Evolutionary diversity in polystomatids infecting tetraploid and octoploid *Xenopus* in East African highlands: biological and molecular evidence

J. A. JACKSON[1]* *and* R. C. TINSLEY[2]

[1] *School of Biology, University of Nottingham, Nottingham NG7 2RD, UK*
[2] *School of Biological Sciences, University of Bristol, Bristol BS8 1UG, UK*

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SUMMARY

Species of *Protopolystoma* are monogenean flukes that only infect allopolyploid hosts in the anuran genus *Xenopus*. Multivariate analyses of morphometric sclerite characters in the nominal species *Protopolystoma simplicis* suggest that morphologically distinguishable populations occur in the tetraploid host, *Xenopus laevis victorianus*, and in each of the octoploid hosts, *X. vestitus* and *X. wittei*. The species-level divergence of a lineage specific to *X. laevis* is supported by sequence variation in the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene. *Protopolystoma simplicis* from *X. laevis* is redesignated *P. microsclera* n. sp., with *P. simplicis* being retained for populations in octoploid hosts. This division is consistent with large differences in egg hatching schedule, fixed differences at the mannose-6-phosphate isomerase and fumarate hydratase loci, and host-specificity in experimental analyses. Although the respective *P. simplicis* populations in *X. vestitus* and *X. wittei* also show significant diversity in allozyme expression, morphometrics and egg hatching schedule, they are retained in the same species because their level of mitochondrial DNA divergence is similar to that found within other *Protopolystoma* species. The consequences of splitting *P. simplicis* for a recent interpretation of the origin of *Protopolystoma* faunas in octoploid *Xenopus* spp. is discussed.

Key words: allopolyploidy, host-specificity, resistance, susceptibility, vertebrate, monogenean, *Protopolystoma*, mitochondrial DNA, allozyme, morphometrics.

INTRODUCTION

There is some evidence to suggest that allopolyploid evolution in the anuran genus *Xenopus* has directed patterns of host resistance/susceptibility to parasites, including the polystomatid monogenean *Protopolystoma* (see Tinsley and Jackson, 1998*a*; Combes, 2001; Jackson and Tinsley, 2003). An improved picture of the biological diversity and host-specificity characteristics of *Protopolystoma* species in polyploid hosts may help understand this interesting host-parasite coevolutionary interaction. *Protopolystoma simplicis* was originally described from tetraploid *Xenopus laevis* subspecies, and the octoploids *X. vestitus* and *X. wittei* (the type-host) in East Africa (Tinsley and Jackson, 1998*b*) on the basis of morphology. In this description, it was noted that individuals in *X. laevis* were divergent, in some respects, from those in *X. wittei* and *X. vestitus*. On the other hand, there were substantial inter-population overlaps in the potentially diagnostic characters, and the respective hosts can sometimes occur in sympatry. Therefore, a conservative classification was initially adopted that included the parasites in different host populations in a single species (Tinsley and Jackson, 1998*b*). However, subsequent observations have continued to suggest that *P. simplicis* in *X. laevis* (this host also harbours another morphospecies, *Protopolystoma xenopodis*) show significant differences in their biological characteristics compared with *P. simplicis* in *X. wittei* and *X. vestitus*. An initial aim of the present study was to reassess the taxonomic status of these within-host populations, based on a larger morphometric data set. This was supported by an analysis of sequence variation in mitochondrial DNA (mtDNA), cross-infection experiments, assays of variation in egg hatching schedules, and a survey of protein expression (mannose-6-phosphate isomerase and fumarate hydratase) amongst populations. We then considered how the present results, and new molecular phylogenetic studies of *Xenopus* (see Evans *et al.* 2004, 2005), affect a recent hypothesis for the origin of *Protopolystoma* spp. faunas in octoploid *Xenopus* spp. (Tinsley and Jackson, 1998*a*; reviewed by Combes, 2001). This 1998 interpretation had assumed conspecificity between sympatric *P. simplicis*-like populations in *X. laevis*, *X. wittei* and *X. vestitus* (see Tinsley and Jackson, 1998*a*).

* Corresponding author: School of Biology, University of Nottingham, Nottingham NG7 2RD, UK. Tel: +44 115 9513188. Fax: +44 115 9513188. E-mail: Joseph.Jackson@Nottingham.ac.uk

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Table 1. Details of the parasite material studied

No.	Locality	Grid reference	Year	Host*	Morphological specimens (1998 taxon)	GenBank Accession numbers
L1	Kaimosi area, Kenya	—	1980	LV	5 *simplicis s.l.* 5 *xenopodis*	
L2	Kaimosi area, Kenya	+0·17° +34·93°	1982	LV	3 *simplicis s.l.* 1 *xenopodis*	
L3	Kaimosi area, Kenya	—	1980	LV	4 *simplicis s.l.* 16 *xenopodis*	
L4	Kaimosi area, Kenya	+0·13° +34·85°	1982	LV	10 *simplicis s.l.* 3 *xenopodis*	
L5	Kaimosi area, Kenya	+0·13° +34·87°	1982	LV	18 *simplicis s.l.*	
L6	Kaimosi area, Kenya	+0·12° +34·97°	1982	LV	4 *simplicis s.l.*	
L7	Tambach area, Kenya	+0·67° +35·58°	1982	LV	1 *simplicis s.l.*	
L8	Kapchorwa, Uganda	+1·3725° +34·4139°	2001	LV	1 *simplicis s.l.*	EF379997
L9	Mabira Forest, Uganda	+0·50° +33·00°	2001	LV	3 *simplicis s.l.*	EF379998
L10	Mukono, Uganda	+0·3633° +32·6967°	2000	LV	10 *simplicis s.l.* 1 *xenopodis*	EF379994–6 EF380004
L11	Masindi, Uganda	+1·6947° +31·4903°	2001	LV	11 *xenopodis*	EF380007–8
L12	Masindi, Uganda	+1·7219° +31·5472°	2001	LV	5 *simplicis s.l.*	
L13	Masindi, Uganda	+1·9705° +31·7059°	2001	LV	3 *xenopodis*	EF380005–6
L14	L. Chahafi, Uganda	−1·35° +29·78°	1996	LV	5 *simplicis s.l.* 12 *xenopodis*	
L15	Kabale-Kisoro, Uganda	−1·1906° +29·8462°	2001	W	1 *simplicis s.l.*	
L16	Kabale-Kisoro, Uganda	−1·1622° +29·8009°	2001	LV	1 *simplicis s.l.*	EF379999
L17	L. Bunyonyi, Uganda	−1·3521° +29·8891°	2001	W	6 *simplicis s.l.*	EF380000–1
L18	Echuya Forest, Uganda	−1·28° +29·82°	1996	W	2 *simplicis s.l.*	
L19	L. Mulehe, Uganda	−1·22° +29·72°	1996	V	35 *simplicis s.l.*	
L20	L. Mutanda, Uganda	−1·22° +29·71°	1996	V	23 *simplicis s.l.*	
L21	L. Mutanda, Uganda	−1·2156° +29·7073°	2001	V	24 *simplicis s.l.*	EF380002
L22	Kigali, Rwanda	−1·93° +30·07°	1991–2	LV	3 *simplicis s.l.* 4 *xenopodis*	
L23	Cyangugu, Rwanda	−2·56° +28·98°	1992	W	7 *simplicis s.l.* 31 *fissilis*	
L24	Bitale, Rwanda	-	1992	W	9 *simplicis s.l.*	

* LV = *Xenopus laevis victorianus*, V = *X. vestitus*, W = *X. wittei*.

MATERIALS AND METHODS

General

Details of hosts and localities are shown in Table 1. In sections of this paper preceding the taxonomic revision given below, parasite specimens falling within our previous concept of *P. simplicis* (see Tinsley and Jackson, 1998*b*) are referred to as *P. simplicis sensu lato* (*s.l.*).

Parasite material for morphological study

Some parasite specimens were dissected from hosts fixed and stored in 4% formal amphibian saline. Others were removed from freshly-killed hosts and fixed under moderate cover-slip pressure in 4% formal amphibian saline. Following storage (in 4% formalin), specimens were dissected with insect pins and level sclerite preparations made by flattening fragments of tissue in small drops of glycerol under cover-slip pressure. Genital spines were prepared as described by Tinsley and Jackson (1998*b*). Further specimens were measured live, or after freezing at −20 °C, under strong cover-slip pressure. Storage and fixation effects on sclerite measurements under these different conditions are negligible (Tinsley and Jackson, unpublished data). Eschewing the use of traditional whole-mounting techniques allowed a full set of accurate measurements for all of the smaller sclerites to be taken for each individual, thus enabling the inclusion of these characters in multivariate analyses. Meristic data on gut morphology (Tinsley and Jackson, 1998*b*) are not included in the present study, as most specimens were fixed within the host and thus preserved in irregular conformations, preventing accurate records.

Morphometric analysis

Sclerite measurements are modified from Tinsley and Jackson (1998*b*), with an additional measurement from the tip of the large hamulus point to the apex of the ventral root (Fig. 1). Measurements (μm) were taken with digital Vernier callipers from drawing-tube outlines made under the 40× (hamuli) or 100× (genital spines, marginal hooklets) objectives of a

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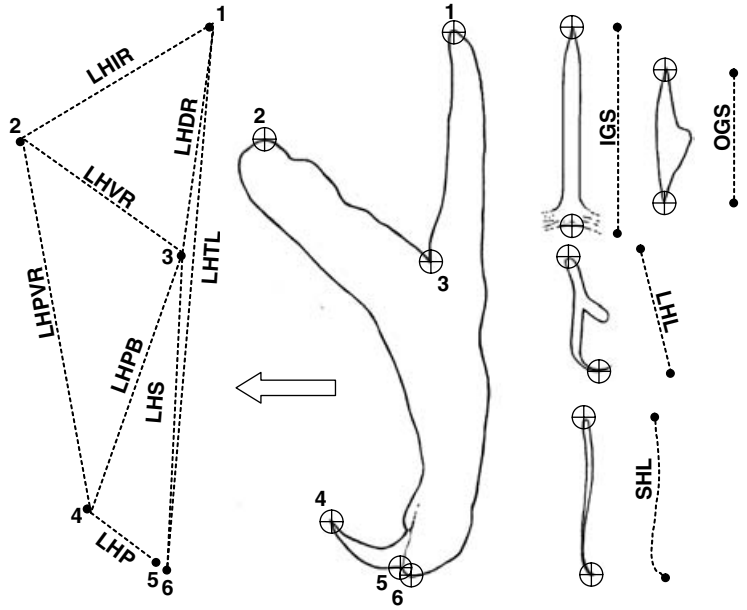

Fig. 1. Measurements (μm) taken on sclerites. LHTL, large hamulus total length; LHP, large hamulus point length; LHS, large hamulus shaft length; LHPB, distance between the point tip and the bifurcation in the large hamulus; LHDR, large hamulus dorsal root length; LHVR, large hamulus ventral root length; LHIR, distance between the root apices in the large hamulus; LHPVR, distance between the point tip and the ventral root apex in the large hamulus; SHL, small hamulus length; LHL, larval hooklet length; IGS, inner genital spine length; OGS, outer genital spine length.

compound light microscope (Nikon). The inclusion of measurements from all sclerite types (large hamulus + small hamulus + larval hooklets + genital spines) in our analyses (see above) allowed greater resolution of groupings than in previous multivariate analyses including only large hamulus sclerite measurements (Tinsley and Jackson, 1998*b*). Principal components analysis (PCA) on the correlation matrix of log-transformed characters was used as an ordination technique to assess the existence of data groupings and the contribution of different characters to these groupings (indicated by character coefficient loadings on individual principal components). Extracted components were retained for further analysis if their eigenvalue approached or exceeded 1 (Kaiser rule). Principal component score variables, excluding component 1 as this mainly reflected growth stage, were subjected to linear discriminant analysis (LDA) in order to objectively assess their ability to classify observations into putative groups. A cross-validation procedure was adopted to give a realistic misclassification rate: each observation was, in turn, removed from the LDA and classified on the basis of all others. PCA and LDA were carried out using the statistical package Minitab 14.2. A problem with the use of LDA above is that it does not take into account the lack of independence of the data from individual localities and hosts. To assess differences in individual principal component variables between groups, whilst allowing for within-site and within-host correlation, we also applied linear mixed models (LMM) employing the statististical package SPSS 13.0.1. The only fixed model term was 'group membership', with the random model including terms for locality and host individual nested within locality.

Allozyme analysis

An electrophoretic survey of *Protopolystoma* specimens in natural populations, originally designed to find polymorphic markers for use in laboratory breeding studies, produced data relevant to the present analysis. Methods were adapted from Richardson *et al*. (1986) and Hebert and Beaton (1993). All parasite samples were run alongside host blood and urinary bladder tissue samples and reference samples of known mobility. Of an initial 5 enzyme systems trialled (isocitrate dehydrogenase, E.C.1.1.1.42; malic enzyme, E.C.1.1.1.43; glucose phosphate isomerase, E.C.5.3.1.9; fumarate hydratase, E.C.4.2.1.2 [FUM]; mannose-6-phosphate isomerase, E.C.5.3.1.8 [MPI]), only MPI and FUM provided simple interpretation and consistently detectable bands which did not co-migrate with host bands. Host blood was collected and centrifuged (2 min) in heparinized microhaematocrit centrifuge tubes; subsequently, the cellular portion was homogenized in cold amphibian phosphate-buffered saline (APBS). For parasite specimens, small portions of tissue, including those containing the genital spines and haptoral armature, were retained for

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subsequent morphometric and molecular analysis. Large adult worms and host urinary bladder tissues were also homogenized in cold APBS. Homogenization and all preparatory steps were carried out on chilled glass or Perspex surfaces. Tissue homogenates were applied to cellulose acetate gels and run at 200 V for 20 min at 4 °C (in Tris-glycine buffer for FUM and MPI).

Experimental cross-infections

Hosts. The following groups of laboratory raised, naïve, full siblings were used in infection experiments. All were first-generation progeny of captive animals taken from natural populations. 1. *X. vestitus* (cross: ML1♂ × ML2♀) from L. Mulehe, Uganda (−1·22° +29·72°). 2. *X. wittei* (CY1♂ × CY2♀) from Cyamudongo, Cyangugu Prefecture, Rwanda (−2·56° +28·98°). 3. *X. l. victorianus* (KG1♂ × KG2♀, see Jackson and Tinsley 2002) from Kigali, Rwanda (−1·93° +30·07°). All hosts were post-metamorphic juveniles at the start of experiments.

Parasites. The following parasite isolates were used in experiments. 1. *P. simplicis s.l.* from *X. l. victorianus* in Mukono District Uganda (Pm1) (+0·3633° +32·6967°). 2. *P. simplicis s.l.* from *X. wittei* near the Kabale-Kisoro road in the L. Bunyonyi area, Uganda (Psw1) (−1·1906° +29·8462°). All infective stages used were the first-generation progeny of worms in naturally infected hosts maintained in the laboratory. Parasite eggs were collected from hosts by filtration of their tank water and were then incubated at 25 °C.

Protocol. Hosts were isolated and exposed to infection following the methods described recently (Jackson *et al*. 2006). Exposures were either to 15 Pm1 (Exp. 1) or to 30 Psw1 eggs (Exp. 2). Following exposure, hosts were maintained in a single controlled temperature room in 90 l aquaria at 25 ± 0·5 °C. Results are presented as the prevalence of adult infection at dissection 160 days p.i. (no juveniles survived by this time). Continuous screening for parasite egg production (Jackson and Tinsley, 2001) from 90 days p.i. confirmed that no infections had matured before 160 days p.i. in the zero or low prevalence groups.

Variation in egg hatching schedules

Egg hatching schedules in *P. xenopodis* (northern form) in *X. l. victorianus* from Masindi district, Uganda (+1·6947° +31·4903°), *P. simplicis s.l.* in *X. l. victorianus* from Mukono district, Uganda (+0·3633° +32·6967°), *P. simplicis s.l.* in *X. vestitus* from L. Mutanda, Uganda (−1·2156° +29·7073°) and *P. simplicis s.l.* in *X. wittei* from the L. Bunyonyi area, Uganda (−1·1906° +29·8462°) were assayed. *Protopolystoma xenopodis* (southern form) and *Protopolystoma orientalis* eggs were also included in assays to provide comparability with previous studies (Jackson *et al*. 2001). Eggs were collected from naturally infected hosts maintained in the laboratory at 25 ± 0·5 °C. Methods follow the protocol of Jackson *et al*. (2001), except that assays were carried out in flat-bottomed 96-well microplates (Nunc). Individual eggs were randomly assigned to single wells containing 200 μl of unchlorinated springwater (from a single batch). Microplates were stacked on the same shelf in a cooled incubator (Gallenkamp) running at 25 ± 0·5 °C (photoperiod: 12 h light/12 h dark) and their spatial positions randomized daily. Recording of embryonation, time to hatching and mortality was as described by Jackson *et al*. (2001). The distributions of hatching data allowed analysis by ANOVA (residuals not differing from a normal distribution, $P > 0{\cdot}05$), with Tukey *post hoc* pairwise comparisons.

Sequencing of mtDNA

The sequencing of mtDNA was selected as an efficient molecular approach for detecting cryptic or phenotypically similar species (Hebert *et al*. 2003*a*, *b*; Vilas *et al*. 2005). Whole specimens or tissue samples for molecular analysis were stored in 90% ethanol at 4 °C for 2–5 years. Genomic DNA was extracted (Qiagen DNeasy® Tissue Kit) and a target fragment (~400 bp) amplified by PCR from the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene. Amplification was with the primers 5′TTTTTTGGGCATCCTGAGGTTTAT3′ and 5′TAAAGAAAGAACATAATGAAAATG3′ (Littlewood *et al*. 1997). Amplicons were cleaned (Promega Wizard® SV) and sequenced directly (MWG Biotech) in both directions. Sequence quality was assessed visually using Chromas 1.45 and data were recorded for regions with concordant, unambiguous 'base calls' for sequences in both directions. Sequences were aligned unambiguously with Clustal X (1.81) (Thompson *et al*. 1997). Other polystomatid *cox1* sequences available in GenBank (*cf*. Littlewood *et al*. 1997) were also used as trial outgroups and for a comparative assessment of evolutionary divergence within *Protopolystoma*. All alignments shared the same amino acid translation reading frame as published sequences for *Polystomoides* and *Neopolystoma* (see Littlewood *et al*. 1997). Phylogenetic analysis was carried out by maximum likelihood (ML), maximum parsimony (MP) and minimum evolution neighbour joining (ME) methods using the program PAUP* (4.0b10) (Swofford, 1998). Bayesian analysis (BA) was also carried out using MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). For MP, an heuristic search with the tree-bisection-reconnection (TBR) algorithm was performed, using random stepwise addition

Table 2. Overall sclerite morphometric variation amongst 5 putative lineages of *Protopolystoma* occurring in *Xenopus* spp. in East African highlands

(Mean with standard error (in parentheses) and range.)

Character	*P. simplicis s.l.* in *X. wittei*	*P. simplicis s.l.* in *X. vestitus*	*P. simplicis s.l.*in *X. laevis victorianus*	*P. xenopodis* (northern form)	*P. fissilis*
LHTL	149·3 (5·1) 89–205	141·5 (3·5) 85–213	158·5 (3·8) 104–243	148·6 (3·7) 95–203	151·4 (3·3) 106–187
LHP	41·5 (0·7) 35–47	44·5 (0·2) 42–48	38·6 (0·1) 35–42	44·8 (0·3) 39–49	31·7 (0·2) 30–33
LHS	84·2 (1·4) 66–94	83·7 (0·8) 69–104	87·8 (1·3) 67–121	81·3 (0·9) 70–105	88·3 (1·8) 56–107
LHPB	76·9 (1·3) 61–87	78·1 (0·7) 53–94	81·1 (1·1) 61–109	78·1 (0·8) 65–91	77·1 (1·4) 51–93
LHDR	65·2 (4·5) 21–117	59·3 (3·0) 11–124	71·7 (2·7) 31–123	68·8 (3·3) 23–120	63·0 (2·2) 47–87
LHVR	67·3 (3·7) 35–101	56·8 (1·8) 26–93	70·7 (2·2) 31–114	67·9 (2·0) 39–106	53·5 (1·3) 38–72
LHIR	69·7 (2·8) 37–98	60·7 (2·2) 31–111	69·1 (2·4) 35–118	69·8 (2·2) 41–113	70·7 (2·4) 41–93
LHPVR	110·6 (3·6) 74–141	104·1 (1·9) 69–138	116·7 (2·5) 78–168	106·8 (2·1) 77–139	92·4 (1·7) 75–111
SHL	27·2 (0·7) 22–36	36·0 (0·6) 25–56	35·2 (1·0) 21–80	29·0 (0·4) 21–36	35·1 (1·4) 22–63
LHL	11·8 (0·1) 11–13	12·5 (0·1) 12–14	13·4 (0·1) 12–15	12·6 (0·1) 11–13	13·0 (0·1) 12–13
IGS	20·5 (0·1) 19–21	18·9 (0·1) 18–20	17·7 (0·1) 15–20	30·4 (0·2) 27–33	22·6 (0·2) 20–25
OGS	13·4 (0·1) 12–14	13·0 (0·1) 12–15	13·1 (0·1) 12–14	22·8 (0·1) 21–25	14·4 (0·1) 14–16
N	25	82	73	56	31

(100 replicates) and random starting trees. For ME, a Jukes-Cantor distance measure was used, with random tie-breaks. The robustness of tree structures inferred by ME and MP was assessed by bootstrapping (2000 replicates) in each case. Prior to ML analysis, Model test 3.7 (Posada and Crandall, 1998) was used to identify the most appropriate DNA substitution model using the Akaike Information Criterion (AIC) and hierarchical likelihood ratio tests (hLRTs). All models within AIC Δ5 of the best AIC value were run, in addition to the optimal model selected by hLRTs. Phylogenetic inferences were not sensitive to the substitution model used and are presented for K81uf+G. Bootstrap values for ML trees are for 100 replicates. Bayesian analyses were run using a general time reversible (GTR) setting, with zero invariant sites. Two Metropolis coupled Markov chain Monte Carlo analyses were run for 15×10^6 generations, with 1 cold and 5 heated chains, and all other settings at default. Trees sampled pre-stationarity were discarded as burn-in.

Taxonomic sections

Type specimens have been deposited at The Natural History Museum, London (BMNH). Where a new taxon is described, each morphometric datum (μm) for the holotype is followed by the range for all conspecific specimens studied. Characters marked with an asterisk are not correlated with growth stage in late juveniles and older (see Tinsley and Jackson, 1998*b*).

RESULTS

Morphometric analysis

Analyses were carried out on the set of 12 sclerite characters (Fig. 1) summarized in Table 2. All individual specimens were urinary bladder stages with rooted (bifid) large hamuli. An initial PCA (Fig. 2A, Table 3) included 267 individuals comprising new collections of *P. simplicis s.l.* and *P. xenopodis*, and a re-measured sample of *Protopolystoma fissilis* (reported by Tinsley and Jackson, 1998*b*). These specimens represent all of the forms known to occur in the highlands of south-western Uganda and Rwanda. The first principal component (PC1) in the analysis showed large coefficients of the same sign for measurements involving elements of the large hamulus body distal to the point (Table 3). This is consistent with PC1 largely representing growth-stage variation in the distal large hamulus (Jackson and Tinsley, 1995; Tinsley and Jackson, 1998*b*). All other sclerites, and the large hamulus point (LHP), have already reached full length in urinary bladder stages with bifid large hamuli (Tinsley and Jackson, 1998*b*). The second component (PC2) represented negative covariation between genital spine lengths (OGS and IGS) and the length of the small hamulus (SHL), the third component (PC3) was dominated by large hamulus point length, and the fourth component (PC4) represented negative covariation between measurements respectively involving the large hamulus shaft (LHS) and roots (LHDR and LHVR). LDA on component scores for PC2–PC4, excluding PC1 as primarily describing ontogenetic stage, with the taxonomic designations of Tinsley and Jackson (1998*b*) as the grouping variable, correctly identified 100% of *P. xenopodis* and *P. fissilis* specimens, and 97% of *P. simplicis s.l.* specimens when cross-validation was used (Table 4). All LDA misclassifications were of *P. simplicis s.l.* specimens as *P. fissilis*. LMM analyses (Fig. 3A) indicated that PC2-PC4 varied significantly between the groups, except for PC2 between *P. simplicis s.l.* and *P. fissilis*. The latter is explained by similar genital spine measurements in the two groups (Table 2) and the high influence of these characters on PC2. Univariate

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Table 3. Results of the principal components analysis (PCA)

(PCA1, including *Protopolystoma simplicis s.l.*, *P. xenopodis* (northern form) and *P. fissilis*. PCA2, including *P. simplicis s.l.* in *Xenopus laevis victorianus*, *Xenopus wittei* and *Xenopus vestitus* (reduced dataset from PCA1). Characters shown in bold are elements of the large hamulus that continue to grow throughout life in urinary bladder stages (all other characters have ceased to increase in length by the time a worm has migrated to the urinary bladder).)

Analysis:	PCA1, $n=267$				PCA2, $n=180$			
Component:	PC1	PC2	PC3	PC4	PC1	PC2	PC3	PC4
Eigenvalue	5·49	2·44	1·08	0·92	5·98	1·61	1·26	1·03
Percentage variation	45·7	20·3	9·0	7·7	49·9	13·4	10·5	8·6
Character coefficients								
LHTL	**0·41**	0·05	0·08	0·09	**0·39**	0·16	0·00	0·07
LHP	0·00	0·27	−0·84	0·10	−0·06	0·28	−0·70	−0·14
LHS	**0·32**	−0·16	0·02	−0·52	**0·33**	0·01	−0·03	−0·28
LHBP	**0·31**	−0·07	−0·22	−0·59	**0·31**	−0·05	−0·10	−0·36
LHDR	**0·39**	0·11	0·12	0·30	**0·37**	0·19	0·03	0·20
LHVR	**0·38**	0·16	−0·03	0·33	**0·37**	0·16	0·08	0·20
LHIR	**0·37**	0·13	0·17	0·10	**0·36**	0·25	0·03	0·15
LHPVR	**0·38**	0·02	−0·25	0·11	**0·38**	−0·03	−0·08	−0·03
SHL	0·09	−0·35	−0·21	−0·09	0·10	−0·31	−0·53	−0·28
LHL	0·20	−0·18	0·24	−0·07	0·22	−0·45	0·14	−0·19
IGS	−0·05	0·58	0·17	−0·25	−0·17	0·63	−0·06	−0·12
OGS	−0·02	0·59	0·08	−0·26	−0·04	0·25	0·42	−0·73

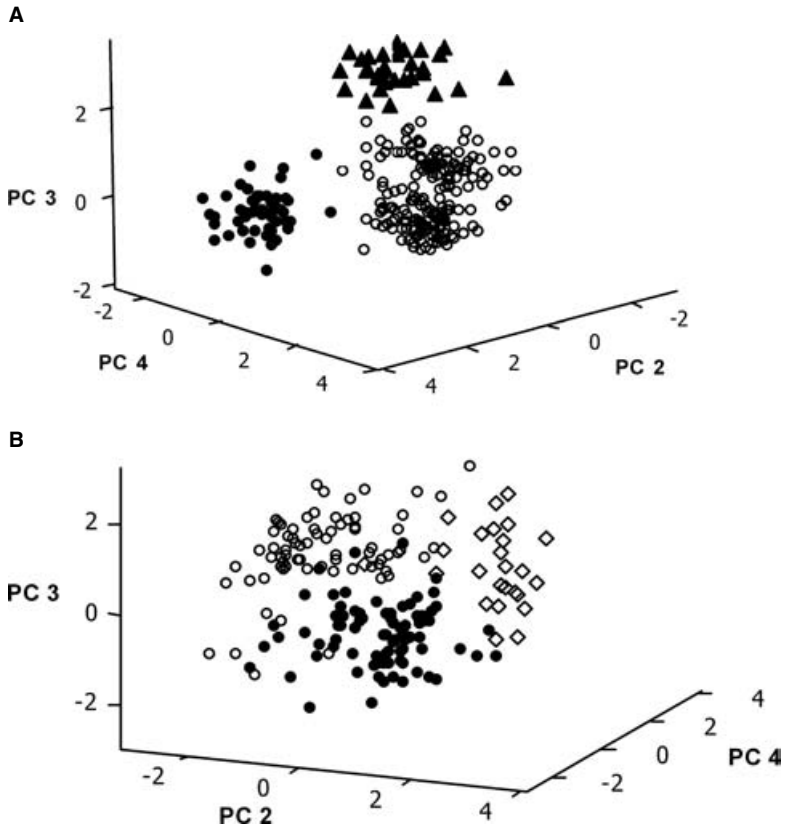

Fig. 2. Principal components analysis (PCA) of morphometric variation in sclerites of *Protopolystoma* spp. Individual scores against principal components (PCs) 2–4 shown on a 3-D scatterplot. PC1 is excluded as it mainly represents ontogenetic variation in the shaft and roots of the large hamulus (A) Variation in the species occurring in East African highlands ($n=267$). Specimens identified following the taxonomic designations by Tinsley and Jackson (1998*b*): *P. xenopodis* (●), *P. fissilis* (▲), *P. simplicis s.l.* (○). (B) Variation in *P. simplicis s.l.* (according to the species concept of Tinsley and Jackson, 1998*b*) in different polyploid *Xenopus* species: tetraploid *X. laevis victorianus* (○), octoploid *X. wittei* (◇), octoploid *X. vestitus* (●).

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Table 4. Morphometric classification of *Protopolystoma* specimens occurring in *Xenopus* spp. in East African highlands

(Based on linear discriminant analysis (LDA) of principal component scores (using cross-validation).)

PCA1, western rift valley highland species (groups according to Tinsley and Jackson, 1998*b*)				PCA2, *P. simplicis s.l.* (groups according to host taxon)			
	True group				True host		
Predicted group	*P. simplicis s.l.*	*P. xenopodis*	*P. fissilis*	Predicted host	*X. wittei*	*X. vestitus*	*X. l. victorianus*
P. simplicis s.l.	174	0	0	*X. wittei*	24	3	4
P. xenopodis	0	56	0	*X. vestitus*	0	73	6
P. fissilis	6	0	31	*X. l. victorianus*	1	6	63
Total *n*	180	56	31		25	82	73
% correct	96·7	100	100		96·0	89·0	86·3

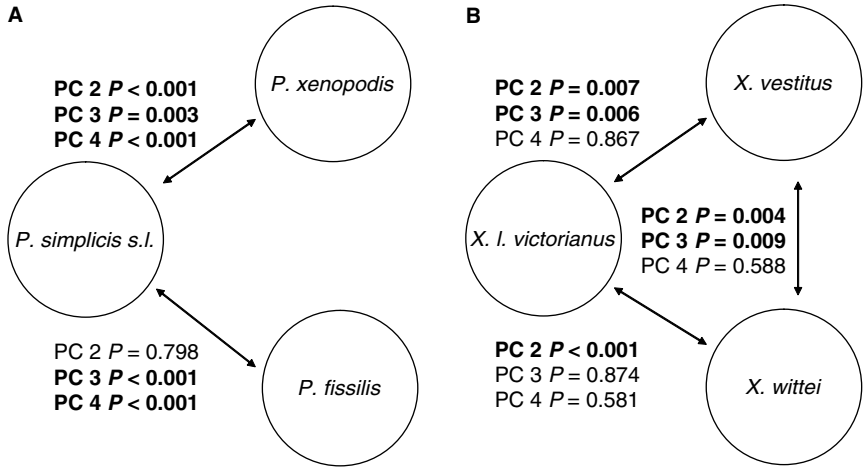

Fig. 3. Linear mixed model (LMM) analysis of between-group variation in morphometric principal component (PC) variables. *P* values are for *post hoc* least significant difference multiple comparisons following the separate analysis of each PC. (A) Data for a principal components analysis (PCA) of species occurring in East African highlands, with groups corresponding to the taxonomy described by Tinsley and Jackson (1998*b*). (B) Data for a PCA of *P. simplicis s.l.* (according to the species concept of Tinsley and Jackson, 1998*b*), with groups corresponding to host identity.

analysis of genital spine lengths in *P. xenopodis* and *P. simplicis s.l.* in *X. l. victorianus* also confirmed the discrete bimodal distributions reported by Tinsley and Jackson (1998*b*) (see Fig. 4). Furthermore, a near discrete distribution for large hamulus point length was found in these samples (Fig. 4).

In order to further assess the differentiation amongst the host-related forms of *P. simplicis s.l.* a PCA was repeated on these samples alone (Fig. 2B). The structure of the coefficients for PC1 was comparable to that in the initial analysis (Table 3), although there were some changes in PC2–PC4, suggesting an altered inter-character correlational structure caused by the removal of *P. xenopodis* and *P. fissilis* specimens from the data set. PC2 now represented negative covariation of the inner genital spine with the larval hooklet (LHL) and small hamulus. PC3 reflected negative covariation of the large hamulus point and small hamulus with respect to the outer genital spine. PC4 mainly represented variation in outer genital spine length whilst also still being influenced by contrasting measurements within the large hamulus body. When LDA (Table 4) was carried out on PC2-PC4 scores for the second PCA, with host identity as the grouping variable, parasites from *X. wittei*, *X. vestitus* and *X. l. victorianus* were classified with 96, 89 and 86% efficiency, respectively. The LMM analyses indicated a lack of significant variation in PC4 between the groups, but that PC2 and PC3 varied very significantly, except in the case of PC3 between the *X. l. victorianus* and *X. wittei* groups (Fig. 3B). The latter was attributable to extensive between-group overlap (Table 2) in large hamulus point length measurements (a strong influence on PC3).

Expression of the enzymes MPI and FUM

The expression of MPI and FUM phenotypes in individuals of *Protopolystoma* spp. from Ugandan

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Table 5. Frequency of mannose-6-phosphate isomerase (MPI) and fumarate hydratase (FUM) electromorphs in populations of *Protopolystoma* spp.

Host	Parasite	Locality	MPI[a]* FUM[d]	MPI[b] FUM[c]	MPI[c] FUM[a]	MPI[d] FUM[b]
X. l. victorianus	*P. xenopodis*	L10			7	
		L11			12	
		L13			3	
X. l. victorianus	*P. simplicis s.l.*	L8	1			
		L9	3			
		L10	12			
		L12	5			
		L16	1			
X. vestitus	*P. simplicis s.l.*	L21		22		
X. wittei	*P. simplicis s.l.*	L17				3

* Protein bands of different mobility are indicated by a different superscript (a–d).

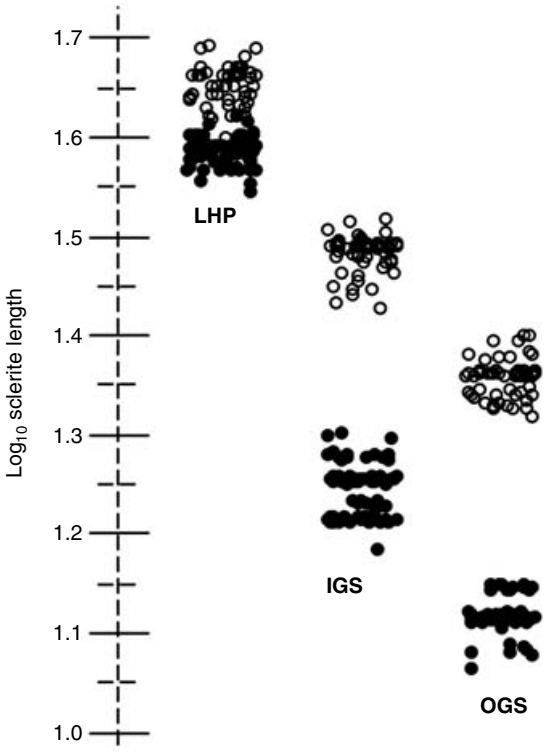

Fig. 4. Variation in diagnostic sclerite measurements between *Protopolystoma xenopodis* (A) and *P. simplicis s.l.* (B) in *Xenopus laevis victorianus*. Vertical scatterplots of log-transformed large hamulus point (LHP), inner genital spine (IGS) and outer genital spine (OGS) lengths.

populations is summarized in Table 5. Fixed differences at both loci were found between 4 groups of samples: (1) *P. simplicis s.l.* from *X. wittei* in south-western Uganda; (2) *P. simplicis s.l.* from *X. vestitus* in south-western Uganda; (3) *P. simplicis s.l.* from *X. l. victorianus* in south-western, south-central, south-eastern and north-western Uganda; (4) *P. xenopodis* from south-central and north-western Uganda. Within these groups variation was monomorphic at both loci.

Experimental host-specificity

Exp. 1: an isolate of *P. simplicis s.l.* (Pm1) from *X. l. victorianus* in south-central Uganda was infective (5/18) to Rwandan *X. l. victorianus*. There was no evidence of infectivity to Ugandan *X. vestitus* (0/15) and Rwandan *X. wittei* (0/20). Exp. 2: a Ugandan isolate (Psw1) of *P. simplicis* from *X. wittei* was of very low infectivity to Rwandan *X. wittei* (1/36), and there was no evidence of infectivity to Rwandan *X. l. victorianus* (0/20). In both experiments early spot checks (< 5 weeks p.i.) were carried out on a few additional individuals from each host group. These checks indicated that larvae always invaded any host and were also able to develop to the blood-feeding early juvenile stage in any host. Failure to develop to the adult stage can therefore be assumed to result from host-parasite incompatibilities rather than a failure of larval invasion or non-viability in the parasite isolate.

Egg hatching schedules

Overall variation in time to hatching amongst the *Protopolystoma* spp. isolates from *X. l. victorianus*, *X. wittei* and *X. vestitus* was highly significant (ANOVA, $F_{3,334} = 551{\cdot}57$, $P < 0{\cdot}0005$) (Fig. 5). Hatching windows were near identical for northern *P. xenopodis* (days 19–26, $n = 107$) and *P. simplicis s.l.* in *X. l. victorianus* (days 19–26, $n = 68$), and hatch time did not vary significantly ($P = 0{\cdot}562$) between these. Hatching windows occurred later in *P. simplicis s.l.* in *X. wittei* (days 26–33, $n = 41$) and *P. simplicis s.l.* in *X. vestitus* (days 25–35, $n = 122$) and there were highly significant pair-wise differences between hatching time in each of these and the 2 parasites in *X. l. victorianus* ($P < 0{\cdot}0005$). There was a much smaller, but significant ($P = 0{\cdot}028$) difference between hatch time in *X. wittei* and *X. vestitus* parasites (eggs from *X. wittei* showing the higher mean hatch time).

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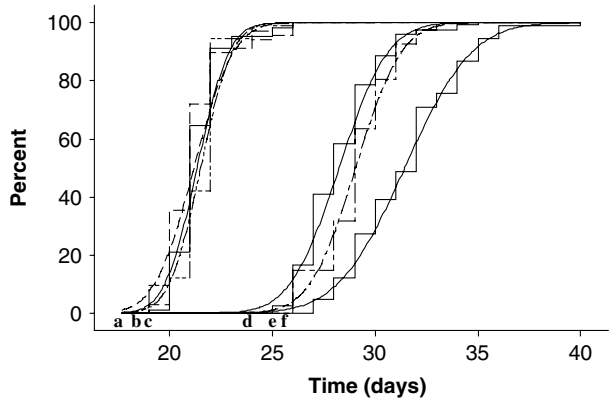

Fig. 5. Egg hatching schedules in different populations of *Protopolystoma* spp. Cumulative percentage hatch against time: observed data (stepped lines) with fitted cumulative distribution functions (smooth lines) for a normal distribution (hatching schedules in the present assays approximated normal distributions). *Protopolystoma simplicis s.l.* in *Xenopus laevis victorianus* (a); *P. xenopodis* (northern form) in *X. l. victorianus* (b); *P. xenopodis* (southern form) in *X. l. laevis* (c); *P. simplicis s.l.* in *X. vestitus* (d); *P. simplicis s.l.* in *X. wittei* (e); *P. orientalis* in *X. muelleri* (f).

Phylogenetic analysis of cox1 *sequence data*

Protopolystoma DNA sequences were obtained for 306 bp of the mitochondrial *cox1* gene. These sequences represented 14 distinct haplotypes from across southern and western Uganda (Fig. 6, Table 1). The set of *Protopolystoma* sequences analysed (the Ugandan haplotypes + a *P. orientalis* haplotype from Swaziland) contained 94 variable and 78 parsimony informative sites. Initial analyses using *Polystomoides* and *Neopolystoma* outgroups suggested that the ingroup phylogeny was disrupted by the inclusion/non-inclusion of the outgroup. There was also evidence of saturated nucleotide variation, with reduced transition: transversion ratios ($\approx$0·5) at inter-generic levels of divergence (Fig. 6C). MP, ML and BA were therefore carried out for the ingroup alone (Fig. 6). Phylogenetic analysis by different methods (ME, MP, ML, BA) consistently indicated strong bootstrap and posterior probability support (100%) for a clade containing *P. simplicis s.l.* in *X. l. victorianus* (node *1*, Fig. 6A, B). The distinct haplotypes, differing by $<$2% sequence divergence, were derived from localities across south-eastern, south-central and south-western Uganda. A distinct clade for *P. xenopodis* populations in *X. l. victorianus* was also well supported by all methods (82–100%) (node *3*, Fig. 6A, B). Haplotypes in the *P. xenopodis* clade were from north-western and south-central Uganda (in sympatry with *P. simplicis s.l.* haplotypes at L10) and varied by up to 10% in nucleotide composition. Only ME (94%) and MP (76%) supported a clade for *P. simplicis s.l.* in the octoploid hosts (node *2*, Fig. 6A, B). Sequences diverged by 12% between parasites in *X. wittei* and *X. vestitus*. In comparison, pair-wise lineage divergence amongst the 3 possible clades (issuing from nodes *1–3* in Fig. 6) was 13–19%, and there were 25–32% divergences from *Polystomoides* and *Neopolystoma* species. Nucleotide variation may have been partly saturated at levels of divergence found between the main *Protopolystoma* clades (Fig. 6C), and their deeper branching interrelationships were unresolved.

Taxonomic revision

On the basis of the variation seen in *cox1* and differences in biological characteristics, the following taxonomic changes are recommended.

Protopolystoma microsclera *n. sp.*

Type-host and locality. *Xenopus laevis victorianus* Ahl, in borrow pits near the Kampala-Jinja road, Mukono district, Uganda, +0·3633° +32·6967° (L10).
Type series. Holotype BMNH 2007.1.31.1; paratypes BMNH 2007.1.31.2–5.
Other museum material. BMNH 1997.7.17.10–11 (deposited as *P. simplicis*).
Other localities and material studied. Specimens at L1–9, L12, L14, L16, L22 (see Table 1).
Previous literature records assignable to P. microsclera. Records of *P. simplicis* in *X. l. victorianus* and *X. l. poweri* in Tinsley and Jackson (1998*b*). *Protopolystoma* species A in *X. l. victorianus* in Jackson *et al.* (1998); *Protopolystoma* sp. in Jackson and Tinsley (2005) and Jackson *et al.* (2006).

Description. With characters of genus (Tinsley and Jackson, 1998*b*). Genital spines short, outer circle (OGS) 14, 12–14*, inner circle (IGS) 19, 15–20*. Large hamulus roots simple; LHTL 190, 104–243, LHP 40, 35–42*, LHS 103, 67–121, LHPB 88, 61–109, LHDR 90, 31–123, LHVR 70, 31–114, LHIR 88, 35–118, LHPVR 121, 78–168. Small hamulus prone to extreme variation in a few individuals; SHL 31, 21–80*. LHL 14, 12–15*. Adults infecting *X. laevis* subspecies, but not *X. l. laevis*. *Cox1* mtDNA variants relatively close to GenBank sequences EF379994–9.

Remarks. This taxon includes *Protopolystoma* species with small genital spines (OGS $\sim$13 μm, IGS $\sim$18 μm) from *X. l. victorianus* and other *Xenopus laevis* ssp. in east and central Africa that were previously classified in *P. simplicis*. Present experimental host-specificity results are consistent with a negligible ability to infect *X. wittei* (the type-host of *P. simplicis*) and *X. vestitus*. Jackson and Tinsley (2005) found that *P. microsclera* (reported as *Protopolystoma* sp.) was also of low laboratory infectivity to an *X. l. laevis* sibship from the Western Cape, South Africa and 5 other *X. l. laevis* sibships

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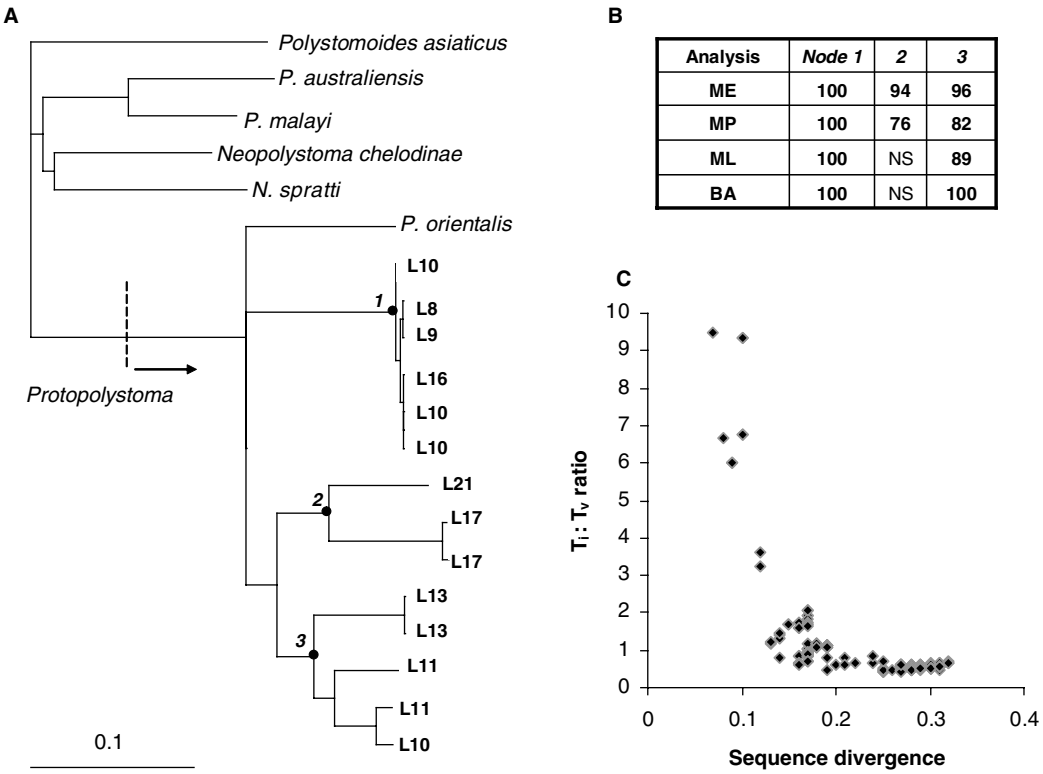

Analysis	*Node 1*	*2*	*3*
ME	100	94	96
MP	100	76	82
ML	100	NS	89
BA	100	NS	100

Fig. 6. Phylogenetic analysis of *cox1* mtDNA sequence data for *Protopolystoma* species. (A) Minimum evolution (ME) tree (50% majority-rule consensus topology), including related polystomatids, with terminal branches for material from the present study labelled by sampling locality (see Table 1). Node *1* gives rise to *P. simplicis s.l.* haplotypes in tetraploid *X. laevis victorianus* hosts, node *2* to *P. simplicis s.l.* haplotypes in octoploid *X. wittei* and *X. vestitus* hosts, and node *3* to *P. xenopodis* haplotypes. Scale bar indicates 0·1 nucleotide substitutions per site. Sequences for outgroup polystomatids used in tree construction are: *Polystomoides asiaticus* (GenBank Accession number: Z83009), *P. malayi* (Z83011), *P. renschi* (Z83015), *P. australiensis* (Z83013), *Neopolystoma spratti* (Z83007), *N. chelodinae* (Z83005) (see Littlewood *et al.* 1997). A sequence for *Protopolystoma orientalis* in *Xenopus muelleri* in Swaziland (−26·0514°+31·6597°) (Accession number: EF380003) is also included. (B) Support for identified nodes from minimum evolution (ME), maximum parsimony (MP), maximum likelihood (ML), and Bayesian analysis (BA). Tabulated values are % bootstrap support (ME, MP or ML) or posterior probabilities (BA). Bootstrap values and posterior probabilities <75% were considered non-significant (NS). (C) Plot of transition to tranversion rate ratio ($T_i : T_v$) against sequence divergence, omitting data for divergences of <5%.

from Mpumalanga province, eastern South Africa. On the other hand, *P. microsclera* isolates usually show significant laboratory infectivity to *X. l. victorianus* sibships from East Africa (Jackson and Tinsley, 2005; Jackson *et al.* 2006).

Protopolystoma simplicis *Tinsley and Jackson, 1998*

Type-host and locality. *Xenopus wittei* Tinsley, Kobel and Fischberg at Cyamudongo Forest, Cyangugu Prefecture, Rwanda (Tinsley and Jackson, 1998*b*).
Type series. BMNH 1997.16.1–4 (see Tinsley and Jackson, 1998*b*).
Other hosts, localities and material studied. *X. wittei*, specimens at L15, L17–18, L23–24 (Table 1). *X. vestitus* Laurent, specimens at L19–21 (Table 1).
Previous literature records assignable to P. simplicis. Records of *P. simplicis* in *X. wittei* and *X. vestitus* in Tinsley and Jackson (1998*b*). *Protopolystoma* species A in *X. wittei* and *X. vestitus* in Jackson *et al.* (1998).

Description. With characters of genus (Tinsley and Jackson, 1998*b*). Genital spines short, outer circle (OGS) 12–15*, inner circle (IGS) 18–21*. Large hamulus roots simple; LHTL 85–213, LHP 35–48*, LHS 66–104, LHPB 53–94, LHDR 11–124, LHVR 26–101, LHIR 31–111, LHPVR 69–141. Short hamulus prone to extreme variation in a few individuals; SHL 22–56*. LHL 11–14*. Adults infecting *X. wittei* and *X. vestitus*. *Cox1* mtDNA variants relatively close to GenBank sequences EF380000–2.

Remarks. *Protopolystoma simplicis* now only includes specimens with simple large hamulus roots in *X. wittei* and *X. vestitus*. Populations in *X. vestitus* and *X. wittei*, although showing some phenotypic differences, are not recognized as distinct species because their level of mtDNA divergence is comparable to that found within the northern form of *P. xenopodis*. There remains some question as to the status of genetically uncharacterized populations

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outside of south-western Uganda. We note that our experimental infections showed very low compatibility of *P. simplicis* from *X. wittei* in western Uganda with *X. wittei* hosts from southern Rwanda. Therefore, there may be significant host-specificity differences in *P. simplicis* from amongst different host populations.

DISCUSSION

Diversity in tetraploid hosts

On the grounds of morphological similarity, *Protopolystoma* with short genital spines and simple hamulus roots in *X. wittei*, *X. vestitus* and *X. l. victorianus* were previously placed in a single taxon, *P. simplicis* (see Tinsley and Jackson, 1998*b*). These populations are clearly distinguished from the sympatric northern form of *P. xenopodis*, that infects *X. laevis* ssp., by non-overlapping variation in genital spine length amongst individuals (Tinsley and Jackson, 1998*b*; confirmed in present study). However, Tinsley and Jackson (1998*b*) noted that *P. simplicis* populations in the tetraploid host, *X. l. victorianus*, and the octoploid hosts, *X. vestitus* and *X. wittei*, also showed variation in copulatory spine length and inter-caecal anastomosis number. The present multivariate analysis confirms that the respective '*P. simplicis*' populations from *X. l. victorianus*, *X. wittei* and *X. vestitus* are morphologically distinguishable at the population level, although there may be some individual overlap. On this basis, material from *X. l. victorianus* is named *P. microsclera* n. sp. This division is strongly supported by *cox1* sequence data: *P. microsclera* haplotypes across southern Uganda form a divergent monophyletic group with respect to the haplotypes of *X. vesititus* and *X. wittei* parasites in south-western Uganda. Evidence for variation in MPI and FUM electromorphs also suggests that fixed allelic differences occur between allopatric populations in *X. laevis* and *X. wittei*/*X. vestitus*. This is consistent with, although insufficient in itself to prove (Richardson *et al*. 1986), the reproductive isolation of populations in the respective hosts. The distinctness of *P. microsclera* was also consistent with laboratory tests of host-specificity. An isolate of this morphospecies from south-central Uganda was infective to an *X. l. victorianus* sibship from central Rwanda, but not an *X. wittei* sibship from southern Rwanda, nor an *X. vestitus* sibship from south-western Uganda. Meanwhile, a *Protopolystoma simplicis* isolate from *X. wittei* in south-western Uganda was not infective to the Rwandan *X. l. victorianus* sibship. A further biological difference is that the larval hatching schedule in *P. microsclera* is very significantly accelerated compared with that in parasites in *X. wittei* and *X. vestitus*. Taken together, the accumulated data therefore strongly support the species-level distinction of *P. microsclera* in the tetraploid *X. laevis* from *P. simplicis* in octoploid species (*X. wittei* + *X. vestitus*) in western Uganda.

Protopolystoma xenopodis (northern form) (Tinsley and Jackson, 1998*b*) and *P. microsclera* share the same species-specificity, co-occurring in *X. laevis* populations across East Africa (present study; Jackson *et al*. 2006), and have very similar larval hatching schedules. Individuals are unambiguously differentiated because of the discrete, bimodal distribution of genital spine length, strongly suggesting that 2 genuine species are involved. However, the morphological data alone allow a hypothetical possibility that spine length variation could be determined by polymorphic alleles segregating within a single genetic species. The present study establishes unequivocally that *P. microsclera* and the sympatric northern form of *P. xenopodis* are genetically distinct species. A fixed allelic difference occurred at both the MPI and FUM loci in samples from a single local habitat, and each morphotype is associated with a divergent, monophyletic *cox1* mtDNA clade across southern and western Uganda. Laboratory coinfection studies (Jackson *et al*. 2006) have confirmed that the apparently extensive and stable geographical coexistence of the two species occurs in the face of within-host competitive interactions so intense that heterospecific adults do not normally coincide in the same host individual. Despite this extremely antagonistic fine-scale interaction, coexistence at the population level and above may be promoted by context-dependent heterogeneity in the dynamics of infection within host individuals. One source of variability is phenotypic plasticity in inferior competitors that allows them to delay development and avoid fatal interactions with adults of the other species, depending on infection sequence. Also important may be the temporal development of species-specific induced resistance, the temporal influence of bystander infections on competitive dominance, and genetic variation in the genotype-specific susceptibility-infectivity characteristics of the hosts and parasites (Jackson *et al*. 2006).

Diversity in octoploid hosts

Specimens in *X. vestitus* at L. Mutanda showed MPI and FUM phenotypes that were distinct from those of specimens in *X. wittei* at L. Bunyonyi. These findings are consistent with the reproductive isolation of the parasites in the respective hosts, but could equally reflect a level of restricted gene flow or differing selection pressures amongst populations in the Kabale and Kisoro district lakes. The present specimens in *X. vestitus* in 2 adjacent lakes (Mutanda and Mulehe) were also morphologically distinguishable from samples in *X. wittei* in the L. Bunyonyi area (15–30 km distant) and in southern Rwanda (~170 km distant), to a significant extent. Variation in egg hatching schedule between geographical

isolates of *P. simplicis* in *X. wittei* and *X. vestitus* was also statistically significant, but modest compared with the difference between each isolate and *P. microsclera*. The present data might, therefore, indicate that *P. simplicis* in *X. wittei* and *X. vestitus* represent independently evolving lineages. However, whilst the host-specific populations contained substantially different *cox1* haplotypes, these diverged at a level similar to that seen within *P. xenopodis*. At present, it therefore seems most appropriate to retain the 2 host-specific forms in 1 species. A question also remains regarding the relationship between *P. simplicis* in Uganda and *X. wittei* in southern Rwanda, given that the Rwandan material in this study was not characterized genetically. This uncertainty is emphasized by the very low infectivity obtained in the cross-infection of southern Rwandan *X. wittei* by *P. simplicis* isolated from *X. wittei* in south-western Uganda.

Implications for the historical formation of Protopolystoma *faunas in octoploid hosts*

Both *Xenopus wittei* and *X. vestitus* are octoploids that have been thought to each descend from one ancestor in a tetraploid lineage of the *X. fraseri* group, and another in the tetraploid *X. laevis* group (Tymowska, 1991). This set of relationships was assumed in the interpretation of host-parasite associations for *Protopolystoma* and *Xenopus* advanced by Tinsley and Jackson (1998*a*) and discussed by Combes (2001). The different *Protopolystoma* species involved were formally described and named, or redescribed, by Tinsley and Jackson (1998*b*). In the 1998 interpretation, the 4 *Protopolystoma* species present in the *X. laevis* and *X. fraseri* lineages were thought to have been inherited differentially by *X. vestitus* and *X. wittei*. Both *X. wittei* and *X. vestitus* were considered to have inherited 1 species (*P. simplicis*) present in *X. laevis*. In the case of *X. wittei*, a further species (*P. fissilis*, present in some local populations) was also thought to have been inherited from *X. fraseri*, reflecting the hybrid ancestry of the host. We now consider how this analysis is complicated by new molecular studies of the host group (Evans *et al*. 2004, 2005) and our present results on relationships within *Protopolystoma*.

Recently, the most comprehensive molecular phylogenetic analyses of *Xenopus* species to date have been reported for mtDNA (Evans *et al*. 2004) and the nuclear recombination-activating gene, *RAG-1* (Evans *et al*. 2005). These studies support a maternal ancestor of *X. wittei* in the *X. fraseri* group, and a paternal ancestor from a lineage arising from an unresolved polytomy involving *X. laevis* and the recently described *X. largeni* (see Evans *et al*. 2005). Either the paternal or maternal ancestor of *X. vestitus* was very closely related to the paternal ancestor of *X. wittei*, and both of the *X. vestitus* parents were also closely related to each other (all presumably stemming from the polytomy with *X. laevis* and *X. largeni*). Interestingly, the lack of an *X. fraseri*-lineage parent for *X. vestitus* is consistent with the absence of a *P. fissilis*-like parasite in this species. The relationships considered by Tinsley and Jackson (1998*a*) are further complicated by the present analysis of East African *Protopolystoma* spp. As discussed above, it appears that the single species assumed to have been shared by *X. wittei*, *X. vestitus* and *X. laevis* is in fact 2, or possibly more, discrete host-specific evolutionary entities: *P. simplicis* in *X. wittei* and *X. vestitus*, which perhaps comprises distinctive lineages, and *P. microsclera* in *X. laevis*. The present study of mtDNA sequence variation suggests that *P. simplicis* haplotypes did not diverge from within the radiation of *P. microsclera* haplotypes currently found in Uganda. However, the deeper branching relationships amongst the different lineages of *Protopolystoma* species remain unresolved. Therefore, the data do not refute the monophyletic relationship between *P. simplicis*-like morphotypes in *X. wittei*, *X. vestitus* and *X. laevis* assumed by Tinsley and Jackson (1998*a*), but also allow that *P. simplicis* in the octoploids may, in fact, have split most recently from another lineage within *Protopolystoma*. Neither possibility can be confirmed until further studies are carried out, which should include expanded target DNA sequences and data for parasites in a more complete set of host species. To unequivocally establish the relationships of *Protopolystoma* species in octoploid hosts, a more detailed molecular phylogenetic analysis is now in progress. This will be based on multiple genes with dense sampling of parasites in hosts of the *X. fraseri* complex, and also the *X. laevis* and *X. largeni* lineages close to the ancestors of *X. vestitus*.

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