Correlating Early Evolution of Parasitic Platyhelminths to Gondwana Breakup

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Abstract.—Investigating patterns and processes of parasite diversification over ancient geological periods should involve
comparisons of host and parasite phylogenies in a biogeographic context. It has been shown previously that the geographi-
cal distribution of host-specific parasites of sarcopterygians was guided, from Palaeozoic to Cainozoic times, mostly by evo-
lution and diversification of their freshwater hosts. Here, we propose phylogenies of neobatrachian frogs and their specific
parasites (Platyhelminthes, Monogenea) to investigate coevolutionary processes and historical biogeography of polystomes
and further discuss all the possible assumptions that may account for the early evolution of these parasites. Phylogenetic
analyses of concatenated rRNA nuclear genes (18S and partial 28S) supplemented by cophylogenetic and biogeographic
vicariance analyses reveal four main parasite lineages that can be ascribed to centers of diversity, namely Australia, India,
Africa, and South America. In addition, the relationships among these biogeographical monophyletic groups, substantiated
by molecular dating, reflect sequential origins during the breakup of Gondwana. The Australian polystome lineage may
have been isolated during the first stages of the breakup, whereas the Indian lineage would have arisen after the com-
plete separation of western and eastern Gondwanan components. Next, polystomes would have codiverged with hyloid
sensu stricto and ranoid frog lineages before the completion of South American and African plate separation. Ultimately,
they would have undergone an extensive diversification in South America when their ancestral host families diversified.
Therefore, the presence of polystome parasites in specific anuran host clades and in discrete geographic areas reveals the
importance of biogeographic vicariance in diversification processes and supports the occurrence and radiation of amphib-
ians over ancient and recent geological periods. [Amphibia; codivergence; coevolution; cophylogeny; Gondwana breakup;
neobatrachia; platyhelminthes; polystomatidae; vicariant biogeography.]

Investigating patterns and processes of parasite evo-
lution over geological time involves comparison of
host and parasite phylogenies to document the his-
tory of the association (Page 1993a). Because of the
scarcity of the fossil record, especially within organ-
isms like platyhelminths with poor instances of soft-
bodied tissue preservation (Poinar 2003), measuring
the extent of congruence between host and parasite
phylogenetic branching patterns provides valuable in-
formation to assess past geographical, climatic, and
ecological features that may have shaped their evo-
lution. In this context, the approach of cophylogeny
mapping (Paterson and Banks 2001) evaluates all kinds
of evolutionary events that produced speciation and/or
diversification of parasites. Processes can be divided
into four main categories: cospeciation (evolution by

descent), host switching (evolution by colonization),
duplication (intrahost divergence), and extinction (sort-
ing events) (Page and Charleston 1998; Johnson et al.
2003; Banks and Paterson 2005). On the other hand,
host phylogeny supplies a powerful timeframe to in-
spect parasite evolution, even more so when the host
fossil record is well calibrated. With the advance of co-
phylogenetic tools (reviewed in Stevens 2004), various
host–parasite associations have been investigated in the
past two decades to explore the evolutionary processes
of parasite diversification, among them viruses, bac-
teria, protozoans, crustaceans, insects, platyhelminths,
and their vertebrate or invertebrate hosts (Paterson
and Poulin 1999; Clark et al. 2000; Ricklefs and Fallon
2002; Jackson and Charleston 2004; Huyse and Volckaert
2005, Banks et al. 2006; Takiya et al. 2006; Hughes et al.

2007; Light and Hafner 2007). Within the phylum Platy-helminthes, for instance, only three genera of the class Monogenea, *Lamellodiscus* (see Desdevises et al. 2002), *Dactylogyrus* (see Simkova et al. 2004), and *Gyrodactylus* (see Huyse and Volckaert 2005), have been investigated. Results revealed numerous occurrences of duplication and host-switching events and very few events of cospe-ciation shaping the evolution of these strictly teleostean fish parasites. Cophylogenetic studies thus constitute a fundamental tool to determine how parasites have evolved and radiated during host evolution. On the other hand, parasites may provide additional data that can, in some cases, be very helpful to investigate the evolutionary ecology of the host (Whiteman and Parker 2005; Nieberding and Olivieri 2007). This is the case, for instance, when two divergent and nonsympatric host species are infected by two sister parasite species, which indicates that donor and receiving host lineages must have occurred in the same area at some point in time.

Anurans (frogs and toads) form the largest group of extant amphibians (Frost et al. 2006). Although studied extensively during the past decade using molecular ap-proaches (e.g., Hay et al. 1995; Ruvinsky and Maxson 1996; Feller and Hedges 1998; Bossuyt and Milinkovitch 2001; Biju and Bossuyt 2003; Vences et al. 2003; Hoegg et al. 2004; San Mauro et al. 2005; Van der Meijden et al. 2005; Bossuyt et al. 2006), numerous issues are still in question, including phylogenetic relationships of the basal frog lineages within the Neobatrachia (see Biju and Bossuyt 2003; San Mauro et al. 2005; Frost et al. 2006; Roelants et al. 2007; Wiens 2007). At this stage, the most relevant phylogenetic studies that address frogs in major families of the Neobatrachia reveal five main lineages whose biogeographic and phylogenetic pat-terns may reflect disintegration of the Gondwana super-continent in the Mesozoic era (see Biju and Bossuyt 2003): (i) the Hyloidea sensu stricto with the Bufonidae, Hylidae, Leptodactylidae, and South American fami-lies; (ii) the Ranoidea with the Ranidae, Rhacophoridae, and major African and Madagascan families; (iii) the Australian hyloids with the Myobatrachidae and two related taxa from southernmost South America (i.e., *Tel-matobufo venustus* and *Caudiverbera caudiverbera*); (iv) the South African Heleophrynidae; and (v) a clade associ-ating the Sooglossidae and Nasikabatrachidae, which are restricted to the Seychelles and India, respectively. Such a correlation between plate tectonics and neoba-trachian frog relationships is of particular interest, not only for knowledge of amphibian evolution but also for subsequent dating of major speciation events within the Neobatrachia. However, in the absence of high res-olution within basal groups of the Neobatrachia, their phylogenetic relationships are still viewed as a poly-tomy (San Mauro et al. 2005). This may be explained by the relatively fast speciation processes that led to the major frog lineages in the Middle/Late Jurassic and Early Cretaceous periods (Biju and Bossuyt 2003).

The Monogenea (Platyhelminthes) includes tens of thousands of parasite species that infest primarily chondrichthyan and actinopterygian fish but also to a lesser extent sarcopterygians, such as the Australian lungfish, amphibians, freshwater turtles, and the African hippopotamus. Among Monogenea, the Polystomati-dae is the most diverse family with about 150 species classified in 23 genera of unequal diversity, *Polystoma* of anurans being the most diversified genus. Like all other monogeneans, polystomatids display a direct life cycle, which facilitates parasite transmission from host to host in aquatic environments. They are generally host and site specific (Murith 1979; Du Preez and Kok 1997; Tinsley 2004). In fact, although they can be found either in the urinary bladder, the pharyngeal cavity, or the conjunctival sacs of their chelonian hosts, polystomes of amphibians are mainly found in the urinary bladder. A few reports have mentioned the presence of more than one polystome species within the same amphibian host species (Bourgat and Murith 1980; Du Preez and Kok 1992), but in most cases, a single parasite species is found per host species. Finally, they show a large diversity of reproductive strategies that range from ovoviviparity to developmental plasticity depending on host ecology (Kearn 1994; Rohde 1994; Whittington 1997; Cribb et al. 2002; Tinsley 2004; Badets and Verneau 2009).

It has been established that phylogenetic relation-ships of parasites of the Polystomatidae are linked with key events in host evolution, such as the verte-brates' transition to land, the lissamphibians' origin, and the freshwater turtles' diversification (Verneau et al. 2002). Subsequently, it was shown that the present day geographical distribution of *Polystoma* of anurans was guided mostly by past dispersals affecting their hyloid hosts during the Tertiary period (Bentz et al. 2006). Given all these findings and the worldwide dis-tribution of polystomes of the Neobatrachia, we aimed to investigate whether evolution of this unique group of parasites may be correlated with the early evolution of their amphibian host species as well as to plate tec-tonics in the Early Jurassic. The parasite phylogeny was generated from analysis of nuclear 18S and partial 28S rRNA genes, whereas the host phylogeny was inferred from analysis of nuclear rhodopsin and mitochondrial 12S and 16S rRNA genes because these markers were shown to be the most suitable for frogs (e.g., Vences et al. 2003; Hoegg et al. 2004; Van der Meijden et al. 2005). Cophylogenetic and biogeographic vicariance analyses were conducted to investigate evolutionary processes of polystome diversification, which in turn may provide valuable insights about host evolutionary ecology.

MATERIALS AND METHODS
Parasite Sampling

Three main reasons explain our relatively small para-site sampling: (i) amphibians are declining more rapidly than any other vertebrate group, which complicated the ability to secure collection permits for frogs that are

threatened with extinction and therefore their parasites; (ii) in contrast to the high diversity of frog species (more than 5000 described species, see Frost et al. 2006), very few host anuran species (i.e., no more than 100) are currently known to be infected by polystomes (see Verneau 2004); and (iii) prevalence of infected hosts is usually very low, rarely >20%. Thus, our molecular data set (Table 1) integrated 20 polystome species from hyloid and ranoid host species, sampled from all continental regions including India, and three other polystome species infecting pelobatid and pipid frogs from the basal archaeobatrachian anurans for outgroup comparisons. Sampling also incorporated the polystome species from the Australian lungfish (i.e., *Concinnocotyla australensis*), the most basal species within the Polystomatidae and two monogenean parasite species of the Infrasubclass Oligonchoinea infecting teleost fishes (see Boeger and Kritsky 2001; Verneau et al. 2002), all of which were used in a global phylogenetic analysis for molecular dating.

Host Sampling

Sequences of all frog species infected by polystomes investigated in the present study were recovered from GenBank (Table 1). We first selected complete or partial 12S and 16S mitochondrial genes that were sequenced for almost all the infected frog species. We also obtained data for part of exon 1 of the nuclear rhodopsin gene that was sequenced for two-thirds of the host taxa. Because several host species had not been sequenced for all three genes, some species were replaced by phylogenetically closely related species according to the classification of Frost et al. (2006) (Table 1). Hence, seven frog species were substituted in the rhodopsin and two in the (12S + 16S) data sets to produce the most comprehensive data sets. This strategy of exchanging host species with close relatives had no impact on the resultant scenarios because phylogenies inferred from both mitochondrial and nuclear data sets were congruent and because we only examined evolutionary processes at the earliest stages of evolution in this host–parasite association. Thus, subsequent cophylogenetic analyses conducted with TreeMap, version 2.02β (Charleston and Page 2002), were simply done with the host phylogeny inferred from the nuclear rhodopsin data set.

Molecular Experiments

All methods used for DNA extraction, amplification, and sequencing are described elsewhere (Bentz et al. 2006). The complete 18S rRNA gene was amplified in one round with primers F18, 5′-ACCTGGTTGATCCT GCCAGTAG-3′ and IR5, 5′-TACGGAAACCTTGTTAC GAC-3′, yielding a polymerase chain reaction (PCR) product of about 2 kb that was subsequently sequenced with the same primers and also the following internal

primers: 18F1, 5′-GTTGTGTCGTGTTGACTCTG-3′; 18F2, 5′-GGAGGGCAAGTCTGGTGCCAG-3′; 18F3, 5′-GGACGGCATGTTTACTTTGA-3′; 18RA, 5′-GCCC-GCGGGGACGATATGTAC-3′; 18RB, 5′-TGCTTTGAGC-ACTCAAATTT-3′; 18RC, 5′-TACGAGCTTTTTAACTG-CAG-3′; and 18RG, 5′-CTCTCTTAACCATTACTTCGG-3′. The partial 28S rRNA gene corresponding to the 5′ terminal end was amplified with primers LSU5′, 5′-TAGGTCGACCCGCTGAAYTTAAGCA-3′ and LSU3′, 5′-TAGAAGCTTCCTGAGGGAAACTTCGG-3′ (Snyder and Loker 2000), yielding a PCR product of about 1.4 kb that was subsequently sequenced with the same primers and also the following internal primers: IF13, 5′-AGCAAACAAGTACCGTGAGGG-3′; IF15, 5′-GTCTGT GGCGTAGTGGTAGAC-3′; IR13, 5′-GTCGTGGCTTAC ACCCTGAGG-3′; and IR14, 5′-CATGTTAAACTCCTT GGTCCG-3′.

Phylogenetic Analyses

Parasite tree reconstructions.—The secondary structure of the small subunit (SSU) ribosomal RNA of *Calicophoron calicophorum* (L06566) was first recovered from the European Ribosomal RNA database (http://www.psb.ugent. be/rRNA/) and aligned with the *Polystoma gallieni* sequence using DCSE v2.6 software (De Rijk and De Wachter 1993). Although most stems and loops in *P. gallieni* were inferred from conserved aligned regions, a few in hyper variable and insertion regions were determined from a search of common motifs in the most distant polystome species with the aid of Mfold software, using default parameters (http://www.bioinfo.rpi.edu/) (Zuker 2003). This concerned helices E10_1, 11, 12, E23_1, E23_2, E23_5, E23_6, E23_7, 43, and 49 (see Van de Peer et al. 1999 for the nomenclature of RNA secondary structures). The 18S sequences of all other parasite species were aligned subsequently according to the structural constraints of the *P. gallieni* sequence. The large subunit (LSU) ribosomal RNA structure of *P. gallieni* was inferred following the same procedure as described above with regard to the RNA secondary structure of *Caenorhabitis elegans* (X03680) and *Dugesia tigrina* (U78718). The 28S sequences of all other parasite species were thus aligned according to the structural constraints of the *P. gallieni* sequence. The C and D5 regions were not constrained due to the high level of divergence within polystomes and the lack of common motifs after Mfold reconstructions. They were therefore treated as loop regions in phylogenetic analyses.

The incongruence between 18S and 28S data sets was first measured by the incongruence length difference (ILD) test implemented in PAUP* 4.0b9 (Swofford 2002). Because no conflicting signal was observed ($P = 0.515$; 1000 replicates), genes were combined for subsequent phylogenetic analyses. The combined data set, including 4160 characters from both nuclear rRNA genes of the 23 anuran polystome species, was partitioned into stem and loop regions for the Bayesian analysis. The Xstem software (Telford et al. 2005) was used to extract

TABLE 1. Parasite species investigated, their host species, geographical origin, and GenBank accession numbers (polystomes: 18S and partial 28S sequences; frogs: partial Rhodopsin, 12S, and 16S sequences)

Parasite species	Host species	Origin	Accession numbers		
			18S and partial 28S	Rhodopsin	12S and 16S
Polystomatidae from neobatrachian hosts					
Diplorchis ranae	Rana rugosa	Japan	AM157184[a] & AM157198[a]	DQ283837[b]	AB430353[c]
Eupolystoma alluaudi	Bufo sp.	Togo	AM051066 & AM157199[a]	DQ283858[b]	DQ158485[b,c]
Eupolystoma vanasi	Schismaderna carens	South Africa	AM157185[a] & AM157200[a]	DQ284027	AY325997[c]
Parapolystoma bulliense	Litoria gracilenta	Australia	AM157186[a] & AM157202[a]	AY844684[b]	DQ116853 & AF136319
Polystoma cuvieri	Physalaemus cuvieri	Paraguay	AM051068 & AM157203[a]	AY844717	AY843729[c]
Polystoma dawiekoki	Ptychadena anchietae	South Africa	AM051069 & AM157204[a]	DQ283760[b]	AF261249 & AF261267
Polystoma floridana	Hyla cinerea	USA	AM157188[a] & AM157211[a]	AY844597	AY680271[c]
Polystoma gallieni	Hyla meridionalis	France	AM051070 & AM157205[a]	—	AY819370 & AY523763
Polystoma indicum	Rhacophorus maximus	India	AM157193[a] & AM157216[a]	EU924552	EF564476 & EF564548
Polystoma integerrimum	Rana temporaria	France	AM051071 & AM157206[a]	U59920	AY326063[c]
Polystoma lopezromani	Phrynohyas venulosa	Paraguay	AM051072 & AM157207[a]	AY364396	AY549362[c]
Polystoma marmorati	Hyperolius marmoratus	South Africa	AM051073 & AM157208[a]	AY323740[b]	AY603987[b,c]
Polystoma naevius	Smilisca baudinii	Costa Rica	AM157187[a] & AM157209[a]	AY844749	AY843762[c]
Polystoma nearcticum	Hyla versicolor	USA	AM051074 & AM157210[a]	AY844675	AY843682[c]
Polystoma testimagna	Strongylopus fasciatus	South Africa	AM157194[a] & AM157217[a]	DQ019574	DQ019594 & AF215412
Polystoma sp.	Rhacophorus omeimontis	China	AM157189[a] & AM157212[a]	EU215565	EU215535[c]
Polystoma sp.	Rhacophorus arboreus	Japan	AM157190[a] & AM157213[a]	AY880653	AF458142[c]
Polystoma sp.	Rhacophorus viridis	Japan	AM157191[a] & AM157214[a]	—	—
Polystoma sp.	Leptodactylus mystaceus	French Guiana	AM157192[a] & AM157215[a]	DQ284015[b]	AY905717 & AY911286
Wetapolystoma almae	Bufo margaritifer	French Guiana	AM051081 & AM157220[a]	—	AY819331 & AF375514
Polystomatidae from archaeobatrachian hosts					
Neodiplorchis scaphiopi	Spea bombifrons	USA	AM051067 & AM157201[a]	DQ283870[b]	AY819327 & AY236818
Protopolystoma xenopodis	Xenopus laevis	South Africa	AM051078 & AM157218[a]	U23808	M10217[c]
Pseudodiplorchis americanus	Scaphiopus couchii	USA	AM051079 & AM157219[a]	DQ283850	DQ283150[c]
Polystomatidae from Dipnoi					
Concinnocotyla australensis	Neoceratodus forsteri	Australia	AM157183[a] & AM157197[a]		
Monogeneans from teleost fishes					
Microcotyle erythrinii	Pagellus erythrinus	France	AM157195[a] & AM157221[a]		
Pseudaxine trachuri	Trachurus trachurus	France	AM157196[a] & AM157222[a]		

[a] Refers to this study; [b] refers to sequences of frog species which are closely related to the actual host species: from top to bottom, taxa substituted for actual host species: *Rana japonica*, *Bufo regularis*, *Litoria aurea*, *Ptychadena aurea*, *Ptychadena mascareniensis*, *Hyperolius viridiflavus*, *Leptodactylus fuscus*, and *Spea hammondii*; [c] refers to accession numbers that are for both 12S and 16S sequences; — indicates that no sequence was available.

the RNA secondary structure information of the DCSE alignment and to convert it into a nexus file format. A GTR + I + Γ model was selected for the loop regions by the Akaike Information Criterion (AIC) implemented in the program Modeltest 3.06 (Posada and Crandall 1998). A doublet model was preferred for the stem partitions as recommended by Telford et al. (2005). Bayesian analysis was conducted using the software MrBayes 3.04b (Huelsenbeck and Ronquist 2001), with four chains running for million generations, sampling each 100 cycles. Bayesian posterior probabilities were computed after removing the first 1000 trees as the burn-in phase. Bayesian Inferences (BI) were run three times independently to assess for convergence, using the Tracer software available at http://tree.bio.ed.ac.uk/software. The maximum-likelihood (ML) analysis was performed on 3125 characters without partitioning and with gaps excluded. A search for the best ML tree was done using the GTR + I + Γ model selected by the AIC in Modeltest and following a heuristic procedure under the Tree Bisection and reconnection (TBR) branch swapping option with PAUP* 4.0b9. ML bootstrap support values were calculated with the same model of sequence evolution under the Nearest Neighbor Interchange (NNI) branch swapping option using 1000 replicates. The maximum parsimony (MP) analysis was performed with PAUP* 4.0b9 (Swofford 2002) following a branch-and-bound search on 593 equally weighted informative characters, with gaps considered as missing data. Percentage support values were calculated following heuristic search and stepwise addition with 1000 bootstrap replicates. Finally, a global phylogenetic analysis was conducted on all polystomatid and fish monogenean species to produce the topology used as support for molecular calibrations (26 taxa).

Host tree reconstructions.—Rhodopsin sequences were aligned with Clustal W (Thompson et al. 1994), which is implemented in MEGA version 4 (Tamura et al. 2007). Alignment revealed 282 characters, of which 89 were parsimony informative (20 taxa). Because rhodopsin is a protein-coding gene, the complete alignment was partitioned for Bayesian analysis and ML according to codon Positions 1, 2, or 3 that may evolve at different evolutionary rates. The most appropriate models of evolution were selected independently for each position categories using the AIC in Modeltest. BI was obtained using a GTR model for Positions 1 and 2 allowing rate variation across sites, and a GTR + I + Γ model for Position 3, after assessing convergence with the aid of the software Tracer. Bayesian posterior probabilities were thus computed after removing the first 1000 trees as the burn-in phase. ML analysis was conducted with a GTR model allowing rate variation across Partitions 1, 2, and 3. The best ML tree was recovered following a heuristic search under the TBR branch swapping option, and ML bootstrap support values were calculated under the NNI branch swapping option, using 1000 bootstrap replicates. The MP analysis was performed following a branch-and-bound search on all equally weighted informative characters and the percentage support values

were calculated following the same procedure with 1000 bootstrap replicates. The procedure of host tree reconstruction from mitochondrial markers is available as Supplementary Methods (see Supplementary material available from http://www.sysbio.oxfordjournals.org). Assuming that actual and substituted host species may be interchanged, the ILD test was also performed between rhodopsin and (12S + 16S) data sets. Once phylogenetic incongruence was excluded ($P = 0.87$; 1000 replicates), a Bayesian analysis was conducted on the complete combined data set using the different models of evolution as selected for nuclear (rhodopsin) and mitochondrial (12S and 16S) partitions.

Cophylogeny

The mathematical procedure developed by Charleston (1998), which is implemented in the algorithm of TreeMap, version 2.02β (Charleston and Page 2002), explores all the possibilities of mapping an associate tree onto a host tree, by minimizing the number of noncodivergence events (i.e., duplication, horizontal transfer, and sorting events). A statistical test is implemented to test whether the most parsimonious reconstructions are significantly better than reconstructions inferred from 1000 randomized associate phylogenies (Charleston and Robertson 2002). Regarding the phylogenetic congruence between rhodopsin and (12S + 16S) host trees, the cophylogenetic analysis was conducted only from comparisons of nuclear host and parasite phylogenies. Host and parasite trees inferred from ML analyses were thus reconciled, after having excluded the most divergent taxa in both phylogenetic trees, namely archaeobatrachian hosts and their parasites that were used only for tree rooting. Due to uncertainties in nuclear and mitochondrial host trees, we computed separate TreeMap analyses on nine alternative topologies within host assemblage following inversion of the three main groups within the Hyloidea and Ranoidea lineages, respectively. Consequently, the cophylogenetic structure was evaluated for each of the nine combinations without the use of the branch length option. Costs for the different processes were settled by setting default at zero for cospeciation and at one for all three other types of events. Preliminary analyses on this host–parasite association revealed that host-switching events were negatively correlated with the number of extinction and duplication events (data not shown). Taking biological features of polystomes into consideration (i.e., a single polystome species per host species in most cases), assuming duplication therefore seems less likely than host switching. The search of the most parsimonious reconstructions was thus performed constraining the maximum number of host-switching events to four due to computing limitations.

The ParaFit approach (Legendre et al. 2002) was also used to assess coevolution between hosts and their parasites. This method which is implemented in Copycat

(Meier-Kolthoff et al. 2007) tests the significance of a global hypothesis of coevolution within host–parasite assemblages from the patristic distances among host and parasite species. This test was computed over three host-parasite combinations, the first resulting from distance comparisons of nuclear ML host and Bayesian parasite trees (17 taxa per tree) and the second and the third resulting from distance comparisons of mitochondrial ML host and nuclear Bayesian parasite trees, with 17 and 19 taxa per tree, respectively.

Vicariant Biogeography

To investigate the historical biogeography of neobatrachian polystomes, and to more precisely explore links between the parasite phylogenetic tree and the Earth's history at the stage of Gondwana breakup, two kinds of procedures, a posteriori and a priori methods, were pursued.

The first approach, DIVA for Dispersal-Vicariance Analysis (Ronquist 1997), is based on a posteriori assumptions. It minimizes dispersal and extinction events and allows multiple and reticulate relationships among areas (Ronquist 1997). Thus, ancestral distributions of organisms are hypothesized without constraining geographical area relationships a priori. In that context, costs are assigned to each kind of speciation event and a three-dimensional cost matrix is built to reconstruct the most parsimonious ancestral distributions of taxa (Ronquist 1997). As different equally parsimonious distributions may be inferred for specific nodes within the tree, the optimal ancestral distributions must be considered a posteriori, according to the current scenarios of plate tectonics (see for instance Bossuyt et al. 2006). In order to reduce hypotheses of ancestral distributions within the polystome tree, we considered the lineage including European and African *Polystoma* species to have originated in Eurasia (Bentz et al. 2001). Similarly, we considered the lineage including *Polystoma* species from Central and North America to be of South American origin (Bentz et al. 2006). DIVA version 1.1 (Ronquist 1996) was run on a Macintosh and costs were set at zero for allopatric (i.e., biogeographic vicariance) and sympatric speciation and at one for dispersal and extinction.

The second approach we used to reconstruct ancestral distributions within the polystome tree was TreeMap, version 2.02β (Charleston and Page 2002). All its principles are the same as those described above for cophylogenetic studies, but host phylogeny is substituted by an area cladogram. In that context, it is based on a priori assumptions of plate tectonic relationships. The most parsimonious reconstructions are then inferred and likewise, one statistical test based on 1000 randomizations of the associate phylogeny gives the significance for the results. For our analyses, we considered for the same reasons as mentioned above the clade of European and African *Polystoma* species of Eurasian origin (Bentz et al. 2001) and the group associating Central

and North American *Polystoma* species of South American origin (Bentz et al. 2006). Costs were set at zero by default for biogeographic vicariance (similar to cospeciation) and at one for sympatric speciation (similar to duplication), dispersal (similar to host switching), and extinction. Contrary to cophylogenetic analysis, no constraint was fixed for the number of dispersal events. The polystome tree was then reconciled to two successive area cladograms, according to the current hypotheses of plate tectonics. The first area cladogram considers a sister relationship between South American and African plates and Indian and Australian plates. Indeed, it is well accepted that the landmasses of Gondwana began to separate in the Early Jurassic, approximately 180–160 Ma, leading to western (South America and Africa) and eastern (India, Madagascar, Seychelles, Antarctica, and Australia) Gondwanan components (Storey 1995, Chatterjee and Scotese 1999; Lawver et al. 1999; Briggs 2003). During subsequent stages of rifting in the Early Cretaceous, the block uniting the Seychelles, Madagascar, and India separated from Antarctica–Australia about 130 Ma (Briggs 2003), and South America may have diverged from Africa at about the same time (Storey 1995; Lawver et al. 1999; Macdonald et al. 2003). The second area cladogram considers the Australian plate as the most basal, the Indian plate being more closely related to the South American and African continents. Indeed, though the first stages of Gondwana breakup were initiated following seafloor spreading in the Somali, Mozambique, and Weddell Sea basins (Storey 1995), it has been proposed from analysis of the seismic structure and sediment distribution in the western Weddell Sea that this region was the site of the initial breakup of Gondwana (Rogenhagen and Jokat 2000). Afterwards, the Australian plate could have first diverged in its austral part from the South American and African plates at a time when India was still joined to the western part of Gondwana and Australia. Because the area cladogram (5 "taxa") is smaller than the parasite phylogeny (20 taxa), randomizing tests may seriously increase the significance of outputs and lead to subjective conclusions. Hence, to test for the reliability of results, analyses were also conducted with both area cladograms and a phylogenetic representation of a restricted subsample of parasite species, which was deduced from the parasite ML tree. The selection of eight polystome species was made according to their phylogenetic position within the phylogenetic tree (i.e., at least one species was selected from the main lineages and sublineages) and their occurence in specific biogeographic areas.

Molecular Calibrations

Because the constancy of the molecular clock was rejected within the Polystomatidae (see Verneau et al. 2002), we used estbranches and multidivtime implemented in the multidistribute package (Thorne et al.

1998; Thorne and Kishino 2002) to infer divergence time estimates within polystomes of the Neobatrachia. The instructions described in the manual of Rutschmann (2005) were followed to perform the molecular dating. The topology used as a constraint for molecular calibrations is a global phylogenetic tree including all polystomatid species, fish monogenean species being used as an outgroup (for a total of 26 taxa). It was inferred from a Bayesian analysis after selecting a doublet model for the stem regions and, by the AIC, a GTR + I + Γ model for the loop regions. ML parameters were estimated for that topology under the F84 + Γ model with BASEML (Yang 1997). ML branch lengths and their variance–covariance matrix were estimated with the estbranches program (Thorne et al. 1998). Rates of molecular evolution and divergence time estimations with their 95% confidence intervals were calculated with multidivtime using the MCMC approach (Thorne et al. 1998; Kishino et al. 2001). Before running the analyses, the following parameters were fixed regarding the origin of the Polystomatidae and according to authors' recommendations: (i) The mean of the prior distribution for the time separating the ingroup root from the present (rttm) was set to 437 Ma (sd = 12). It corresponds to the largest window of time that separates the Actinopterygii and Sarcopterygii (Janvier 1998; Ahlberg 1999) and, consequently, their respective monogenean parasites under hypothesis of coevolution (Verneau et al. 2002). (ii) The mean rate of molecular evolution (rtrate) was estimated from the distance tree computed with estbranches and set at 0.0004819. Finally, (iii) brownmean and brownsd were set at 0.0048 following advice in Rutschmann (2005) and big time, which corresponds to the maximum expected origin of the ingroup, was set to 450 Ma.

Three calibration procedures depending on selected calibration points were then used to infer divergence time estimates. Procedure 1 attempts to fix two calibration points onto the global phylogenetic tree. The separation between *C. australensis* and the polystomes of tetrapods at about 410–415 Ma (Verneau et al. 2002) corresponds to the divergence time between Dipnoi and tetrapods (Janvier 1998; Zhu et al. 2001). The colonization of Africa by *Polystoma* at about 5–25 Ma corresponds to the largest window of time for vertebrate dispersal between Southern Europe and North Africa during the Miocene (Rage 1988). Indeed, though Bentz et al. (2001) assumed that ancestors of *Pelobates* frogs could have been potential hosts that carried polystomes from Eurasia to Africa about 5 Ma, via a terrestrial dispersal route on the western extremity of the Mediterranean Basin by the Upper Miocene (see Rage 1988), another temporal scheme could have been considered because a dispersal route for vertebrates was also invoked between Eurasia and Africa in the eastern Mediterranean area by the Lower Miocene, about 25 Ma (Rage 1988). Procedures 2 and 3 reestimate these divergence times with one single calibration point, either the separation between *C. australensis* and the polystomes of tetrapods at about 410–415 Ma or the colonization of Africa by *Polystoma* at about 5–25 Ma. This allows the detection of whether multidivtime is wrongly influenced by one of the two calibration points.

RESULTS

Alignment of Nuclear Ribosomal Genes

All monogenean species used in this study are listed in Table 1, and new sequences of the complete 18S and partial 28S rRNA genes are deposited in GenBank under accession numbers AM157183 to AM157222. Secondary structures of the complete SSU and partial LSU ribosomal sequences of *P. gallieni* are shown as Supplementary Appendices 1 and 2, respectively. These models served as a template to shape the secondary structure of all other polystomatids and fish monogeneans. This allowed us to refine an alignment according to structural constraints (i.e., loops and stems).

Polystomatid Phylogeny

The MP analysis, which was conducted on the 23 taxa, yields a single parsimonious tree (tree length = 1135; consistency index = 0.67), this being identical to the Bayesian consensus and ML trees (ML tree score = 13266.3028). For this reason, we only present the ML tree with bootstrap values inferred from ML and MP analyses and Bayesian posterior probabilities (Fig. 1). Phylogenetic relationships of polystomes reveal four main lineages, whose distributions can be ascribed to restricted geographical areas. The first group associates two species, *Parapolystoma bulliense* and *Diplorchis ranae*, found, respectively, on hylid frogs (Pelodryadinae) of Australia (i.e., *Litoria gracilenta*) and ranid frogs of Japan (i.e., *Rana rugosa*). The second lineage encompasses four species of *Polystoma*. All are recovered from *Rhacophorus* species in India, China, and Japan. The third clade is composed of two species of *Eupolystoma* sampled from African bufonids and the last lineage unites *Wetapolystoma almae* and most of the *Polystoma* species. These parasites, which are found from both hyloid sensu stricto and ranoid frog lineages, are distributed worldwide except in Australia and Madagascar. For convenience, we will call the monophyletic group that includes *W. almae* and all species of *Polystoma* infecting non-*Rhacophorus* hosts, the *Polystoma* sensu stricto lineage. Results also reveal that American polystomes do not form a clade, the strict Central and North American polystome group being the most basal taxon among the *Polystoma* sensu stricto lineage. Concerning the phylogenetic relationships between the four main parasite lineages, the "*Parapolystoma/Diplorchis*" lineage appears most basal among neobatrachian polystomes and the lineage including Indian and South-eastern Asian rhacophorid polystomes is related to the stem branch of the closely related *Eupolystoma* and *Polystoma* sensu stricto lineages (Fig. 1). Finally, the global phylogeny that includes 26 taxa confirms the monophyly of the Polystomatidae, with *C. australensis* being the most basal taxon within polystomes (results not shown).
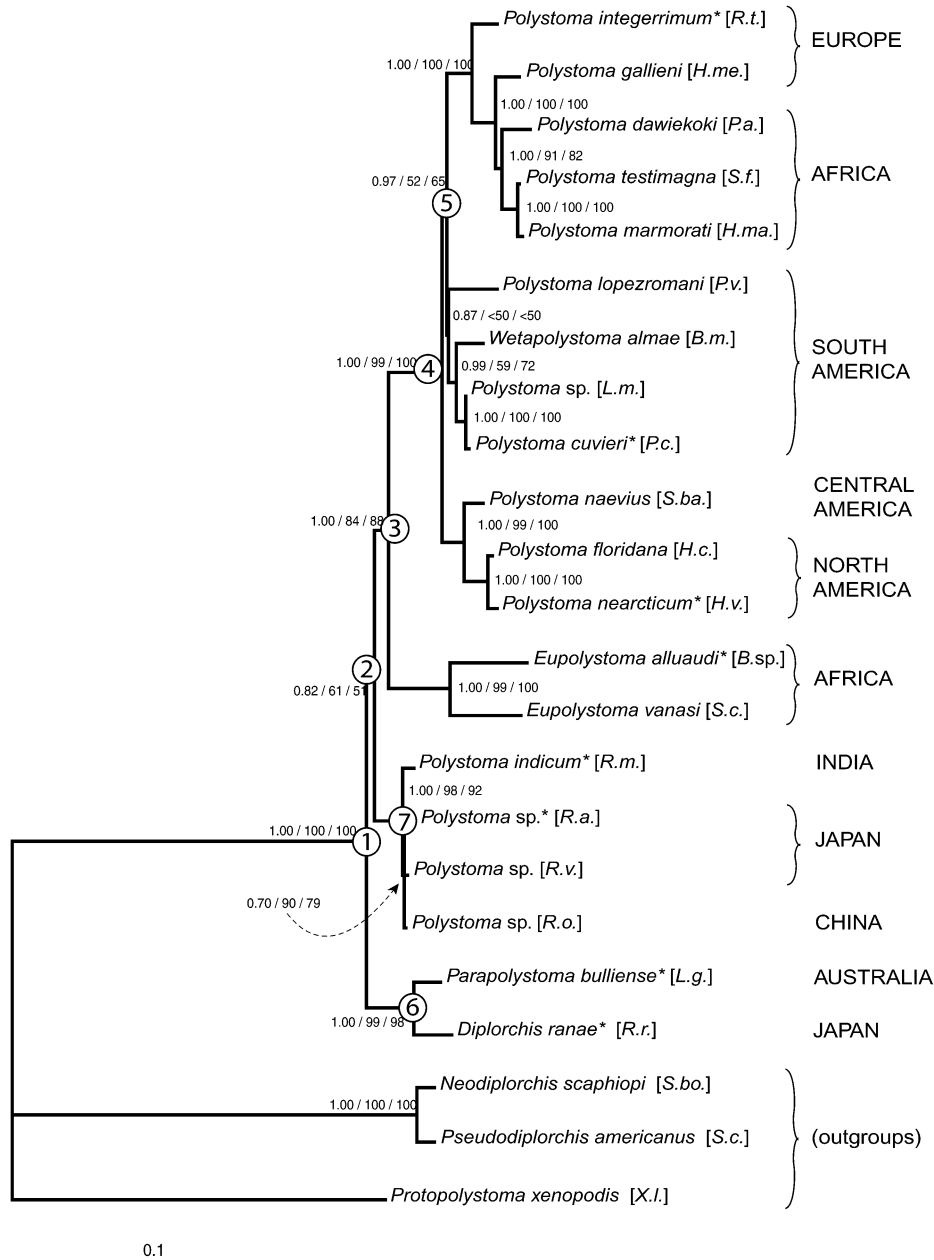
FIGURE 1. Parasite ML tree inferred from analysis of 3125 characters (complete 18S and partial 28S). Abbreviations in brackets refer to host species, from top to bottom: *R.t.* = *Rana temporaria*; *H.me.* = *Hyla meridionalis*; *P.a.* = *Ptychadena anchietae*; *S.f.* = *Strongylopus fasciatus*; *H.ma.* = *Hyperolius marmoratus*; *P.v.* = *Phrynohyas venulosa*; *B.m.* = *Bufo margaritifer*; *L.m.* = *Leptodactylus mystaceus*; *P.c.* = *Physalaemus cuvieri*; *S.ba.* = *Smilisca baudinii*; *H.c.* = *Hyla cinerea*; *H.v.* = *Hyla versicolor*; *B.*sp. = *Bufo* sp.; *S.c.* = *Schismaderma carens*; *R.m.* = *Rhacophorus maximus*; *R.a.* = *Rhacophorus arboreus*; *R.v.* = *Rhacophorus viridis*; *R.o.* = *Rhacophorus omeimontis*; *L.g.* = *Litoria gracilenta*; *R.r.* = *Rana rugosa*; *S.bo.* = *Spea bombifrons*; *S.c.* = *Scaphiopus couchii*; *X.l.* = *Xenopus laevis*. Numbers at nodes indicate, from left to right, Bayesian posterior probabilities, MP, and ML percentage bootstrap support values with 1000 replicates. Labelled nodes 1–7 correspond to the speciation events discussed in the text (see Table 2 for molecular dating). Asterisks indicate species that were selected to build a restricted phylogenetic representation of polystomes used in vicariance analyses.

Frog Phylogeny

Because no incongruence is revealed between rhodopsin and (12S + 16S) data sets, we only present the ML tree inferred from analysis of the rhodopsin alignment (Fig. 2), with Bayesian posterior probabilities and branch support values provided from ML and MP analyses. Posterior probabilities obtained from the Bayesian analysis of the complete combined data set (12S, 16S, and rhodopsin) are also indicated. Nuclear and mitochondrial host trees are very similar to each other and to the most recent phylogenetic hypotheses of amphibian families and subfamilies (Darst and
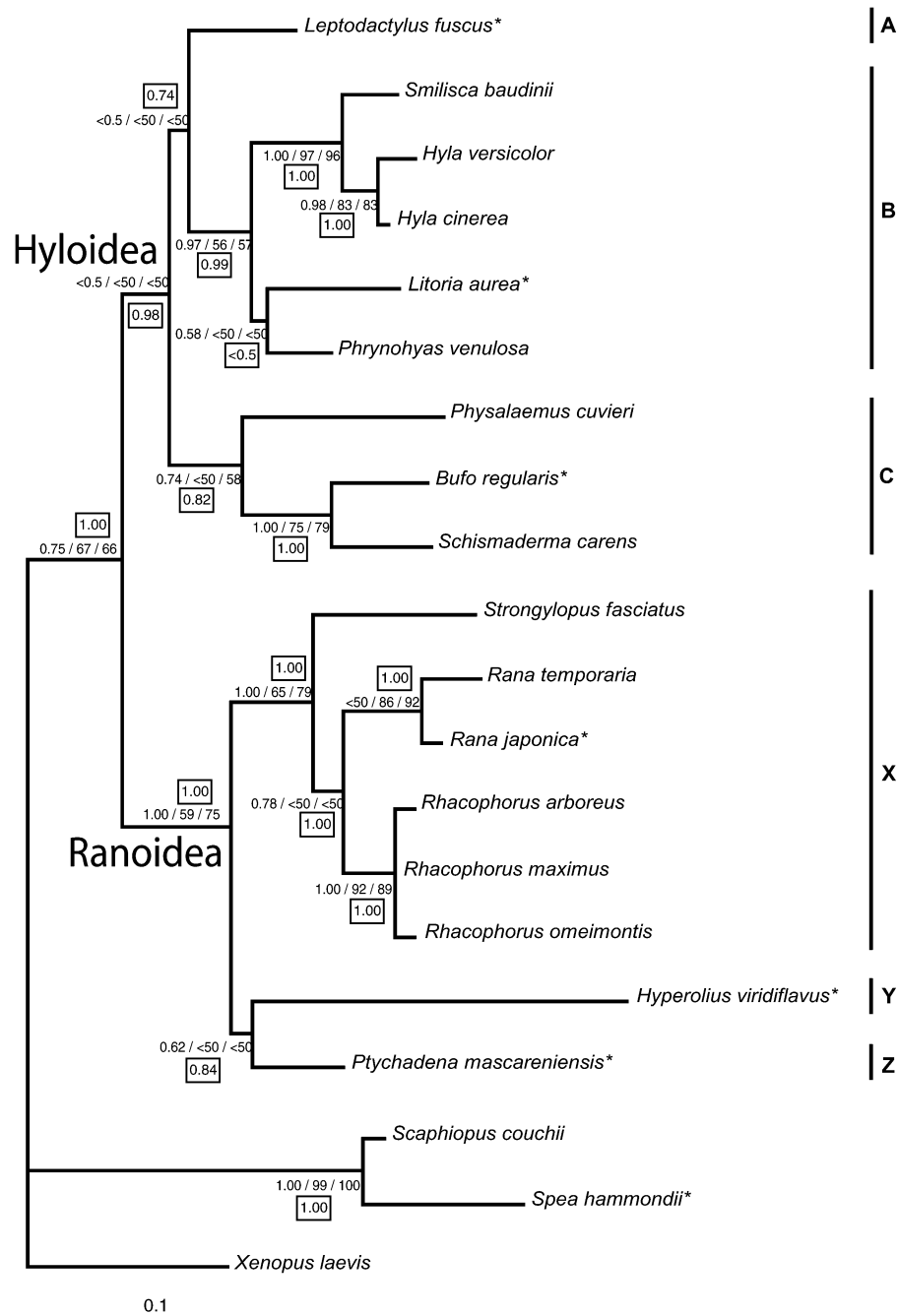
FIGURE 2. Amphibian ML tree inferred from analysis of 282 characters (nuclear rhodopsin). Numbers indicate from left to right Bayesian posterior probabilities, MP, and ML percentage bootstrap support values with 1000 replicates. Framed values correspond to Posterior probabilities resulting from the Bayesian analysis of the complete data set (combined 12S, 16S, and rhodopsin markers). Asterisks indicate taxa substituted for actual host species. Letters on the right-hand side show the six lineages that were inverted for the cophylogenetic analyses.

Cannatella 2004; Pauly et al. 2004; Wiens et al. 2005; Roelants et al. 2007). The Hyloidea sensu stricto and Ranoidea lineages are indeed monophyletic; the Hylinae, Bufonidae, Ranidae, and the Rhacophoridae are each monophyletic; and the two latter families are sister taxa (Fig. 2).

Cophylogeny

TreeMap analysis.—For the cophylogenetic analyses, we retain the ML host tree inferred from the rhodopsin data set (without outgroups) because it allows comparisons of host–parasite relationships that each results from nuclear gene analysis. As TreeMap requires fully

resolved phylogenies, we considered nine topologies within the host tree by exchanging branching patterns within Hyloidea (i.e., between *Leptodactylus fuscus* (A), Hylinae (B), and Bufonidae (C)) and within Ranoidea (i.e., between the group uniting *Strongylopus fasciatus*, Ranidae and Rhacophoridae (X), *Hyperolius viridiflavus* (Y), and *Ptychadena mascareniensis* (Z)) (see Fig. 2). All of these arrangements were successively compared with the polystome topology after having excluded *P. gallieni*, *W. almae*, and outgroups (Fig. 1). A total of 162 significant cophylogenetic reconstructions, depending on the host cladogram selected for TreeMap analysis, are recovered differing by their global cost, the direction, and number of host-switching events and the number of codivergence, duplication, and extinction events. A tanglegram showing frog and polystome phylogenetic relationships and their interactions is shown in Figure 3 based on the host phylogeny in Figure 2 and on the parasite phylogeny in Figure 1. Among the five significant reconstructions ($P < 0.01$), the most parsimonious regarding the cost scheme designed for the different processes (duplication, horizontal transfer, and sorting events) involves a total cost of 30 (Fig. 4). It suggests

four host switching, 22 codivergence, 10 duplication, and 16 extinction events and illustrates one of the numerous scenarios that may account for the evolution of polystomes within amphibians. Host-switching events would have occured from *L. fuscus* to the ancestral stock of Ranoidea, from *L. fuscus* to *Physalaemus cuvieri*, from *L. fuscus* to *Phrynohyas venulosa*, and from *H. viridiflavus* to *S. fasciatus* (Fig. 4).

ParaFit analysis.—Among the three host–parasite combinations that were tested, none of them is significant ($P > 0.05$), suggesting that hosts and polystomes could be randomly associated. These results match well with TreeMap reconstructions which propose either a large number of ancient and recent duplications followed by extinction events or numerous cases of host-switching events, to reconcile host and parasite phylogenies.

Vicariant Biogeography

DIVA, like TreeMap, requires fully resolved phylogenies. Thus, we used the polystome phylogeny de-

FIGURE 3.   Tanglegram for frogs (left; from Fig. 2) and their polystomes (right; from Fig. 1). Parasites are linked to their specific or substituted hosts by lines. Asterisks indicate taxa substituted for actual host species. Abbreviations in brackets refer to host species from top to bottom: *R.o.* = *Rhacophorus omeimontis*; *R.a.* = *Rhacophorus arboreus*; *L.m.* = *Leptodactylus mystaceus*. This figure is available in black and white in print and in color at *Systematic Biology* online.
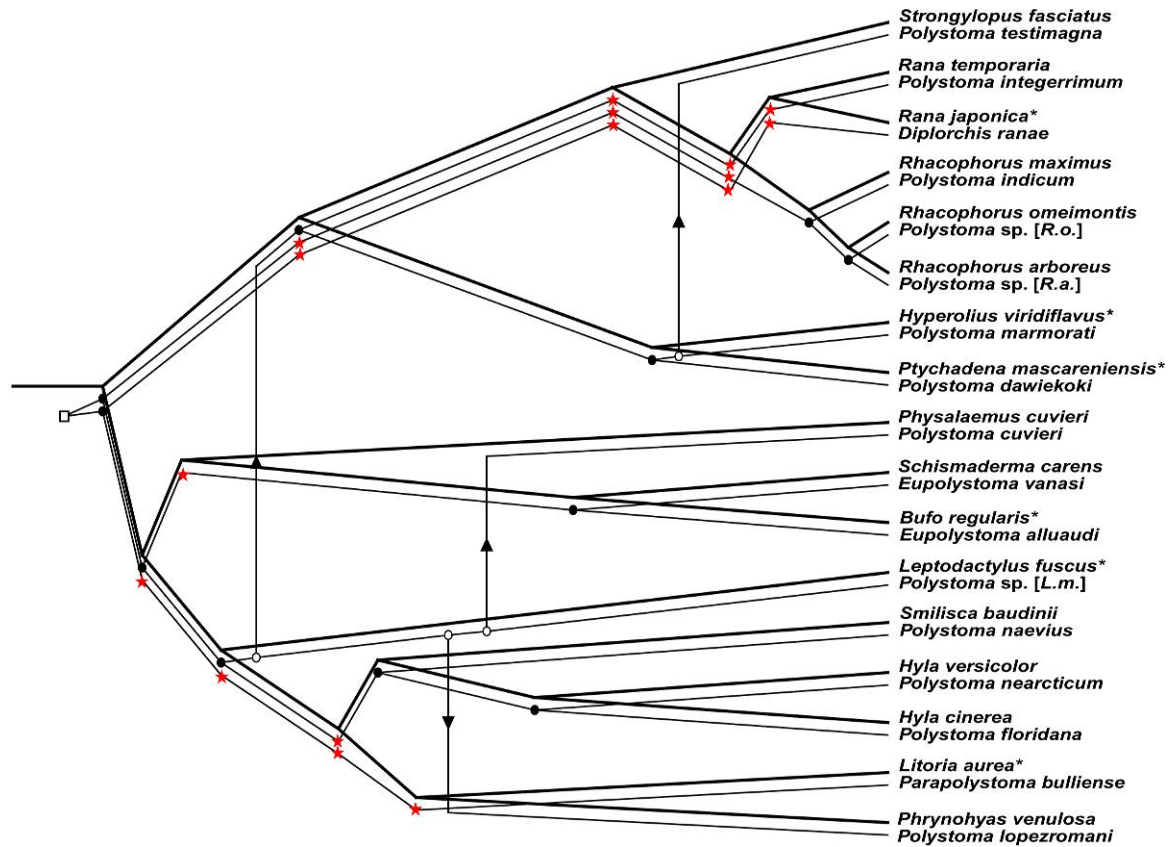
FIGURE 4.   Cophylogenetic reconstruction inferred from a TreeMap analysis (total cost = 30). Key to symbols: open squares and circles refer to duplication (10 events), filled black circles to codivergence (22 events), stars to extinction (16 events), and filled triangles to host switching (4 events). Asterisks indicate taxa substituted for actual host species. For abbreviations in brackets, refer to Figure 3. This figure is available in black and white in print and in color at *Systematic Biology* online.

picted in Figure 1 as input for analysis and considered *Polystoma* sp. of *Rhacophorus viridis* and *R. omeimontis* as sister species. Similarly, we also assumed a Eurasian origin for the lineage including European and African *Polystoma* species and a South American origin for the clade including *Polystoma* species from Central and North America (see Bentz et al. 2006).

DIVA analysis.—This gives one reconstruction with several hypotheses of distribution for the two most basal nodes (Fig. 5). Five different geographical distributions are hypothesized for the ancestral polystome lineage: one corresponding to Gondwana and the other four to blocks associating Eurasia with three of the four remaining plates (Fig. 5). Similarly, six distributions are hypothesized for the ancestral group uniting *Eupolystoma*, *Polystoma*, and *W. almae*, three of them associating Eurasia with two of the four other plates to the exclusion of Australia and the three others associating India to South America, India to Africa, and India to the "South American–African" block. On the other hand, only one geographical distribution is proposed for all other nodes (Fig. 5). If, then, 30 different scenarios can be proposed to account for the distribution of ancestral stocks of polystomes, most of them can be

rejected due to the current knowledge of plate tectonics and reticulate relationships among areas.

TreeMap analysis.—This was conducted using two different area cladograms. In the first scenario (see Tanglegram of Fig. 6), when a sister relationship between, respectively, South American and African plates and Indian and Australian plates is considered, six different reconstructions are proposed ($P < 0.01$). Five of them suggest a Pangaean polystome origin and differ from each other by the number of dispersals and extinctions. In addition, three of those five reconstructions suggest duplication events in the most ancestral distribution (i.e., in Pangaea). Only the sixth reconstruction (Fig. 7) proposes a different origin for polystomes that would be centred on the "Indian–Australian" block (i.e., in the eastern part of Gondwana). After divergence of the Australian and Indian polystomes, following plate tectonics, dispersal would have occurred from the "Indian" plate to the "South American–African" block (i.e., to the western part of Gondwana) and from the "Australian" plate to Eurasia. A third polystome dispersal would have occurred from South America to Eurasia after codivergence of African and South American polystomes
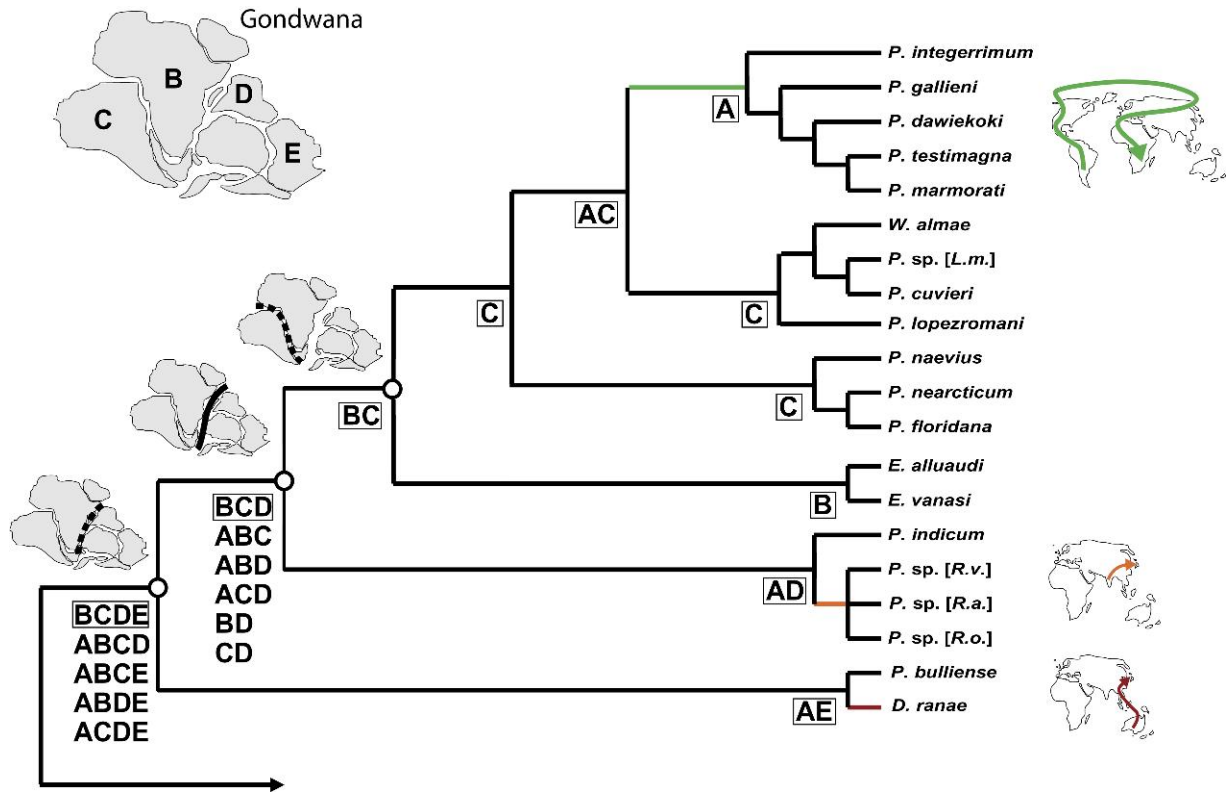
FIGURE 5.   All ancestral distributions inferred from DIVA analysis. A = Eurasia; B = Africa; C = South America; D = India; E = Australia. When several hypotheses for ancestral distributions are hypothesized, the most parsimonious is framed (see text for more details). Open circles at nodes refer to biogeographic vicariance, whereas coloured tree branches and matching arrows indicate dispersal from one area to the other. Dashed and solid lines on the graphic represent incomplete and complete fault lines between western and eastern Gondwanan components on one side and between South America and Africa on the other. Abbreviations in brackets refer to host species from top to bottom: *L.m.* = *Leptodactylus mystaceus*; *R.v.* = *Rhacophorus viridis*; *R.a.* = *Rhacophorus arboreus*; *R.o.* = *Rhacophorus omeimontis*.

with their respective continents. In the second scenario (see Tanglegram of Fig. 8), if the Australian plate is considered as the most basal and the Indian plate intermediate between closely related South American and African continents, five different reconstructions are proposed ($P < 0.01$). Four of them suggest a Pangaean origin for polystomes and differ from each other by the number of dispersals and extinctions. In addition, all of them suggest duplication events (two to eight) in Pangaea. On the other hand, the fifth reconstruction suggests a polystome origin in Gondwana. Six codivergence events are proposed (biogeographic vicariance) following separation of geographical blocks and three dispersal events, respectively, from Australia, India, and South America to Eurasia (Fig. 9). When a phylogenetic representation of a restricted subsample of polystome species (species marked with an asterisk in Fig. 1) is compared with both area cladograms, the same parsimonious reconstructions are found. Whereas ten of them are not significant ($P > 0.2$), the single significant reconstruction ($P < 0.05$) also suggests a Gondwanan origin for polystomes and variance patterns following plate tectonics (Supplementary Appendix 3).

Molecular Dating

We kept a tree for molecular dating whose branching patterns within neobatrachian polystomes are identical to the one depicted in Figure 1. The lungfish parasite, namely *C. australensis*, is placed at the base of the Polystomatidae, and the archaeobatrachian polystomes are considered as a monophyletic sister group of neobatrachian polystomes. Calibration procedure 1 produces more recent estimates than procedures 2 and 3 (Table 2). Because procedures 2 and 3 present related estimates, it appears that multidivtime gives consistent estimates using either calibration point individually. Thus, molecular estimates inferred from procedure 1 can be considered for molecular dating. These estimates show an age of about 194 Ma for the split between "*Parapolystoma/Diplorchis*" and "*Eupolystoma/Polystoma/W. almae*" lineages, an age of about 177 Ma for the divergence between the rhacophorid polystomes and the "*Eupolystoma/Polystoma* sensu stricto" clade, an age of about 156 Ma for the divergence between *Eupolystoma* and *Polystoma* sensu stricto lineages, an age of about 71–79 Ma for the American polystomes diversification, an age of about 106 Ma for the split between *Parapolystoma*
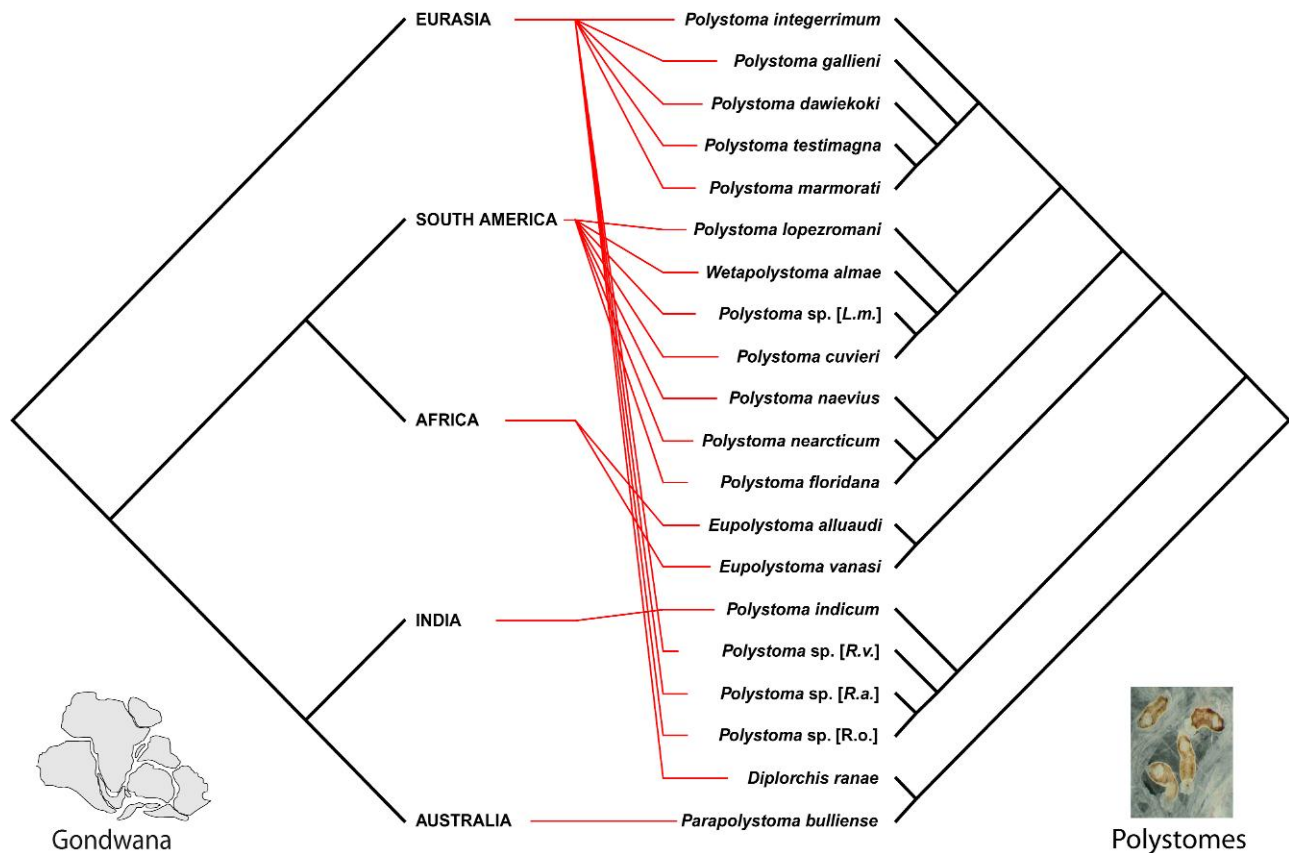
FIGURE 6. Tanglegram generated from TreeMap analysis for Gondwanan areas (left) and polystomes (right) when a sister relationship is assumed between India and Australia on one hand and between South America and Africa on the other. Parasites are linked to their geographical area by lines. For abbreviations in brackets, refer to Figure 5. This figure is available in black and white in print and in color at *Systematic Biology* online.

and *Diplorchis*, and finally an age of about 86 Ma for the Indian–South-eastern Asian rhacophorid polystome divergence.

DISCUSSION

Coevolution

The cophylogenetic approach based on the comparison of phylogenetic trees of hosts and their parasites has been largely developed by Page (1990, 1993b, 1994, 1995) and expanded from the study of pocket gophers and their chewing lice (Hafner and Page 1995; Page and Charleston 1998). Whereas cospeciation has been globally hypothesized for vertebrates and their strict host-specific ectoparasites (i.e., lice) with a direct life-cycle (e.g., Hafner and Nadler 1988; Hafner et al. 1994; Page et al. 1998; Clayton and Johnson 2003; Banks et al. 2006), it appears to be less prevalent within monogeneans and their fish host species (Desdevises et al. 2002; Simkova et al. 2004; Huyse and Volckaert 2005). Polystomes are globally distributed host-specific monogenean mesoparasites and are, in most cases, represented by a single parasite species per host species (Murith 1979; Du Preez and Kok 1997, Tinsley 2004; Verneau 2004). They also have a direct

life cycle involving free swimming larvae in aquatic environments. Therefore, investigating the coevolutionary processes that account for the intimate amphibian and polystome relationships may provide relevant insights about parasite diversification and host evolutionary ecology.

According to our cophylogenetic analyses on different combinations of host topology, the most parsimonious reconstructions (i.e., those which show the minimal total cost) imply three or four host-switching events in most cases. Unfortunately, it is impossible to find other solutions involving more host switching due to computation limitations. Furthermore, most reconstructions show ancient duplication events that appear to have occurred within the ancestral stock of neobatrachians and/or within ancestral hyloid and ranoid hosts (Fig. 4). Several duplication events within ancestral neobatrachian lineages imply that some divergent parasite lineages should co-occur within extant host species, which is actually not the case. A few reports have indeed mentioned the presence of more than one polystome species within the same host species (Bourgat and Murith 1980; Du Preez and Kok 1992; Ibikounle M., Sakiti N., and Verneau O., unpublished observations). However, the three last authors showed that this parasite diversity was the result of recent duplications in the
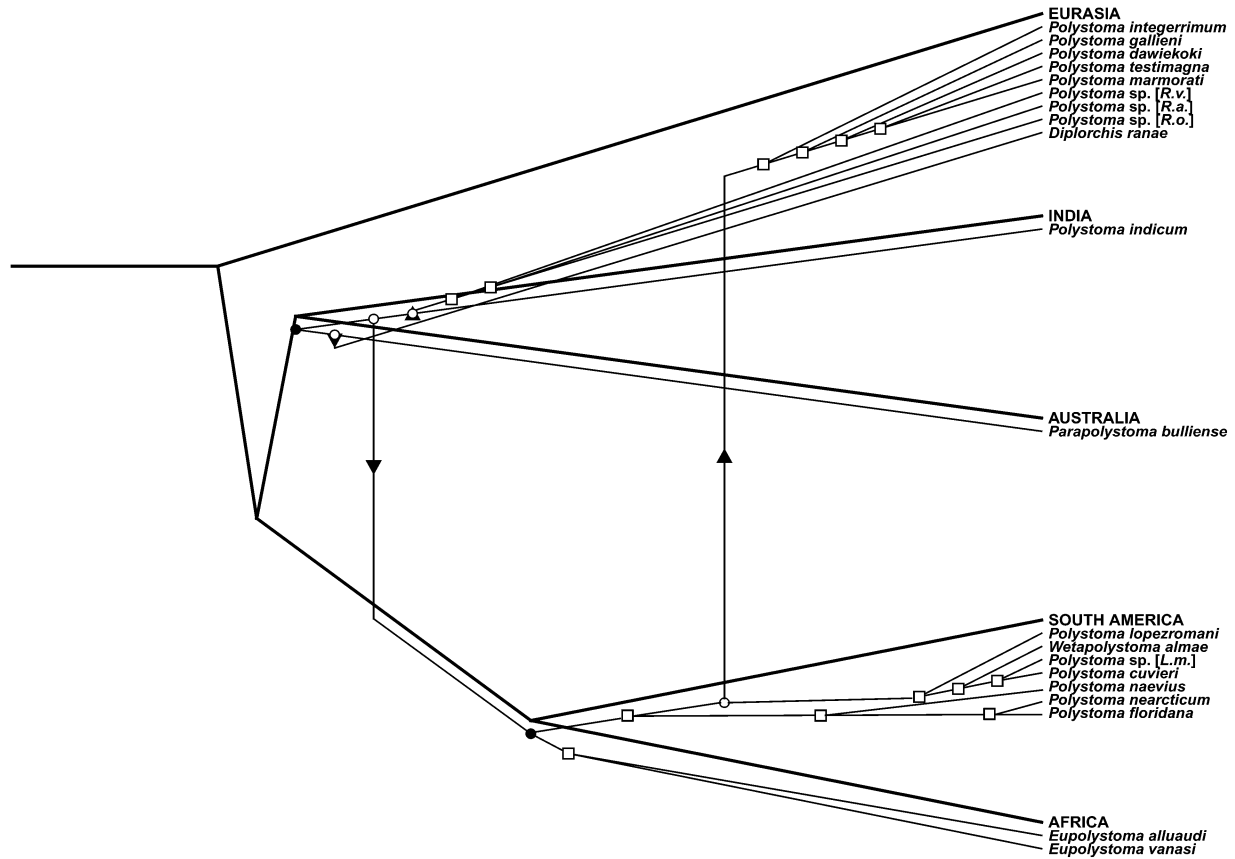
FIGURE 7.   One possible area-polystome historical reconstruction inferred from a TreeMap analysis on the Tanglegram shown in Figure 6. Key to symbols: open squares and circles refer to sympatric speciation, filled circles to biogeographic vicariance, and filled triangles to dispersal. For abbreviations in brackets, refer to Figure 5.

host (unpublished data). Even though extinction processes cannot be rejected, either for host and or parasite lineages, assuming the occurrence of several polystome duplication events within the ancestral frog lineage or within ancestral hyloids, seems very unlikely. Similarly, if *Diplorchis* and *Parapolystoma* genera originated in ancestral neobatrachian frogs, they must have arisen in Gondwana, which is considered the centre of origin for neobatrachians (Zhang et al. 2005). Thus, how can we explain that the survivors of these two polystome lineages are only found in South-eastern Asia and Australia, respectively (see Verneau 2004)? The same question arises with the clade of rhacophorid polystomes, which is considered of the same geographical and temporal origin, but is only distributed in India and Southeastern Asia. On the other hand, host-switching events from hyloid lineages to an ancestral stock of ranoids are highlighted for every most parsimonious cophylogenetic reconstruction (see Fig. 4). Such events imply that extant African *Polystoma* species have originated in ancestral ranoids on Gondwana, thus challenging the Eurasian origin hypothesis inferred from phylogenetic analyses (Bentz et al. 2001).

Regardless of the coevolutionary processes invoked to explain the polystome diversification across host

evolution (i.e., ancestral duplication and host-switching events), neither process accounts for the biology and recent evolutionary history of polystomes. Weckstein (2004) showed within the toucan (bird)—chewing louse (insect) association that biogeography could be more important than host associations in structuring parasite populations and species. In contrast, polystomes are mesoparasites that parasitize the urinary bladder of frogs. Therefore, polystome diversification has necessarily been constrained by host evolutionary ecology. Thus, two nonexclusive hypotheses may be advanced at this stage to explain the nonreliability of such cophylogenetic scenarios. (i) There is a bias in our parasite sampling that does not reflect the overall diversity of neobatrachian polystomes. Because only 20 frog species were surveyed, new polystome species may be expected to be found, especially in poorly studied geographic areas like Asia and Australia, which will reinforce the proposed cophylogenetic scenarios. (ii) Some codivergence events may have been hidden by subsequent ancestral parasite and/or host extinctions. Due to the poor evidence in the fossil record for platyhelminths (Poinar 2003), this hypothesis may be extremely difficult to demonstrate.
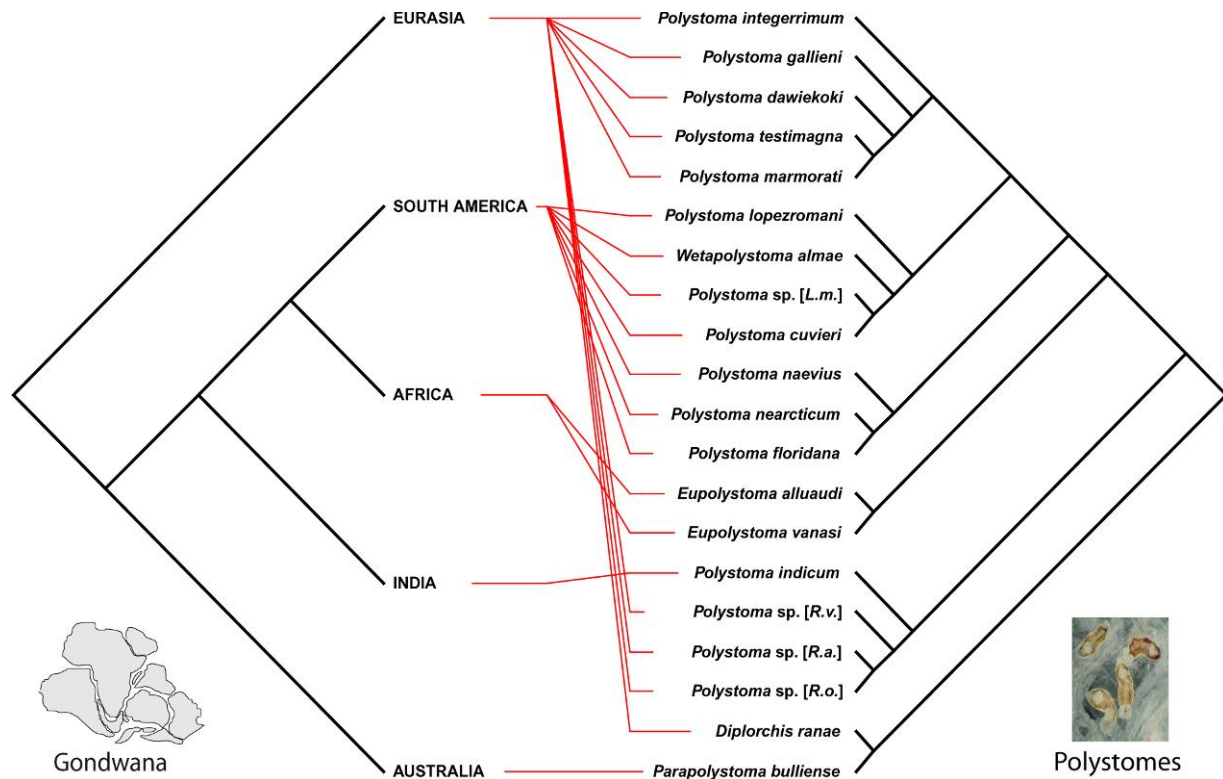
FIGURE 8.   Tanglegram generated from TreeMap analysis for Gondwanan areas (left) and polystomes (right) when a basal position is assumed for Australia and an intermediate one for India. Parasites are linked to their geographical area by lines. For abbreviations in brackets, refer to Figure 5. This figure is available in black and white in print and in color at *Systematic Biology* online.

Historical Biogeography of Neobatrachian Polystomes

According to plate tectonic and reticulate relationships among areas, DIVA and TreeMap analyses show very similar results that indicate a Gondwanan origin for neobatrachian polystomes. Vicariance biogeography would be at the origin of the earliest divergences, and following subsequent continental drifting and colliding, terrestrial dispersal would have been possible in the new areas. Because Australia could have been isolated during the first stages of Gondwana breakup from South America and Africa at a geological period where India was still bound to the western part of Gondwana, the ancestral "*Parapolystoma/Diplorchis*" lineage would have originated in Australia and the ancestral "*Eupolystoma/Polystoma/W. almae*" lineage in the block associating India, Africa, and South America. This hypothesis that implies one biogeographic vicariance for the ancestral stock of polystomes instead of extra dispersal from plate to plate fits well with an Africa-India origin proposed for advanced frogs (Zhang et al. 2005). It also concurs well with our molecular dating at approximately 194 Ma (Table 2), which corresponds to the period of intensive sea-floor spreading in the Somali, Mozambique, and Weddell Sea basins (Storey 1995) that could have isolated Australia from the western part of Gondwana (Rogenhagen and Jokat 2000). Next, the ancestral stock of the "*Eupolystoma/Polystoma* sensu stricto" lineage would have diverged from the ancestral

stock of rhacophorid polystomes by vicariance, the first clade evolving in the western part of Gondwana and the second in India. This second codivergence is dated about 177 Ma, which is approximately the period when eastern (i.e., India, Madagascar, Seychelles, Antarctica, and Australia) and western (i.e., South America and Africa) Gondwanan components were fully separated (Storey 1995; Chatterjee and Scotese 1999; Lawver et al. 1999; Briggs 2003). Finally, the ancestral distribution suggested for the *Polystoma* sensu stricto lineage, which is centred in the South American plate, is also in accordance with biogeographic vicariance. Following the separation of South America from Africa, the *Polystoma* sensu stricto and *Eupolystoma* lineages would have been isolated in South America and in Africa, respectively. This third codivergence is estimated about 156 Ma, which is more or less close to the separation of the South American and African plates (Storey 1995; Lawver et al. 1999; Macdonald et al. 2003).

According to this proposed scheme for polystome evolution, several dispersals would have occurred. (i) From Australia to Eurasia, when *Parapolystoma* and *Diplorchis* diverged. The *Parapolystoma–Diplorchis* divergence dated at about 106 Ma suggests that *Diplorchis* dispersal could have taken place at this period of time or more recently. Because Australia and Asia are separated by a network of deep ocean trenches, dispersal from one continent to another, even from step to
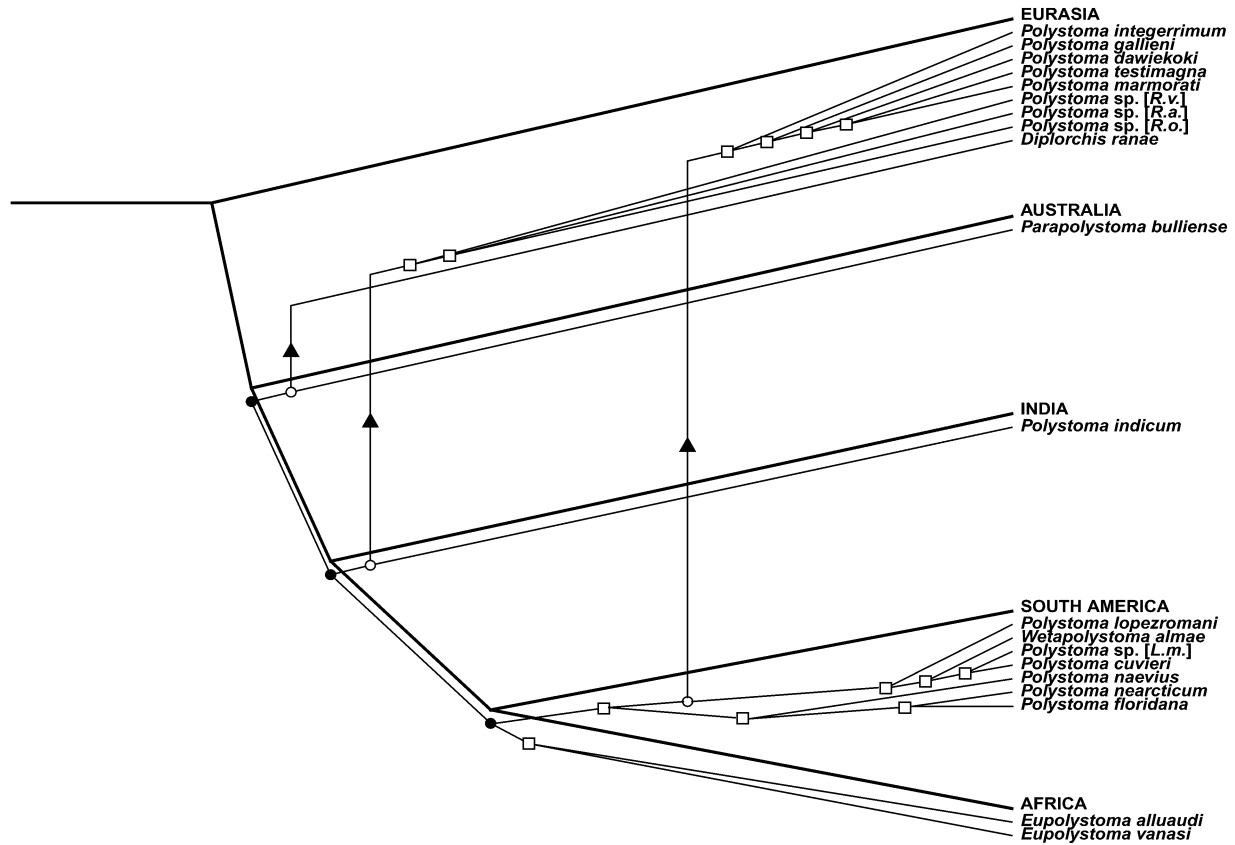
FIGURE 9.   One possible area-polystome historical reconstruction inferred from a TreeMap analysis on the Tanglegram shown in Figure 8. Key to symbols: open squares and circles refer to sympatric speciation, filled circles to biogeographic vicariance, and filled triangles to dispersal. For abbreviations in brackets, refer to Figure 5.

step, is not obvious even though it was illustrated for dispersal across the Wallace's and Huxley's lines for fanged frogs of the ranid *Limnonectes* (see Evans et al. 2003). On the other hand, to explain the distribution and phylogenetic relationships within microhylids, Van Bocxlaer et al. (2006) suggested a dispersal route from Australia to Eurasia following a Late Cretaceous connection of Indo-Madagascar and Australia-New Guinea with Southeast Asia. Accordingly, assuming polystome dispersal from Australia to Eurasia is not unrealistic. (ii) From India to Southeast Asia when Indian and Southeastern Asian rhacophorid polystomes diverged. This dichotomy, which is dated about 86 Ma at a period near the collision of India with Asia (Briggs 2003), allows ample time for terrestrial polystome invasions. Finally, (iii) from South America to "Eurasia" in a stepwise manner. It would have started approximately from 79 to 71 Ma, which corresponds with a period of intensive diversification of polystomes in South America. Following land connections between South and North America *via* a central corridor in the Palaeocene and between North America and Eurasia *via* Beringia in the same period of time, polystomes would have first dispersed to Eurasia and next to Africa (Fig. 5; Bentz et al. 2001, 2006).

In conclusion, the phylogenetic branching patterns and divergence time estimates presented here for polystomes support vicariant clades that probably arose during the breakup of Gondwana. Polystome lineages may therefore reflect rifting and drifting of ancient and present continents and coevolution between ancestral host lineages and their parasites.

Correlating Vicariance of Polystomatids to the Early Evolution of Frogs

Due to their direct life cycle which requires freshwater environments for egg laying, larval development, and infection process, polystome dispersal can only occur through amphibian dispersal. Hence, polystome vicariance necessarily implies host vicariance, a process which is similar to host–parasite codivergence following continental drift. One may question, therefore, which of the main stem lineages of the Neobatrachia carried polystomes to their definitive continents and subcontinents. When host and parasite phylogenetic branching patterns at lower levels are compared, some discrepancies are apparent. The Australian polystome lineage appears most basal in the parasite tree (Fig. 1), whereas the Australian frog subfamily Pelodryadinae from which *Parapolystoma bulliense* was recovered falls within the primarily South American hyloid lineage

TABLE 2. Molecular dating estimates (million years) for the numbered speciation events shown in Figure 1, with their standard deviation and 95% confidence intervals

	Procedure 1	Procedure 2	Procedure 3
Node 1	194 ± 31 (133, 257)	216 ± 32 (152, 278)	217 ± 31 (156, 278)
Node 2	177 ± 29 (120, 238)	200 ± 32 (137, 263)	197 ± 30 (137, 259)
Node 3	156 ± 28 (104, 213)	181 ± 32 (120, 245)	174 ± 29 (119, 234)
Node 4	79 ± 17 (49, 118)	104 ± 26 (58, 163)	87 ± 18 (55, 127)
Node 5	71 ± 15 (44, 106)	95 ± 25 (52, 152)	78 ± 16 (49, 115)
Node 6	106 ± 31 (53, 174)	123 ± 35 (62, 199)	119 ± 34 (61, 191)
Node 7	86 ± 27 (38, 146)	107 ± 34 (47, 178)	97 ± 30 (42, 162)

Procedure 1 fixed two calibration points onto the global phylogenetic tree, whereas procedures 2 and 3 reestimated the divergence times with only one of the two calibration points.

(Fig. 2; San Mauro et al. 2005; Frost et al. 2006; Roelants et al. 2007; Wiens 2007). According to vicariance biogeographic evidence and molecular dating of polystomes, it seems very unlikely that pelodryadine hylids coevolved with *Parapolystoma*. Therefore, occurrence of the basal Australian polystome lineage within frogs of South American origin likely indicates ancestral host switching. It would have occurred either from uninvestigated extant Australian host taxa (see below) or extinct host species to pelodryadine frogs when the latter colonized Australia *via* an archipelago that is supposed to have connected the South America and Antarctica/Australia supercontinent (see Ruvinsky and Maxson 1996). If extant Australian myobatrachids are those hosts, they should be basal within the neobatrachian tree, but instead, they seem to be related to South American hyloids (Roelants et al. 2007; Wiens 2007). However, the early history of the Neobatrachia is still regarded as a polytomy by some authors (San Mauro et al. 2005) and probably warrants a more in depth study. Moreover, though no polystome species have so far been described from myobatrachids, it must be remembered that almost all anurans in Australia, that is, myobatrachid, pelodryadine, and microhylid frogs, have not been investigated for polystomes. An alternative hypothesis to reconcile the deepest host and parasite branching patterns is to consider gaps within the Australian fossil record. Finding older Australian frog fossils might indeed challenge current hypotheses about the early neobatrachian evolution, as has been the case following the recent discovery of a Late Cretaceous fossil in Madagascar that showed strong affinities with South American hyloids (Evans et al. 2008). Finally, hylids and ranids have an almost worldwide distribution but with a single exception (i.e., *R. daemeli*), ranids are absent from Australia. On the other hand if the Pelodryadinae is endemic to the Australo-Papuan region, its nearest relative, that is the Phyllomedusinae (see Wiens et al. 2005; Wiens 2007), is distributed across South America. Thus, the ancestral host that disseminated Australian polystome species to Southeast Asia still remains in question. It could be microhylid frogs of the Asterophryinae that show strong phylogenetic affinities with Madagascan, Indian, and South–Eastern Dyscophinae and Microhylinae frog species.

Subsequently, the stem branch of the "Indian" frog lineage (i.e., the Nasikabatrachidae and Sooglossidae)

would have carried ancestral polystomes to the "supercontinent" Madagascar-Seychelles-India that was still joined to the Antarctican–Australian block. Polystomes would have secondarily switched to rhacophorid hosts when their distant ancestors invaded India, probably from Africa or Madagascar (see Bossuyt et al. 2006), and would have invaded Asia after India collided with Southeast Asia. Indeed, it was suggested that rhacophorine tree frogs dispersed out of India and subsequently radiated outward in Southeast Asia (Bossuyt and Milinkovitch 2001). It should be noted that this hypothesis concurs well with the molecular dating proposed for the origin of the "Sooglossidae/ Nasikabatrachidae lineage" (Biju and Bossuyt 2003) though the placement of the latter clade within the Neobatrachia is still disputed (Biju and Bossuyt 2003; San Mauro et al. 2005; Frost et al. 2006; Roelants et al. 2007; Wiens 2007). Thus, if our hypothesis about the earlier evolution of polystomes at the period of the breakup of Gondwana is correct, a survey of frogs of Myobatrachidae and Microhylidae in Australia, Nasikabatrachidae in India, and Sooglossidae in the Seychelles should reveal new polystome species intimately related to the Australian and Indian parasite lineages, respectively.

Despite good correspondence between the relationships of hyloid sensu stricto and ranoid frog lineages and the separation of South American and African plates, it is now considered that these two lineages have diverged about 150–160 Ma (Biju and Bossuyt 2003; San Mauro et al. 2005; Roelants et al. 2007), most likely *before* complete separation of the plates and not at the time of their separation about 130 Ma as was suggested by Feller and Hedges (1998). According to molecular dating, the *Polystoma* sensu stricto and *Eupolystoma* lineages would have codiverged with hyloid sensu stricto and ranoid frogs, respectively. *Eupolystoma* would have secondarily undergone extinction within ranoids and switched to bufonoids, at a period close to the separation of the South American and African plates. Following the complete separation of the two continents, the *Polystoma* sensu stricto and *Eupolystoma* lineages would have been isolated in South America and Africa, respectively. Ultimately, the period of diversification of polystomes in South America, dated approximately about 71–79 Ma, relates well to the presumed diversification of their ancestral-specific host

families (Estes and Reig 1973; Sanchiz and Rocek 1996). Indeed, studies of frog specimens close to extant genera from South America have extended fossil records of the Bufonidae, Hylidae, and Leptodactylidae in the Palaeocene of Brazil (Estes and Reig 1973). These last results corroborate our findings on the early historical biogeography of neobatrachian polystomes because once again, they demonstrate the link between host and parasite diversification. Furthermore, they validate a scheme of dispersal by amphibians from South America to North America in the Palaeocene, which would have brought polystomes from South America, *via* North America, to Eurasia and later to Africa (Fig. 5; Bentz et al. 2006).

CONCLUSIONS

The close correspondence between the early polystome dichotomies and plate tectonics, substantiated by molecular dating, illustrates vicariance events within flatworm parasites. This implies ancestral host–parasite codivergence following continental drift, although such processes have not been identified from cophylogenetic analyses that suggest numerous ancestral duplication and host-switching events. Several hypotheses may be developed to explain the incongruence between cophylogenetic and biogeographic analyses. (i) The parasite phylogeny is not accurate because of the "limited parasite data set". This has been well documented with the improvement of taxon sampling through phylogenetic studies (e.g., Heath et al. 2008a, 2008b). However, if this hypothesis is correct, the parasite branching patterns presented here would, by chance, reveal a strong relationship with the Earth's early history, which is very unlikely. (ii) There is a bias in our parasite sampling which would explain incongruent phylogenies between hosts and parasites at deeper levels. (iii) Codivergence events may have been hidden by subsequent ancestral parasite and/or host extinctions. Then the presence of polystome parasites in specific anuran host clades and in discrete geographic areas indicates the occurrence and radiation of amphibians over ancient and recent geological periods and thus provide promising biological tags for host evolutionary ecology in the Mesozoic period, especially when the fossil record is quite limited.

Regarding these hypotheses, a more thorough parasite sampling should help to propose a more robust polystome phylogeny. However, regarding biogeographic scenarios, one may expect to find new polystome species from frogs of the Myobatrachidae and Microhylidae in Australia, Nasikabatrachidae in India, and Sooglossidae in the Seychelles.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at http://www.sysbio.oxfordjournals.org/. Alignments and trees for both host and parasite can also be retrieved under Accession Number 11352 in TreeBASE (http://www.treebase.org/treebase-web/home.html).

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