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Trypanosoma (*Megatrypanum*) *melophagium* in the Sheep Ked *Melophagus ovinus* from Organic Farms in Croatia: Phylogenetic Inferences Support Restriction to Sheep and Sheep Keds and Close Relationship with Trypanosomes from Other Ruminant Species

FRANJO MARTINKOVIĆ,[a] KREŠIMIR MATANOVIĆ,[b] ADRIANA C. RODRIGUES,[c] HERAKLES A. GARCIA[c,d] and MARTA M. G. TEIXEIRA[c]

[a]*Department for Parasitology and Parasitic Diseases with Clinics, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10000, Zagreb, Croatia, and*
[b]*Department for Microbiology and Infectious Diseases with Clinic, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10000, Zagreb, Croatia, and*
[c]*Instituto de Ciências Biomédicas, Universidade de São Paulo, Avenue Professor Lineu Prestes 1374, 05508-900, São Paulo, SP, Brazil, and*
[d]*Departamento de Patologia Veterinaria, Faculdad de Ciencias Veterinarias, Universidad Central de Venezuela, Maracay, Venezuela, and*

ABSTRACT. *Trypanosoma* (*Megatrypanum*) *melophagium* is a parasite of sheep transmitted by sheep keds, the sheep-restricted ectoparasite *Melophagus ovinus* (Diptera: Hippoboscidae). Sheep keds were 100% prevalent in sheep from five organic farms in Croatia, Southeastern Europe, whereas trypanosomes morphologically compatible with *T. melophagium* were 86% prevalent in the guts of the sheep keds. Multilocus phylogenetic analyses using sequences of small subunit rRNA, glycosomal glyceraldehyde-3-phosphate dehydrogenase, spliced leader, and internal transcribed spacer 1 of the rDNA distinguished *T. melophagium* from all allied trypanosomes from other ruminant species and placed the trypanosome in the subgenus *Megatrypanum*. Trypanosomes from sheep keds from Croatia and Scotland, the only available isolates for comparison, shared identical sequences. All biologic and phylogenetic inferences support the restriction of *T. melophagium* to sheep and, especially, to the sheep keds. The comparison of trypanosomes from sheep, cattle, and deer from the same country, which was never achieved before this work, strongly supported the host-restricted specificity of trypanosomes of the subgenus *Megatrypanum*. Our findings indicate that with the expansion of organic farms, both sheep keds and *T. melophagium* may re-emerge as parasitic infections of sheep.
Key Words. Evolution, host-specificity, molecular taxonomy, morphology, phylogeny, artiodactyls, *Trypanosoma theileri*.

TRYPANOSOMA (*Megatrypanum*) *melophagium* is a non-pathogenic parasite of domestic sheep (*Ovis aries*) transmitted by the sheep ked *Melophagus ovinus*. This flagellate, first recorded in sheep keds from Germany in 1905, was thought to be a monoxenous insect trypanosomatid named *Crithidia melophagia*. The relationship between this ked-trypanosomatid and sheep was proven using laboratory-bred keds for the xenodiagnosis of sheep inoculated with trypanosomatids from sheep keds. The trypanosome isolated from the gut of sheep ked and blood of sheep was named *T. melophagium* (Hoare, 1923a,b, 1972).

Sheep keds infected with *T. melophagium* have been found in temperate zones of Europe (i.e. Germany, England, Scotland, Holland, and Yugoslavia), Asia (i.e. Russia, China, and Pakistan), Africa (i.e. Tunisia, South Africa), Canada, and South America (i.e. Argentina and Brazil) (Costa et al. 1983; Gibson, Pilkington, and Pemberton 2010; Hoare 1923a,b, 1972; Lu 1975; Mackerras 1959; Nelson 1981; Talat, Khanum, and Hayat 2005; Turner and Murnane 1930).

Prior to insecticide control, sheep keds used to be of veterinary importance as they cause massive skin lesions and dermatitis in infected sheep and are vectors of several diseases (Small 2005). The distribution of sheep keds overlapped that of its hosts, the European sheep, except in tropical areas (Bequaert 1952). The sheep ked *M. ovinus* is an obligatory, bloodsucking ectoparasite of the Hippoboscidae, a family of wingless Diptera strictly associated with sheep for their entire life; there are no free-living stages, and transmission occurs via intimate body contact in sheep herds (Bequaert 1952; Small 2005). Hippoboscidae of the genera *Melophagous* and *Lipoptena* are known to act as vectors of *T. melophagium* (vector: sheep ked), *Trypanosoma theodori* (vector: goat ked), and *Trypanosoma cervi* (vector: deer ked). These three trypanosome species are all members of the subgenus *Megatrypanum*. Hippoboscids of other genera are vectors of avian trypanosomes (Böse and Petersen 1991; Hoare 1972; Mansfield 1977; Molyneux 1977; Molyneux and Stiles 1991; Votýpka et al. 2002).

Trypanosoma melophagium was originally known from the developmental forms in the gut of sheep keds, where multiplication of flagellates and development of infective forms were restricted to the digestive tubes. Despite causing heavy infections, *T. melophagium* are apparently harmless to sheep keds, and transmission from insect to insect does not occur. Infection occurs when sheep eat infected keds. Due to the extremely low parasitemia in the infected sheep, blood trypomastigotes are rare, and the only reliable method of diagnosing *T. melophagium* infection in sheep is by blood culture. Cryptic infection may be detected by cultures of total blood samples or peripheral blood lymphocytes (Hoare 1923a,b, 1972). *Trypanosoma melophagium* and *Trypanosoma theileri* (the latter is the type species of the subgenus *Megatrypanum*, and is a cattle parasite that is transmitted by tabanids), are the only species of *Megatrypanum* whose life cycles have been studied in vertebrate and invertebrate hosts. The separation of these two species takes into account the lack of natural and experimental cross-infection between trypanosomes of cattle and sheep, in addition to the host-restriction of sheep keds (Hoare 1923a,b, 1972; Molyneux 1977; Molyneux and Stiles 1991; Wells 1976).

After a long absence from the literature, *T. melophagium* was recently reported in the blood of domestic sheep in Pakistan (Talat et al. 2005), Turkey (Nalbantoglu and Karaer 2008), and in keds from feral sheep in Scotland (Gibson et al. 2010). One isolate of *T. melophagium* recently obtained from

Corresponding Author: Marta M. G. Teixeira, Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, SP, 05508-900, Brazil—Telephone number: 55 11 3091 7268; FAX number: 55 11 3091 7417; e-mail: mmgteix@icb.usp.br

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Scottish keds, which was described as a lineage of *T. theileri,* slightly diverged on partial small subunit (SSU) rRNA and spliced leader (SL) sequences when compared with *T. theileri* from cattle and a *Megatrypanum* sp. from deer (Gibson et al. 2010). However, the positioning of *T. melophagium* in the phylogenetic tree of *Trypanosoma* and its relationships with other species has not yet been addressed. The phylogenetic analyses of trypanosomes of the subgenus *Megatrypanum* from cattle, water buffalo, deer, and antelopes revealed two main lineages and several, apparently host-specific genotypes (Garcia et al. 2011a,b; Rodrigues et al. 2003, 2006, 2010a,b).

In this study, we carried out a survey of sheep keds in sheep herds from organic farms in Croatia and obtained a culture of trypanosome from this fly, which was classified as *T. melophagium* according to its morphology in both the sheep ked gut and culture, together with its positioning in the phylogenetic tree of *Trypanosoma*. We compared the Croatian *T. melophagium* with a previously reported isolate of this species from Scotland, and evaluated the host-restriction of this species by inferring its phylogenetic relationships with allied trypanosomes of the subgenus *Megatrypanum* from other ruminants using sequences from SSU rRNA, glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH), SL, and internal transcribed spacer 1 (ITS1) of the rDNA.

MATERIALS AND METHODS

Survey of trypanosomes in sheep and sheep keds by culture. This study was carried out from March to May in 2005 and 2006 on clinically healthy, adult sheep that were not treated with insecticides and that were living in five organic farms in different regions of Croatia: Goljak (45°56′S, 15°6′E), Josani (46° 63′S, 23°13′E), Raducic (44°04′S, 16°07′E), Kuterevo (44°82′S, 15°14′E), and Cakovci (45°23′S, 19°05′E). Sheep keds *M. ovinus* were manually collected from the fleece of sheep, dissected, and their midguts and hindguts were examined by microscopical analysis. Blood samples collected from the jugular vein of randomly chosen sheep using EDTA as anticoagulant were smeared in glass slides, stained with Giemsa and microscopically examined for trypanosomes. The gut contents of trypanosome-positive keds were individually inoculated into culture tubes containing Biphasic Chocolate Agar Medium for Trypanosomatids (BCAT) medium (Franjo Martinković in prep.) [Correction made here after initial online publication.]. The BCAT medium consists of a solid phase of chocolate agar (3.0% (w/v) of Blood Agar Base containing 10.0% (v/v) of canine blood autoclaved for 20 min) overlaid with a liquid phase of Peptone-Yeast extract (P-Y) medium (Limoncu et al. 1997), supplemented in grams/liter of medium by 15 g peptone; 2.5 g yeast extract; 8 g NaCl; 7.5 g Na_2HPO_4; 1.0 g glucose; 0.4 g KCl; 0.06 g $Na_2HPO_4{\cdot}12H_2O$; 0.06 g KH_2PO_4; 0.1 g $MgSO_4{\cdot}7H_2O$; 0.1 g $MgCl_2{\cdot}6H_2O$; 0.15 g $CaCl_22H_20$, and 5.0% (v/v) fresh human urine sterilized by filtration in a 0.22 μm membrane. To control bacterial contamination, 1.0 mg/ml of streptomycin and 1,000 IU/ml of penicillin were added to this medium.

The cultures were incubated at 25 °C and checked microscopically every 3 d until the 30th day of incubation. After successive passages, one established culture, named as *T. melophagium* TmHR1, was expanded for DNA preparation, and preserved in liquid nitrogen in the Trypanosomatid Culture Collection (code number TCC1993), Department of Parasitology, University of São Paulo, São Paulo, SP, Brazil.

Growth behavior and morphological characterization of the sheep ked trypanosomes. The growth behavior of the trypanosome from the sheep ked was compared by co-culturing the trypanosomes in BCAT medium and with a monolayer of Hi-5 insect cells (*Trichoplusia ni*) overlaid with liquid TC100 medium (=Grace's medium) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hi5-TC100) as employed for other trypanosomes (Rodrigues et al. 2003; Viola et al. 2009). Cultures were incubated at 25–28 °C and checked twice weekly for 20 d to assess growth and differentiation of the flagellates. Epimastigotes from Hi5-TC100 cultures were transferred to monolayers of mammalian HeLa cells cultured with Roswell Park Memorial Institute (RPMI) medium supplemented with 5% FBS and incubated at 37 °C with 5% CO_2.

For morphological analysis of the trypanosomes, ked guts were dissected, divided into anterior (midgut) and posterior (hindgut) portions, and smeared on glass slides. Smears were also prepared from different culture conditions. Smears on glass slides were fixed with methanol, stained with Giemsa, and photographed.

Barcoding and phylogenetic inferences using sequences from SSU rRNA, gGAPDH, ITS rDNA, and SL. Trypanosomes used in this study are listed in Table 1. DNA of cultured trypanosomes was obtained by classical phenol-chloroform extraction. For the DNA barcoding, we employed ~ 900-bp DNA fragment consisting of V7–V8 region of SSU rRNA, PCR-amplified and sequenced as described previously, as was the entire SSU rRNA from *T. melophagium* determined in this study (GenBank accession no: HQ664912) (Ferreira et al. 2007; Rodrigues et al. 2006). The gGAPDH sequences from *T. melophagium* (GenBank accession no: HQ664807) was obtained as previously described (Hamilton, Gibson, and Stevens 2007; Viola et al. 2009).

Sequences were aligned (CLUSTALX) (Thompson et al. 1997) and manually refined. Three alignments consisting of independent and combined data of these two genes were created based on a previous alignment (Garcia et al. 2011b; Hamilton et al. 2007) : (1) V7–V8 SSU rRNA (~ 730 bp) sequences from 23 isolates of *Megatrypanum* representative of all genotypes and sequences determined in this study from trypanosomes of sheep ked, cattle, and deer (GenBank accession no listed in Table 1) from Croatia, using trypanosomes of the clade *Trypanosoma cyclops* as the outgroup for the subgenus *Megatrypanum* (769 characters, 75 parsimony informative); (2) gGAPDH sequences (~ 847 bp) of the sheep ked isolate, and sequences from 18 representative of all *Megatrypanum* genotypes (847 characters, 126 informative); (3) alignment 1 (V7–V8 SSU rRNA) concatenated with gGAPDH sequences (1,960 characters, 920 informative) from the new isolate of sheep ked plus 23 isolates from cattle, water buffalo, antelopes, and fallow deer of the subgenus *Megatrypanum* spp., and 28 species representatives of all major clades of *Trypanosoma*. Parsimony (P) and bootstrap (100 replicates) analyses were carried out using PAUP* 4.0b10 (Swofford 2002) with 100 replicates of random addition sequences followed by branch swapping (RAS-TBR). ML analyses were performed using RAxML v.7.0.4 (Stamatakis 2006), and tree searches were conducted using GTRGAMMA with 500 maximum parsimony starting trees. Model parameters were estimated in RAxML over the duration of the tree search. Nodal support was estimated using 500 bootstrap replicates in RAxML with GTRGAMMA and maximum parsimony starting trees. Bayesian analysis was performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). For tree searches, we employed GTR + gamma and a proportion of invariable sites. The first 25% of the trees from 100,000.000 generations were discarded as burn-in (Viola et al. 2009).

Length and sequence polymorphisms of ITS1 rDNA were assessed for *T. melophagium* and *T. theileri* from Croatia (GenBank accession no: HQ664850–HQ664855) as described

Table **1.** Host and geographic origin of trypanosomes of the subgenus *Megatrypanum* and Genbank accession numbers of sequences included in this study.

				GenBank acession number			
Trypanosome isolate	TCC [a]	Host origin	Geographic origin	V7–V8 SSU	gGAPDH	ITS1rDNA	SL
Trypanosoma melophagium							
StKilda	–	Sheep ked	Scotland	FN666409	–	–	FN666410
TmHR1	1993	Sheep ked	Croatia	**HQ664912**[b]	**HQ664807**[b]	**HQ664850–HQ664852**[b]	**HQ664890–HQ664892**[b]
Trypanosoma theileri							
TthHR1	1992	Cattle	Croatia	**JN798503**[b]	–	**HQ664853–HQ664855**[b]	**HQ664893–HQ664894**[b]
Tthc1	–	Cattle	Brazil	HQ664905	HQ664792	HQ664817	HQ664863
Tthc2	171	Cattle	Brazil	AY773679	–	AY773707	HQ664864
Tthc3	161	Cattle	Brazil	AY773681	HQ664793	AY773698	GQ162125
Tthc8	–	Cattle	Brazil	AY773682	–	–	–
Tthc9	–	Cattle	Brazil	AY773683	–	–	–
Tthc10	–	Cattle	Brazil	AY773684	–	–	–
Tthc5	–	Cattle	Brazil	AY773689	–	AY773711	–
Tthc12	–	Cattle	Brazil	AY773690	–	–	–
Tthc14	–	Cattle	Brazil	AY773692	–	–	–
Tthc16	–	Cattle	Brazil	AY773687	–	–	–
Tthc29	1459	Cattle	Brazil	GQ176155	HQ664799	HQ664818	–
Tthc30	1460	Cattle	Brazil	GQ176157	HQ664801	–	GQ162134
Tthc32	1462	Cattle	Brazil	GQ176158	HQ664802	–	HQ664882; GQ162136
Tthc37	1787	Cattle	Brazil	GQ176159	HQ664803	–	HQ664883
Tthc38	1788	Cattle	Brazil	GQ176160	–	–	HQ664866; HQ664867
Tthc39	–	Cattle	Brazil	HQ664906	HQ664794	HQ664823	HQ664868; HQ664869 HQ162160
Tthc40	–	Cattle	Brazil	HQ664907	HQ664795	HQ664825	HQ664870–HQ664872 HQ162164
Tthc41	–	Cattle	Brazil	HQ664908	HQ664796	HQ664827	HQ664873–HQ664875
TthcV2	–	Cattle	Venezuela	HQ664909	–	HQ664829	HQ664878; HQ664879
TthcV4	–	Cattle	Venezuela	HQ664910	HQ664797	HQ664830	HQ664880; HQ664881
TthcV5	–	Cattle	Venezuela	HQ664911	HQ664798	–	–
K127	–	Cattle	Germany	AJ009164	AJ620282	–	–
TREU124	–	Cattle	Scotland	AJ009163	HQ664800	–	HQ664876; HQ664877
Tth KM	–	Cattle	Japan	AB007814	–	–	–
Trypanosoma theileri-like							
Tthb3	163	Buffalo	Brazil	AY773674	–	–	–
Tthb4	162	Buffalo	Brazil	AY773675	–	AY773701	–
Tthb6	165	Buffalo	Brazil	AY773676	–	AY773699	GQ162118
Tthb10	166	Buffalo	Brazil	HQ664895	HQ664784	HQ664808	HQ664857
Tthb12	–	Buffalo	Brazil	AY773677	–	AY773702	–
Tthb13	–	Buffalo	Brazil	AY773678	–	AY773703	GQ162121
Tthb14	1858	Buffalo	Brazil	HQ664896	HQ664785	HQ664809	HQ664859
Tthb19	1855	Buffalo	Brazil	HQ664899	HQ664788	HQ664811	–
CepCamp4	–	Duiker	Cameroon	FM202491	HQ664804	HQ664832–HQ664836	HQ664889
CepCamp5	–	Duiker	Cameroon	FM202490	HQ664805	HQ664837–HQ664841	HQ664888
SitaBip1	–	Sitatunga	Cameroon	FM202489	FM164792	HQ664842–HQ664844	HQ664886; HQ664887
D30	–	Deer	Germany	AJ009165	HQ664806	AY773714	HQ664884; HQ664885
TC3	1959	Deer	Croatia	**JN798599**[b]	–	**JN798602**[b]	–

[a]TCC, code number at Trypanosomatid Culture Collection from the Department of Parasitology, University of São Paulo, São Paulo, Brazil.
[b]Sequences determined in the present study are in bold.

before (Maia da Silva et al. 2004; Rodrigues et al. 2006). Amplification and sequencing of SL genes from the trypanosomes of sheep ked (~ 900 bp) and cattle (~ 880 bp) from Croatia (GenBank accession no: HQ664890–HQ664894) were performed in this study as described previously (Rodrigues et al. 2010a). Sequences of 3–5 clones of each gene from each isolate were determined, and those representing polymorphisms were used for phylogenetic inferences. Sequences of ITS1 rDNA (~ 267 bp) and partial SL sequences (~ 400 bp of transcript plus partial intergenic regions) were aligned with sequences from other *T. theileri* trypanosomes from GenBank (accession no in Table 1) and employed for Network analysis using the Neighbor-Net method with the Kimura 2-parameter as implemented in Splits Tree4 V4.10 (Huson and Bryant 2006). Internode support was estimated by 100 bootstrap replicates using the same parameters optimized for network inferences. All alignments employed in this study are available from the corresponding author by request.

RESULTS

Prevalence of sheep keds and detection of trypanosomes infecting sheep keds and sheep. All sheep examined in Croatia that were not treated with insecticides against ectoparasites and that were bred in natural conditions on five organic farms had sheep keds in their fleece during our surveys in 2005 and 2006. We detected trypanosomes in 372 of 433 sheep keds from 134 sheep sampled at all farms with an overall preva-

lence of 86.0% (372/433). The prevalence of trypanosomes in sheep keds was always high and varied in the farms analyzed: minimum of 50.0% in Cakovci, 66.6% in Raducic, 72.0% in Josani, 83.3% in Kuterevo, to a maximum of 94.5% in Goljak. Blood smears from 134 sheep infested with sheep keds microscopically examined for trypanosomes were always negative, despite 86% prevalence of sheep keds infected with trypanosomes.

Developmental and morphological analysis of trypanosomes in the gut of sheep keds. Morphological analysis of Giemsa-stained smears of gut contents of sheep keds from Croatia allowed following the development of *T. melophagium* in these vectors as reported by Hoare (1923a), who showed by detailed descriptions with drawings, the development stages of this species in sheep keds. Here, we selected microphotographs of *T. melophagium* found in naturally infected sheep keds to illustrate the development of this species in its vector (Fig. 1–14).

Analysis of Giemsa-stained flagellates in smears of the gut contents from sheep keds disclosed developing forms similar to those reported by Hoare (1923a,b). The midguts of most sheep keds were heavily infected with great masses of epimastigotes attached to the gut epithelium by their flagella (Fig. 1, 2), and arranged in large agglomerates in the lumen, showing flagellates multiplying by binary fission (Fig. 3). A large

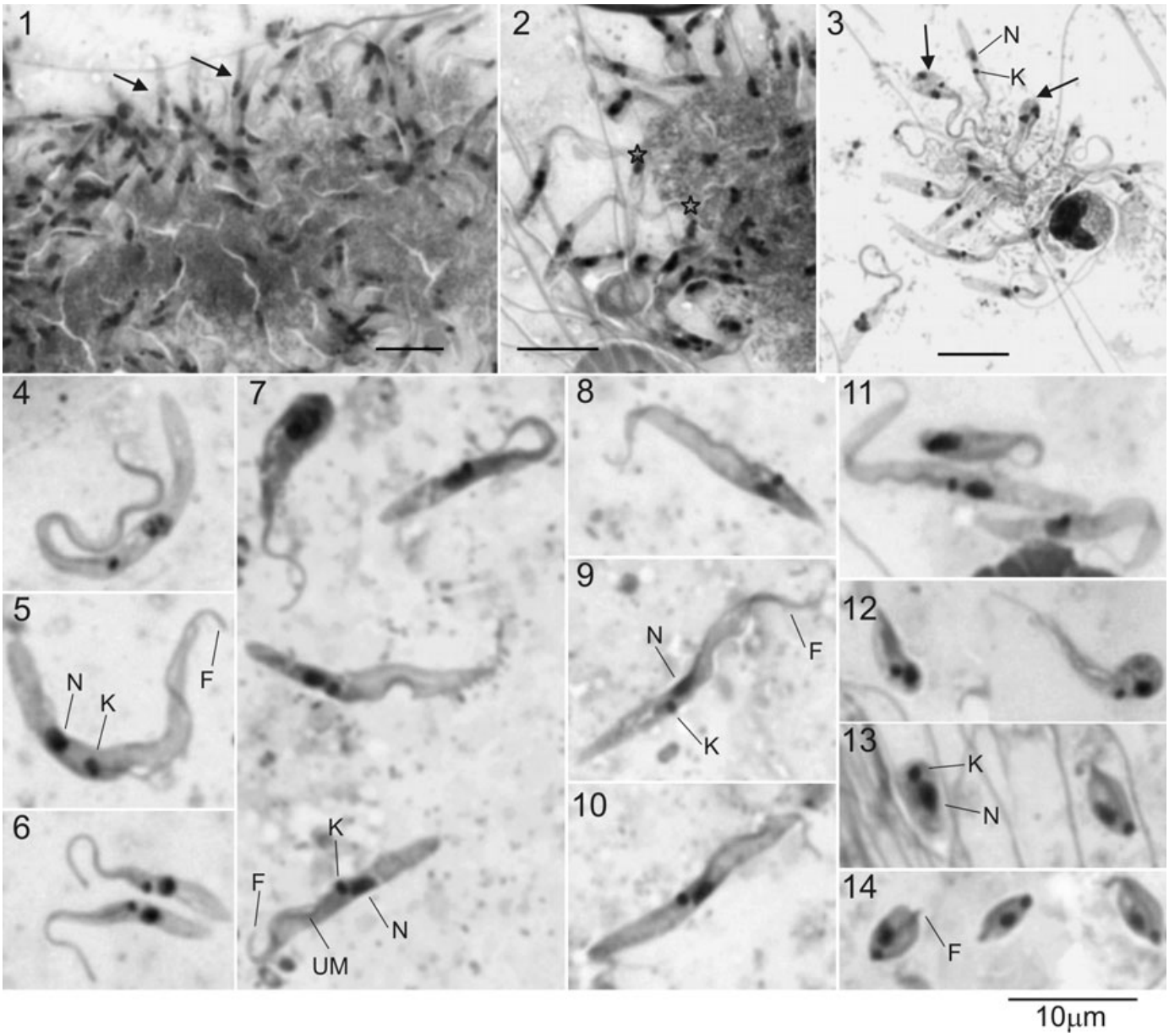

Fig. **1–14.** Developmental stages of *Trypanosoma melophagium* observed in the digestive tract of sheep keds. **1, 2.** Large numbers of epimastigotes (arrows) attached to the midgut epithelium by their flagella (stars). **3.** Agglomerates of epimastigotes in the lumen of the gut showing dividing flagellates (arrows). **4–7.** Free epimastigotes of variable size with the kinetoplast (K) close to the nucleus (N), thin undulating membrane (UM) and short-free flagellum. **8.** Transition form of epimastigote to trypomastigote. **9**, **10.** Slender trypomastigotes. **11, 12.** Short epimastigotes, probably, transition forms to metacyclic trypomastigotes. **12–14.** Elliptical metacyclic trypomastigotes in the final portion of the guts with the flagellum running internally along the body and showing a short-free portion.

number of free epimastigotes varying in shape and size with body length ranging from 14 to 37.6 μm were common in the guts (Fig. 4–7). Most epimastigotes were long and slender, with a rounded posterior end, the kinetoplast very close to the nucleus, thin undulating membrane closely adhered to the body, and short-free flagellum (Fig. 7). Some transition forms between trypo- and epimastigotes, as indicated by the lateral position of the kinetoplast (Fig. 8), and slender trypomastigotes (Fig. 9, 10) were found mixed with the epimastigotes.

In the hindguts, we observed transition forms between epimastigotes and trypomastigotes (Fig. 11, 12), and high relative abundance of metacyclic trypomastigotes (Fig. 12–14). Metacyclic forms are oval with the kinetoplast relatively large and disposed at the extreme end of the body; the flagellum runs internally along the body showing a short-free portion (Fig. 12–14). These forms, which clearly differed from metacyclic trypomastigotes of *T. theileri*, were found adhered to the chitinous layer of the intestine walls before being eliminated with the feces, thus closing the development of *T. melophagium* in the sheep ked (Hoare 1923a).

Growth behavior in distinct media and culture conditions. A culture of sheep ked trypanosomes was obtained from the gut of one fly collected from a sheep from Goljak, Croatia, using BCAT medium. The behavior and cell differentiation of this sheep ked isolate was investigated from the logarithmic phase to the 15th day of stationary phase using BCAT medium and cocultivation with monolayers of Hi5 cells in TC100 medium. Both culture conditions allowed successive cultures of flagellates showing some differences (Fig. 15–28). In BCAT cultures, at 25 °C we obtained the largest number of flagellates. Epimastigotes multiplied by binary division, and their kinetoplast is generally positioned adjacent to the nucleus, the undulating membrane is not well developed, and they have long flagella (Fig. 15). In stationary cultures, some small trypomastigotes were observed (Fig. 16, 17). BCAT cultures incubated at 37 °C showed some relatively large trypomastigotes, besides small forms (Fig. 18–20). In the Hi5-TC100 cultures at 28 °C, flagellates initially multiplied as small epimastigotes adhering to the cell surface, which gradually became large epimastigotes free in the supernatant (Fig. 21); trypomastigotes were not observed. Typical metacyclic trypomastigotes, as observed in the gut of sheep keds, were not observed in any culture.

When epimastigotes from Hi5 cultures were transferred to monolayers of HeLa cells, incubated at 37 °C, they initially became very large and dividing epimastigotes (Fig. 21–24); after the 4th day, most forms differentiated to trypomastigotes of variable length (Fig. 25–28). Some trypomastigotes in the supernatant of the HeLa cell culture were very large and pointed at the posterior end; the kinetoplast was not adjacent to the nucleus, and they showed a conspicuous undulating membrane and long-free flagellum (Fig. 25). These large forms resembled the trypomastigotes in the blood of sheep (Hoare 1972).

DNA barcoding of the sheep ked trypanosome. A BLAST search of the V7–V8 rRNA sequence determined in this study for the new trypanosome from Croatian sheep ked (GenBank accession number: HQ664912) showed 100.0% match with sequences from *T. melophagium* from Scottish sheep ked (accession number: FN666409) (Gibson et al. 2010). The sequence of *T. melophagium* is more similar to the sequences of trypanosomes from deer (from Germany and Croatia), antelopes, and cattle of the lineage TthII (~ 99.4% of sequence similarity) than the sequences of the isolates from cattle and water buffalo of the lineage TthI (~ 97.8%). *Trypanosoma melophagium* from Croatia are 0.96% divergent in the V7–V8 rRNA sequences from the cattle *T. theileri* from Germany, Scotland, and Brazil, and substantial divergence (~ 2.2%) from the genotype found in cattle from Croatia, which sequence is identical to those from other cattle isolates (accession number: AY773681) all assigned to the TthIB, comprising isolates from Brazil, the USA, and Japan of the lineage TthI (Fig. 29A).

Phylogenetic positioning of *Trypanosoma melophagium* based on SSU rRNA and gGAPDH gene sequences. Corroborating results from barcoded V7–V8 SSU rRNA and divergences of whole SSU rRNA sequences confirmed that *T. melophagium* is more related to cattle and deer trypanosomes of the lineage TthII. However, these sequences are highly conserved among these trypanosomes and, hence, generated poorly resolved phylogenetic relationships among these trypanosomes.

The gGAPDH sequences previously determined for trypanosomes from cattle, water buffalo, deer, sitatunga, and duiker (Garcia et al. 2011b; Hamilton et al. 2009) were aligned with sequences determined in this study for *T. melophagium*. Unfortunately, the gGAPDH sequence is not available for the Scottish isolate of *T. melophagium* (Gibson et al. 2010). The Croatian sheep ked isolate was confirmed to be within the lineage TthII of the subgenus *Megatrypanum* and was separated by large nucleotide divergences from all other trypanosomes within this lineage: 4.25% divergence from both German isolates from cattle (*T. theileri* K127) and fallow deer (D30), 4.5% and 4.6% divergence, respectively, from cattle *T. theileri* from Scotland and Brazil, 4.5% divergent from the isolate of the sitatunga antelope, and 5.8% and 6.0% divergent from the duiker isolates CepCamp4 and CepCamp5, respectively. Large divergences (minimum 8.3% divergence) separate *T. melophagium* from trypanosomes of cattle, including one isolate from Croatia (8.4%) nested into the lineage TthI.

A combined data set of V7–V8 rRNA and gGAPDH genes was employed to infer phylogenetic relationships aiming to provide better support for the positioning of *T. melophagium* in the phylogenetic tree of *Trypanosoma* and its relationships with other *Megatrypanum* trypanosomes. All inferences (i.e. MP, ML, and BI analyses) generated congruent phylogenetic trees strongly supporting the positioning of *T. melophagium* within the lineage TthII. The topology generated by the combined data set was concordant with divergences among the trypanosomes, but the values supporting the internal branching patterns were still insufficient to resolve the relationships among the trypanosomes within TthII (Fig. 29B). Nevertheless, divergences are enough to warrant the status of a separate species for *T. melophagium*.

Molecular diagnosis and phylogenetic analysis using ITS1 rDNA. Molecular diagnosis of the sheep ked trypanosome was carried out by PCR amplification of the ITS1 rDNA that generated a fragment that shows a small amount of length polymorphism among species of the subgenus *Megatrypanum* (Fig. 30A). This method is useful to distinguish these species from all pathogenic trypanosomes that share ruminant hosts (Rodrigues et al. 2006). The length of amplified and sequenced ITS1 rDNA from *T. melophagium* was slightly smaller (285 bp) than the ITS1 rDNA of fallow deer (290 bp), cattle (305–317 bp), and water buffalo (305 bp) isolates.

Network analysis of polymorphic ITS1 rDNA sequences clearly separated *T. melophagium* from all other trypanosomes of the subgenus *Megatrypanum* corroborating SSU rRNA and gGAPDH data (Fig. 30B). Sequences of ITS1 rDNA exhibited higher divergences of *T. melophagium* compared with the trypanosomes of the TthI (43.0%) than to TthII (~ 23.0%) lineages with comparable divergences from cattle of genotypes IIA (~ 22.0%) and IIB (~ 22.7%), duikers (~ 2.0–24.0%), deer

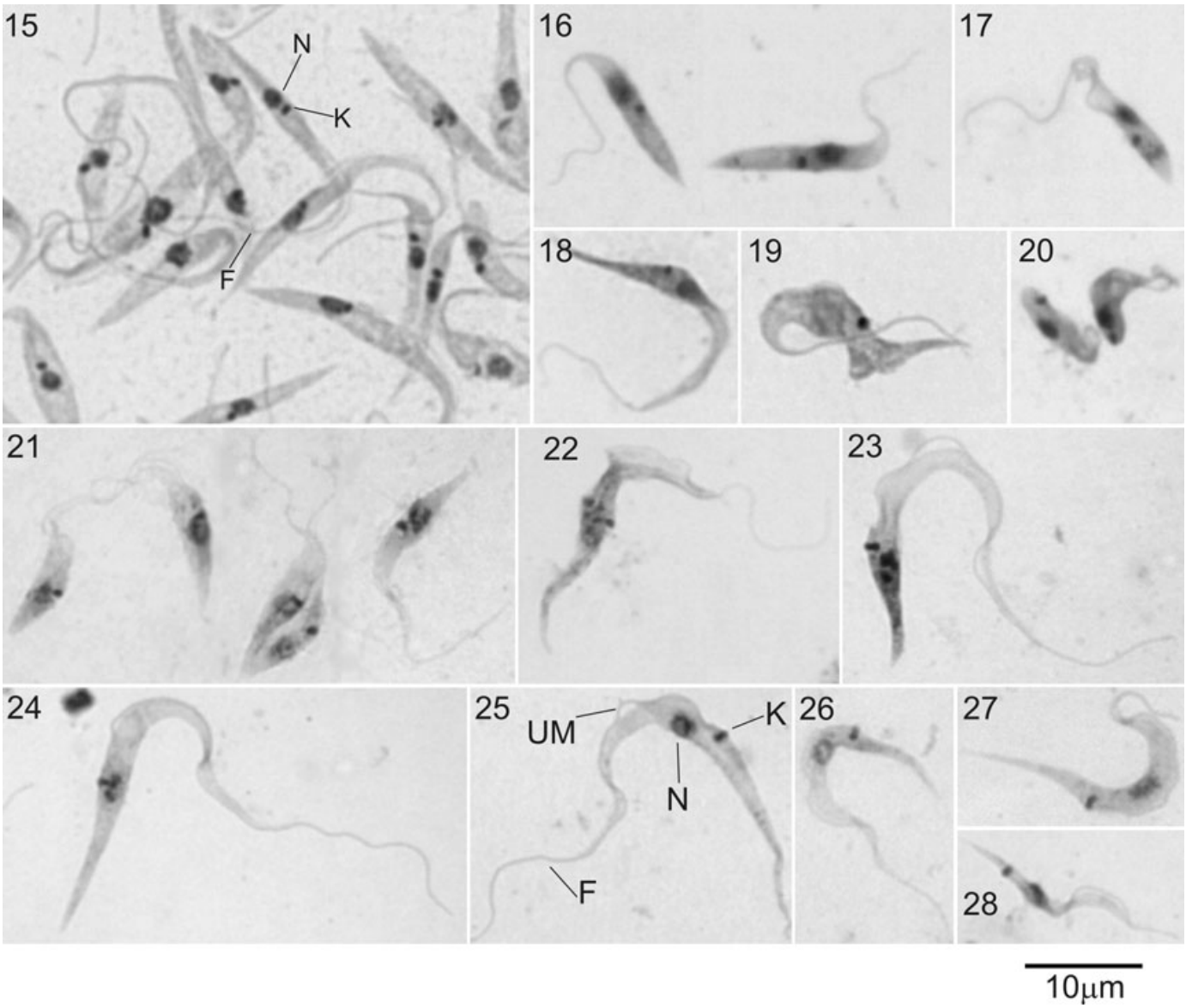

Fig. **15–28.** Forms of *Trypanosoma melophagium* from distinct media and culture conditions. **15.** Epimastigotes from logarithmic culture in Biphasic Chocolate Agar Medium for Trypanosomatids (BCAT) medium at 25 °C showing the kinetoplast (K) adjacent to the nucleus (N) and a long-free flagellum (F). **16, 17.** Trypomastigotes at stationary culture phase. **18–20.** Trypomastigote forms in BCAT cultures at 37 °C. **21.** Epimastigotes from Hi5-TC100 cultures at 28 °C. **22–24.** Transition forms of epimastigotes to large trypomastigotes in the supernatants of HeLa cells at 37 °C showing large trypomastigotes variable in length. **25–28.** Trypomastigotes pointed at the posterior end, showing noticeable undulating membrane (UM) and long-free flagellum (F) resembling trypomastigotes reported in blood of sheep.

(~ 24.0%), and sitatunga (~ 23.0%). Sequences from cattle (TthHR1) and deer isolates from Croatia were identical to sequences from previously characterized isolates from cattle (TthIB) and deer (D30), respectively (Table 1).

Characterization of SL gene repeats from *Trypanosoma melophagium* and phylogenetic analysis. We initially investigated the polymorphisms of whole SL gene repeats from both sheep ked and cattle isolates from Croatia. The sequence divergence among the four to five cloned copies of SL repeats within the same isolate was small for both *T. melophagium* (~ 0.7%) and *T. theileri* (~ 0.8%). Alignment of the transcript (exon and intron) plus partial intergenic sequences (~ 400 bp) of these isolates with available SL sequences from *Megatrypanum* spp. (Garcia et al. 2011b; Gibson et al. 2010; Rodrigues et al. 2010a) revealed identical sequences for the two isolates of *T. melophagium,* whereas they clearly diverged from all other trypanosomes in the intergenic regions. Despite large divergences, the SL-network confirmed the closest relationship of *T. melophagium* to TthII (i.e. IIA, 18.0%; IIB, 22.0%; and IIC, 22.0%) compared with TthI (~ 41.0%) genotypes (Fig. 31A).

Furthermore, *T. melophagium* and *T. theileri* exhibited a copy of the 5S rRNA gene inserted into the intergenic region of the SL repeats and almost identical secondary structures (Fig. 31B). In agreement with data from SL and other genes compared in this study, the 5S rRNA sequence of *T. melophagium* was identical to sequences from the cattle

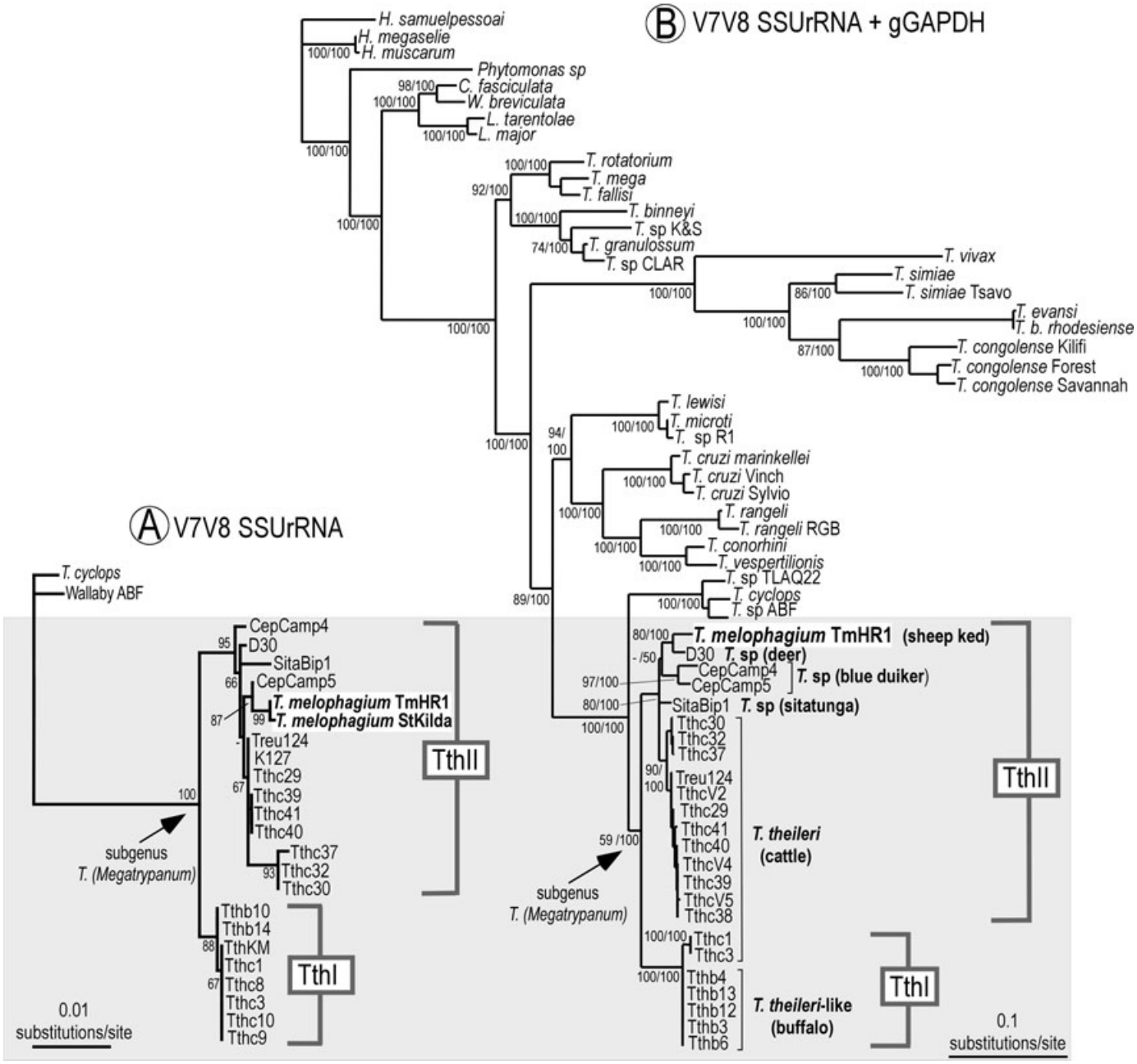

Fig. **29.** Barcoding and phylogenetic inferences of *Trypanosoma melophagium*. (**A**) Phylogram inferred by maximum likelihood using V7–V8 SSU rRNA sequences of 17 isolates of the subgenus *Megatrypanum,* including isolates representative of the two phylogenetic lineages (TthI and TthII) and genotypes (TthIA-C and TthIIA-G). The numbers at nodes are bootstrap values derived from 500 replicates. (**B**) Phylogenetic tree inferred by maximum likelihood based on concatenated V7–V8 rRNA and (gGAPDH) sequences (1,329 characters, −Ln = 25680.127478) from 23 trypanosomes of *Megatrypanum* and 28 other trypanosomes representatives of all major clades within the genus *Trypanosoma* using nontrypanosomes trypanosomatids as outgroups. Values at nodes are ML/MP support values derived from 500 replicates (−support value < 50).

genotypes of TthII while it diverged ~ 28% from the sequence of the cattle isolate from Croatia that was placed within the TthI lineage.

DISCUSSION

In this study, *T. melophagium,* a trypanosome of sheep transmitted by sheep keds, was positioned through multilocus analysis in the phylogenetic tree of *Trypanosoma* for the first time confirming its affiliation with trypanosomes of cattle, water buffalo, deer, and antelopes. It nested together with *T. theileri* in the subgenus *Megatrypanum* (Garcia et al. 2011b; Hoare 1972; Rodrigues et al. 2006). The inclusion of *T. melophagium* in the subgenus *Megatrypanum* provided additional support to the observation that the trypanosomes nesting in the monophyletic assemblage containing *T. theileri* from cattle,

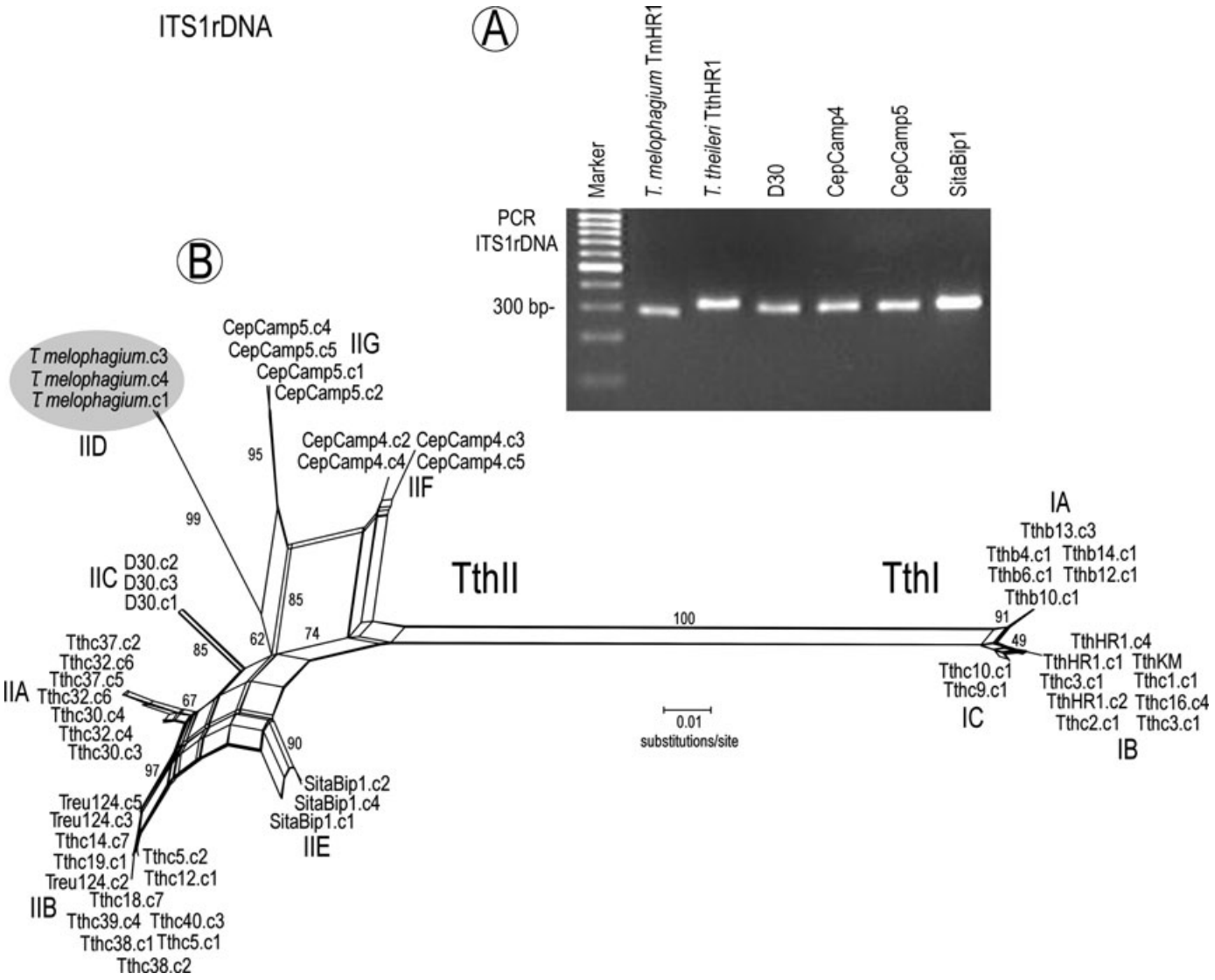

Fig. **30.** Molecular diagnosis of *Trypanosoma melophagium* and phylogenetic analysis using internal transcribed spacer 1 (ITS1) rDNA sequences. (**A**) Agarose gel (2%) stained with ethidium bromide of PCR-amplified ITS1 rDNA sequences from *T. melophagium* (sheep ked) and other trypanosomes of the subgenus *Megatrypanum: T. melophagium* TmHR1 from sheep ked; *T. theileri* TthHR1 from cattle, D30 from fallow deer, *Trypanosoma* sp. CepCamp4 and CepCamp5 from blue duikers and *Trypanosoma* sp. SitaBip1 from Sitatunga. (**B**) Network genealogy of ITS1 rDNA sequences from *T. melophagium* and 30 trypanosomes from other ruminant species representative of all genotypes within the subgenus *Megatrypanum* inferred using the Neighbor-Net method with the Kimura 2-parameter model. The codes used for ITS1 rDNA sequences indicate the isolates of *Trypanosoma theileri* followed by the number of cloned copy of ITS1 rDNA sequence. Trypanosomes of this subgenus were distributed in two lineages, TthI and TthII, comprising the genotypes TthIA-C and TthIIA-G.

the type species of the subgenus *Megatrypanum*, are exclusive of ruminants, domestic or wild, and widespread throughout the world (Hamilton et al. 2009; Rodrigues et al. 2006, 2010a, b). The complexity within the subgenus *Megatrypanum* by phylogenetic analyses disclosed by analyses of SSU rRNA, gGAPDH, ITS rDNA, SL, and cathepsinL-like) sequences revealed two main lineages (TthI and TthII) and 10 genotypes associated with the host species examined: four genotypes from cattle, one from water buffalo, one from deer, two from duikers, and one from sitatunga (Garcia et al. 2011a,b; Rodrigues et al. 2006, 2010a,b). We demonstrated that *T. theileri* from cattle and water buffalo are different, even when obtained from animals sharing the same pastures, supporting host-restriction (Garcia et al. 2011b; Rodrigues et al. 2006, 2010a,b). In contrast to studies that compared several isolates from cattle and water buffalo, only one isolate from each other ruminant species has been molecularly analyzed so far, thus hindering the understanding of genotypes, distribution, and reliability of hypotheses of host-association and evolutionary history of *Megatrypanum* spp.

Although it has been known for more than 100 yr that sheep and sheep keds are hosts of *T. melophagium*, data are mostly restricted to morphology in sheep keds and short-term cultures. The only available molecular data were from a Scottish sheep ked isolate (Gibson et al. 2010). Surveys on the prevalence of *T. melophagium* in sheep require haemoculturing, and cryptic infection is thought to occur in all temperate zones of the world where sheep are infested with keds

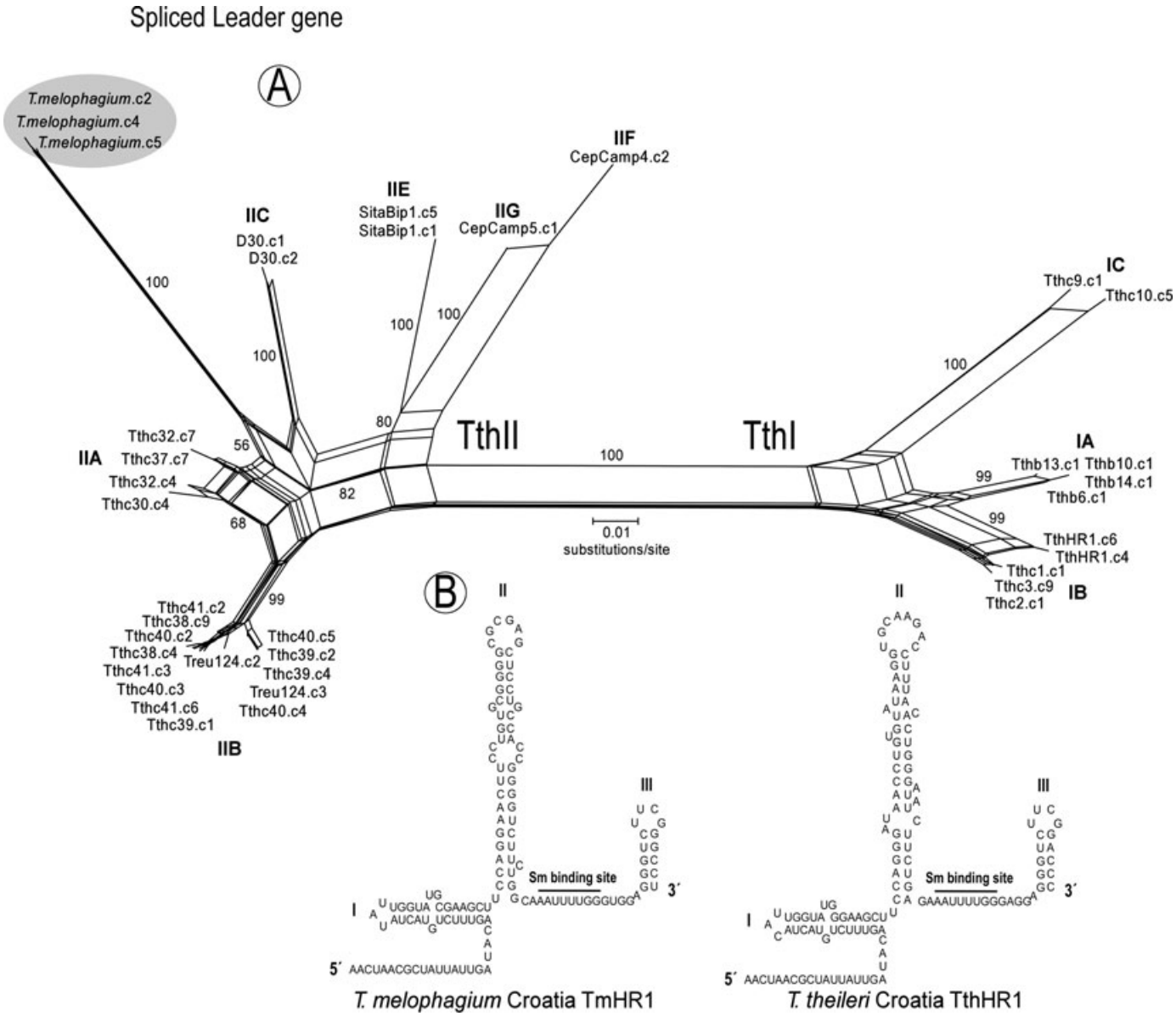

Fig. **31.** Characterization of splice leader (SL) gene from *Trypanosoma melophagium* and phylogenetic analysis. (**A**) Network genealogy of SL sequences corresponding to intron and partial intergenic regions (538 characters) from *T. melophagium* and 35 sequences from 22 isolates of *Trypanosoma theileri* trypanosomes inferred using the Neighbor-Net method with Kimura 2-parameter model. *Trypanosoma melophagium* sequences are in a gray circle. The codes used for SL sequences indicate the names of *T. theileri* isolates, followed by the number of cloned and sequenced SL gene. (**B**) SL RNA structure of *T. melophagium* and *T. theileri* from Croatia.

(Hoare 1972). Increased parasitemias were only found in splenectomized and cortisone-treated sheep infested with sheep keds infected with *T. melophagium* (Büscher and Friedhoff 1984).

Although sheep keds have almost been eradicated from domestic sheep by treatments against their ectoparasites (Small 2005), they were recently reported from feral sheep in Scotland (Gibson et al. 2010). In the survey we carried out in Croatia, we found sheep keds infected by *T. melophagium* in all organic farms investigated. With the expansion of organic farms and the prohibition of insecticides, the sheep ked and its parasite *T. melophagium* may be re-emerging. Our attempts to detect trypanosomes on blood of sheep were unsuccessful, despite the fact that the sheep were heavily infested with keds carrying *T. melophagium*. These results agreed with previous attempts to detect *T. melophagium* in sheep blood samples (Hoare 1923a,b, 1972; Wells 1976).

The developmental forms of *T. melophagium* were reported from the guts of sheep keds corresponding to those described by Hoare (1923a), including the large number of long epimastigotes attached to the gut wall by their flagella. Electron microscopy studies revealed that the epimastigotes of *T. melophagium* were attached to the ked gut wall by hemidesmosomes, and showed a fibrous material surrounding the flagellates (Büscher and Friedhoff 1984; Heywood and Molyneux 1985; Molyneux 1975; Molyneux, Selkirk, and Lavin 1978). The behavior of the flagellates we have observed in cultures with or without mammalian cells agree to previously

reported for *T. theileri* and *T. melophagium* (Gibson et al. 2010; Hoare 1972; Rodrigues et al. 2003).

In the present study, phylogenetic analysis using SSU rRNA, gGAPDH, ITS1 rDNA, and SL sequences strongly supported *T. melophagium* as a separate species and members of the subgenus *Megatrypanum*. The polymorphic sequences of V7–V8 SSU rRNA and SL gene of *T. melophagium* from Croatia and Scotland are identical, whereas they largely differed from *T. theileri* isolates from the cattle from Croatia and Scotland. In fact, *T. melophagium* was more related to deer than to cattle isolates from Croatia. As in this study, a previous study of *Megatrypanum* distinguished between Scottish *T. theileri* and *T. melophagium* through differences in DNA buoyant densities (Newton 1971). The phylogenetic relationship of trypanosomes from sheep, cattle, and deer from the same country, which was never addressed before this study, strongly supported the host-restricted specificity of these trypanosomes.

Multilocus phylogenetic analyses positioned *T. melophagium* distant from all other trypanosomes of the subgenus *Megatrypanum* supporting its taxonomic status as a separate species, not a simple genotype of *T. theileri*. On the other hand, even the analysis of the polymorphic ITS1 rDNA and SL sequences failed to distinguish the sheep ked isolates from Scottish feral sheep (Gibson et al. 2010) and domestic sheep from Croatia. Investigation of isolates from other regions will be important to determine whether *T. melophagium* dispersed accompanying the worldwide dispersion of domestic sheep, which is thought to have been domesticated in the Near East at the middle of the 11th millennium, and then dispersed to Europe and elsewhere (Vigne 2011).

Findings from this study support the restriction of *T. melophagium* to sheep and, especially, to sheep keds. However, incongruence between phylogenies from the species of the subgenus *Megatrypanum* and their ruminant hosts suggests that switches of trypanosomes between ruminant hosts played an important role in the evolutionary history of the subgenus *Megatrypanum*. The data suggest the origin of *T. melophagium* from a trypanosome that adapted to transmission by sheep keds and evolved to be a parasite specific for sheep, as a consequence of the host-restriction of sheep keds to sheep. This hypothesis is also supported by the lack of evidence of infections by this trypanosome in other host species (Hoare 1923a, b, 1972; Mansfield 1977; Molyneux 1977; Wells 1976). This vector-parasite relationship is in accordance with the co-evolution reported for hippoboscid species and other hosts (Dittmar et al. 2006; Petersen et al. 2007). Phylogenetic studies of trypanosomes from ruminants and their respective keds would be valuable for the understanding of the probably ancient relationships and co-divergent history between host-restricted ectoparasites and species of the subgenus *Megatrypanum* parasitic in sheep, goat, deer, and wild bovids of temperate regions (Dirie et al. 1990; Molyneux 1977).

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