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Three monophyletic clusters in *Retortamonas* species isolated from vertebrates

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ABSTRACT

Retortamonas spp. has been reported as an intestinal parasite among various host organisms, including humans; however, its intra-genus molecular diversity has not yet been elucidated. Haplotypes of the 18S small subunit ribosomal RNA locus (1836–1899 bp) of *Retortamonas* spp. from humans (n = 8), pigs (n = 6), dogs (n = 1), goats (n = 16), water buffalos (n = 23), cattle (n = 7), rats (n = 3), and chickens (n = 5) were analyzed with references isolated from non-human mammals, amphibians, and insects. Phylogenetic and network analyses revealed a statistically supported three cluster formation among the vertebrate-isolated haplotypes, while insect-isolated haplotypes were independently clustered with *Chilomastix*. In the clade of vertebrate isolates, assemblage A (amphibian genotype), which included the amphibian references, was addressed as an out-group of the other clusters. Assemblage B (mammalian and chicken genotype) included most haplotypes from various mammals including humans with the haplotypes isolated from a chicken. Human isolates were all classified into this assemblage, thus assemblage B might correspond to *R. intestinalis*. Assemblage C (bovine genotype), which included specific haplotypes from water buffalos and cattle, was addressed as a sister lineage of assemblage B. Among the diversified haplotypes of assemblage B, a specific haplotype, which was identified from multiple host mammals (humans, dogs, pigs, cattle, water buffalos, elks, goats, and rats), indicates the potential zoonotic transmission of the *Retortamonas* among them. The genotyping classification of retortamonads could contribute to a better understanding of its molecular epidemiology, especially among humans and related host organisms.

1. Introduction

Although the first case of *Waskia* (Syn. *Retortamonas*) *intestinalis* was reported from a soldier in Alexandria, Egypt in 1917 [1], and the subsequent case of *Embadomonas* (Syn. *Retortamonas*) *intestinalis* was reported from a returnee from the Far East in 1922 [2], *Retortamonas* was rarely detected in clinical samples of diarrheal patients [3]. Therefore, after the revision of those species names to *Retortamonas* [4], which has been considered to be a harmless commensal protozoan [5]. Mainly due to the lack of pathogenicity, reports of retortamonads from humans have been still rare; however, microscopic determination of 0.1–10.7% prevalence [6–8] and the detection of *R. intestinalis* in the pancreatic juice of an obstructive cholangitis case [9] have been reported.

Retortamonas spp. can parasitize the intestine of a wide variety of vertebrates (mammalian, avian, and amphibian organisms) and invertebrates (insects) [5], and thus far, > 20 species names have been proposed for the genus based on morphologic features and host specificities [10]. However, with regard to GenBank data, the available genetic references of *Retortamonas* spp. are only the ones from sheep, guinea pig, goat, elk, and amphibians (poison arrow frog and giant salamander) [11,12]. Partial sequences of 18S small subunit ribosomal RNA (18S rRNA) of *Retortamonas* spp. from insects are also available, though all the references are directly submitted ones [5]. It should be noted that there is no information about *Retortamonas* spp. from humans in GenBank. Due to the lack of molecular reference, especially *R.*

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intestinalis from humans, *Retortamonas* spp. are excluded from various research topics of human subjects, such as the eukaryotome survey of gut microbiota [13] and molecular epidemiological evaluations to address those commensal or beneficial features of intestinal protozoan parasites [14].

To elucidate the molecular taxonomy of genus *Retortamonas* in detail, we collected *Retortamonas* spp. from humans and closely related organisms in Indonesia and addressed their molecular haplotypes phylogenetically.

2. Materials and methods

2.1. Sample collection

Ethical review was performed, and approval for this study in Indonesia was granted by the ethics committee of the Faculty of Medicine, Hasanuddin University, Makassar, Indonesia and Kanazawa University in Japan. Fecal samples were collected from humans, dogs (*Canis lupus familiaris*), rats (*Rattus exulans*), goats (*Capra hircus*), pigs (*Sus crofa domesticus*), water buffalos (*Bubalus bubalis*), cattle (*Bos taurus*), and chickens (*Gallus gallus domesticus*) from 2013 to 2015 at Wainyapu village (Kera-Panba school: 9°38′36.13“S, 119° 0′58.05”E), Sumba Islands, Indonesia. Human stools were collected using stool bags. Stools from pigs, dogs, and goats were taken by rectal enemas, and stools from chickens and rodents were collected by intestinal dissection.

2.2. Culture examination

All collected fecal materials were assessed by culture examination, which employed modified Tanabe-Chiba medium [15], including Ringer solution with 10% inactivated bovine serum and 0.1% Asparagine with 0.05% rice starch. Briefly, 0.2 g stool was inoculated into 1.0 mL medium in a 1.5 mL screw-capped tube. The tubes were incubated at room temperature, and the media were renewed every three days. At 7–14 days after inoculation, the culture sediment was examined microscopically to detect trophozoites or cysts of *Retortamonas* species. Subsequently, 200 μL of *Retortamonas*-positive culture samples were preserved with 600 μL of DNAzol® reagent (Molecular Research Center, Inc., OH, US) at 4 °C for further analyses.

2.3. PCR screening

Genomic DNA was extracted from DNAzol® sample mixtures according to the manufacturer's instructions with some modification [16]. The final products were suspended in 80 μL of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA and preserved at −30 °C for further analyses.

PCR screening targeting 18S rRNA gene locus of *Retortamonas* spp. was designed as a nested PCR (see Table 1). The optimized PCR conditions were as follows. One μL of purified genomic DNA solution was used as a template in a 10 μL reaction mixture containing 0.4 μM of each primer, 0.8 mM of each deoxynucleotide triphosphate (dNTP), 0.5 U of LA Taq® DNA polymerase (Takara Bio Inc., Shiga, Japan), and 0.1% DMSO. The cycle parameters for the first round of PCR were as follows: initial denaturation at 94 °C for 1 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 2 min, and a final extension at 72 °C for 5 min. For the second round of PCR, 0.5 μL of the PCR mixture from the initial PCR round was used as the template with a similar reaction mixture. The cycle conditions used for the second round were also the same except for the annealing temperature, which was decreased to 55 °C. The PCR products were electrophoresed on 1.0% L03 agarose (TaKaRa Bio Inc.) with ethidium bromide and visualized on an ultraviolet trans-illuminator, Gel Doc™ EZ Imager (BioRad Laboratories, Tokyo, Japan).

Table 1
List of primers used for the molecular screening of 18S rRNA gene locus in this study.

Primer	Nucleotide sequence	Product size
1st PCR		1963 bp
TN21′	5′-^{*39}TAAAGATTAAGCCATGCATGTSK^{61}-3′	
TN14′	5′-^{2001}GATACCTTGTTACGACTTCTY^{1981}-3′	
2nd PCR		1942 bp
TN167	5′-^{51}CATGCATGTCGAAGTATAAAC^{71}-3′	
TN112	5′-^{1992}TTACGACTTCTCCTTCCTCTA^{1972}-3′	

* Position numbers are according to the reference sequence of AF439346.

2.4. DNA sequencing analysis

Target band was excised from the gel and purified using the Fastgene® gel PCR extraction kit (Nippon Genetics, Tokyo, Japan) according to the manufacturer's instructions. Direct sequencing of each purified PCR product was conducted with the ABI Prism Big Dye V3.1 Cycle sequencing kit (Life Technologies Japan, Tokyo, Japan) on an Applied Biosystems 3130 Genetic Analyzer (Life Technologies Japan). All nucleotide sequences were confirmed using both forward and reverse of the second PCR primers. To build the long sequences of *Retortamonas* spp., additional sequencing primers TN205 (5′-CTGTTA TTGCCTCTACCTTC) and TN204 (5′-CGCGAAAATTACCCAATGTAC) were used in the sequencing process. When direct sequencing could not confirm the DNA sequences of those PCR amplicons due to heterogeneity of the sequencing results, a sub-cloning method was applied for analysis. Cloning was performed using the Takara TA-Mighty Cloning Kit (Takara Bio Inc.) according to the manufacturer's instructions. The recombinant plasmids (pMD20-T) were transformed into *Escherichia coli* DH5-α and screened on Luria Broth (LB) agar plates supplemented with 100 mg/L of Ampicillin. The positive colonies were cultured in LB liquid medium overnight and then purified using the QIAGEN® plasmid mini kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions. Their full-length insertion DNA was sequenced using M13 primer RV and M13 primer M4. All confirmed sequences of the 18S rRNA gene locus of *Retortamonas* spp. were registered into the DDBJ/EMBL/GenBank data library as accession numbers LC422138–LC422197 and LC422253–LC422279.

2.5. Phylogenetic analyses

As reference sequences, the 18S rRNA gene sequences of representative species of Fornicata including *Retortamonas* spp. were used. The DDBJ accession numbers of *Retortamonas* spp. from insects (large crane fly, hissing cockroach, pine chafer beetle, and scarab beetle) and other representative *Fornicata* spp. were shown in Fig. 1. The ones of *Retortamonas* spp. from sheep, guinea pig, goat, elk, and amphibians (poison arrow frog and giant salamander) were shown in Fig. 2A.

All the reference sequences and the sequences confirmed in this study were phylogenetically analyzed. For comparative analyses of phylogenetic reconstructions, Maximum Likelihood (ML) and Maximum Parsimony (MP) methods were conducted by MEGA7 [17], and the Bayesian Inference (BI) method was conducted using Geneious 10.2.3 (Biomatters Ltd., Auckland, New Zealand). For statistical assessments of the ML and MP trees and BI tree, bootstrap values calculated with 1000 replications and posterior probability values were used, respectively. To characterize the haplotype diversity of *Retortamonas* spp., a network phylogenetic analysis using the Network 5.1.3 software (Fluxus Technology Ltd., Suffolk, UK) under the median joining parameter was also performed. The figure was drawn using the Network Publisher add-on (Fluxus Technology Ltd.).

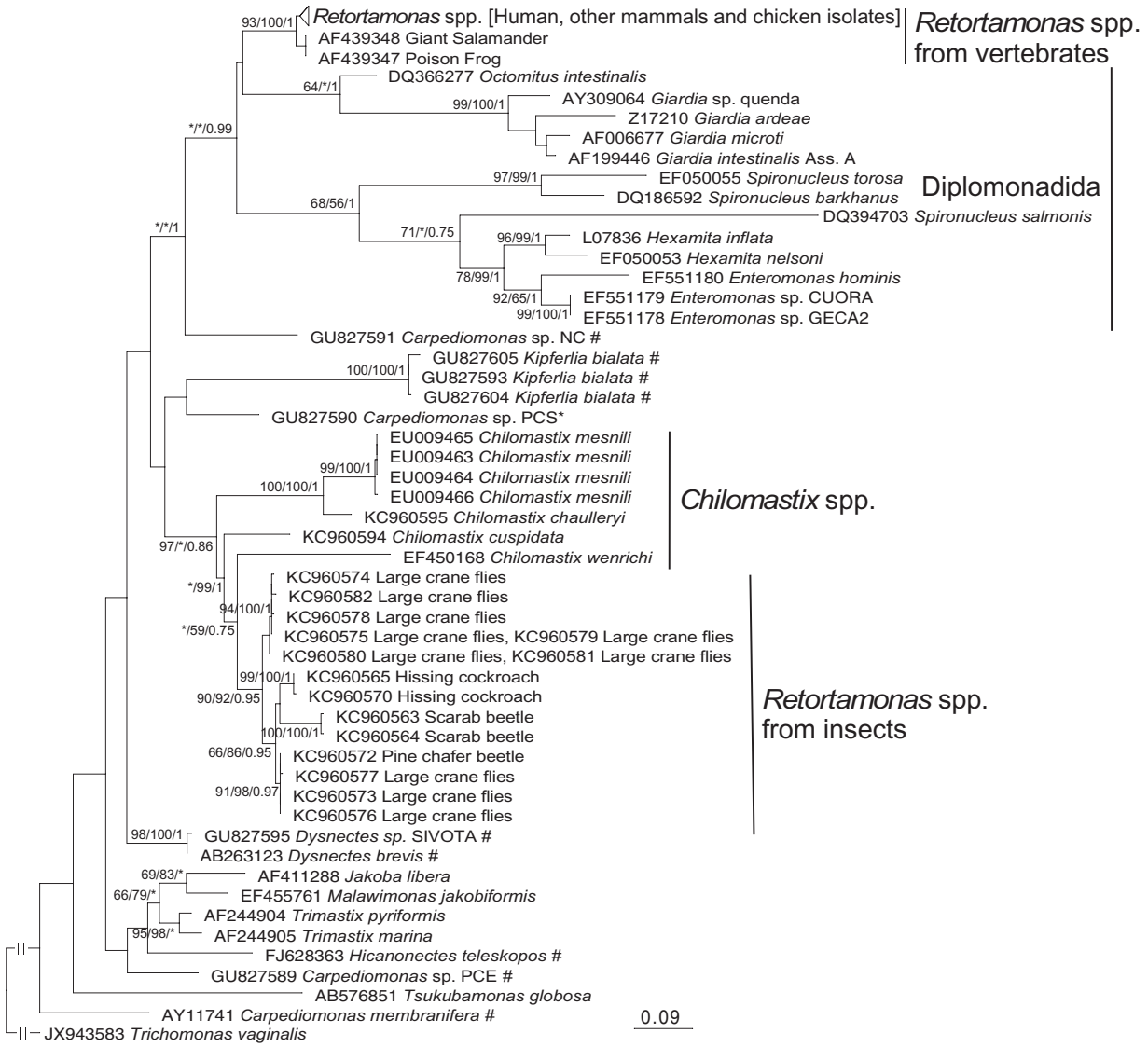

Fig. 1. Genetic diversity of diplomonads, Carpediemonas-like organisms, and retortamonads among Fornicata. Phylogenetic tree inferred using 18S rRNA sequences of 124 isolates from various organisms and *Trichomonas vaginalis* as an outgroup. All insertion and deletion in the nucleotide alignment were removed, and, in total, 318 nucleotide positions were used as the final data set. The best tree of the ML analysis using the Kimura-2 parameter model is shown with bootstrap proportion values (BP) for the ML and MP methods along with the Posterior Probability (PP) value for Bayesian Inference analysis. BP values < 50% and PP < 0.5 were shown by asterisk (*), while the value was not shown when all the values did not satisfy the criteria. Hash symbol (#) indicates the member of Carpediomonas-like organisms.

3. Results and discussion

3.1. Presence of Retortamonas spp. among various host organisms

Due to the rare presence of *Retortamonas* spp. even at the parasites-endemic investigation site, sample collections were conducted by chance through repeated fieldwork, thus it was difficult to determine prevalence among the total samples. A representative investigation, which was conducted in September 2013, revealed the following prevalences of *Retortamonas* spp. in various host organisms: humans 1.4% (4/290), dogs 7.1% (1/14), cattle 100% (1/1), water buffalos 29.0% (9/31), and chickens 2.2% (1/45). Other hosts, such as pigs (n = 31), horses (n = 7), goats (n = 3), rats (n = 12), and ducks (n = 3), were all negative for *Retortamonas* spp. (data not shown).

The partial 18S rRNA gene segments (1836–1899 bp) were successfully amplified from 37 (8-human, 4-dog, 4-pig, 3-goat, 1-cattle, 9-water buffalo, 7-rat, and 1-chicken) samples. Within those amplicons, 23 nucleotide sequences were confirmed by the direct sequencing method, and the remaining 14 amplicons were analyzed using a sub-cloning methodology. In total, 87 (23 direct and 64 sub-cloned) nucleotide sequences, 8-human, 1-dog, 6-pig, 16-goat, 7-cattle, 23-water buffalo, 3-rat, and 5-chicken DNA haplotypes, which were unique in each host category, were confirmed. As mentioned above, there is no registration of *Retortamonas* spp. from humans, dogs, pigs, cattle, water buffalos, rats and also chickens in GenBank thus far; therefore, those novel haplotypes confirmed in this study could provide wider insight into the host range of the *Retortamonas* species.

3.2. Genetic diversity of retortamonads among Fornicata species

Fornicata was a clade defined under a major supergroup Excavata [18]. Fornicates are unicellular heterotrophic flagellates with 1–4 flagella, and the clade consists of three lineages [19], e.g., Diplomonadida [20], Carpediemonas-like organisms [21], and Retortamonadida [22]. Regarding Retortamonadida, the polyphyletic characteristics of retortamonads have been discussed. The close relationship of mammalian

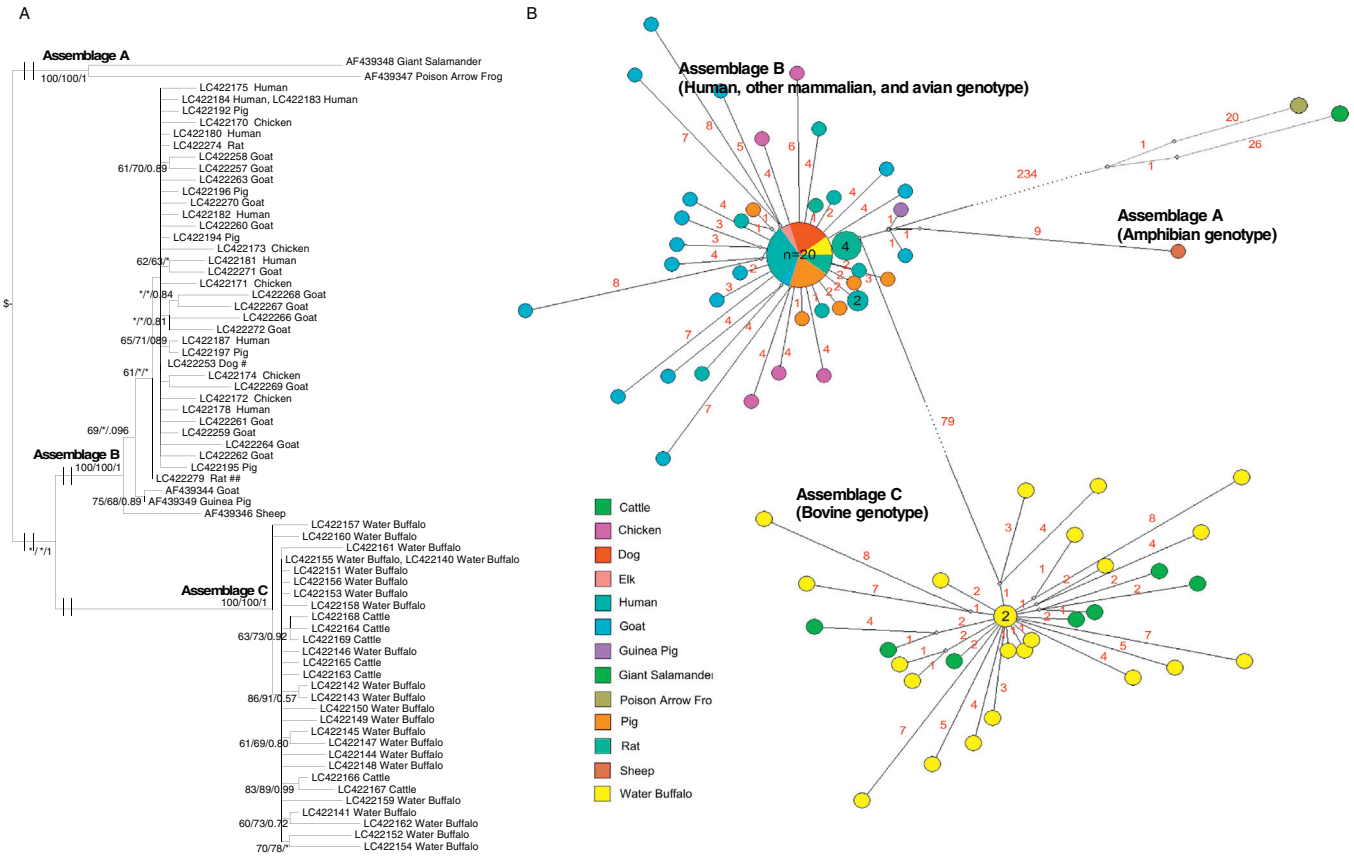

Fig. 2. 18S rRNA gene diversity of *Retortamonas* species from vertebrates. (A) Phylogenetic tree was inferred using 18S rRNA sequences of newly confirmed haplotypes of mammalian and chicken isolates (n = 69) and mammalian and amphibian references (n = 6) from GenBank. In total, 1710 nucleotide positions were used as the final data set. The sequence preparations and analyses were conducted in the same manner as in Fig. 1. Hash symbol (#) indicates the specific identical haplotype, including 20 isolates: buffalo (LC422139, LC422138), dog (LC422253, LC422255, LC422254, LC422256), human (LC422176, LC422179, LC422188, LC422185, LC422177, LC422186), pig (LC422190, LC422191, LC422189, LC422193), rat (LC422273, LC422276), goat (LC422265), and elk (AF439345). Double hash symbol (##) indicates identical haplotype, including four isolates from rat (LC422279, LC422278, LC422277, LC422275). Although this phylogeny was analyzed as an unrooted tree to maximize the nucleotide positions of the final data set, the deduced root (shown as "$") was estimated by the phylogenetic analysis with *Dysnectes brevis* (AB263123) as an outgroup (Data not shown).
(B) In the network analysis, 484 variable sites (Haplotype diversity, hd, 0.95) in the 1710 nucleotide positions were used.

and amphibian retortamonads and diplomonads was first indicated [11,23]. Further, recent molecular phylogenetic analyses have shown that the clade of Retortamonadida seems to split into a cluster of the retortamonads from vertebrates with diplomonads [24–26] and an another putative cluster, which was suggested to consist of insect retortamonads with the *Chilomastix* species [5]. Evidence of the latter cluster has not yet been confirmed, since only the directly submitted unpublished reference sequences (by Cepicka et al.) of retortamonads from insects are available in GenBank.

All newly confirmed haplotypes in this study and the reference sequences of retortamonads from vertebrates and insects were applied to a phylogenetic reconstruction with representative references of Fornicata as mentioned in the Materials and Methods. Due to the length limitation of some reference sequences, the analyses were performed using comparatively short sequences including 318 substituted nucleotide positions of 18S rRNA gene locus; however, the reconstructed phylogenetic tree revealed the split features of Retortamonadida among the clades in Fornicata (Fig. 1).

The monophyly of mammalian, avian, and amphibian-isolated retortamonads was strongly supported (ML 96%, MP 100%, BI 1), while the cluster of this monophyletic group and diplomonads, such as *Giardia* spp. and *Enteromonas* spp., was not supported by ML and MP, but BI 0.99. These results are consistent with early reports, which indicated the close relation between retortamonads and diplomonads with low statistical supports [11,23]. However, the analysis resolution could be improved using multigene phylogenies [26,27], as also in this case. It might be noteworthy that the cluster of retortamonads and diplomonads was strongly supported (ML 99%, MP 96%, BI 1), when only the representative longer sequences (1,163 nucleotide positions) of 18S rRNA gene locus were used (supplementary data 1).

The monophyly of retortamonads from insects (large crane fly, hissing cockroach, pine chafer beetle, and scarab beetle) was also well supported (ML 90%, MP 92%, BI 0.95), and the monophyly of this cluster and *Chilomastix* spp. was moderately supported (ML 97%, MP < 50%, BI 0.86).

Considering the original definition of Retortamonadida, which included two genera *Chilomastix* and *Retortamonas* [22], the insect isolates apparently could fulfill the criteria to be Retortamonadida. The result is consistent with the recent ultrastructural finding that both retortamonads from insects and *Chilomastix* spp. possess a complete microtubular corset, which is absent in the retortamonads from mammalians and amphibians [28]. In contrast, the presence of the monophyletic cluster of retortamonads from vertebrates and diplomonads suggests the requirement of revision for the genus name of the vertebrate-derived "*Retortamonas*" as a member of Diplomonadida. However, as well known, the sampling of those organisms are quite limited so far, thus the revision should wait for the enrichment of related molecular taxonomic references.

3.3. 18S rRNA gene diversity of vertebrates-isolated Retortamonas species

The haplotype variations of retortamonads from mammalian, avian, and amphibian hosts were phylogenetically addressed into three monophyletic clusters (Fig. 2A), which were all robustly supported (ML 100%, MP 100%, BI 1). Therefore, we propose three assemblages (A, B, and C) based on the clusters of the 18S rRNA gene locus. The assemblage A (amphibian genotype), which includes the amphibian references, is addressed as an sister group of the clade consisted of the other assemblages. Assemblage B (mammalian and chicken genotype) includes most haplotypes from various mammals including humans with the haplotypes isolated from a chicken. Assemblage C (bovine genotype) includes specific haplotypes of water buffalo and cattle, and the assemblage is addressed as a sister lineage of assemblage B.

Some sub-clusters within the assemblage B were also observed. Except the haplotype of elk isolate, which was 100% matched to a haplotype (shown as # in Fig. 2A) confirmed in this study, all the reference haplotypes (from goat, guinea pig, and sheep) were addressed as outgroups to the cluster of the newly isolated haplotypes, though the relationship was not clearly supported by bootstrap or posterior probability values (61/*/*). This outgroup topology did not appear in BI analysis. Instead, the cluster of reference haplotypes isolated from goat, guinea pig, and sheep was positioned within the in-group of newly identified haplotypes. In the latter case, the cluster formation as [sheep (goat, guinea pig)] of reference haplotypes was observed. The whole clustering, and also the inside clustering of goat and guinea pig were both strongly supported (BI 0.98 and 0.96, respectively) (data not shown).

The unique clustering of those reference haplotypes isolated from various host mammals suggests the presence of geographical variations of those haplotypes. Although, a wider geographic sampling is required to verify this hypothesis, all the reference haplotypes were reported from the United States and Europe, while this sampling was done in Indonesia, thus such geographical effect could take place. Meanwhile, host specific cluster formation among haplotypes in assemblage B was not confirmed, thus the geographical effect to those haplotype variations seems to be stronger than the effect of host specificities. Taken the stronger effect of geographical variations together with the fact that the hosts of assemblage B includes almost all haplotypes isolated from those vertebrates, a comparatively loose host specificity of assemblage B might be considered.

The results of phylogenetic network analysis (Fig. 2B) showed the presence of a specific haplotype (shown as # in Fig. 2A, and as the most major pie chart in Fig. 2B), detected from various host organisms (humans, dogs, pigs, cattle, water buffalos, elks, goats, and rats) in assemblage B. Within the 59 haplotypes of assemblage B, including 55 haplotypes confirmed in this study and 4 reference haplotypes, 20 haplotypes were identified as this specific haplotype, and only chickens, guinea pigs, and sheep did not have the haplotype. The specific haplotype, which circulated among humans and various vertebrates, indicates that the haplotype could be maintained by zoonotic transmission among them.

As host specific genotypes, assemblage A (amphibian genotype) and assemblage C (bovine genotype) were also observed. In the investigation site, human and related animals are closely related in their daily life, thus, at least, assemblage C might be considered to possess strict host specificity to bovine (cattle and water buffalos).

Even early molecular epidemiological work, the genetically distant cluster of amphibian isolates were recognized and assumed to be other species of *Retortamonas* [11]. Furthermore, in this study, the presence of robustly supported molecular taxonomic multiple clusters among vertebrate-derived retortamonads emphasizes the need to rearrange or revision the complicated species names of *Retortamonas* spp. [10] by comparative evaluation of morphological and molecular classifications.

The haplotypes isolated from humans were only detected from assemblage B, thus assemblage B might correspond to *R. intestinalis*. The haplotypes from cattle, goats, and water buffalos (assemblages B or C) might be related to *R. ovis* (hosts: sheep and cattle) or *R. ruminatum* (Indian bulls). The haplotypes from rats (assemblage B) might be related to *R. caviae* (guinea pig) or *R. kirbii* (American woodchuck). Without checking the relation of genetic haplotypes and previous species names one by one, it might be difficult to confirm those molecular classifications; however, most of those species names are considered to be synonyms of *R. intestinalis* (assemblage B), and the other names might be designated to other specific genotypes. It is also of interest to know whether the haplotypes from a chicken are related to the recently reported ostrich strain of *Retortamonas* [29]. If so, it could be identified as *R. intestinalis* (assemblage B).

In this study, we confirmed the polyphyletic features of current *Retortamonas* spp. and revealed at least four lineages, including three clusters (assemblages A–C) of genetic haplotypes from vertebrates, which are closely related to diplomonads, and one cluster of haplotypes from insects, which is considered to fulfill the original criteria of Retortamonadida with the *Chilomastix* species. The genotyping classification of retortamonads could contribute to a better understanding of its molecular epidemiology, especially among humans and related host organisms.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2018.12.004.

References

[1] O.C.F. Wenyon, CM, Human Intestinal Protozoa in the Near East, JOHN BALE, SONS & DANIELSSON, LTD, London, 1917.
[2] W. Broughton-Alcock, J.G. Thomson, *Embadomonas intestinalis* (Wenyon and O'Connor), 1917; description of the cysts and free forms found in a case in England, Proc. R. Soc. Med. 15 (1922) 8–13 Sect Trop Dis Parasitol.
[3] M.J. Hogue, Four races of *Retortamonas (Embadomonas) Intestinalis*, Am. J. Epidemiol. 23 (1) (1936) 80–84.
[4] D.H. Wenrich, The Relation of the Protozoan Flagellate, *Retortamonas gryllotalpae* (Grassi, 1879) Stiles, 1902 to the Species of the Genus *Embadomonas* Mackinnon, 1911, Trans. Am. Microsc. Soc. 15 (4) (1932) 225–238.
[5] E.N. Jaroslav Kulda, Ivan Cepicka, Retortamonadida (with notes on *Carpediemonas*-like organisms and caviomonadidae), in: A.G.B.S. John M. Archibald, Claudio H. Slamovits (Eds.), Handbook of the Protists, Springer, 2017.
[6] J.M. Goldsmid, S. Rogers, K. Mahomed, Observations on the intestinal protozoa infecting man in Rhodesia, S. Afr. Med. J. 50 (40) (1976) 1547–1550.
[7] G. Cancrini, A. Bartoloni, L. Nunez, F. Paradisi, Intestinal parasites in the Camiri, Gutierrez and Boyuibe areas, Santa Cruz Department, Bolivia, Parassitologia 30 (2–3) (1988) 263–269.
[8] H.H. Kasssem, H.A. Zaed, G.A. Sadaga, Intestinal parasitic infection among children and neonatus admitted to Ibn-Sina Hospital, Sirt, Libya, J. Egypt Soc. Parasitol. 37 (2) (2007) 371–380.
[9] O. Kawamura, Y. Kon, A. Naganuma, T. Iwami, H. Maruyama, T. Yamada, K. Sonobe, T. Horikoshi, M. Kusano, M. Mori, *Retortamonas intestinalis* in the pancreatic juice of a patient with small nodular lesions of the main pancreatic duct, Gastrointest. Endosc. 53 (4) (2001) 508–510.
[10] M.A. Ansari, The genus *Retortamonas* Grassi; its cultivation and development, Pak. J. Health 5 (4) (1956) 189–195.
[11] J.D. Silberman, A.G. Simpson, J. Kulda, I. Cepicka, V. Hampl, P.J. Johnson, A.J. Roger, Retortamonad flagellates are closely related to diplomonads–implications for the history of mitochondrial function in eukaryote evolution, Mol. Biol. Evol. 19 (5) (2002) 777–786.
[12] L.A. Amaral Zettler, T.A. Nerad, C.J. O'Kelly, M.T. Peglar, P.M. Gillevet, J.D. Silberman, M.L. Sogin, A molecular reassessment of the Leptomyxid amoebae, Protist 151 (3) (2000) 275–282.
[13] L.O. Andersen, H. Vedel Nielsen, C.R. Stensvold, Waiting for the human intestinal

Eukaryotome, ISME J. 7 (7) (2013) 1253–1255.
[14] J. Lukes, C.R. Stensvold, K. Jirku-Pomajbikova, L. Wegener Parfrey, Are human intestinal eukaryotes beneficial or commensals? PLoS Pathog. 11 (8) (2015) e1005039.
[15] M. Tanabe, E. Chiba, A new culture medium for *Entamoeba histolytica*, Acta Med. Keijo 11 (1928) 1–4.
[16] M. Kamaruddin, M. Tokoro, M.M. Rahman, S. Arayama, A.P. Hidayati, D. Syafruddin, P.B. Asih, H. Yoshikawa, E. Kawahara, Molecular characterization of various trichomonad species isolated from humans and related mammals in Indonesia, Kor. J. Parasitol. 52 (5) (2014) 471–478.
[17] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (7) (2016) 1870–1874.
[18] A.G. Simpson, Cytoskeletal organization, phylogenetic affinities and systematics in the contentious taxon Excavata (Eukaryota), Int. J. Syst. Evol. Microbiol. 53 (Pt 6) (2003) 1759–1777.
[19] S.M. Adl, A.G. Simpson, C.E. Lane, J. Lukes, D. Bass, S.S. Bowser, M.W. Brown, F. Burki, M. Dunthorn, V. Hampl, A. Heiss, M. Hoppenrath, E. Lara, L. Le Gall, D.H. Lynn, H. McManus, E.A. Mitchell, S.E. Mozley-Stanridge, L.W. Parfrey, J. Pawlowski, S. Rueckert, L. Shadwick, C.L. Schoch, A. Smirnov, F.W. Spiegel, The revised classification of eukaryotes, J. Eukaryot. Microbiol. 59 (5) (2012) 429–493.
[20] G. Brugerolle, Contribution ál'étude cytologique et phylétique des Diplozoaires (Zoomastigophorea, Diplozoa Dangeard 1910), VI: Caractéres généraux des Diplozoaires, Protistologica 11 (1975) 111–118.
[21] M. Kolisko, J.D. Silberman, I. Cepicka, N. Yubuki, K. Takishita, A. Yabuki, B.S. Leander, I. Inouye, Y. Inagaki, A.J. Roger, A.G.B. Simpson, A wide diversity of previously undetected free-living relatives of diplomonads isolated from marine/saline habitats, Environ. Microbiol. 12 (10) (2010) 2700–2710.
[22] P.P. Grassé, Ordre des Retortamonadines nov, Traité de Zoologie. Phylogénie, Protozoaires: Généralités, Flagellés, Masson and Cie, Paris, 1952, pp. 824–835.
[23] M. Kolisko, I. Cepicka, V. Hampl, J. Kulda, J. Flegr, The phylogenetic position of enteromonads: a challenge for the present models of diplomonad evolution, Int. J. Syst. Evol. Microbiol. 55 (4) (2005) 1729–1733.
[24] M. Kolisko, I. Cepicka, V. Hampl, J. Leigh, A.J. Roger, J. Kulda, A.G.B. Simpson, J. Flegr, Molecular phylogeny of diplomonads and enteromonads based on SSU rRNA, alpha-tubulin and HSP90 genes: Implications for the evolutionary history of the double karyomastigont of diplomonads, BMC Evol. Biol. 8 (1) (2008).
[25] I. Cepicka, M. Kostka, M. Uzlikova, J. Kulda, J. Flegr, Non-monophyly of Retortamonadida and high genetic diversity of the genus *Chilomastix* suggested by analysis of SSU rDNA, Mol. Phylogenet. Evol. 48 (2) (2008) 770–775.
[26] K. Takishita, M. Kolisko, H. Komatsuzaki, A. Yabuki, Y. Inagaki, I. Cepicka, P. Smejkalova, J.D. Silberman, T. Hashimoto, A.J. Roger, A.G. Simpson, Multigene phylogenies of diverse *Carpediemonas*-like organisms identify the closest relatives of 'amitochondriate' diplomonads and retortamonads, Protist 163 (3) (2012) 344–355.
[27] M.M. Leger, M. Kolisko, R. Kamikawa, C.W. Stairs, K. Kume, I. Čepička, J.D. Silberman, J.O. Andersson, F. Xu, A. Yabuki, L. Eme, Q. Zhang, K. Takishita, Y. Inagaki, A.G.B. Simpson, T. Hashimoto, A.J. Roger, Organelles that illuminate the origins of *Trichomonas* hydrogenosomes and *Giardia* mitosomes, Nat. Ecol. Evol. 1 (4) (2017).
[28] G. Brugerolle, The symbiotic fauna of the African termite *Hodotermes mossambicus* identification of four flagellate species of the genera *Spironympha*, Trichomonoides and *Retortamonas*, Parasitol. Res. 98 (3) (2006) 257–263.
[29] R.A. Martinez-Diaz, A.T. Castro, S. Herrera, F. Ponce, First report of the genus *Retortamonas* (Sarcomastigophora: Retortamonadidae) in birds, Mem. Inst. Oswaldo Cruz 96 (7) (2001) 961–963.