ELSEVIER

International Journal for Parasitology 31 (2001) 697–705

INTERNATIONAL Journal for PARASITOLOGY

www.parasitology-online.com

# Origin and evolution of African *Polystoma* (Monogenea: Polystomatidae) assessed by molecular methods ☆

Sophie Bentz[a,*], Stéphanie Leroy[a], Louis du Preez[b], Jean Mariaux[c], Claude Vaucher[c], Olivier Verneau[a]

[a]*Centre de Biologie et d'Ecologie Tropicale et Méditerranéenne, UMR 5555 du CNRS, Université de Perpignan, Avenue de Villeneuve, F-66860 Perpignan Cedex, France*

[b]*School of Environmental Science and Development, Potchefstroom University, Private Bag X6001, Potchefstroom 2520, South Africa*

[c]*Museum of Natural History, P.O. Box 6434, 1211, Geneva 6, Switzerland*

Received 29 November 2000; received in revised form 15 February 2001; sccepted 15 February 2001

**Abstract**

Among Polystomatidae (Monogenea), the genus *Polystoma*, which mainly infests neobatrachian hosts, is the most diverse and occurs principally in Africa, from where half the species have been reported. Previous molecular phylogenetic studies have shown that this genus originated in South America, and later colonised Eurasia and Africa. No mention was made on dispersal corridors between Europe and Africa or of the origin of the African *Polystoma* radiation. Therefore, a molecular phylogeny was inferred from ITS1 sequences of 21 taxa comprising two species from America, seven representatives from Europe and 12 from Africa. The topology of the phylogenetic tree reveals that a single event of colonisation took place from Europe to Africa and that the putative host carrying along the ancestral polystome is to be found among ancestral pelobatids. Percentage divergences estimates suggest that some presumably distinct vesicular species in unrelated South African anurans and some neotenic forms found in several distinct hosts in Ivory Coast, could, in fact, belong to two single polystome species parasitising divergent hosts. Two main factors are identified that may explain the diversity of African polystomes: (i), we propose that following some degree of generalism, at least during the juvenile stages of both hosts and parasites, distinctive larval behaviour of polystomes engenders isolation between parasite populations that precludes sympatric speciations; (ii), cospeciation events between *Ptychadena* hosts and their parasites are another factor of diversification of *Polystoma* on the African continent. Finally, we discuss the systematic status of the Madagascan parasite *Metapolystoma*, as well as the colonisation of Madagascar by the host *Ptychadena mascareniensis*. 

*Keywords*: Polystomatidae; *Polystoma*; Amphibia; Phylogeny; ITS1; Africa

## 1. Introduction

Polystomatids are endoparasitic in aquatic tetrapods, and the majority of them are found in anurans and fresh water turtles. Their global distribution, low mobility of their hosts, biological life-cycle of the anuran parasites, involving gill cycles on tadpoles (fish-like) and vesicular cycles on adults, as well as the almost strict host-specificity of the parasites provide clues to the antiquity of the family (Tinsley, 1981; Prudhoe and Bray, 1982; Batchvarov et al., 1995). So far, 19 extant genera have been recognised within the Polystomatidae, if *Sphyranura* is included as suggested by Sinnappah et al. (2001), representing about 200 species. Among the Polystomatidae, the widespread genus *Polystoma* is found mostly on neobatrachians, principally in Africa where it is most diverse. Prudhoe and Bray (1982) suggested that the genus originated during the Early Cretaceous, i.e. some 140 Myr ago, and that isolation following continental fragmentation and drifting had been the major event in the subsequent evolution of *Polystoma*. By contrast, biogeographical interpretations inferred from molecular phylogenies suggest that *Polystoma* originated in South America and subsequently colonised North America, Europe and Africa (Sinnapah N.D., Phylogeography of Monogenean Polystomatidae; A molecular approach to infer the evolutionary history of this group of parasites, thesis 1998, University

☆ Note: Nucleotide sequence data reported in this paper are available in the EMBL databases under the accession numbers AJ301685, AJ301686, AJ301687, AJ301688, AJ301689, AJ301692, AJ301693, AJ310396, AJ310397, AJ310398, AJ310399, AJ310400, AJ310401, AJ310402, AJ310403, AJ310404, AJ310405, AJ310406, AJ310407, AJ310408, AJ310409, AJ310410, AJ310411.

* Corresponding author. Tel.: +33-4-6866-2050; fax: +33-4-6866-2281.
*E-mail address:* bentz@univ-perp.fr (S. Bentz).

0020-7519/01/$20.00 
PII: S0020-7519(01)00179-5

of Perpignan). The monophyly of African *Polystoma* species with respect to the paraphyly of American and European taxa suggested that Africa was the final continent invaded, though only few representatives were included in the analysis (Sinnapah N.D., thesis 1998). Concerning the diversity of African *Polystoma*, Tinsley (1974) pointed out that there was a need for a systematic revision of some presumably distinct species, because of the difficulty in identifying species without knowing both host and locality. He was followed by Murith (1981a) who defined several groups of related parasite species. However, species included in these groups are to date not considered as conspecific. The last polystome to be described from Africa was *Polystoma claudecombesi* by Du Preez and Kok (1995). It represented the 32nd described species from this continent, while a dozen presumably distinct species remain unnamed. Africa, then, seems to have the greatest diversity of *Polystoma* compared with Eurasia and America, although this could be an overestimate. Moreover, the concept of host-specificity of African *Polystoma* is contentious (Prudhoe and Bray, 1982; Batchvarov et al., 1995). Experimental infections carried out with natural and substitute hosts (Bourgat and Salami-Cadoux, 1976; Du Preez and Kok, 1993, 1997; Du Preez et al., 1997) did not produce general conclusions.

A molecular phylogeny, inferred from ITS1 sequences, assessed two major points: what were the colonisation routes of polystomes from Europe to Africa and which hosts were involved? What are the factors which led to the diversification of *Polystoma* on the African continent? The latter point is discussed with special emphasis on both systematics and host-specificity.

## 2. Materials and methods

### 2.1. Sampling

Twenty-three taxa were included in the analysis. Host and parasite species were carefully examined by the different people who collected the specimens on the field. Some of them are unidentified species and some are individuals of the same species taken from the same host species from different geographical areas. Species names when known, authorities, host species and family and country of sampling are listed in Table 1

### 2.2. DNA sequencing

DNA extractions from tissues preserved in 95% ethanol were performed using a standard procedure seen in Hillis et

Table 1
Taxonomic samples with indication of authorities, hosts species and family and country of sampling

| *Polystoma* species[a] | Authorities | Host species | Family | Country |
|---|---|---|---|---|
| *Polystoma marmorati*[b] | van Niekerk et al., 1993 | *Hyperolius m. marmoratus* | Hyperolidae | South Africa |
| *Polystoma australis*[b] | Kok and van Wyk, 1986 | *Semnodactylus wealii* | Hyperolidae | South Africa |
| *Polystoma umthakathi*[b] | Kok and Seaman, 1987 | *Natalobatrachus bonebergi* | Ranidae | South Africa |
| *Polystoma testimagna*[b] | Du Preez and Kok, 1993 | *Strongylopus f. fasciatus* | Ranidae | South Africa |
| *Polystoma* sp. (1)[b] | | *Cacosternum nanum* | Ranidae | South Africa |
| *Metapolystoma brygoonis*[b] | Euzet and Combes, 1964 | *Ptychadena mascareniensis* | Ranidae | Madagascar |
| *Polystoma* sp. (2)[c] | | *Hemisus marmoratus* | Ranidae | Ivory Coast |
| *Polystoma* sp. (3)[c] | | *Ptychadena maccarthiensis* | Ranidae | Ivory Coast |
| *Polystoma baeri*[c] | Maeder et al., 1970 | *Ptychadena maccarthiensis* | Ranidae | Ivory Coast |
| *Polystoma mangenoti*[c] | Gallien, 1956 | *Ptychadena superciliaris* | Ranidae | Ivory Coast |
| *Polystoma* sp. (4)[b] | | *Ptychadena anchietae* | Ranidae | Tanzania |
| *Polystoma* sp. (5)[b] | | *Ptychadena anchietae* | Ranidae | South Africa |
| *Polystoma integerrimum*[b] | Froelich, 1791 | *Rana temporaria* | Ranidae | France GB[d] |
| *Polystoma integerrimum*[b] | Froëlich, 1791 | *Rana temporaria* | Ranidae | France DR[d] |
| *Polystoma integerrimum*[b] | Froëlich, 1791 | *Rana temporaria* | Ranidae | France PT[d] |
| *Polystoma combesi*[b] | Batchvarov, 1982 | *Rana graeca* | Ranidae | Bulgaria |
| *Polystoma* sp. (6)[b] | | *Rana chensinensis* | Ranidae | Russia |
| *Polystoma* sp. (7)[b] | | *Bufo gargarizans* | Bufonidae | Russia |
| *Polystoma gallieni*[b] | Gallien, 1947 | *Hyla meridionalis* | Hylidae | France |
| *Polystoma fuscus*[b] | Biserkov and Hadjinikolova, 1993 | *Pelobates fuscus* | Pelobatidae | Bulgaria |
| *Polystoma pelobatis*[c] | Euzet and Combes, 1966 | *Pelobates cultripes* | Pelobatidae | France |
| *Wetapolystoma almae*[b] | Gray, 1993 | *Bufo typhonius* | Bufonidae | French Guyana |
| *Polystoma nearcticum*[b] | Paul, 1938 | *Hyla versicolor* | Hylidae | USA |

[a] Vouchers numbers for *Polystoma* sp. (2): 30763, 30764, 30765–30775INVE; *Polystoma* sp. (3): 30777INVE; *Polystoma baeri*: 30778–30780; *Polystoma mangenoti*: 30781INVE; *Polystoma* sp. (4): 30776INVE (Museum of Natural History of Geneva). *Polystoma australis*, *Polystoma* sp. (1) and *Polystoma testimagna* are available for studies in Potchefsroom University, South Africa; *Polystoma nearcticum* in Bristol University, UK, and all others except *Polystoma* sp. (6) and *Poystoma* sp. (7) are available at the University of Perpignan, France. *Poystoma* sp. (6) and *Poystoma* sp. (7) are currently not available.
[b] Vesicular form.
[c] Branchial form.
[d] GB, DR and PT refer to different areas in France: Verrière du Gros Bois (Jura), Drambon (Jura) and Porté (Pyrénées Orientales), respectively.

al. (1996). Terminal 18S rDNA plus the complete ITS1 region were PCR-amplified for most of the samples in one round using different combinations of primers S1-H7 or L7-H7 as reported in Verneau et al. (1997) and Sinnappah et al. (2001). ITS1 for four other polystomes (*Polystoma baeri*, *Polystoma* sp. (3), *Polystoma* sp. (4) and *Polystoma mangenoti*) was amplified in two overlapping fragments (IF6-IR7 + IF4-H7). Thus, the partial 18S rDNA was not included in our analysis for two reasons: (i), we did not get its sequence for the four latter taxa; and (ii), it did not provide much information for the group of interest due to the very low variability it exhibits. Purified PCR products were cloned into pGEM-T vector (Promega) using *Escherichia coli* JM109 supercompetent cells for transformation (Promega). Where possible, three clones were manually sequenced following the T7 DNA polymerase protocol (Pharmacia) using $^{35}$S-dATP. Sequencing was performed with the universal plasmid SP6 and T7 primers, as well as internal primers (IF6, IF4, IR7 and IR4). All sequences were obtained for both strands and the consensus sequences reported.

Fig. 1 shows the position of primers used both for PCR and sequencing. IF4 (5′-GGG CAA GGC GTA AAG AAG CT-3′), IR7 (5′-ATG CAA AAT GGT AGA GCT AAC-3′), IR4 (5′-GGT ACA GGA ACC GGA ATG AG-3′) and IF6 (5′-CCA AAC TTG ATC ATT TAG AGG-3′) have been defined for this study.

### 2.3. Sequence analysis

Sequences were aligned by eye using ED editor (MUST package; Philippe, 1993). When necessary, blocks of gaps were introduced to optimise alignment, but indels as well as undetermined sites were removed for analysis. The alignment is available at EMBL under the accession number: ALIGN-000042. Gene heterogeneity was estimated with the $\alpha$ parameter worked out in the program Puzzle (Strimmer and von Haeseler, 1996) and pairwise percentage differences of transitions were plotted against pairwise percentage differences of transversions to evaluate the degree of substitutional saturation.

### 2.4. Phylogenetic reconstruction

Phylogenetic analysis based on neighbour-joining (NJ; Saitou and Nei, 1987) and maximum-parsimony (MP) methods comprised all the taxa but *Polystoma integerrimum* sampled in Porté and Verrières du Gros-Bois (France). Trees were rooted with two American representatives, namely *Wetapolystoma almae* and *Polystoma nearcticum*, as an unpublished analysis showed them to be basal with respect to the Eurafrican clade.

The NJ method was applied to the distance of Tajima–Nei (Tajima and Nei, 1984) extracted from MEGA (Kumar et al., 1993). The robustness of nodes was estimated by bootstrap proportions (BP; Felsenstein, 1985) after 1000 replicates with the program MEGA. Additionally, a distance matrix (Table 2) was worked out for the 23 taxa.

MP analyses were performed with PAUP version 4.0b4a (Swofford, 1998) using a heuristic search with random addition of taxa (10 replicates), tree bisection reconnexion (TBR) branch-swapping, and MULPARS options. Only equally weighted informative sites were considered. The robustness of nodes was assessed with BP (1000 replicates with single addition of taxa).

## 3. Results

### 3.1. Analysis of ITS1 sequences

The alignment of ITS1 sequences for 21 taxa represents 933 positions, among which 513 were retained for analysis after removal of indels. There was a bias in nucleotide frequencies, with C and G exhibiting a deficit (Pi[A] = 25.6, Pi[C] = 17.5, Pi[G] = 20.4, Pi[T] = 36.5) and $\alpha = 2.30$, thus approaching the case where a uniform rate of substitution along the sequence can be stated. The estimated transition/transversion ratio was equal to 1.6.

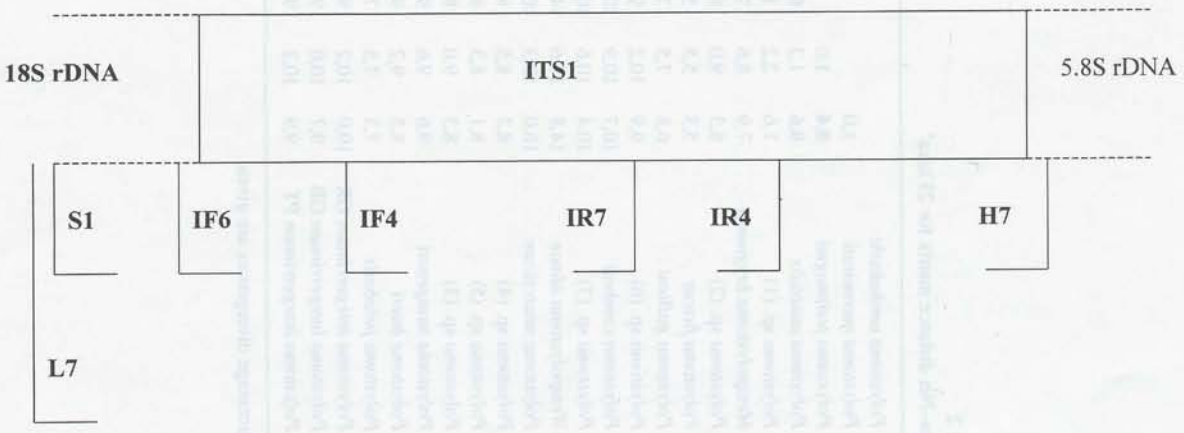


Fig. 1. Primers used for PCR and sequencing. IF, internal forward; IR, internal reverse.

Table 2
Tajima–Nei distance matrix for 23 taxa[a]

| | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *Polystoma umthakathi* | | | | | | | | | | | | | | | | | | | | | |
| 2 | *Polystoma marmorati* | 1,0 | | | | | | | | | | | | | | | | | | | | |
| 3 | *Polystoma testimagna* | **0,4** | 1,0 | | | | | | | | | | | | | | | | | | | |
| 4 | *Polystoma australis* | **0,6** | 1,2 | **0,6** | | | | | | | | | | | | | | | | | | |
| 5 | *Polystoma* sp. (1) | 1,6 | 2,2 | 1,6 | 1,8 | | | | | | | | | | | | | | | | | |
| 6 | *Metapolystoma brygoonis* | 7,9 | 8,6 | 7,9 | 7,9 | 9,0 | | | | | | | | | | | | | | | | |
| 7 | *Polystoma* sp. (2) | 8,3 | 9,0 | 8,8 | 9,0 | 9,4 | 8,8 | | | | | | | | | | | | | | | |
| 8 | *Polystoma fuscus* | 5,3 | 5,5 | 5,3 | 5,1 | 6,4 | 8,1 | 8,8 | | | | | | | | | | | | | | |
| 9 | *Polystoma gallieni* | 6,8 | 7,5 | 7,3 | 7,5 | 7,9 | 10,2 | 9,5 | 4,9 | | | | | | | | | | | | | |
| 10 | *Polystoma* sp. (6) | 9,9 | 10,2 | 9,9 | 9,9 | 11,1 | 12,1 | 11,8 | 8,2 | 8,9 | | | | | | | | | | | | |
| 11 | *Polystoma combesi* | 10,7 | 10,9 | 10,7 | 10,7 | 11,6 | 13,3 | 12,5 | 8,9 | 8,2 | 4,1 | | | | | | | | | | | |
| 12 | *Polystoma* sp. (7) | 10,4 | 10,6 | 10,4 | 10,4 | 11,5 | 12,5 | 12,2 | 8,6 | 9,3 | **0,4** | 4,5 | | | | | | | | | | |
| 13 | *Wetapolystoma almae* | 14,8 | 15,6 | 14,8 | 14,8 | 15,5 | 16,8 | 17,3 | 14,3 | 15,6 | 14,7 | 15,8 | 15,2 | | | | | | | | | |
| 14 | *Polystoma nearcticum* | 16,0 | 17,3 | 16,5 | 16,3 | 17,5 | 19,4 | 20,6 | 16,8 | 17,3 | 16,0 | 16,2 | 16,4 | 15,0 | | | | | | | | |
| 15 | *Polystoma* sp. (4) | 8,3 | 8,5 | 8,3 | 8,5 | 9,0 | 8,6 | 3,8 | 8,3 | 9,5 | 11,7 | 12,5 | 12,2 | 17,5 | 20,8 | | | | | | | |
| 16 | *Polystoma* sp. (5) | 8,1 | 8,3 | 8,1 | 8,3 | 8,7 | 8,4 | 3,6 | 8,1 | 9,2 | 11,5 | 12,5 | 12,0 | 17,0 | 20,8 | **0,8** | | | | | | |
| 17 | *Polystoma* sp. (3) | 8,3 | 9,0 | 8,8 | 9,0 | 9,4 | 8,8 | **0,4** | 8,8 | 9,5 | 11,8 | 12,5 | 12,2 | 17,3 | 20,6 | 3,8 | 3,6 | | | | | |
| 18 | *Polystoma mangenoti* | 9,6 | 9,6 | 9,6 | 9,9 | 10,3 | 10,6 | 4,7 | 10,3 | 11,5 | 13,6 | 14,6 | 14,1 | 19,5 | 23,3 | 4,6 | 4,0 | 4,7 | | | | |
| 19 | *Polystoma baeri* | 8,5 | 9,2 | 9,0 | 9,2 | 9,7 | 9,0 | **0,6** | 9,0 | 9,7 | 12,0 | 12,7 | 12,4 | 17,3 | 20,6 | 4,0 | 3,8 | **0,6** | 4,9 | | | |
| 20 | *Polystoma pelobatis* | 7,3 | 7,5 | 7,3 | 7,5 | 8,2 | 9,8 | 10,0 | 4,7 | 6,4 | 9,1 | 9,8 | 9,5 | 13,8 | 18,1 | 9,5 | 9,3 | 10,0 | 11,6 | 10,2 | | |
| 21 | *Polystoma integerrimum* GR | 10,0 | 10,2 | 9,9 | 10,0 | 10,8 | 12,3 | 11,5 | 8,2 | 7,9 | 3,8 | 1,8 | 4,3 | 15,8 | 15,7 | 11,8 | 11,8 | 11,5 | 13,9 | 11,7 | 9,1 | |
| 22 | *Polystoma integerrimum* GB | 9,7 | 10,0 | 9,7 | 9,7 | 10,6 | 12,3 | 11,5 | 8,0 | 7,7 | 3,6 | 1,6 | 4,0 | 15,5 | 15,4 | 11,5 | 11,5 | 11,5 | 13,6 | 11,7 | 8,9 | **0,2** |
| 23 | *Polystoma integerrimum* PT | 9,9 | 10,2 | 9,9 | 9,9 | 10,8 | 12,5 | 11,7 | 8,2 | 7,9 | 3,4 | 1,4 | 3,8 | 15,2 | 16,0 | 11,7 | 11,7 | 11,7 | 13,8 | 12,0 | 9,1 | **0,8** |

[a] Percentage divergences are given.

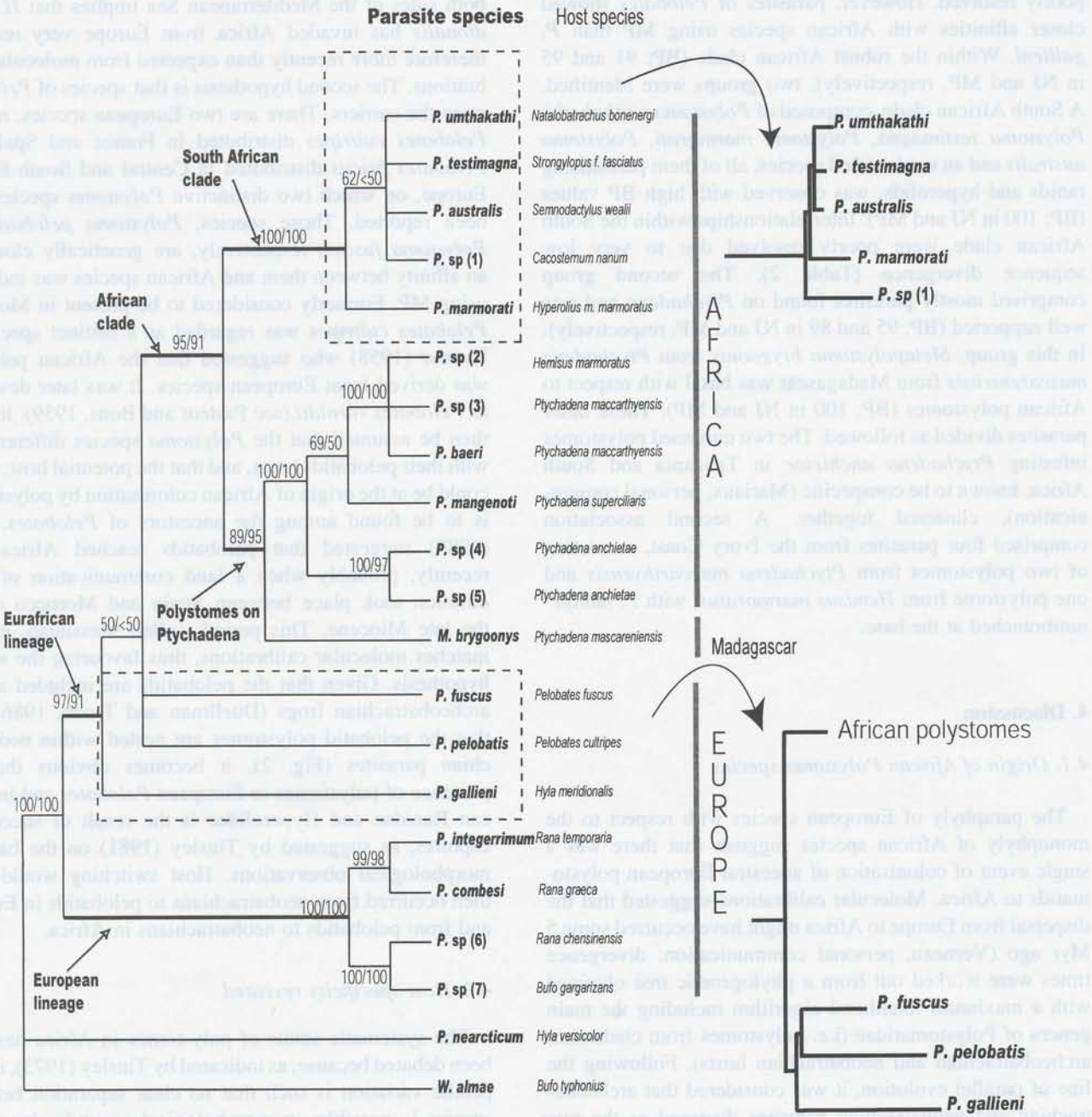


Fig. 2. Maximum-parsimony 50% majority-rule bootstrap consensus tree. NJ tree details are reported on the right side. Bootstrap values are indicated on the MP tree: MP bootstrap/NJ bootstrap.

The plot of transition percentages against transversion percentages was linear and no saturation bias was observed.

### 3.2. Phylogenetic reconstructions

No major discrepancies between the two methods of reconstruction were observed. Using MP, three shortest trees were retained (tree length: 194) with a consistency index of 0.696. The results are presented in Fig. 2. We observed two distinct lineages. One is strictly European and includes *Polystoma* of Ranidae and Bufonidae. It is assessed by high BP values in NJ and MP. The second comprises European *Polystoma* species of Hylidae and Pelobatidae that are basal with respect to an African clade. This Eurafrican clade was robust whichever the method was used (BP: 91 and 97 in NJ and MP, respectively). Interrelationships within the three European polystomes, *Polystoma pelobatis*, *Polystoma fuscus* and *Polystoma gallieni*, at the base of the African clade are

poorly resolved. However, parasites of *Pelobates* showed closer affinities with African species using MP than *P. gallieni*. Within the robust African clade (BP: 91 and 95 in NJ and MP, respectively), two groups were identified. A South African clade, composed of *Polystoma umthakathi*, *Polystoma testimagna*, *Polystoma marmorati*, *Polystoma australis* and an unidentified species, all of them parasitising ranids and hyperolids, was observed with high BP values (BP: 100 in NJ and MP). Interrelationships within the South African clade were poorly resolved due to very low sequence divergence (Table 2). The second group comprised mostly parasites found on *Ptychadena* and was well supported (BP: 95 and 89 in NJ and MP, respectively). In this group, *Metapolystoma brygoonis* from *Ptychadena mascareniensis* from Madagascar was basal with respect to African polystomes (BP: 100 in NJ and MP). These latter parasites divided as followed. The two unnamed polystomes infesting *Ptychadena anchietae* in Tanzania and South Africa, known to be conspecific (Mariaux, personal communication), clustered together. A second association comprised four parasites from the Ivory Coast, consisting of two polystomes from *Ptychadena maccarthyensis* and one polystome from *Hemisus marmoratus*, with *P. mangenoti*branched at the base.

## 4. Discussion

### *4.1. Origin of African Polystoma species*

The paraphyly of European species with respect to the monophyly of African species suggests that there was a single event of colonisation of ancestral European polystomatids to Africa. Molecular calibrations suggested that the dispersal from Europe to Africa might have occurred some 5 Myr ago (Verneau, personal communication: divergence times were worked out from a phylogenetic tree obtained with a maximum likelihood algorithm including the main genera of Polystomatidae (i.e. polystomes from chelonian, archeobatrachian and neobatrachian hosts). Following the line of parallel evolution, it was considered that archeobatrachian and neobatrachian parasites diverged as the two anuran lineages split. The calibration point was then assumed to be 180 Myr. Similar evolutionary rates among neobatrachian parasites allowed taking the average branch length to work out an age estimation), thus much more recently than previously thought (Prudhoe and Bray, 1982; Batchvarov et al., 1995). Furthermore, the potential hosts which might have brought the first *Polystoma* to the African continent could be either *Hyla* or some species of *Pelobates*. Indeed, parasite representatives of these genera are found at the base of the African clade, but no clear pattern is observed. *Polystoma gallieni* has been reported both from Western Europe (Gallien, 1947) and Morocco (Euzet and Combes, 1975) from *Hyla meridionalis*. Assuming that the same host species harbours the same polystome species on both sides of the Mediterranean Sea implies that *H. meridionalis* has invaded Africa from Europe very recently, therefore more recently than expected from molecular calibrations. The second hypothesis is that species of *Pelobates* were the carriers. There are two European species, namely *Pelobates cultripes* distributed in France and Spain and *Pelobates fuscus* distributed in Central and South-Eastern Europe, on which two distinctive *Polystoma* species have been reported. Those species, *Polystoma pelobatis* and *Polystoma fuscus*, respectively, are genetically close and an affinity between them and African species was indicated using MP. Formerly considered to be present in Morocco, *Pelobates cultripes* was regarded as a distinct species by Pasteur (1958) who suggested that the African pelobatid was derived from European species. It was later described as *Pelobates varaldii* (see Pasteur and Bons, 1959). It could then be assumed that the *Polystoma* species differentiated with their pelobatids hosts, and that the potential host, which could be at the origin of African colonisation by polystomes, is to be found among the ancestors of *Pelobates*. Rage (1988) suggested that pelobatids reached Africa only recently, probably when a land communication of short duration took place between Spain and Morocco during the late Miocene. This period, called Messinian, exactly matches molecular calibrations, thus favouring the second hypothesis. Given that the pelobatids are included among archeobatrachian frogs (Duellman and Trueb, 1986), and that the pelobatid polystomes are nested within neobatrachian parasites (Fig. 2), it becomes obvious that the presence of polystomes in European *Pelobates* and in African Ranidae and Hyperolidae is the result of successive captures, as suggested by Tinsley (1981) on the basis of morphological observations. Host switching would have then occurred from neobatrachians to pelobatids in Europe, and from pelobatids to neobatrachians in Africa.

### *4.2. Host-specificity revisited*

The systematic status of polystomes in Africa has long been debated because, as indicated by Tinsley (1973), intraspecific variation is such that no clear separation between species is possible on morphological grounds alone. The first attempt to clarify *Polystoma* systematics was the proposal of an *africanum* species complex grouping all *Polystoma* species which could not be identified without knowing both hosts and localities (Tinsley, 1974). This concept was subsequently modified by Murith (1981a) with the addition of a *togoensis* species complex. The need for revision is necessary to tackle questions of host-specificity.

ITS1 sequences of three individuals of *P. integerrimum* sampled from *Rana temporaria* from separate areas in France (see Table 1) and two individuals of undescribed species from *P. anchietae* in Tanzania and South Africa were compared to work out intraspecific divergence. The results tend to suggest that the threshold under which individuals can be considered conspecific is around 1% diver-

gence (Table 2). Given this, the South African *P. australis*, *P. testimagna* and *P. umthakathi* from *Semnodactylus wealii, Strongylopus f. fasciatus* and *Natalobatrachus bonebergi*, respectively, could perhaps be considered as populations of the same species rather than distinct species. However, this needs to be studied in greater depth. Furthermore, no clear relationships can be defined among these three taxa whichever phylogenetic method was used, whereas *P. marmorati* falls outside this cluster (Fig. 2). Even though some morphometric differences were reported as diagnostic in distinguishing parasite species (Du Preez and Kok, 1993, 1997), *P. australis* can develop in *N. bonebergi*, the natural host of *P. umthakathi*, and vice versa, but can not infest *Hyperolius marmoratus*, the host for *P. marmorati* (see Du Preez and Kok, 1997). In addition, it was reported that *P. testimagna* could not develop in *H. marmoratus* (see Du Preez and Kok, 1993). Rather than postulating that polystomes do not exhibit the same degree of host-specificity (Du Preez and Kok, 1997), it could be suggested that some polystomes are generalists rather than specialists. The difference in host ecology (reproduction period, habitat, tadpole longevity) and physiology might then result in isolation (temporal and/or behavioural) and precludes the speciation of polystomes. Du Preez et al. (1997) suggested that the natural host was recognised at first contact between oncomiracidium and tadpole. These results, based on behavioural observations of *P. umthakathi*, *P. australis* and *P. marmorati* larvae, contrast with our results unless we suggest that *P. umthakathi* and *P. australis* are undergoing speciation and that oncomiracidium behaviour is the first process involved in speciation.

There is another group of three parasites, the two forms from *Ptychadena maccarthiensis*, i.e. *P. baeri* and an undetermined species, and the neotenic form found on *H. marmoratus*, in which pairwise percentage divergences between species are below the threshold of 1% divergence. Murith (1981a) suggested from both striking morphological similarities between the parasite found on *Hemisus* tadpoles and neotenic forms of *P. baeri* and *P. mangenoti*, and from the occurrence of several distinct polystome species on gills of a single species of tadpole (Murith, 1979), that gill forms might develop on non-specific tadpoles while vesicular forms exhibited a strict host-specificity. Bourgat and Salami-Cadoux (1976) also suggested this on the basis of experimental observations. Here, we confirm that a single polystome species can inhabit different host species, at least during the juvenile period of the host. Since vesicular parasites were never found on *H. marmoratus*, we suggest that either it is due to sampling bias, or that the phenomenon we observed in the Ivory Coast is the first stage in the capture process, the second being the process discussed above with parasites being able to conduct vesicular cycles on two distinct hosts species. The degree of generalism of polystome relative to distinct amphibian host species, which could be generated through neotenic forms to maintain parasitism, would enhance lateral transfer events. The isolation within non-closely related hosts would then generate speciation. This hypothesis would reconcile both behavioural and experimental observations on polystomes and molecular phylogenetic analysis.

### 4.3. Evolution of Polystoma species on Ptychadena hosts

Diversification of African polystomes might also be related to host diversification by parallel evolution. Our phylogeny shows a robust association of parasites that were found in *Ptychadena* host species from the Ivory Coast, Tanzania, South Africa and Madagascar. *Ptychadena* has been cited as the most suitable host for polystomes because it fulfils the ecological and physiological requirements for completion of the polystomatid life-cycle (Du Preez and Kok, 1992a). Moreover, radiation of the genus *Polystoma* could mirror the evolution of the genus *Ptychadena*, which, according to Perret (1979), is undergoing an explosive radiation. The fact that the systematic status of some of these frogs is not clarified, for example *P. anchietae* has been synonymised with *Ptychadena superciliaris*, the host of two unnamed polystomes and *Polystoma mangenoti*, respectively, might illustrate very recent divergence and thus testify to the incipient phase of polystomes evolution. The three individuals of *Polystoma integerrimum* from France exhibit some level of genetic divergence with respect to each other, the two representatives collected in Jura (East of France) being more closely related than they are from the form of the Pyrénées (South of France; Table 2). This is probably due to the separation of *Rana temporaria* populations on isolated mountainous countries. The Tanzanian polystome of *P. anchietae* also shows some divergence relative to the South African form. We thus suggest that geographical dispersal of hosts carrying along vesicular polystomes might play a major role in concomitant host and parasite speciation.

*Metapolystoma brygoonis*, discovered in the urinary bladder of *P. mascareniensis*, was first described as a species of *Polystoma* (Euzet and Combes, 1964), although it differs from all congeners in the presence of a posterior ovary and a long uterus. *Metapolystoma cachani* was also included in the genus *Polystoma* (Gallien, 1956). A new genus was later created by Combes (1976) to group those specimens sharing this type of female organ morphology. A third species, *Metapolystoma porosissima*, was later described and placed in the genus (Du Preez and Kok, 1992b). Given the placement of *M. brygoonis* in our tree (Fig. 2), we do not consider the genus *Metapolystoma* to be valid. A large uterus might be the result of adaptation of the parasite to the ecology of the host (Kok and Seaman, 1987; Murith, 1981b; Tinsley, 1983). Furthermore, Tinsley (1974) suggested that the uterine structure in *M. cachani* and *M. brygoonis* had been achieved convergently. Therefore, *Metapolystoma* would be based on homoplastic characters. However, this can only be validated by adding other members of *Metapolystoma*.

*Ptychadena mascareniensis* is found throughout Africa, South of the Sahara, in Madagascar, where it is the sole non-endemic frog, and neighbouring islands (Perret, 1966). This host has been cited to harbour, in addition to *M. brygoonis*, other species of *Polystoma*. *Polystoma africanum* was reported from Zaïre (Vercammen-Grandjean, 1960), Uganda and Kenya (Tinsley, 1974), *Polystoma aethiopiense* from Ethiopia (Mezcal, 1970) and *Polystoma togoensis* from Togo (Bourgat, 1977), Cameroon (Murith et al., 1978) and Ivory Coast (Murith, 1981b). Specificity, apparently operating below the species level of the host, might also reveal that the host itself is undergoing speciation. Indeed, *P. mascareniensis* was subdivided into two subspecies (Perret, 1979), and it was also claimed that the Madagascan species was not conspecific with African species (Glaw and Vences, 1994). Additional phylogenetic analysis, including additional polystomes from *P. mascareniensis*, would provide relevant information on host evolution, thus allowing an accurate estimate of the date of colonisation of Madagascar by the frog carrying this polystome along. To date, we can only suggest that the presence of both *Ptychadena* and its polystome on the island is a recent event, with a dating of less than 5 Myr.

## Acknowledgements

V. Biserkov, G. Batchvarov, F. Catzeflis, E. Faliex, R. Guyétant, E. Jobet, B. Lebedev, B. Sellin and R. Tinsley are sincerely thanked for their contribution in the tissue sampling. The 'Ministère de l'Enseignement Supérieur de la Recherche et de L'Innovation Technologique' in Abidjan allowed J.M., C.V. and O.V. to investigate polystomes in the Ivory Coast in the summer of 1998. These authors are particularly indebted to the 'Centre Suisse de Recherches Scientifiques d'Adiopodoumé' and the 'Station Biologique' in Lamto for facilities and co-operation during the field work. The collections of J.M. in Tanzania were supported by the Swiss National Science Foundation grant, 3100-055710.98. The authors are also very grateful to N.D. Sinnappah for sharing unpublished sequences and to two anonymous referees for their comments. This work was supported financially by a grant from Actions Concertées Coordonnées (ACC SV7: Systématique et Biodiversité).

## References

Batchvarov, G.K., 1982. *Polystoma combesi* n. sp. (Polystomatidae Gambl, 1896) a parasite on *Rana graeca* Boulenger, 1897, in Bulgaria. C.R. Acad. Bulg. Sci. 35, 249–251.

Batchvarov, G., Combes, C., Fournier, A., Guyétant, R., 1995. Les Polystomatidae (Monogènes parasites d'amphibiens) en Europe. Bull. Soc. Herp. Fr. 73–74, 60–63.

Biskerov, V.Y., Hadjinikolova, R.I., 1993. *Polystoma fuscus* n. sp. (Polystomatidae) from the common spadefoot *Pelobates fuscus* (Pelobatidae) in Bulgaria. Syst. Parasitol. 25, 145–151.

Bourgat, R., 1977. Etude comparative des Polystomes (Monogènes) de ranidés (Anoures) du Sud Togo, description de *Polystoma togoensis* n. sp. Bull. Mus. Natl. Hist. Nat. 312, 447–63.

Bourgat, R., Salami-Cadoux, M.L., 1976. Recherches expérimentales sur la spécificité parasitaire des Polystomes de *Bufo regularis*, *Rana galamensis* et *Hylarana albolabris* au Togo. Rev. Sci. Méd. Biol. Togo 1, 41–42.

Combes, C., 1976. World biogeography of polystomatid monogeneans. Proc. Inst. Biol. Pedol. Far. East. Sci. 34, 55–69.

Duellman, W.E., Trueb, L., 1986. Biology of Amphibians. McGraw–Hill, New York.

Du Preez, L.H., Kok, D.K., 1992a. Syntopic occurrence of new species of *Polystoma* and *Metapolystoma* (Monogenea: Polystomatidae) in *Ptychadena porosissima* in South Africa. Syst. Parasitol. 22, 141–50.

Du Preez, L.H., Kok, D.K., 1992b. The frog genus *Ptychadena* as host for polystomatid (Monogenea) parasites in Africa. J. Herpetol. Assoc. Afr. 40, 47–49.

Du Preez, L.H., Kok, D.J., 1993. Polystomatidae (Monogenea) of Anura in southern Africa: *Polystoma testimagna* n. sp. parasitic in *Strongylopus f. fasciatus* (Smith, 1849). Syst. Parasitol. 25, 213–9.

Du Preez, L.H., Kok, D.J., 1995. Polystomatidae (Monogenea) of southern African Anura: *Polystoma claudecombesi* n. sp. parasitic in *Rana angolensis* Bocage. Syst. Parasitol. 30, 223–31.

Du Preez, L.H., Kok, D.J., 1997. Supporting experimental evidence of host specificity among southern African polystomes (Polystomatidae: Monogenea). Parasitol. Res. 83, 558–62.

Du Preez, L.H., Kok, D.J., Seaman, M.T., 1997. Host recognition behaviour of polystome oncomiracidia (Polystomatidae: Monogenea) in contact with natural and substitute anuran hosts. Zool. Afr. 111, 47–55.

Euzet, L., Combes, C., 1964. Sur un Polystomatidae (Monogenea) récolté à Madagascar chez *Rana mascareniensis* Duméril et Bribon. Bull. Soc. Zool. Fr. 4, 392–401.

Euzet, L., Combes, C., 1966. *Polystoma integerrimum pelobatis* n. subsp. (Monogenea) parasite de *Pelobates cultripes* (Cuvier, 1829). Ann. Parasitol. 41, 109–18.

Euzet, L., Combes, C., 1975. Présence au Maroc de *Polystoma gallieni* Price, 1939 (Monogenea) chez *Hyla meridionalis* Boettger, 1874. Bull. Mus. Natl. Hist. Nat. 302, 655–7.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–91.

Froëlich von, J.A., 1791. Beitrage zur naturgeschichte der eingeweidewürmer. Naturfoscher, halle 25, 52–113.

Gallien, L., 1947. Description de *Polystoma gallieni* E.W. Price (Monogenea, Polystomatidae) parasite de *Hyla arborea* var. *meridionalis* Boettger. Ann. Parasitol. Hum. Comp. 22, 24–29.

Gallien, L., 1956. Deux espèces nouvelles de polystomes Africains. Bull. Soc. Zool. Fr. 5–6, 369–74.

Glaw, F., Vences, M., 1994. A Fieldguide to the Amphibians and Reptiles of Madagascar, 2nd Edition. Moos Druck, Leverkusen and Farbo, Köln, Germany.

Gray, M.E., 1993. *Wetapolystoma almae* n. gen., n. sp. (Monogenea: Polystomatidae) parasite of *Bufo typhonius* (Linnaeus, 1758) (Amphibia: Bufonidae) from tropical Peru. Trans. Kans Acad. Sci. 96, 181–85.

Hillis, D.M., Moritz, C., Mable, B.K., 1996. Molecular Systematics, 2nd Edition. Sinauer Associates, Sunderland, MA.

Kok, D.J., Seaman, M.T., 1987. Polystomatidae (Monogenea) parasitic in the anuran genus *Natalobatrachus* in South Africa. S. Afr. J. Zool. 22, 258–63.

Kumar, S., Tamura, K., Nei, M., 1993. MEGA: Molecular Evolutionary Genetics Analysis, Version 1.01, The Pennsylvania State University, University Park, PA 16802.

Maeder, A.-M., Euzet, L., Combes, C., 1970. Espèces nouvelles du genre *Polystoma* (Monogenea) en Afrique Occidentale. Z. Parasitenk. 35, 140–55.

Mezcal, F.H., 1970. Trematodes of anurans from Ethiopia. Arb. Univ. Bergen, Mat.-Nat. Vitensk. Ser. 1, 1–73.

Murith, D., 1979. Identité des larves de polystomes (Monogenea) parasites du tétard de *Dicroglossus occipitalis* (Günther) en Côte-d'Ivoire. Z. Parasitenkd. 59, 187–94.