Parasitol Res (2005) 95: 79–89
DOI 10.1007/s00436-004-1262-x

ORIGINAL PAPER

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Validity reassessment of *Trichobilharzia* species using *Lymnaea stagnalis* as the intermediate host

Received: 21 September 2004 / Accepted: 27 September 2004 / Published online: 4 December 2004

Abstract The systematics within the genus *Trichobilharzia* is complicated. After the description of the type species *Trichobilharzia ocellata*, the name was routinely used for nearly all European findings of ocellate furcocercariae. *T. ocellata* was also described from North America and Japan. However, the identity of *T. ocellata* remains questionable. Comparison of data from the literature showed differences among various *T. ocellata* isolates and led us to the conclusion that the North American and the Japanese findings are not identical with European *T. ocellata*. In addition, the description of *T. szidati* corresponds with the recently reported European *T. ocellata* isolates. Sequence analysis of the ITS region confirmed that they are identical.

Introduction

The genus *Trichobilharzia* includes more than 40 species, but the validity of some is contested (Blair and Islam 1983). Therefore, the systematics within the genus remains a complicated matter. *Trichobilharzia ocellata*, as the type species, was originally characterized at the larval stage as *Cercaria ocellata* La Valette de St. George, 1855 found in the snail *Lymnaea stagnalis* in the Berlin area (Germany). Later, the genus *Trichobilharzia* was established by Skrjabin and Zakharov (1920), who described *T. kossarewi*. Brumpt (1931) used cercariae determined as *C. ocellata* for experimental infection of ducks and found the adult worms to be identical to those of *T. kossarewi*. According to the law of priority, he introduced the name *T. ocellata* (La Valette de St. George, 1855) Brumpt, 1931.

After this initial period, *C. ocellata* was reported from other European countries. Nevertheless, cercariae routinely determined as *C. ocellata* differed in their intermediate hosts, behaviour and dimensions, which led to doubts on the identity of these isolates (Szidat and Wigand 1934; Szidat 1942), and to the description of three new cercaria species: *C. pseudocellata* Szidat, 1942, *C. neocellata* Szidat, 1934 (both from *Stagnicola palustris*) and *C. parocellata* Szidat, 1942 (from *Radix peregra*). Finally, Neuhaus (1952) determined the complete life cycle of *C. parocellata* and established a new species sharing some important features with *T. ocellata*. As the name *T. parocellata* had been already occupied (Johnston and Simpson 1939), he named it *T. szidati* Neuhaus, 1952. Contrary to this, most authors tended to use the name *T. ocellata* widely for nearly all European findings of ocellate furcocercariae. The recent discovery of three distinct species can serve as an argument against such an approach: *T. franki* Müller and Kimmig, 1994 developing in *Radix auricularia* (Germany), *T. regenti* Horák, Kolářová and Dvořák, 1998 developing in *R. peregra* and *R. ovata* (Czech Republic), and *T. salmanticensis* Simon-Martin and Simon-Vicente, 1999 developing in *R. ovata* (Spain).

In North America, *C. elvae* Miller, 1923 was described from *L. stagnalis apressa* and *L. s. perampla*. McMullen and Beaver (1945) followed the life cycle of this schistosome and, based on egg morphology, they synonymized it with *T. ocellata*. In Japan, Chikami (1961) examined ducks infected by species of *Trichobilharzia*, which he regarded to be identical with *T. elvae*. He followed McMullen and Beaver (1945) and renamed this species *T. ocellata*.

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Farley (1971) and Blair and Islam (1983) proposed that *T. ocellata* represents a complex of species. This proposal was recently confirmed by recognizing *T. franki*, *T. regenti* and *T. salmanticensis*. Odening (1996) claimed that, according to the International Code of Zoological Nomenclature, one species from the *T. ocellata* complex should be assigned to the name *T. ocellata*; *T. szidati* Neuhaus, 1952 might be the candidate, as both species have the same intermediate host, type location and nearly identical morphology (McMullen and Beaver 1983; Odening 1996).

In the first part of this paper, we address the validity of various morphological characters for the determination of *T. ocellata*, *T. elvae* and *T. szidati* by reviewing data from the literature. In the second part, we present an analysis of the relationships and validity of the species of *Trichobilharzia* by using molecular techniques. By combining these approaches, we intend to clarify the confused taxonomy and nomenclature of the genus with particular respect to *T. ocellata*.

Materials and methods

For a comparison of morphology, behaviour and life cycles, we used articles on *T. ocellata* (La Valette de St. George 1855; Brumpt 1931; McMullen and Beaver 1945; Sluiters 1983; Chikami 1961; Kock and Böckeler 1998; Gay et al. 1999; Kock 2000, 2001,), *T. kossarewi* (Skrjabin and Zakharov 1920) and *T. szidati* (Neuhaus 1952; Kolářová and Horák 1996).

Parasites

For the experimental evaluation of the identity of *T. ocellata* isolates, sequencing of the internal transcribed spacer (ITS) region of DNA (including part of 18S rDNA, ITS1, 5.8S rDNA, ITS2 and part of 28S rDNA) was used. Cercariae of 12 bird schistosome isolates were analysed; DNA from *Dendritobilharzia pulverulenta* adults was used for comparison (Table 1).

Isolation of genomic DNA

After emerging from snails, the cercariae were centrifuged, fixed in 96% ethanol and stored at room temperature. Before DNA extraction, the cercariae were washed three times in sterile PBS buffer, pH 7.4. The genomic DNA was isolated using the High Pure DNA Template Preparation Kit (Roche).

PCR amplification of the ITS region

Polymerase chain reaction was carried out in a reaction volume of 20 µl, according to Dvořák et al. (2002). Specific primers its4Trem (5′-TCCTCCGCTTATTGATATGC-3′), complementary to the conserved region at the 5′-end of the 28S rRNA gene, and its5Trem (5′-GGAAGTAAAAGTCGTAACAAGG-3′), complementary to the conserved region at the 3′-end of the 18S rRNA gene, were used for amplification of the whole ITS region, which includes ITS1, 5.8S rRNA gene and ITS2 sequences. The reaction mixture contained 1 µl of the DNA template, 6 µM of each primer (Generi Biotech), 1× *Taq* polymerase buffer (Perkin Elmer), 3 mM $MgCl_2$ (Perkin Elmer), 400 µM dNTP (Promega) and 2 units of *Taq* polymerase (MBI). The reaction temperature profile included the initial denaturation step at 95°C for 5 min followed by 35 cycles (denaturation at 95°C for 60 s, annealing at 50°C for 45 s and extension at 72°C for 120 s) and a final extension step at 72°C for 10 min. Polymerase chain reactions were carried out in a PTC-200 thermal cycler (MJ Research).

Cloning

The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) from the PCR mixture. If the PCR resulted in more types of products, they were separated in agarose gel and the band of the expected size was isolated using the QIAquick Gel Extraction Kit (Qiagen). The PCR products were inserted into the pCR

Table 1 Isolates used for sequence analysis of ITS region. *ND* indicates not determined

Isolate	Determination	Host	Origin
Tsz	*Trichobilharzia szidati*	*Lymnaea stagnalis*	Laboratory strain, Prague, Czech Republic
ToA	*Trichobilharzia ocellata*	*L. stagnalis*	Laboratory strain, Amsterdam, The Netherlands
ToE	*T. ocellata*	*L. stagnalis*	Laboratory strain, Erlangen, Germany
Ls1	ND	*L. stagnalis*	Pond Schwarzenberg, Czech Republic
Ls2	ND	*L. stagnalis*	Pond of the village Běstvina, Czech Republic
Ls3	ND	*L. stagnalis*	Pond Smyslov, Czech Republic
Ls4	ND	*L. stagnalis*	Lake Kolbudy, Poland
Ls5	ND	*L. stagnalis*	Lake Soczewka, Poland
Ra1	*Trichobilharzia franki*	*Radix auricularia*	Pond Podkadovský, Czech Republic
Ra2	ND	*R. auricularia*	Lake Gorskie, Poland
Ra3	ND	*R. auricularia*	Lake Kujan, Poland
Pf	ND	*Physa fontinalis*	Pond Žabinec, Czech Republic
Dp	*Dendritobilharzia pulverulenta*	*Gallus gallus*	Experimental infection, New Mexico, USA

2.1-TOPO cloning vector and amplified in chemically competent *Escherichia coli* cells which were transformed using the TOPO TA Cloning Kit (version K2, Invitrogen). The preparation of plasmid DNA was performed using the QIAquick Spin Miniprep Kit (Qiagen).

Sequencing

Sequencing was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's protocol. Two clones from two independent amplification reactions of each DNA isolate were sequenced. To further substantiate sequence comparisons between species, three clones from three independent amplification reactions were sequenced. Sequencing primers M13F and M13R (Sigma) annealing to plasmid sites and specific primers annealing to the DNA insert—its4Trem, its5Trem, its2Trem (5′-GCTGCACTCTTCATCGACGC-3′) and its3Trem (5′-GCGTCGATGAAGAGTGCAGC-3′) were used for the sequencing reactions. The products of these reactions were analysed on an ABIPrism 310 automated sequencer (Perkin Elmer). The sequences were deposited in the GenBank database under the accession numbers AY713961–AY713973.

Phylogenetic analysis

Phylogenetic analysis was performed using the new sequences and sequences of *T. regenti*, *T. franki* and *T. szidati* downloaded from the database (accession numbers AF356845, AF263829, AF263828, respectively). Sequences of the genus *Schistosoma* (accession numbers AF531314, AF531312, Z21718, Z21716, Z21717, AY197345, AY197344, AY197343) were used as outgroups. Sequences were aligned in ClustalX 1.81 and the alignment was manually refined in Bioedit 5.0.9. The region comprising a part of ITS1, the whole 5.8S rDNA, ITS2 and part of 28S rDNA (852 positions, Fig. 1) was used for tree construction.

The phylogenetic analyses using maximum likelihood, maximum parsimony and distance methods were performed using PAUP4.0 (Swofford 2002). The tree space was searched by ten replicates of heuristic search, with the starting tree being constructed by random taxa addition and TBR swapping. The model for maximum likelihood (TrNef+I+Γ) and its parameters were chosen using the hierarchical likelihood ratio test implemented in Modeltest 3.06 (Posada and Crandall 1998). A distance tree was constructed using the Fitch-Margoliash method with logdet distances. Constant sites were excluded before the Fitch-Margoliash analysis in order to lower the rate-across-sites heterogenity. Node support was assessed by bootstrapping (100 replicates for maximum likelihood and 1,000 replicates for each maximum parsimony and distance analysis).

The phylogenetic analysis using the Bayesian method was performed with MrBayes 3.0 (Huelsenbeck and Ronquist 2001). Four simultaneous Markov-Monte Carlo chains (temperature 0.2) were run for 200,000 generations under the covarion GTR+I+Γ substitution model and sampled every 100 generations. The first 500 trees were discarded as the 'burnin'.

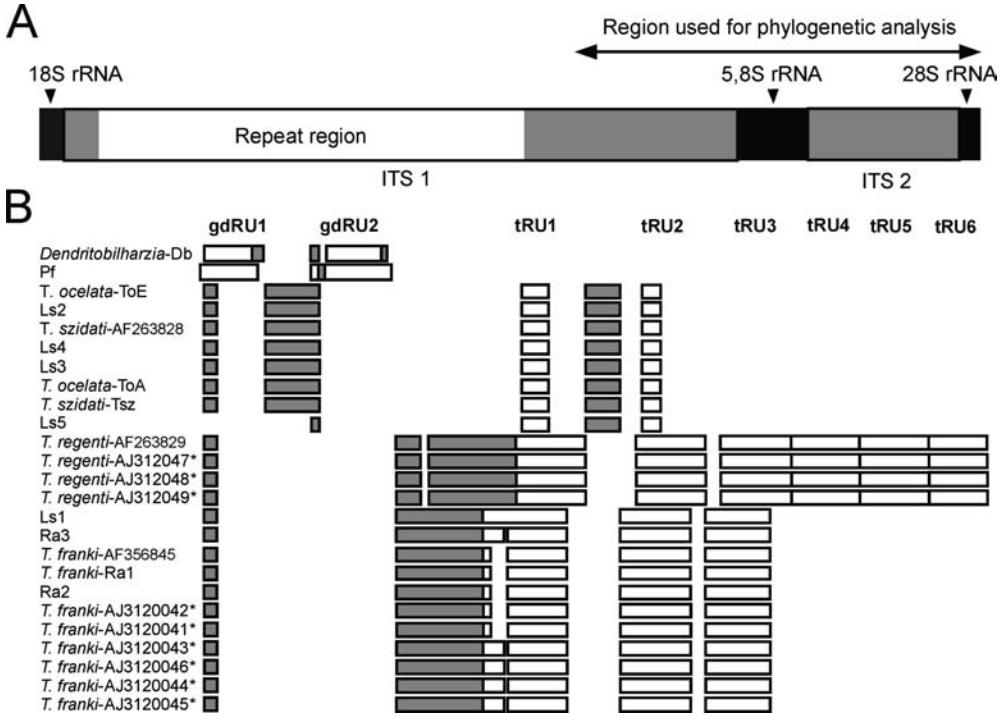

Fig. 1A, B Scheme of the ITS region of schistosome isolates. **A** Overall map of the region. *White* indicates repeat region, *grey* unique region of ITS1 and ITS2, and *black* rDNAs. The region used for phylogenetic analysis is marked by *arrows*. **B** A detailed scheme of the structure of the repeat region for isolates of *Trichobilharzia*, *Dendritobilharzia* and putative *Gigantobilharzia*. An *asterisk* indicates isolates of Picard and Jousson (2001) that were not included in the phylogenetic analysis, *white* indicates repeat units, *grey* unique sequences, *tRU Trichobilharzia* repeat unit, and *gdRU Gigantobilharzia* and *Dendritobilharzia* repeat unit. The alignment is available upon request (vlada@natur.cuni.cz)

Results and discussion

Morphology and ecology

Eggs

All authors have described the eggs of *T. ocellata* and *T. szidati* isolates as spindle shaped with a spine at one end and the second end rounded. The dimensions of the eggs described are similar (Table 2).

Eggs were found in the distal 3/5 of the small intestine and in the large intestine (Brumpt 1931), or in the whole intestine (McMullen and Beaver 1945). Neuhaus (1952) did not mention in what part of the intestine the eggs occurred. As he found the adults in the middle part of the intestine, the eggs probably came from the same location. In all of the above-mentioned cases, the eggs occurred in the intestinal wall. We suppose that the differences in egg location can be caused by migration of adults and are not important for species determination.

There are no significant differences in egg morphology between particular isolates. This led McMullen and Beaver (1945), Chikami (1961) and Sluiters (1983), whose determination of their *Trichobilharzia* isolates was based exclusively on egg morphology, to the conclusion that the isolates represent a single species. This approach is in our opinion problematic, as some *Trichobilharzia* species produce eggs similar to those of *T. ocellata* (e.g. *T. aureliani*, *T. spinulata*: Fain 1956), suggesting that egg morphology alone does not provide enough criteria for species determination.

Miracidia

The European and Japanese *T. ocellata* isolates differed in their miracidial characters: the pattern of ciliated plates was 9:6:4:3 = 22 in European (Kock 2000) and 5:7:6:4 = 22 in Japanese (Chikami 1961) isolates, and germinal cells occurred in a single cluster in European (Kock 2000) and were divided into two groups in Japanese (Chikami 1961) isolates. Whereas the number of clusters of germinal cells is probably of little importance for species determination, the arrangement of the ciliated plates may represent a valid criterion (Horák et al. 2002). Therefore, the difference between the two isolates implies that they do not belong to the same species.

Table 2 Dimensions of eggs of different *Trichobilharzia ocellata* and *T. szidati* isolates (in μm)

Author	Eggs	Length	Width
Brumpt[a] 1931		214–271	43–60
McMullen and	Newly laid eggs	160	28
Beaver 1945	Older eggs	220	60
Chikami 1961		206	56
Sluiters 1983	Fertile eggs	226 (178–273)	54 (40–67)
	Infertile eggs	205 (158–260)	46 (36–58)
Kock 2000		205–216	42–51
Neuhaus 1952		197–210	37–65

[a]No measurements are mentioned by the author; dimensions are based on comparison of the size of drawings and scales given in the paper. *T. kossarewi* eggs are not known. Talbot (1936) and La Valette de St. George (1855) observed only cercariae

Mother and daughter sporocysts

No clear characters of mother and daughter sporocysts were described as useful for species determination within *Trichobilharzia*. Exceptionally, spination of the anterior end is mentioned (Neuhaus 1952).

Cercariae

Intermediate host specificity In Europe, *T. ocellata* has been reported not only from *L. stagnalis* (e.g. Germany La Valette de St. George 1855, France Brumpt 1931, the Netherlands Sluiters 1983), but also from snails belonging to other species, genera and families (e.g. *Radix ovata*, *Galba* (= *Stagnicola*) *palustris*, and even *Anisus leucostomus*, *Planorbarius corneus*, *Bithynia leachi*: Kilias and Frick 1964). As far as is known, species of *Trichobilharzia* do not use snails from the families Planorbidae and Bithyniidae. In addition, findings from lymnaeids other than *L. stagnalis* probably belong to other schistosome species [e.g. *T. regenti* and *T. salmanticensis* from *R. ovata* (Horák et al. 1998; Simon-Martin and Simon-Vicente 1999), *T. franki* from *R. auricularia* (Müller and Kimmig 1994)]. The intermediate hosts of *C. elvae* in North America are *L. stagnalis appressa* and *L. stagnalis perampla* (Talbot 1936). In Japan, Chikami (1961) showed that *L. japonica* is a suitable intermediate host for *T. ocellata* (according to the new classification, this snail species belongs to the genus *Radix*).

The situation of *T. szidati* is somewhat complicated. Originally, *C. parocellata* Szidat, 1942 was only described from *R. peregra*. However, Neuhaus (1952) determined *C. parocellata* cercariae from both *L. stagnalis* and *R. ovata* (*R. peregra* and *R. ovata* are sometimes considered as identical species, e.g. Odening 1996), because they were indistinguishable based on their morphology and dimensions. He did not mention the snail species from which the cercariae were obtained for experimental infection of final hosts; he simply stated that only *L. stagnalis* was used for reinfection by miracidia of *T. szidati*. Therefore, some authors suppose that Neuhaus used only *L. stagnalis* for the life cycle of *T. szidati* (Odening 1996). This approach is also accepted in our work.

As far as is known, the intermediate host specificity of *Trichobilharzia* is narrow. As most species with known life cycles use only one snail species, intermediate host specificity seems to be an important character. Narrow specificity was determined under laboratory conditions (Kalbe et al. 1997, 2000; Kock 2001). These observations

suggest that different snail species probably host different species of *Trichobilharzia*. Thus, determining isolates from various snails species as *T. ocellata* (e.g. Voronin et al. 2002; Źbikowska 2004) is rather risky. The term *Trichobilharzia* sp. should be used in case the identity of cercariae cannot be proved using experimental infections or molecular analysis. Knowledge of the intermediate host narrows the spectrum of potential *Trichobilharzia* species found, but the exact determination needs to be experimentally confirmed, as one snail species can harbour a number of schistosome species (e.g. *T. salmanticensis* and *T. regenti* both use *R. ovata* (Horák et al. 1998; Simon-Martin and Simon-Vicente 1999), *L. lessoni* is used by *T. australis* (Blair and Islam 1983) and *T. parocellata* (Islam and Copeman 1986), and even *L. stagnalis* can host *T. szidati* (Neuhaus 1952) and *Trichobilharzia* sp. (from the pond Schwarzenberg, Czech Republic), as confirmed by gene sequencing (see below).

In view of host specificity, we suggest that the Japanese *T. ocellata* is not the same as *T. ocellata* sensu stricto, because *L. stagnalis* is not the intermediate host. The North American isolates of *T. ocellata* (synonym *T. elvae*) use only certain subspecies of *L. stagnalis*; this particular snail population is most likely geographically isolated from the European *L. stagnalis*. Therefore, we believe the North American and the European *T. ocellata* isolates do not belong to the same species. Contrary to this, *T. szidati* has the same intermediate host as the European *T. ocellata*, which supports our view that these species should be considered identical.

Morphology One of the most important characters of cercariae is the morphology of the excretory system. Except for two species, all *Trichobilharzia* have the same flame cell formula: 2 [3 + 3 + (1)] = 14. This formula was also reported for the Japanese and the North American *T. ocellata* (Talbot 1936; Chikami 1961) and the European *T. ocellata* (Kock 2000) and *T. szidati* (Neuhaus 1952; Kolářová and Horák 1996). The information on flame cells is missing in the work of La Valette de St. George (1855).

Meyer and Dubois (1954) determined ocellate furcocercariae based on the structure of the penetration glands and the position of eye spots. According to their system, *C. ocellata* La Valette de St. George, 1855, *C. elvae* Miller, 1923 and *C. szidati* Neuhaus, 1952 belong to the same group. Voronin et al. (2002) described variation in the arrangement of the excretory system, eyes and openings of the penetration glands between various *T. ocellata* populations, but the validity of these criteria has not been confirmed. All *Trichobilharzia* species possess five pairs of penetration glands (Blair and Islam 1983; Horák et al. 2002): two pairs of circumacetabular and three pairs of postacetabular penetration glands. The only exception is *C. ocellata* described by La Valette de St. George (1855): in the drawing and the text, only three pairs of postacetabular penetration glands are reported. In terms of the currently accepted genus morphology (Horák et al. 2002), this organism cannot belong to *Trichobilharzia*.

The dimensions of the cercariae of *T. szidati* and the European, the North American and the Japanese *T. ocellata* did not differ markedly (Table 3); the cercariae of *Trichobilharzia* are, however, contractile and, therefore, the fixative-dependent variability of the dimensions within one species may be high (Meyer and Dubois 1954). Cercariae of many species have dimensions similar to those of *T. ocellata* (e.g. *T. parocellata*, Islam and Copeman 1986). Therefore, the dimensions are of little importance for species determination.

Table 4 shows minor differences in cercarial chaetotaxy (characterization of surface papillae by staining with silver nitrate) among the isolates of *T. ocellata* and *T. szidati*. Unfortunately, the taxonomic value of chaetotaxy and papillae patterns is still unclear. Chaetotaxy enables species determination within the genus *Schistosoma* (Richard 1971). In the case of *Trichobilharzia*, however, it probably cannot be used for distinct species determination, because the differences in papillae patterns are minor. For *T. franki* and *T. ocellata* cercariae, Kock and Böckeler (1998) observed the same papillae pattern, while Gay et al. (1999) found one papilla in a different position.

Table 3 Dimensions of cercariae of different *T. ocellata* and *T. szidati* isolates (in μm). Brumpt (1931), McMullen and Beaver (1945) and Sluiters (1983) did not mention the dimensions of cercariae; cercariae of *T. kossarewi* are not known

	La Valette de St. George 1855	Talbot 1936	Chikami 1961	Kock 2000	Kolářová and Horák 1996	Neuhaus 1952
Body length	200–440	307 ± 9.7	295	272.3 ± 2.02	290–390	305.7
Body width	50–100	67 ± 6.5	67	50.4 ± 0.83	70–80	72.3
Anterior end-eye	130			106.9 ± 0.99		124.7
Anterior end-acetabulum				164.2 ± 1.28		190.0
Head organ length		97 ± 3.7	91		83–97	91.6
Head organ width		42 ± 3.5	42		46–59	54.7
Acetabulum	33	34 ± 0.8	24	16.8 ± 0.62	26–42×24–47	30.9
Tail stem length		400 ± 11.4	368	418.9 ± 3.95	330–456	431.0
Tail stem width	33	45 ± 4.1	46	42.9 ± 0.79		44.0
Furca length	300	254 ± 11.4	212	202.8 ± 2.29	192–246	247.0
Furca width	16	23 ± 3.9	30	27.4 ± 0.64	24–28	24.3
Eye	10–66			6.7 ± 0.03	8	8.0

Table 4 Differences in chaetotaxy among various *T. ocellata* isolates and *T. szidati*. Gay et al. (1999) divided the papillae on the ventral side of the furca into two types: FV at the base of the furca (one on each branch) and FT, which are situated terminally (zero to one on each branch). The other authors marked both types as FV. Similarly, Gay et al. marked two of the six papillae on the ventral side of the tail stem (UV) as UL, because they are situated ventrolaterally. Cercariae studied by the other authors differed slightly in the position of these two papillae; they are situated rather medially. However, the total number of ventral papillae on the tail stem is in all cases the same. Eklu-Natey et al. (1985) described the chaetotaxy of *T.* cf. *ocellata*, but as this isolate was found in the snails *Lymnaea* (= *Radix*) *peregra,* we can be sure that it did not belong to *T. ocellata* (intermediate host *L. stagnalis*)

	T. ocellata Richard (1971)	*T. ocellata* Kock and Böckeler (1998)	*T. ocellata* Gay et al. (1999)	*T. szidati* Kolářová and Horák (1996)
CIL	6	7	5–12	6–9
CIIIL	2	3	3	3
SI	3	3	0–2	5
FV	2	4	2–4*	4

Behaviour Cercariae were positively phototactic, as in the case of the Japanese isolate (Chikami 1961), or positively phototactic and negatively geotactic, as in the case of the European isolates (Neuhaus 1952; Kock 2000). In a resting position, *T. szidati* cercariae were attached to the container wall using only the acetabulum (Neuhaus 1952), whereas *T. ocellata* (= *T. elvae*) cercariae used both the acetabulum and oral sucker (McMullen and Beaver 1945). There was also a difference in the daily peak of cercarial emergence from snails: *T. szidati* cercariae had no definite daily peak of emergence (Neuhaus 1952); on the other hand, *T. ocellata* (= *T. elvae*) cercariae mostly emerged during the hours after dawn (Cort and Talbot 1936).

The behaviour of cercariae is probably an important determination character, but the conditions of observation need to be standardized. There are some differences between the European and the North American isolates, but due to uncertain observation conditions the validity of any comparison is questionable.

Adults

Localization Adults were found in the vessels of the inner intestinal wall (Brumpt 1931), in the mesenteric vessels (Brumpt 1931; Chikami 1961), or they occurred in the venous plexus of the submucosa and often extended to the villi (McMullen and Beaver 1945). Some isolates differed in localization, as the adults were found out of the vessels in the intestinal wall tissue (Neuhaus 1952; Kock 2000). Adults of *T. kossarewi* were found in the hepatic vessels (Skrjabin and Zakharov 1920). We suppose that the adults can migrate and, therefore, that their position can vary. As a consequence, the final location is probably of little importance for species determination within the visceral species of *Trichobilharzia*.

Morphology It is believed that the morphology of the digestive system (the position of the caecal bifurcation and reunion) and the morphology of the male reproductive system are the most important determination characters of species within the genus *Trichobilharzia* (Fain 1956; Blair and Islam 1983; Horák et al. 2002). The structure of the body surface and the shape of the posterior end are also used as criteria (Fain 1956). However, the information on the adult morphology of the European *T. ocellata* isolates is incomplete. In Brumpt's text and drawings (Brumpt 1931), no relevant and convincing morphological data on adults (compare with the definition of the genus by Khalil in 2002) were presented; the following structures were not characterized: gynaecophoric canal, acetabulum, genital pore, seminal vesicle, and seminal receptacle. Also, recent descriptions (Sluiters 1983; Kock 2000) do not contain any information on adults. Therefore, a comparison with *T. szidati* (Neuhaus 1952) was not possible.

The basic character of the genus is the presence of a gynaecophoric canal. However, the gynaecophoric canal is absent in Brumpt's drawing (1931). He probably did not observe this structure, as only fragments of adult worms were available for examination. On the other hand, Skrjabin and Zakharov (1920) did not mention the gynaecophoric canal in the description of *T. kossarewi*, but the widened body part described in the text and depicted in the drawing probably represents this structure. Testes in all findings were numerous and organized in one row (Skrjabin and Zakharov 1920; McMullen and Beaver 1945; Neuhaus 1952; Chikami 1961). There were no differences in the morphology of the male reproductive system among the isolates compared.

The isolates of *T. ocellata* (sensu McMullen and Beaver 1945 and Chikami 1961) and *T. szidati* did not differ in the position of the caecal bifurcation. This was situated anteriorly to the acetabulum in both sexes (McMullen and Beaver 1945; Neuhaus 1952; Chikami 1961). In addition, the position of the caecal reunion in males did not differ in these species. It was situated close to the anterior end of the seminal vesicle in all isolates (McMullen and Beaver 1945; Neuhaus 1952; Chikami 1961). Females, however, differed in the position of the caecal reunion. In Japanese and North American *T. ocellata*, it is located near posterior end of the seminal receptacle (McMullen et Beaver 1945; Chikami 1961), while in *T. szidati* it occurs before the seminal receptacle (Neuhaus 1952). Such differences indicate that the

European *T. szidati* and the Japanese and North American *T. ocellata* are not identical species.

The posterior end was depicted as spatulate (McMullen and Beaver 1945), trilobed (McMullen and Beaver 1945; Sluiters 1983), or rounded (Brumpt 1931; Sluiters 1983) in both sexes of the North American *T. ocellata*, and rounded in female and spatulate in male in *T. szidati* (Neuhaus 1952). The shape of the posterior end apparently depends on the state of the individual. The adults carried fine spines on the whole body in the case of *T. szidati* (Neuhaus 1952), or were without spines in the case of the North American *T. ocellata* (McMullen and Beaver 1945). The adults of *T. kossarewi* were without spines except for the acetabulum and the broadened body part (Skrjabin and Zakharov 1920). The value of these two characters (shape of the posterior end, spination) is questionable, because the authors observed the worms under different conditions and, in the case of spination, some fixation methods can lead to the loss of spines.

Some authors distinguished species by the dimensions of the adults (Fain 1956), but the validity of this character is disputable, as worms are very contractile and their dimensions can vary according to the method of fixation. However, the Japanese isolate has larger suckers than the other isolates (Tables 5, 6). This also implies that the Japanese isolate does not belong to *T. ocellata*.

Differences among *T. szidati* and various *T. ocellata* isolates are listed in Table 7.

Molecular analysis

As the cercariae of different *Trichobilharzia* species are indistinguishable, there are two possibilities for species determination: (1) experimental infection of the final host and determination of adults, and (2) sequence analysis of DNA (e.g. the ITS region). As the experimental infections are often unsuccessful, or only fragments of adults are found, sequence analysis is a more suitable approach for this purpose. Until the sequence analysis and/or determination of adults is done, all findings of cercariae should be labelled as *Trichobilharzia* sp.

The sequence of the ITS region seems to be suitable for this purpose (Kane and Rollinson 1994), and it is strongly recommended that characterizing this region forms an integral part of new species descriptions within the genus (e.g. Horák et al. 2002). In addition, sequencing the ITS region is convenient, as several *Trichobilharzia* ITS sequences are available in GenBank

Table 5 Dimensions of adult males of *T. ocellata*, *T. szidati* and *T. kossarewi* (in μm). La Valette de St. George (1855) and Talbot (1936) did not observe the adults; Sluiters (1983) and Kock (2000) did not mention the dimensions of adults

	Brumpt 1931	McMullen and Beaver 1945	Chikami 1961	Neuhaus 1952	Skrjabin and Zakharov 1920
Body length		> 5 mm	2.75 mm	3.05 mm	4 mm
Body width	27–40.5		62	20–34	
Oral sucker		20–32×16–20	45×41	28×18	
Acetabulum		9×18	65×52	19	50
Anterior end-acetabulum		320–400	412	430	690
Es. bifurcation-acetabulum		40–50			
Acetabulum-gyn. canal		30–36		230	
Gyn. canal length		60–100	285	220	
Gyn. canal width		28–40	75	30	
No. of testes			76	~70	
Testes	18–23×36–56	16–28×16–32	19–42×32–55	20	180×500
Seminal vesicle length		200–220	306	200	220
Seminal vesicle width		~120	75	12	

Table 6 Dimensions of adult females of *T. ocellata* and *T. szidati* (in μm). Sluiters (1983) and Kock (2000) did not mention the dimensions of adults. Females of *T. kossarewi* were not found

	Brumpt 1931	McMullen and Beaver 1945	Chikami 1961	Neuhaus 1952
Body length		2.7 mm	4.93 mm	3.0 mm
Body width	36–59 mm		68	20–36
Oral sucker		16–20x18–22	47×65	25×18
Acetabulum			51	16
Anterior end-acetabulum		320–370	766	270–310
Es. bifurcation-acetabulum		48–52	141	
Ootype length		160	169	
Genital pore-ootype		120		
Ovary		Diameter up to 20		340×22
Acetabulum-ovary		240–360		700
Vitelline follicles diameter		16–32		20–25

Table 7 Differences among *T. ocellata* and *T. szidati* isolates

	T. ocellata (Europe)	*T. szidati*	*T. ocellata* (Japan)	*T. ocellata* (North America)
Intermediate host	*L. stagnalis*	*L. stagnalis*	*R.* (*Lymnaea*) *japonica*	*L. s. perampla*L. *s. apressa*
Attaching		Acetabulum		Both suckers
Emerging		No peak		After dawn
Female caecal reunion		Before seminal receptacle	Near posterior end of seminal receptacle	Near posterior end of seminal receptacle

for comparison. Beside ITS, some authors have sequenced nuclear 18S rDNA, the 28S rRNA gene and the mitochondrial cytochrome oxidase I gene (Lockyer et al. 2003), a Sau3A tandemly repeated sequence (Hertel et al. 2002). At present, DNA sequence data are available for only four species: *T. regenti*, *T. szidati*, *T. ocellata* and *T. franki*.

Phylogenetic relationship between isolates of bird schistosomes

We used the ITS region to assess the relationship between 13 isolates of bird schistosomes. A map of the ITS region of *Trichobilharzia* is given in Fig. 1a. This region includes a long repeat region flanked by hypervariable regions. The extensive sequence diversity between isolates and the presence of tandem repeats complicated the alignment of this region and disabled its use for phylogenetic analysis. The phylogenetic analysis was, thus, based upon the unique part of the ITS1, 5.8S rDNA, ITS2 and 28S rDNA (Fig. 1a). The phylogenetic tree including the sequences of our isolates and the database sequences of bird schistosomes is given in Fig. 2. The tree was rooted using *Schistosoma* sequences.

The first clade in the tree was formed by the sequence of the *Dendritobilharzia* isolate (Dp) and the isolate Pf. The bootstrap support of this clade was moderate (58%, 73% and 89% for distance, maximum parsimony and maximum likelihood analysis, respectively) and its Bayesian posterior probability was 0.99. Strong support for the existence of this clade came from the structure of the repeat region (Fig. 1b), as both isolates shared specific homologous repetitions that differed in their location from repetitions in other isolates.

The second clade was formed by all *Trichobilharzia* isolates and the database sequences of *T. szidati*, *T. regenti* and *T. franki* (bootstrap support 86–100%, posterior probability 1.00). The *Trichobilharzia* clade was divided into three main branches: (1) the database sequence of *T. szidati*, isolate Tsz determined as *T. szidati*, isolates ToA and ToE determined as *T. ocellata*, and undetermined isolates Ls2, Ls3, Ls4 and Ls5, (2) the database sequence of *T. regenti*, and (3) the database sequence of *T. franki*, isolate of *T. franki* Ra1 and undetermined isolates Ls1, Ra3, and Ra2. The latter two branches were in a sister relationship. The described topology of the *Trichobilharzia* clade had high nodal support (bootstraps 87–100%, posterior probability 1.00).

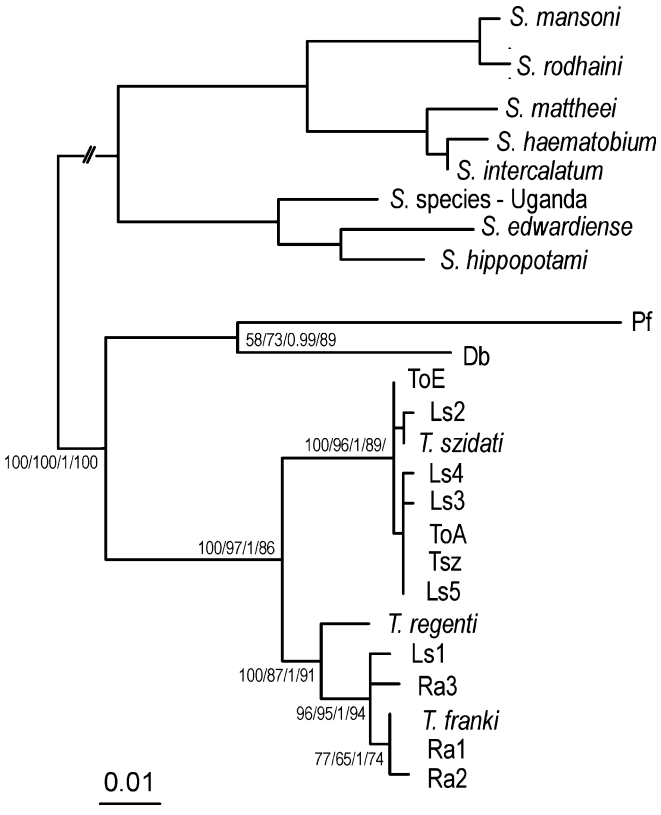

Fig. 2 The phylogenetic tree of isolates of bird schistosomes based on the partial sequence of the ITS region. The sequences of the genus *Schistosoma* were used as outgroups. The tree was constructed using the maximum likelihood method in PAUP 4.0 (TrNef + I + Γ model of substitution). The node support is given in distance bootstraps, maximum parsimony bootstraps, bayesian posterior probabilities and maximum likelihood bootstraps

Species status of the bird schistosome isolates

The sister position of isolate Pf to the *Dendritobilharzia* sequence and its position outside of the *Trichobilharzia* clade indicates that this isolate does not belong to the genus *Trichobilharzia*. Molecular analysis of 18S and 28S rDNA revealed the genus *Gigantobilharzia* as a sister group of the genus *Dendritobilharzia* (Lockyer et al. 2003), which implies that isolate Pf belongs to the genus *Gigantobilharzia*. This point can also be confirmed by the fact that, in addition to *Trichobilharzia*, the only genus of ocellate furcocercariae developing in physid snails is *Gigantobilharzia* (Farley 1971).

All other isolates belonged to a common clade with database sequences of three species of the genus

Trichobilharzia, suggesting that they represent isolates of this genus. Species determination based only on tree topology is difficult, because the border between species cannot be drawn objectively in the tree. However, in the case of our isolates, discrete species specific characters were found in the structure of the repeat region. The schematic representation of the repeat region is given in Fig. 1b. For the sake of completeness, nine more sequences of isolates of *T. regenti* and *T. franki* (Picard and Jousson 2001) were added to the scheme (asterisk). These isolates could not be used in the phylogenetic analysis, as the sequences of ITS2 and 5.8S rDNA are not available. The structure of the region was fully consistent with the phylogenetic tree. In *T. szidati*, *T. ocellata* and related isolates, the region contains two copies of repetitive elements separated by a unique sequence. In the isolates determined as *T. franki* (*T. franki* and Ra1) and the related isolates Ls1, Ra2, Ra3, the region contained three tandem repeats. In all *T. regenti* isolates, the region contained six tandem repeats. Although they differed in copy number, length and sequence, the single repeat units were homologous in all *Trichobilhazia* isolates. The alignment of the repeat units in the three *Trichobilharzia* species is given in Dvořák et al. (2002).

Repetitive sequences evolve at a higher rate than unique sequences, so that, usually, they differ substantially even between closely related species (Charlesworth et al. 1994). The similarity in the structure of the repeat region within the three described branches of the *Trichobilharzia* clade strongly suggested that these branches represent three distinct species. The isolates Ls1, Ra3, Ra1 and Ra2 are conspecific with the database sequence of *T. franki*, and so can be designated as *T. franki*. None of the isolates sequenced by us belonged to the species *T. regenti*, which in our analysis was represented only by the database sequence. The nomenclature and the acceptance of the conspecifity of the third group of isolates was complicated by the fact that the isolates ToE and ToA were formerly determined as *T. ocellata*, while isolate Tsz and the database sequence were determined as *T. szidati*. All isolates in this branch were very similar in sequence. In the entire ITS region (including the repeat and hypervariable regions), they differed only in 13 characters. The most parsimonious tree based upon these differences is given in Fig. 3. The isolates determined as *T. ocellata* and the isolates determined as *T. szidati* are intermixed in this tree. This indicates, together with the similarity in the repeat region, that the differentiation between *T. ocellata* and *T. szidati* within this group of isolates is groundless, and they all represent a single species.

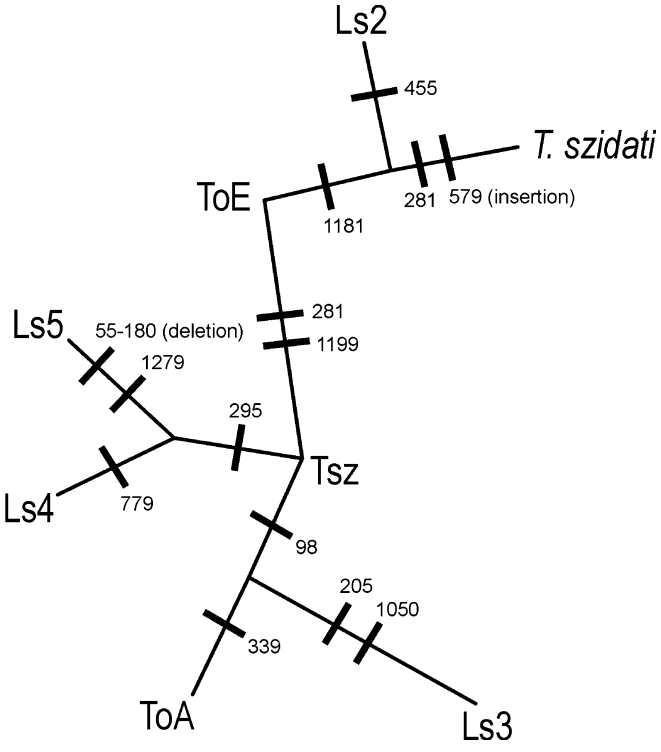

Fig. 3 Phylogenetic tree of the isolates of *Trichobilharzia szidati* and *T. ocellata* constructed on the basis of the complete ITS region using maximum parsimony. Each variable character is marked as a *thick line* with a *number* indicating its position in the alignment. All variable characters except 281 and 1,181 are consistent. Due to the inconsistency between 281 and 1,181, the part of the tree with isolates ToE, Ls2 and *T. szidati* can have two equally parsimonious topologies. In the second one, the isolates ToE and Ls2 are related to the exclusion of *T. szidati*

Conclusions

The analysis of published morphological and life cycle data on *Trichobilharzia* species from *L. stagnalis*, supported by the molecular analysis of selected isolates, shows that it is necessary to reassess our view on the position of *T. ocellata* (presently the type species) within the genus, and to resolve long-held confusion caused by reports of *T. ocellata* in the field. In terms of the validity of species, it can be concluded that:

1. *C. ocellata* La Valette de St. George, 1855 cannot be considered as a member of the genus *Trichobilharzia*, because the cercariae contain an unusual number (only three pairs) of penetration gland cells.
2. *T. kossarewi* Skrjabin and Zakharov, 1920 is the first description of *Trichobilharzia*, although the gynaecophoric canal is not specifically highlighted. Nevertheless, the remaining diagnostic features of the genus are included in the work. Unfortunately, the description of *T. kossarewi* is too general (no species specific structures are mentioned) and, therefore, it cannot be distinguished from the other species. For the reasons outlined under (3) we propose to retain the name of *T. kossarewi*.
3. *T. ocellata* Brumpt, 1931 is probably the most problematic description having caused considerable trouble in *Trichobilharzia* systematics. Although many important data are missing, the description might

represent a *Trichobilharzia* species, but there is no reason to synonymize it with *T. kossarewi* [in our current view there are insufficient data to compare the findings of Brumpt (1931) and Skrjabin and Zakharov (1920)]. There is no description of the cercariae in Brumpt's work and it seems to be doubtful whether cercariae with only three pairs of penetration glands (see the description of La Valette de St. George 1855) were used in the experiment, and adults resembling a *Trichobilharzia* species (namely *T. kossarewi*) were recovered.

4. The recent European *T. ocellata* descriptions (Sluiters 1983; Kock 2000) correspond with *T. szidati* in intermediate host specificity, and the morphology of eggs and cercariae. Sequence analysis of the ITS region showed that both the laboratory isolates from *L. stagnalis* named as *T. szidati* or *T. ocellata*, and the isolates of *Trichobilharzia* sp. from naturally infected *L. stagnalis*, are identical. It is therefore strongly recommended that the name *T. ocellata* be suppressed and the name *T. szidati* be used exclusively for all of these schistosomes, as *T. szidati* represents a morphologically well-defined species. In contrast, the name *T. ocellata* is a freely used conventional term for ocellate furcocercariae or adults of different origin which may belong to different *Trichobilharzia* species.
5. We suggest that the Japanese isolate (Chikami 1961) does not belong to *T. ocellata*. It differs markedly from the European isolates in its intermediate host. Moreover, the Japanese isolate differs from *T. szidati* (identical with *T. ocellata* sensu Sluiters 1983; Kock and Böckeler 1998; Gay et al. 1999; Kock 2000, 2001) in the position of the caecal reunion in females and the dimensions of the suckers in adults.
6. The North American *T. ocellata* isolate differs from *T. szidati* in the position of the caecal reunion in females and the behaviour of cercariae. They develop in certain subspecies of *L. stagnalis* occurring exclusively in North America, probably with no links to the European *L. stagnalis* population. Therefore, we assume that the North American *T. ocellata* isolate is not identical with the European *T. ocellata* (= *T. szidati*).
7. Molecular analysis of the ITS region confirmed the existence of a cryptic *Trichobilharzia* species from *L. stagnalis*, which is indistinguishable based on cercarial morphology and intermediate host specificity, but differs markedly in the ITS region sequence.
8. The structure of the repeat region in ITS1 appears to be a suitable marker for the discrimination of closely related *Trichobilharzia* species.

In order to avoid any confusion, future descriptions of *Trichobilharzia* species should contain at least the following data: a description of egg and adult morphology, intermediate host specificity, and the sequence of rDNA deposited in a suitable database (e.g. GenBank) for future comparison.

Acknowledgements We wish to thank Prof. W. Haas from the University of Erlangen (Germany) and Dr. M. de Jong-Brink from the Vrije Universiteit (The Netherlands) for providing us with *T. ocellata* cercariae and Dr. E. Żbikowska from Nicholas Copernicus University (Poland) for providing us with cercariae from naturally infected snails. This work was supported by the Czech Ministry of Education (grant no. J13/981131-4), by the Czech Ministry of Health (grant no. NJ7545-3/2003) and by The Wellcome Trust (grant no. 072255). The experiments comply with the current laws of the Czech Republic and the United Kingdom.

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