Molecular Diversity of Entodiniomorphid Ciliate *Trogloidytellla abrassarti* and Its Coevolution With Chimpanzees

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ABSTRACT The entodiniomorphid ciliate *Trogloidytellla abrassarti* is a colonic mutualist of great apes. Its host specificity makes it a suitable model for studies of primate evolution. We explored molecular diversity of *T. abrassarti* with regard to large geographical distribution and taxonomic diversity of its most common host, the chimpanzee. We found a very low diversification of *T. abrassarti* in chimpanzees across Africa. Distribution of two types of *T. abrassarti* supports evolutionary separation of the Western chimpanzee, *P. t. verus*, from populations in Central and East Africa. Type I *T. abrassarti* is probably a derived form, which corresponds with the Central African origin of chimpanzees and a founder event leading to *P. t. verus*. Exclusivity of the respective types of *T. abrassarti* to Western and Central/Eastern chimpanzees corroborates the difference found between an introduced population of presumed Western chimpanzees on Rubondo Island and an autochthonous population in mainland Tanzania. The identity of *T. abrassarti* from Nigerian *P. t. elliottii* and Central African chimpanzees suggests their close evolutionary relationship. Although this contrasts with published mtDNA data, it corroborates current opinion on the exclusive position of *P. t. verus* within the chimpanzee phylogeny. The type of

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One avenue of research into a species’ evolutionary history is to investigate the associations between a host and its symbionts. In the broadest meaning here, this includes parasites, commensals, and mutualists. Using the information from coevolving hosts and their symbionts offers a powerful tool for independent evaluation of directly inferred phylogenies of the target organisms (Hafner and Nadler, 1988; Page and Charleston, 1998). The evolutionary history of primates, especially hominids, has been scrutinized by studies surveying their evolution with various pathogens, e.g., viruses and malaria infecting *Plasmodium* species (Mu et al., 2005; Switzer et al., 2005; Liu et al., 2008; Duval et al., 2010). However, pathogens likely evolve through competitive processes with the host that should be quite different from the coevolution of commensal/mutualist organisms (Poulin, 2007).

Entodiniomorphid ciliates (Ciliata: Litostomatea: Entodiniomorphida) are mutualistic protists involved in fermentation processes in the large intestine of various herbivorous mammals (i.e., elephants, rhinoceroses, horses, hippopotamus, and various rodents), which enables the hosts to utilize the dietary fiber otherwise not digestible by animal hydrolytic enzymes (Collet et al., 1984; Moore and Dehorry, 1993; Stevens and Hume, 1998; Strüder-Kypke et al., 2007; Profousová et al., 2011). Among primates, only the African great apes are colonized by entodiniomorphid ciliates, namely by six species from the families Troglodytellidae, Cycloposthiidae, and Blepharocorythidae (Imai et al., 1991; Tokiwa et al., 2010).

**Troglodytellidae aberratti** (Troglodytellidae), the model organism in our study, is typically found in common chimpanzees *Pan troglodytes* (File et al., 1976; Krief et al., 2005; Pomajbíkova et al., 2010), from which it was originally described (Brumpt and Joyeux, 1912). However, this ciliate species was later recorded in gorillas (Goussard et al., 1983; Modrý et al., 2009) and possibly conspecific morphologically indistinguishable ciliates also occur in bonobos (Hasegawa et al., 1983; Dupain et al., 2009; Pomajbíkova et al., 2010).

Recently, phylogenetic analysis using sequences of small subunit ribosomal DNA (SSU rDNA) confirmed the sister position of *T. aberratti* to colon ciliates of the genus *Cycloposthium* (Cycloposthiidae) from horses (Ibris et al., 2008; Modrý et al., 2009), which supports their previously assumed morphology-based classification (Strüder-Kypke et al., 2007). However, both studies neglected to analyze for genetic variability potentially inherent with a wide geographic distribution of the host species. The common chimpanzee is particularly interesting in this regard as its natural range stretches over 5,000 km from Senegal to Tanzania, and several subspecies are recognized in this vast area: *P. t. verus*, *P. t. elliottii* (junior synonym *P. t. vellerosus*), *P. t. troglodytes*, and *P. t. schweinfurthii* (Gonder et al., 2006, 2011; Oates et al., 2009).

In contrast to ciliates of the families Cycloposthiidae and Blepharocorythidae, which are found predominantly in ungulates, members of the family Troglodytellidae are considered specific to African great apes (e.g., Imai et al., 1991; Van Hoven et al., 1998; Muehlenbein, 2005; Ito et al., 2006; Dupain et al., 2009; Modrý et al., 2009; Kaur et al., 2010; Pomajbíkova et al., 2010). There are two anecdotic reports of *T. aberratti* in other primates, namely in orangutans and siamangs (Mortelmans et al., 1970; O’Donoghue et al., 1993), however the infected animals were kept in captivity and in contact with chimpanzees (e.g., sharing cage), which were probably the source of the infection. To our knowledge, *T. aberratti* has never been reported in wild siamangs, orangutans or other primates.

Such a symbiotic association, in which the mutualist has limited dispersal ability and is passed from a host to its offspring, offers a unique opportunity to study the codivergence in the host and its evolutionary partner, the ciliate. Similarly coupled evolutionary history has been recently demonstrated in some species, for instance in lice of anthropoid primates, where splits of lineages leading to extant ape species led to parallel diversification resulting in recent sister species within the lice genera *Pediculus* and *Pthirus* (Reed et al., 2007).

We studied intraspecific sequence variation of *T. aberratti* from both chimpanzee species (*P. troglodytes*, *P. paniscus*) in order to uncover possible evolutionary units within this ciliate species, which may have coevolved in a phylogeographic pattern similar to its host’s. We employed two molecular markers: SSU rDNA and internally transcribed spacer region (ITS). The SSU rDNA enables basic specific assignment and comparison with previously studied entodiniomorphid ciliates. The ITS, a highly variable stretch of DNA separating the genes for small and large subunit rDNA, is considered useful for inferring discriminating intraspecific variation in a wide range of organisms including ciliates (Wright, 1999). If the processes beyond chimpanzee diversification are really reflected in the current intraspecific diversity of *T. aberratti*, the information retrieved from ciliate phylogeny can help us to answer persisting questions about the speciation and systematics of chimpanzees.

**MATERIAL AND METHODS**

**Sampling**

Ciliates were isolated from fecal samples of four subspecies of wild common chimpanzees collected at eleven localities throughout the chimpanzee distribution range. Numbers of successfully processed samples are given in parentheses; for geographical distribution of localities see also Figure 1: *Pan troglodytes schweinfurthii*—Kalinzu Forest Reserve, Uganda (*n* = 3); Kyambura Gorge, Queen Elizabeth National Park, Uganda (*n* = 1); Budongo Forest Reserve, Uganda (*n* = 1); Uganda, Tanzania (*n* = 2); *P. t. troglodytes*—Petit Loango, Gabon (*n* = 1); Goualougo Triangle, Republic of Congo (*n* = 2); *P. t. elliottii*: Gashaka Gumti National Park, Nigeria (*n* = 2); *P. t. verus*: Tai National Park, Côte d’Ivoire (*n* = 1); Bosou, Guinea (*n* = 1); introduced *P. t. verus*: Rubondo Island, Tanzania (*n* = 2). Samples from bonobos, *P. paniscus*, collected at Wamba, Democratic Republic of Congo (*n* = 2), were also included for interspecific comparison. Fecal samples were obtained during tracking of chimpanzees or under nests, and immediately preserved in
96% ethanol or RNAlater (Qiagen, Germany) for molecular analysis and in 20 ml of 10% formaldehyde for microscopic detection of ciliates. Presence of ciliates in formaldehyde samples was microscopically determined by the merthiolate–iodine–formaldehyde concentration (MIFC) method (Blagg et al., 1955). All laboratory procedures were carried out at the Department of Parasitology, University of Veterinary and Pharmaceutical Sciences (UVPS), Brno.

**Extraction and amplification of DNA**

One to three cells of *T. abrassarti* were picked from the ethanol or RNAlater samples under microscope, washed in distilled water, and put into a 0.2 ml tube with 20–30 µl of distilled water. Genomic DNA was extracted using a modified protocol by Regensbogenova et al. (2004). Twenty-five microliters of 10% Chelex (Biorad, CA, USA) were added to ease the lysis of cells, the mixture was briefly vortexed and centrifuged, and then heated at 55°C for 30 min. After the incubation, the mixture was again vortexed and centrifuged, and then heated at 98°C for 2 min at 94°C, 35 cycles of 45 s at 94°C, 45 s at 50°C, and 90 s at 72°C, and terminal elongation for 5 min at 72°C. Products of PCR were then loaded to 1.5% agarose gel to verify success of the PCR. Amplicons of relevant size were excised and purified with QIAEX II Purification Kit (Qiagen). Direct sequencing was performed commercially (Macrogen, Korea) with the same primers on an ABI 3730xl sequencer using the BigDye Terminator Kit (Applied Biosystems, CA, USA).

**DNA sequence analysis**

Sequences were assembled and checked for errors in Sequencer 4.6 (Gene Codes, MI). These sequences were then aligned in BioEdit (Hall, 1999) and compared with previously published sequences of *T. abrassarti* stored in the GenBank: AB437346 and AB437347 (Irbis et al., 2008), EU127481–EU127484 and EU680310 (Modry et al., 2009). Variability of obtained sequences was assessed and related to geographical distribution of sampled localities.

Phylogenetic relationships among obtained sequences was inferred with a selected spectrum of entodiniomorph and vestibuliferid ciliates occurring in the gastrointestinal system of ruminants and perissodactyls, as they were used by Irbis et al. (2008) and Modry et al. (2009): Cycloposthium bipalatum AB530165, Cycloposthium edentatum EF632077, Cycloposthium ishikawai EF632076, Entodinium caudatum U57765, Eudinium maggi U57766, Polyplastron multivesiculatum U57767, Ophryoscolex purkynejii U57768, Dasytricha ruminantium U57769, Isotricha prostoma AF029762, Isotricha intestinalis U57770, Balantidium coli AF029763. Sequences were aligned in ClustalW (Thompson et al., 1994) as implemented in BioEdit under default settings. Maximum parsimony (MP) analysis with all characters equally weighted and equally weighted and taxon-pairwise heuristic search (MP) were used. Nodal support was assessed using non-parametric bootstrapping in 1,000 pseudo-replicates. In order to recover probable ancestral–descendant relationships among the newly obtained sequences, ancestral states of variable sites were inferred under the MP criterion.

**RESULTS**

**Variability of sequences**

We successfully obtained 21 partial sequences of SSU and 12 partial sequences of ITS from the sample set. SSU fragments about 605 and 650 bp long, respectively, were obtained from samples with low quality of DNA. These could be assembled and aligned with other SSU sequences to an alignment of 1,316 bp including a 61 bp gap in the center of the alignment where the bases were missing due to lack of signal in chromatograms from the low quality DNA samples. The first position of the alignment corresponded to position 51 of the SSU sequence of *T. abrassarti* AB437346 and AB437347. All SSU sequences differed in two variable sites: in position 408 of the

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Sequence variability

SSU sequences were identical with or only slightly different from the sequences in the GenBank. Published sequences AB437346 from Sierra Leone and AB437347 from Guinea by Irbis et al. (2008) were identical with the partial SSU rDNA sequences of our T. abrassarti Type I, confined to West African populations of the common chimpanzee. The T. abrassarti Type II sequences showed the same nucleotides on the characteristic positions as sequences of T. abrassarti EU127481–EU127484 from captive gorillas kept in European Zoos and a chimpanzee from a sanctuary in Cameroon (Modry et al., 2009).

Our phylogenetic analysis based on sequences of SSU rDNA agreed with the results of Irbis et al. (2008) and Modry et al. (2009). Sequences of T. abrassarti were placed as a sister clade to the genus Cycloposthium, intestinal ciliates of horse (Struder-Kypke et al., 2007). Proximity of these two ciliate genera both in phylogeny and in ecology could thus enable a relevant comparison forming a clade with T. abrassarti as well as its sister position to Cycloposthium was well supported by bootstrap. Overall, the hindgut and rumen entodiniomorphids formed reciprocally monophyletic clades. Within the Troglodytella clade, Type Ia was the basal lineage, while Type Ib clustered with both variants of Type I (Fig. 2A). MP reconstruction of ancestral states in the recovered trees revealed A as a plesiomorphic character in the position 408 and T in position 433 (Fig. 2B). The variable region in Troglodytella could be identified as coding for a hairpin, where corresponding bases are related according to the Watson–Crick pairing. Due to high variability of this region and obvious pairwise interdependence of sites, the ancestral states were not reconstructed. The variants Type Ib, Type Ia, and Type Ib, however, differed in just two sites in the region (positions 1031 and 1047). This similarity contrasts with eight variable sites, in which Type Ia differs from the other types of T. abrassarti.

Phylogenetic relationships among the SSU sequences

Recovered MP topology (4 trees, 326 steps) placed the T. abrassarti sequences as a sister group to the clade comprising Cycloposthium sequences (Fig. 2A). Monophyly of T. abrassarti as well as its sister position to Cycloposthium was well supported by bootstrap. Overall, the hindgut and rumen entodiniomorphids formed reciprocally monophyletic clades. Within the Troglodytella clade, Type Ia was the basal lineage, while Type Ib clustered with both variants of Type I (Fig. 2A). MP reconstruction of ancestral states in the recovered trees revealed A as a plesiomorphic character in the position 408 and T in position 433 (Fig. 2B). The variable region in Troglodytella could be identified as coding for a hairpin, where corresponding bases are related according to the Watson–Crick pairing. Due to high variability of this region and obvious pairwise interdependence of sites, the ancestral states were not reconstructed. The variants Type Ib, Type Ia, and Type Ib, however, differed in just two sites in the region (positions 1031 and 1047). This similarity contrasts with eight variable sites, in which Type Ia differs from the other types of T. abrassarti.

Geographical distribution of T. abrassarti sequences

Type I SSU sequences of T. abrassarti, characterized by G at position 408 and A at position 433, were revealed in the sampled populations of western chimpanzees from Guinea, Ivory Coast and from introduced chimpanzees on Rubondo Island, Tanzania. Type II sequences, characterized by A at the position 408 and T at the position 433, were found in chimpanzees from Uganda, Republic of Congo, Nigeria, Gabon, and mainland Tanzania (Fig. 1). Sequences of T. abrassarti obtained from bonobos belonged also to Type II. Despite a lower number of obtained ITS sequences in comparison to SSU sequences, the geographical division was identical with that of SSU types; ITS sequences with C at position 65 were found in West African samples, while the ones with T at this position were typical for Central and East African samples.

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DISCUSSION

Ubiquitous occurrence of ciliates of the genus Troglodytella in wild African great apes, their close association with the colonic ecosystem and digestion, and their host specificity create a unique host–symbiont system that provides an exciting approach to study the evolution of African great apes. The Troglodytella-great ape system probably coevolved during the evolution of the family Hominidae and it may also provide a unique contribution to our knowledge of human evolution (Fig. 3).
of their genetic variability. Excluding the variable region in both *Troglodytella* and *Cycloposthium*, Type I and Type II of *T. abrassarti* differ by two substitutions from each other, whereas *C. edentatum* and *C. ishikawai* differ by four substitutions on the 1,316 bp fragment of SSU, *C. bipalmatum* and *C. ishikawai* by two substitutions, and one indel, and *C. bipalmatum* and *C. edentatum* by six substitutions and one indel (Table 1). Interestingly, the variable region was identical in the latter pair of *Cycloposthium* species, which otherwise showed the greatest interspecific difference within included hindgut ciliates (Table 1). Obviously, the significance of substitutions within this variable region remains unclear, and should be interpreted with caution in systematic appraisal. A comparison with SSU sequence of *Saccharomyces cerevisiae* (GenBank accession number AY251636) and analysis of its secondary structure model clearly identified the variable region as the variable V7 hairpin region (Neefs et al., 1993). The hairpin structure of the variable region between positions 926 and 942 could be confirmed in all four *Troglodytella* sequences. In such a structure, substitutions are not independent and invoke compensatory changes, which very likely boost the local substitution rate (Tillier and Collins, 1998). This could be a plausible explanation for the respective variability within Type I and Type II of *T. abrassarti*, which otherwise differ in two point mutations in positions 408 and 433.

While the high variability could be plausibly explained by different substitution rates of SSU regions, simultaneous occurrence of the two variants (“a” and “b”) within each of the types of *T. abrassarti* likely represents intragenomic polymorphism of tandemly organized ribosomal genes, which is known to occur in protists and other groups of organisms (e.g., Alverson and Kolnick, 2005; Simon and Weiss, 2008; Gómez et al., 2009). This is supported by the low number of individuals that were selectively picked from each fecal sample and provided template DNA for PCR amplification, which were unambiguously identified as *T. abrassarti*. Further support for this explanation can be found in the published studies by Irbis et al. (2008) and Modrý et al. (2009). In the first study, the authors sequenced two individual cells and obtained two almost identical sequences, both containing two unresolved bases on the same positions, where our chromatograms showed double peaks A/T and T/A. In the latter study, the published sequences were obtained using cloning of PCR products, and thus clearly identified individual variants in the variable region.

Interestingly, the phylogenetic analysis did not cluster both types of *T. abrassarti* into two reciprocally monophyletic clades, but showed paraphyly in Type II. The rather large difference between variants of the Type II and similarity of Type IIb and both variants of Type I (Table 1, Fig. 2A) further suggest that Type I could have evolved from Type IIb, and current *T. abrassarti* retains ancestral polymorphism of SSU sequences. Such a relationship is supported by reconstructed bases at ancestral nodes in the variable positions identifying Type I and
Type II, where G in position 430 and A in position 456 are likely derived, while A and T, respectively, are ancestral (Fig. 2B).

In contrast to SSU, variability of the ITS region remained surprisingly low, although it similarly corresponded with the division to Type I and Type II. Low genetic differences within a large geographical area were also revealed in the world-wide distributed rumen symbiont *Isotricha prosoma* (Wright, 1998), which showed no ITS variation among cattle and sheep from both North America and Australia. This contrast to the generally presumed high intraspecific diversity of the ITS region may have, however, resulted from the recent introduction of these domestic species, most likely of European origin.

Implication for the evolutionary history of chimpanzee

Low variability of *T. abrassarti* sequences throughout the range of the common chimpanzee suggests the conspecificity of all sequenced ciliates. However, the geographical distribution of both types of *T. abrassarti* basically follows a distributional pattern of chimpanzee evolutionary units. According to mtDNA data, chimpanzee populations confined to West Africa (represented by subspecies *P. t. verus* and *P. t. ellioti*), and Central and East Africa (subspecies *P. t. troglodytes* and *P. t. schweinfurthii*) represent two evolutionary groups (Gonder et al., 2006; Stone et al., 2010; Bjork et al., 2011). Subspecific division within the latter group does not likely reflect phylogenetic relationships, because *P. t. schweinfurthii* lineage falls within *P. t. troglodytes*. Recent nuclear data confirm this relationship between Central and Eastern chimpanzee populations, whereas they deny sister relationship between *P. t. verus* and *P. t. ellioti* (Gonder et al. 2011). The Western chimpanzee *P. t. verus* thus represents the basal lineage of the species, being well defined within chimpanzee phylogeny and deeply divergent from other chimpanzee lineages from the rest of Africa (Morin et al., 1994; Gonder et al., 2006, 2011; Stone et al., 2010; Bjork et al., 2011). This separate position of *P. t. verus* largely corresponds with exclusive presence of *T. abrassarti* Type I in the Western chimpanzee populations. All other lineages, represented by the other three chimpanzee subspecies, are populated by *T. abrassarti* Type II. The observed pattern of genetic distinction of *T. abrassarti* in chimpanzee seems to reflect the geographical separation of the African equatorial forest belt by the Dahomey gap. This stretch of dry savannah spreading from eastern Ghana to Togo and reaching the Bay of Benin has been considered one of the major geographical barriers between Guinean andCongolean rainforests of West and Central Africa, respectively (Robbins, 1978).

Such a barrier may have caused reproductive isolation of the West African *P. t. verus* from Central and East African *P. t. troglodytes* and *P. t. schweinfurthii*, as hypothesized to have occurred in other mammals (Grubb, 1978; Gonder et al., 2011; Bjork et al., 2011).

However, we found Type II of *T. abrassarti* also in Nigeria, in chimpanzee populations recognized as *P. t. ellioti*, supposedly a sister taxon to *P. t. verus* (Gonder et al., 2006; Stone et al., 2010; Bjork et al., 2011). Assuming a coevolution scenario in *T. abrassarti* and the common chimpanzee, our data would suggest that the mtDNA-based phylogeny, which places these two chimpanzee subspecies into the Western evolution-
P. t. schweinfurthii, from which it remains spatially and genetically isolated. Our result supports the West African origin, as T. abrassarti on Rubondo Island is of a different type than the one on the Tanzanian mainland, namely of the West African Type I.

Host specificity of T. abrassarti

Conspecificity of Troglodytella isolates in the common chimpanzee throughout its large range corresponds with previous morphology-based tentative assignment of Troglodytella trophozoites from various natural and captive populations to T. abrassarti (Irbis et al., 2008; Modrý et al., 2009; Kaur et al., 2010; Pomajbíková et al., 2010). In contrast to a previous study expecting the presence of different species of Troglodytella in bonobos (Dupain et al., 2009), sequences retrieved from bonobo samples belonged to Type II, which occurs in the East/Central African common chimpanzee. Because of the assumed geographical isolation of bonobos since the splitting event from a common Pan ancestor about 0.9–2.1 million years ago (Won and Hey, 2005; Wegman and Excoffier, 2010; Stone et al., 2010; Gonder et al., 2011; Fischer et al., 2011), the strictly ape-bound T. abrassarti may theoretically have also evolved in a similar splitting pattern with Troglodytella divergent from both T. abrassarti Type I and Type II.

Although there is only a single report of occurrence of T. abrassarti in wild gorillas (Goussard et al., 1983), molecular data on T. abrassarti from captive gorillas show bases A and T on the respective positions 408 and 433, typical for Type II (Modrý et al., 2009). This affinity leads us to hypothesize about Type II existing in wild gorillas, but further analyses of Troglodytella from both species of wild gorilla must be conducted to clarify the situation. The occurrence of Type II T. abrassarti in bonobos (and perhaps gorillas) can be explained by interchange of intestinal symbiotes in the contact area of the ape species, which could lead to area specific unification of T. abrassarti throughout Central and East Africa. Such a switch from one host species to another was recently discovered in the primate louse genus Pthirus, where a common Pthirus ancestor probably switched from gorillas to humans, and evolved into two extant species P. gorillae in gorillas and P. pubis in humans (Reed et al., 2007). The hypothesized contact between gorillas and humans through sharing nests may technically explain also contact between great ape species and potential interspecific fecal-oral transmission of T. abrassarti. On the other hand, while distribution ranges of common chimpanzee and gorilla have presumably overlapped, the bonobo is assumed to have remained isolated in the Congo basin without contact with other ape species (Eriksson et al., 2004; Won and Hey, 2005). The finding of Type II T. abrassarti in a bonobo population as well as a nearby, but isolated regional population of chimpanzees suggests occasional contact during relatively recent changes in the course of large rivers, generally considered impenetrable barriers for primes (Eriksson et al., 2004). An alternative explanation may be based on the recent study by Wegman and Excoffier (2010), who confirmed relatively intensive historical gene flow between the chimpanze and bonobo populations. After the split from their common ancestor, the lineages evolving into two Pan species likely shared intestinal ciliates, which may have led to maintenance of the same Type II in both extant species. Alternatively, Type II may be the original type of T. abrassarti occurring throughout the Lower Guinean rainforest in their common ancestor and thus now present in both chimpanzee species.

Sharing of the same intestinal symbiotes in syntopic great ape species re-opens the question of the presence of entodiniomorphid ciliates in our human ancestors. It is probable that entodiniomorphid ciliates contributed to fermentation processes in the intestine of early hominids, enabling them to digest large quantities of plant material similar to gorillas and chimpanzees (see a comment by A. Kortlandt in Stahl et al., 1984). If so then the elimination of entodiniomorphid ciliates in hominids might be connected to relatively recent changes in diet after application of fire for food preparation.

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