markers: a new view of an old group. *Diversity*, 6(2): 323–338.

Álvarez-Presas, M., Sánchez-Gracia, A., Carbayo, F., Rozas, J., Riutort, M. 2014. Insights into the origin and distribution of biodiversity in the Brazilian Atlantic Forest hot spot: a statistical phylogeographic study using a low-dispersal organism. *Heredity*, 112(6): 656–665.

Baguñà, J., Carranza, S., Pala, M., Ribera, C., Giribet, G., Arnedo, M.A., Ribas, M., Riutort, M. 1999. From morphology and karyology to molecules. New methods for taxonomical identification of asexual populations of freshwater planarians. A tribute to Professor Mario Benazzi. *Italian Journal of Zoology*, 66: 207–214.

Baguñà, J., Carranza, S., Pops, J., Ruiz-Trillo, I., Riutort, M. 2001. Molecular taxonomy and phylogeny of the Tricladida. *In:* Littlewood, D.T.J., Bray, R.D. (eds.), *The interrelationships of the Platyhelminthes*. Taylor and Francis, London, England. pp. 49–56.

Bohonak, A.J. 1999. Dispersal, gene flow, and population structure. *The Quarterly Review of Biology*, 74(1): 21–45.

Bruvo, R., Michiels, N.K., D'Souza T.G., Schulenburg, H. 2004. A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. *Molecular Ecology*, 13(7): 2101–2106.

Carbayo, F., Leal-Zanchet, A.M., Vieira, M. 2002. Terrestrial flatworm (Platyhelminthes: Tricladida: Terricola) diversity versus man-induced disturbance in an ombrophilous forest in southern Brazil. *Biodiversity and Conservation*, 11(6): 1091–1104.

Carranza, S., Baguñà, J., Riutort, M. 1997. Are the Platyhelminthes a monophyletic primitive group? An assessment using 18S rDNA sequences. *Molecular Biology and Evolution*, 14(5): 485–497.

Carranza, S., Littlewood, D.T.J., Clough, K. A., Ruiz-Trillo, I., Baguñà, J., Riutort, M. 1998. A robust molecular phylogeny of the Tricladida (Platyhelminthes: Seriata) with a discussion on morphological synapomorphies. *Proceedings of the Royal Society B: Biological Sciences*, 265(1396): 631–640.

Charbagi-Barbirou, K., Álvarez-Presas, M., Vila-Farré, M., Gammoudi, M., Tekaya, S., Riutort, M. 2011. Marine triclads (Platyhelminthes, Tricladida, Maricola), a preliminary molecular approach to their phylogeny. *Cahiers de Biologie Marine*. 52: 303–311.

Egger, B., Lapraz, F., Tomiczek, B., Müller, S., Dessimoz, C., Girstmair, J., Škunca, N., Rawlinson, K.A., Cameron, C.B., Beli, E., Todaro, M.A., Gammoudi, M., Noreña, C., Telford, M.J. 2015. A transcriptomic-phylogenomic analysis of the evolutionary relationships of flatworms. *Current Biology*, 25(10): 1347–1353.

Elliott, S.A., Alvarado A.S. 2013. The history and enduring contributions of planarians to the study of animal regeneration. *Wiley Interdisciplinary Reviews: Developmental Biology*, 2(3): 301–326.

Field, K.G., Olsen, G.J., Lane, D.J., Giovannoni, S.J., Ghiselin, M.T., Raff, E.C., Pace, N.R., Raff, R.A. 1988. Molecular phylogeny of the animal kingdom. *Science*, 239(4841): 748–753.

Fonseca, C.R., Ganade, G., Baldissera, R., Becker, C.G., Boelter, C.R., Brescovit, A.D., Campos, L.M., Fleck, T., Fonseca, V.S., Hartz, S.M., Joner, F., Käffer, M.I., Leal-Zanchet, A.M., Marcelli, M.P., Mesquita, A.S., Mondin, C.A., Paz, C.P., Petry, M.V., Piovensan, F.N., Putzke, J., Stranz, A., Vergara, M., Vieira, E.M. 2009. Towards an ecologically-sustainable forestry in the Atlantic Forest. *Biological Conservation*, 142(6): 1209–1219.

Harrath, A.H., Mansour, L., Lagnika, M., Sluys, R., Boutin, C., Alwasel, S., Poch, A., Riutort, M. 2016. A molecular analysis of the phylogenetic position of the suborder Cavernicola within the Tricladida (Platyhelminthes), with the description of a new species of stygobiont flatworm from Benin. *Zoological Journal of the Linnean Society*, 178(3): 482–491.

Hebert, P.D.N., Cywinska, A., Ball, S.L., deWaard, J.R. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences*, 270: 313–321.

Hebert, P.D.N., Gregory, T.R. 2005. The promise of DNA barcoding for taxonomy. *Systematic Biology*, 54(5): 852–859.

Horvat, T., Kalafatić, M., Kopjar, N., Kovacević, G. 2005. Toxicity testing of herbicide norflurazon on an aquatic bioindicator species-the planarian Polycelis felina. *Aquatic Toxicology*, 73(4): 342–352.

Laumer, C.E., Giribet, G. 2014. Inclusive taxon sampling suggests a single, stepwise origin of ectolecithality in Platyhelminthes. *Biological Journal of the Linnean Society*, 111(3): 570–588.

Laumer, C.E., Hejnol, A., Giribet, G., 2015. Nuclear genomic signals of the "microturbellarian" roots of platyhelminth evolutionary innovation. *eLife*, 4: e05503.

Lázaro, E.M., Harrath, A.H., Stocchino, G.A., Pala, M., Baguñà, J., Riutort, M. 2011. *Schmidtea mediterranea* phylogeography: an old species surviving on a few Mediterranean islands? *BMC Evolutionary Biology*, 11(1): 274.

Lázaro, E.M., Riutort, M. 2013. *Dugesia sicula* (Platyhelminthes, Tricladida): the colonizing success of an asexual planarian. *BMC Evolutionary Biology*, 13(1): 268.

Lázaro, E.M., Sluys, R., Pala, M., Stocchino, G.A., Baguñà, J., Riutort, M. 2009. Molecular barcoding and phylogeography of sexual and asexual freshwater planarians of the genus *Dugesia* in the Western Mediterranean (Platyhelminthes, Tricladida, Dugesiidae). *Molecular Phylogenetics and Evolution*, 52(3): 835–845.

Leal-Zanchet, A.M., Carbayo, F. 2000. Fauna de planárias terrestres (Platyhelminthes, Tricladida, Terricola) da Floresta Nacional de São Francisco de Paula, RS, Brasil: Uma análise preliminary. *Acta Biology Leopoldensia*, 22: 19–25.

Levin, M., Pietak, A.M., Bischof, J. 2019. Planarian regeneration as a model of anatomical homeostasis: Recent progress in biophysical and computational approaches. *Seminars in Cell and Developmental Biology*, 87: 125–144.

Michiels, N.K., Beukeboom, L.W., Pongratz, N., Zeitlinger, J. 2001. Parthenogenetic flatworms have more symbionts than their coexisting, sexual conspecifics, but does this support the Red Queen? *Journal of Evolutionary Biology*, 14(1): 110–119.

Morgan, T.H. 1898. Experimental studies of the regeneration of *Planaria maculata*. *Archive für Entwicklungsmechanik der Organismen*, 7: 364–397.

Myers, N., Mittermeier, R.A., Mittermeier, C.G., da Fonseca, G.A.B., Kent, J. 2000. Biodiversity hotspots for conservation priorities. *Nature*, 403(6772): 853–858.

Newmark, P.A., Alavarado, S.A. 2002. Not your father's planarian: A classic model enters the era of functional genomics. *Nature Reviews Genetics*, 3(3): 210–219.

Perissoratis, C., Conispoliatis, N. 2003. The impacts of sea-level changes during latest Pleistocene and Holocene times on the morphology of the Ionian and Aegean seas (SE Alpine Europe). *Marine Geology*, 196(3–4): 145–156.

Pongratz, N., Gerace, L., Michiels, N.K. 2002. Genetic differentiation within and between populations of a hermaphroditic freshwater planarian. *Heredity*, 89(1): 64–69.

Pongratz, N., Storhas, M., Carranza, S., Michiels, N.K. 2003. Phylogeography of competing sexual and parthenogenetic forms of a freshwater flatworm: patterns and explanations. *BMC Evolutionary Biology*, 3(23): 1–15.

Ramachandran, S., Beukeboom, L.W., Gerace, L., Pavlovic, N., Carranza, S., Michiels, N.K. 1997. Isolation and characterization of microsatellite loci from the planarian *Dugesia polychroa* (Schmidt) (Platyhelminthes: Tricladida). *Molecular Ecology*, 6(4): 389–391.

Randolph, H. 1897. Observation s and experiments on regeneration in planarians. *Archiv für Entwicklungsmechanik der Organismen*, 5(2): 352–372.

Reddien, P.W. 2018. The cellular and molecular basis for planrian regeneration. *Cell*, 175: 327–345.

Reddien, P.W., Alvarado, A.S. 2004. Fundamentals of planarian regeneration. *Annual Review of Cell and Developmental Biology*, 20: 725–757.

Riesgo, A., Burke, E.A., Laumer, C., Giribet, G. 2017. Genetic variation and geographic differentiation in the marine triclad *Bdelloura candida* (Platyhelminthes, Tricaldida, Maricola), ectocommensal on the American horseshoe crab *Limulus polyphemus*. *Marine Biology*, 164(5): 111.

Riutort, M., Field, K.G., Raff, R.A., Baguña, J. 1993. 18S rRNA sequences and phylogeny of Platyhelminthes. *Biochemical Systematics and Ecology*, 21(1): 71–77.

Sfenthourakis, S., Legakis, A. 2001. Hotspots of endemic terrestrial invertebrates in Southern Greece. *Biodiversity and Conservation*, 10(8): 1387–1417.

Slatkin, M. 1987. Gene flow and the geographic structure of natural populations. *Science*, 236(4803): 787–792.

Slatkin, M., Slatkin, N., Slatkin, D.N. 1985. Gene flow in natural populations. *Annual Review of Ecology and Systematics*, 16(1): 393–430.

Sluys, R. 1999. Global diversity of land planarians (Platyhelminthes, Tricladida, Terricola): a new indicator-taxon in biodiversity and conservation studies. *Biodiversity and Conservation*, 8(12): 1663–1681.

Solà, E., Álvarez-Presas, M., Frías-López, C., Littlewood, D.T.J, Rozas, J., Riutort, M. 2015. Evolutionary analysis of mtigenomes from parasitic and free-living flatworms. *Plos One*, 10(3): e0120081.

Solà, E., Sluys, R., Gritzalis, K., Riutort, M. 2013. Fluvial basin history in the northeastern Mediterranean region underlies dispersal and speciation patterns in the genus *Dugesia* (Platyhelminthes, Tricladida, Dugesiidae). *Molecular Phylogenetics and Evolution*, 66(3): 877–888.

Sunnucks, P., Blacket, M.J., Taylor, J.M., Sands, C.J., Ciavaglia, S.A., Garrick, R.C., Tait, N.N., Rowell, D.M., Pavlova, A. 2006. A tale of two flatties: different responses of two terrestrial flatworms to past environmental climatic fluctuations at Tallaganda in montane southeastern Australia. *Molecular Ecology*, 15(14): 4513–4531.

Tasaki, J., Uchiyama-Tasaki, C., Rouhana, L. 2016. Analysis of stem cell motility in vivo based on immunodetection of planarian neoblasts and tracing of BrdU-labeled cells after partial irradiation. *Methods in Molecular Biology*, 1365(3): 323–338.

Tautz, D., Arctander, P., Minelli, A., Thomas, R.H., Vogler, A.P. 2002. DNA points the way ahead in taxonomy. *Nature*, 418(6897): 479.

Wagner, D.E., Wang, I.E., Reddien, P.W. 2011. Clonogenic neoblasts are pluripotent adult stem cells that underlie planarian regeneration. *Science*, 332(6031): 811–816.

Weinzierl, R.P., Beukeboom, L.W., Gerace, L., Michiels, N.K. 1999. Spatial and ecological overlap between coexisting sexual and parthenogenetic *Schmidtea polychroa* (Tricladida; Platyhelminthes). *Hydrobiologia*, 392(2): 179–185.

Wu, J.P., Li, M.H. 2018. The use of freshwater planarians in environmental toxicology studies: Advantages and potential. *Ecotoxicology and Environmental Safety*, 161: 45–56.

Yang, H.M. Sluys, R., Kawakatsu, M., Min, G.S. 2018. New molecular sequences for two genera of marine planarians facilitate determination of their position in the phylogenetic tree, with new records for two species (Platyhelminthes, Tricladida, Maricola). *ZooKeys*. 781: 1–17.

Zhang, D.X., Hewitt, G.M. 2003. Nuclear DNA analysis in genetic studies of populations: practice, problems and prospects. *Molecular Ecology*, 12: 563–584.

Zhang, H.C., Liu, T.Y., Shi, C.Y., Chen, G.W., Liu, D.Z. 2017. Genotoxicity evaluation of an urban stream on freshwater planarian by RAPD assay. *Bulletin of Environmental Contamination and Toxicology*, 98(4): 484–488.

Zhang, H.C., Ma, K.X., Yang, Y.J., Shi, C.Y., Chen, G.W., Liu, D.Z. 2018. Molecular cloning, characterization, expression and enzyme activity of catalase from planarian *Dugesia japonica* in response to environmental pollutants. *Ecotoxicology and Environmental Safety*, 165: 88–95.

Zhang, H.C., Ma, K.X., Yang, Y.J., Shi, C.Y., Chen, G.W., Liu, D.Z. 2019. CuZnSOD and MnSOD from freshwater planarian *Dugesia japonica*: cDNA cloning, mRNA expression and enzyme activity in response to environmental pollutants. *Aquatic Toxicology*, 208, 12–19.