An Investigation of the Prevalence of Giardia agilis in Anuran Amphibians Reveals Special Parasitic Adaptations of this Giardia Species

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Research

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Abstract

Background: *Giardia agilis* is a *Giardia* species with a very narrow and elongated body distinct from others. It was first reported in 1882, and was detected in several species of anuran amphibians. Although there were some studies about its morphology, no investigations about its prevalence have ever been reported to date.

Methods: We detected *G. agilis* in frogs and tadpoles from some areas of China based on its distinct morphology. Statistical analysis was performed using mid-P exact probability tests and differences were considered significant when p-values ≤ 0.05 were obtained.

Results: We investigated the prevalence of *G. agilis* in 25 anuran amphibian species and found that 195 of the 463 (42.1%) samples were detected to be positive. Our molecular phylogenetic analysis indicated all the detected *G. agilis* were from the same species. The 195 positive frog samples were from 9 frog species, which are distributed scatteredly in four families that are not closely related rather than just restricted to a narrow lineage. The statistical prevalence among adults of different frog species showed no significant difference, and so did among tadpoles, but the prevalence in the tadpoles is significantly higher than in their adults. More interestingly, although the prevalence in *Kaloula verrucosa* from the same area showed no significant differences between its tadpoles without legs and the ones with two legs, but is significantly higher in these two developmental stages than in the four-legged stage, which is still much higher than in the adults. Moreover, all the positive samples were found to be from the areas with relatively high altitude (more than 870 meters).

Conclusions: *G. agilis* is probably able to infect all anuran amphibians without species-bias. The turning point of prevalence in the period of front leg development might be related with the development of immune system of the hosts. That *G. agilis* tends to infect easily the frogs living in high altitude areas means that it has adapted to the dramatic temperature change in the poikilothermal animal hosts. Therefore, *G. agilis* has evolved some special successful parasitism strategies for parastizing the poikilothermal hosts with metamorphosis development – anuran amphibians.

Background

Giardia spp. are intestinal protozoan parasites found in almost all vertebrates worldwide, and have attracted the attention of scientists for both medical and evolutionary biological reasons in the past 300 years [1]. There are currently eight Giardia species considered to be valid [2]. G. agilis, as one of them, was first reported by Künstler in 1882, and was later detected in the intestines of tadpoles and adults of several anuran amphibian species by other researchers [1, 3]. The morphology of G. agilis have been studied based on interference reflexion microscopy and scanning electron microscopy (SEM), and it was shown that the trophozoites of G. agilis have a narrow and elongated body, and the length of their adhesive discs is about one fifth of their body [4]. Obviously, it has a distinct morphology, and thus is easy to be distinguished from other Giardia species. Currently, molecular identification of Giardia species

is usually based on the following five genes: glutamate dehydrogenase, beta-giardin, elongation factor-1 alpha, triose phosphate isomerase and small subunit rRNA (SSU rRNA) [5]. But except beta-giardin, all the other genes of G. agilis do not have sequence data yet [2].

Investigation of the prevalence of Giardia has been carried out mainly on G. intestinalis, and more than 100 waterborne giardiasis outbreaks had been reported worldwide till 2004 [6]. Potential mechanisms of transmission was one of the important contents of the investigations, including person to person, animal to animal and zoonotic through drinking water or recreational contact (such as in swimming) and foodborne [6, 7, 8]. The host specificity of different G. intestinalis genotypes was another important content, and more than 50 different mammals were investigated, including wild animals, pets and livestock [9, 10]. Epidemiology of G. intestinalis has been a continuous and hot research topic in the protozoan parasite field due to the harm of giardiasis to human and other mammals.

Unfortunately, we know almost nothing about the prevalence of G. agilis though this species has been reported more than one hundred years. In the present study, the prevalence of G. agilis was investigated in many anuran amphibians in China. We also sequenced their SSU rRNA and β-giardin genes to access the molecular identification and phylogenetic analysis to identify the species. The results show that there are no significant differences in prevalence either in the same host specie from different areas or among different host species from a common area, but there were significant differences between adult frogs and their tadpoles; and that interestingly, all positive samples were from high altitude areas, where temperatures are relatively variable. The possible reasons and implications of these observations are discussed.

Material And Method

Sample collection

G. agilis trophozoites were collected from frogs and tadpoles. The frogs and tadpoles were anesthetized to death with 20% ethanol and then their intestines were removed and cut into 0.1 cm segments. The segments of intestines were collected into centrifuge tubes with amphibian normal saline (0.65% sodium chloride). The centrifuge tubes were chilled in ice for more than 30 min. The suspension was briefly centrifuged at 1,000× g for 1s to remove the precipitants of large fragments and the supernatant was collected into new centrifuge tubes. Then centrifuge tubes were centrifuged at 750× g for 5 min and the supernatant was discarded to concentrate the trophozoites. The sediment was re-suspended with amphibian normal saline and kept at room temperature for 30 min. Then the amphibian normal saline was replaced. The centrifuge tubes were chilled in ice for 30 min and centrifuged at 2,000× g for 5 min, to collect the trophozoites. The supernatant was discarded and the precipitated trophozoites were re-suspended with amphibian normal saline and were stored at 4 °C for later use.

The frog samples were collected in some selected areas of Yunnan, Sichuan, Gansu, and Shaanxi provinces, and Shanghai city of China at the time from March of 2014 to May of 2019. We followed the guidelines of The Animal Care and Use Committee of the American Society of Mammalogists for the use

of wildlife in our research [11]. All the experimental procedures and animal care were performed according to the protocols approved by the Institutional Animal Care and Use Committee of the Kunming Institute of Zoology, Chinese Academy of Sciences (PAOKIZ140105, 01/2014).

Morphological identification of *G. agilis*

The slides were prepared with trophozoite supernatant in amphibian normal saline. All slides were examined under oil immersion by 40× and 100× HCX PL APO objectives on a Leica DM2500 microscope (Leica, Wetzlar, Germany). The images were captured by a Leica DFC450 C digital camera. Sample handling and photomicrographs of scanning electron microscope were processed at the Kunming Medical University (Kunming, China) using HITACHI 3700N (Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using mid-P exact probability tests and 95% confidence interval (CI) value was calculated, differences were considered significant when p-values ≤ 0.05 were obtained (https://www.medcalc.org/).

DNA extraction, PCR amplification and sequencing

G. agilis genomic DNA was extracted from feces samples of tadpoles and frogs using the TIANamp Stolol DNA Kit (Tiangen, Beijing, China) according to the manufacturer's protocol. The primers specific to both ends of the sequences of the two genes, *SSU rRNA* and *β-giardin*, were designed according to the conserved sequences of these genes of *G. intestinalis* (50586 isolate) using PRIMER PREMIER program version 5.00 (Biosoft International) [12], and the expected product lengths of the *SSU rRNA* and *β-giardin* were 950 bp and 500 bp, respectively (see primer sequences in Additional file 1). The primers were used to amplify the sequences of the two genes from all fecal genomic DNA samples by PCR. The PCR reactions were set up in 25 μl 2× PCR Taq Plus MasterMix with dye (abm, Canada), 1 μM of each primer and 1-5 μl of DNA sample. Thermocycling conditions were as follows: 94 °C for 10 min followed by 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 90 s, followed by 72 °C for 10 min. The PCR products were purified using the Wizard SV Gel and PCR clean-up system kit (Qiagen), and cloned into pMD-19T vectors using TaKaRa pMD-19T VectorCloning Kit (TaKaRa, Tokyo, Japan). The ligation products were transformed into DH5α chemically competent *E. coli*. Colony PCR was carried out with the vector-specific primers provided in the kit, and colonies were selected and Sanger-sequenced using vector-specific forward and reverse primers by TSINGKE Biological Technology (Kunming, China).

Molecular phylogenetic analysis

In order to perform the phylogenetic analysis, we sequenced, identified and retrieved the *SSU rRNA* and *β-giardin* of *G. agilis* (GenBank accession numbers: MN227552 and MG733773). The other sequences used in the analysis were all retrieved from the GenBank database (see their accession numbers in Additional file 2). The number of isolates of *G. agilis* for each locus sequenced is as follows: 5 for SSU rRNA and 7 for β-giardin. The maximum likelihood phylogenetic trees based on SSU rRNA and β-giardin DNA

sequences were reconstructed by using the PhyML software [13]. The sequence data of the two loci are from 9 and 10 *Giardia* species or isolates. Multiple sequence alignments were performed with ClustalW 2.0 program [14], and the alignments were visually inspected to eliminate poorly aligned positions. The best-fit DNA model used for reconstructing the maximum likelihood phylogeny was selected by the JModelTest software [15, 16]. Trees were constructed using the PhyML 3.0 based on 14 taxa with 817 nt positions for SSU rRNA gene and 16 taxa with 335 nt positions for β-giardin gene. Tree reliability was determined by using bootstrap analyses with 1000 replicates

Results

Microscopy

G. agilis trophozoites from the intestines of all animals were detected and measured by microscopy. Trophozoites have an elongated body with lengths of 20–30 μm and widths of 4–5 μm (Fig. 1). But more accurate measurements could not be reached because *G. agilis* trophozoites are easily distorted morphologically during their purification from intestines.

Prevalence of *G. agilis* in 25 different anuran amphibian species

The investigation data from the following frog samples of 25 species: 19 species with only adult frogs, one species with only tadpoles, and five species with both tadpoles and adult frogs. 195 of all the 463 (42.1%) investigated samples were detected to be positive for *G. agilis*. And the 195 positive samples were from 9 species of the 25 investigated frog species, and the statistical prevalence of *G. agilis* in all these positive host species are shown in Table 1. Tadpole samples of all the six species were detected to be positive. The 16 negative anuran amphibian species and detective samples were shown in Table 2, all negative samples were adult frogs. Though 16 species were detected to be negative, the prevalence of *G. agilis* in positive frogs species showed no significant difference. The prevalence in the tadpoles of the six species was also not significantly different. In the five species samples with both adult frogs and tadpoles, we found that either the respective prevalence between adult frogs and their tadpoles among each frog species or the total prevalence between the adult frogs of all the species (8.9%, 95% CI 2.48 to 21.24%) and their tadpoles (84.6%, 95% CI 77.78 to 89.99%) were significantly different (Table. 3). Furthermore, in Xihuanyuan lake of Kunming (102.7°E, 25.0°N), we collected and detected *G. agilis* in different developmental stages of *Kaloula verrucosa* tadpoles, and found that the prevalence are as follows: tadpoles without legs (Gosner stages 26-30) – 86.4% (95% CI 65.13 to 97.11%); tadpoles with two legs (Gosner stages 31-40) – 100% (95% CI 69.15 to 100.00%) and tadpoles with four legs (Gosner stages 41-46) – 0% (95% CI 0.00 to 60.24%) (Table 4). The prevalence of *G. agilis* in different developmental stages of *Kaloula verrucosa* tadpoles from same area showed no significant differences between tadpoles without legs (Gosner stages 26-30) and tadpoles with two legs (Gosner stages 31-40), but they both are significantly higher than those in tadpoles with four legs (Gosner stages 41-46) which was higher than adult frogs.

Prevalence of *G. agilis* in anuran amphibians from different areas

The 463 frog samples were collected from 14 areas of 5 provinces in China (Fig. 2). For the information of these sampling areas, please see Additional file 3. The prevalence of *G. agilis* in the 25 sampling frog species from these different areas is summarized, please see Additional file 4. All samples from the same area were put together as calculating the prevalence in the area. We found that the prevalence among the positive frog species from areas with higher altitude (equal to or over 870m) showed no significant difference from each other, and that the prevalence in the same positive frog species from these different areas with higher altitude showed much less significant difference (Table. 5). On the contrary, the frog samples from the areas with lower altitude (lower than 870m) were all negative. Interestingly, all the positive samples came from these sampling places where the altitudes are more than 870m. For example, *Rana catesbeiana* is positive in Dali (1505m) and negative in Shanghai (5m), and *Amolops mantzorum* is positive in Deyang (870m) and negative in Chengdu (730m). And the total prevalence of areas in different altitude were significantly different: the total prevalence of areas with higher altitude were significantly higher than the areas with lower altitude (Table. 6).

Phylogenetic analysis of collected *G. agilis* samples

We sequenced a fragment of the *SSU rRNA* and *β-giardin* genes of the collected *G. agilis* samples, respectively. Phylogenetic analysis based on these fragments showed that *G. agilis* we collected is genetically distinct from all other *Giardia* species (see additional file 5), and that all *G. agilis* we detected showed little genetic distance, indicating they might be from one assemblage despite the samples being collected from different hosts and areas (Fig. 3 and Fig. 4).

Discussion

In the present study, the morphological characteristics were used to identify the G. agilis from all the frog samples, and the partial SSU rRNA and β-giardin sequences were used as supplementary identification evidence. The anuran amphibian species were collected as many as possible from 14 areas in China where natural environments are different. We have detected G. agilis in 463 collected individual animals from 25 anuran amphibian species, including in adults and/or tadpoles, and found that 9 species of them have positive individuals and the overall prevalence was 42.1% (195/463). We noticed the prevalence among the adults of the positive frog species showed no significant difference, and all the tadpole samples from the six species were detected to be positive, and the prevalence among them also showed no significant difference. On the other hand, the 25 frog species samples we detected were from seven anuran families, four of them: Microhylidae, Rhacophoridaae, Ranidae and Pipidae were detected to be positive, and three of them: Hylidae, Bufonidae and Pelobatidae were detected to be negative. Obviously, the positive species are not restricted to a narrow lineage but are distributed scatteredly in as many as four families not closely related in the order Anuran [17]. We further noticed that the prevalence in the same positive species from different high-altitude places also showed no significant difference. All these observations imply that G. agilis is probably able to infect all anuran amphibian species without species-bias, and that the species that were detected to be negative might be due to either their small sample size or the absence of tadpole samples. That the prevalence of G. agilis seems to show no preference in

different anuran amphibians might imply that G. agilis has evolved some special parasitic adaptation or host-specific strategy so that it can almost indiscriminately infect all anuran amphibian species.

Interestingly, we found that there were significant differences of G. agilis prevalence between adult frogs and their tadpoles. In five frog species we detected the prevalence of G. agilis both in their adults and tadpoles, and found that either in the individual species or in the total species, the prevalence of G. agilis in tadpoles was much higher than in adult frogs. Furthermore, we found that the prevalence of G. agilis in different developmental stages of Kaloula verrucosa tadpoles from the same area exhibited an interesting feature: there were no significant differences between the tadpoles without legs (Gosner stages 26–30) and the tadpoles with two legs (Gosner stages 31–40), but they both were significantly higher than that in the tadpoles with four legs (Gosner stages 41–46). This suggests that apparently there exist a turning point of prevalence of G. agilis in the period of front leg development of frogs, which might be related with the development of immune system of frogs. Because it is known that during the ontogeny of frogs, tadpoles exhibit changes in immune system cells transforming from larval-type to adult-type, especially changing sharply at the stage of tadpole tail degeneration [18], which is consistent with the finding of “turning point of prevalence”. While, for other known Giardia species, they all infect animals without metamorphosis, such as G. ardeae and G. psittaci in birds, G. microti and G. muris in rodents, G. intestinalis in mammals [10], G. peramelis in Australian bandicoots [19] and G. cricetidarum in hamsters [2], no such a phenomenon has ever been seen. Thus, this observation may imply that the transforms of immune system cells from larval-type to adult-type probably occur while the front legs begin to grow in the development of frogs, and that G. agilis in the developing tadpoles begins to decrease as the transform begins and finally reaches a much less level in the adults. This means that different from other known Giardia species, G. agilis has developed an appreciate parasitic adaptation strategy to the metamorphosis development of frogs

It is also interesting that in our investigation all the 195 positive samples were found to be collected from the areas where the altitudes are above 870 m, including the same positive species from different areas, but all these G. agilis positive frog species are not plateau specific. The average temperatures across areas that positive samples were collected are different [20]. Furthermore, the temperatures often fluctuates dramatically during a short period of time at high altitude areas, and anuran amphibians, as poikilotherms, also often change their body temperatures, therefore that G. agilis parasitizes the frogs living in high altitude areas means that different from other known Giardia spp. this species is often under the pressure of temperature change. Therefore, compared to other known Giardia species, especially those parasitizing homothermal animals, G. agilis might have evolved some special ability to adapt the dramatic change of temperature, which is worthy of further study.

Our analysis at two different loci showed that G. agilis samples from different places showed little genetic distance, and the genetic distances among different assemblages of G. intestinalis are even greater than among them. This means that not only all these G. agilis samples we detected in different anuran amphibians must be from the same species, but also G. agilis might be under a greater selective pressure than G. intestinalis. At least the remarkable immune system transform of the hosts and the

dramatic temperature change of its living environment mentioned above might be two extra resources of such selective pressure. Therefore, G. agilis must have to evolve some special parasitic adaptation or host-specific strategy compared to other known Giardia species.

Conclusion

The prevalence of G. agilis in 25 anuran amphibians species from seven families in China was investigated, and 195 of the 463 (42.1%) samples collected from nine frog species of four families that are not closely related were detected to be positive with similar prevalence. Thus G. agilis is probably able to infect all anuran amphibian species without species-bias at a quite high prevalence. However, the prevalence in adult frogs was always lower than in their tadpoles, and the turning point seems to appear in the period of front leg development, which might be related with the development of immune system of frogs from larval-type to adult-type. Most interestingly, G. agilis tends to infect easily the frogs living in the high altitude areas (about more than 870 m), where the temperatures often fluctuates dramatically. This means that G. agilis in the poikilothermal animal frogs has adapted to the dramatic temperature change. All these findings imply that G. agilis has evolved some special successful parasitism strategies for parastizing the poikilothermal hosts with metamorphosis development – frogs.

Declarations

Ethics approval and consent to participate

We followed the guidelines of The Animal Care and Use Committee of the American Society of Mammalogists for the use of wildlife in our research [11]. All the experimental procedures and animal care were performed according to the protocols approved by the Institutional Animal Care and Use Committee of the Kunming Institute of Zoology, Chinese Academy of Sciences.

Consent for publication

Not applicable.

Availability of data and material

The genetic datasets generated and analysed during the current study are available in the GenBank, https://www.ncbi.nlm.nih.gov/genbank/. *The PRIMER PREMIER* Program version *5.00 is* downloaded from the web, http://www.premierbiosoft.com/products/products. The JmodleTest software is downloaded from the web, http://jmodeltest.org. The clustalW 2.0 program is available in the web, http://www.ebi.ac.uk/Tools/msa/. Statistical analysis was performed in the web, https://www.medcalc.org/.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ZL did the experiments, collected and analyzed the data, and wrote the manuscript; JC carried out the experiments and collected the data; JS, QY and HB took part in the collection of samples; JXW took part in partial data analysis ; JFW made a contribution to conception, design, and writing the manuscript. All authors read and approved the final version of the manuscript.

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Authors' information (optional)

Not applicable.

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Tables

Family	Positive Species	Stage	Positive / Detected (Prevalence)	95% CI
Microhylidae	*Kaloula verrucosa*	Frog & Tadpole	104/126 (82.5%)	74.72 - 88.69%
Rhacophoridaae	*Rhacophorus sp.*	Tadpole	22/22 (100%)	84.56 - 100.00%
Pipidae	*Xenopus laevis*	Frog	2/2 (100%)	15.81 - 100.00%
Ranidae	*Nidirana pleuraden*	Frog	12/66 (18.2%)	9.78 - 29.63%
	Rana chaochiaoensis	Frog	22/29 (75.9%)	65.11 - 95.64%
	Amolops mantzorum	Tadpole	7/15 (46.7%)	21.29 - 73.44%
	Odorrana margaratae	Tadpole	10/19 (52.6%)	28.84 - 75.53%
	Rana catesbeiana	Tadpole	7/25 (28.0%)	12.07 - 49.39%
	Rana quadranus	Tadpole	16/27 (59.2%)	38.74 - 77.56%

Table 1. Prevalence of *G. agilis* in the 9 positive anuran amphibian species.

Family	Frogs	Stage	Detected Samples
Hylidae	*Hyla chinensis*	Frog	2
	Hyla annectans Jerdon		9
Microhylidae	*Calluella yunnanensis*		24
	Microhyla ornata		9
Rhacophoridaae	*Rhacophorus* sp.		1
Bufonidae	*Polypedates chenfui*		1
	Bufo gargarizans andrewsi		2
Ranidae	*Duttaphrynus melanostictus*		10
	Pelophylax plancyi		2
	Pelophylax pleuraden		15
	Rana grahami		8
	Odorrana rodora		5
	Quasipaa spinosa		18
	Paa yunnanensis		2
	Rana rugulosa		8
Pelobatidae	*Oreolalax* sp.		8

Table 2. Detective samples of the 16 *G. agilis* negative anuran amphibian species.

Frogs and Tadpoles		Positive/Detected Samples	Total Prevalence	95% CI
Adult Frog	*Kaloula verrucosa*	4/19 (21%)	8.9%	2.48-21.24%
	Rana catesbeiana	0/3 (0%)		
	Amolops mantzorum	0/8 (0%)		
	Rana quadranus	0/11 (0%)		
	Odorrana margaratae	0/4 (0%)		
Tadpole	*Kaloula verrucosa*	86/92 (93.5%)	84.6%	77.78-89.99%
	Rana catesbeiana	7/22 (31.8%)		
	Amolops mantzorum	7/7 (100%)		
	Rana quadranus	16/16 (100%)		
	Odorrana margaratae	10/12 (83.3%)		

Table 3. Prevalence of *G. agilis* in the 5 anuran amphibian species with both tadpoles and adult frogs.

Kaloula verrucosa Tadpole in Kunming	Prevalence (Positive/Detected Samples)	95% CI
Without legs	86.4%(19/22)	65.13 - 97.11%
With two legs	100%(10/10)	69.15 - 100.00%
With four legs	0%(0/4)	0.00 - 60.24%

Table 4. Prevalence of *G. agilis* in three different developmental stages of *Kaloula verrucosa* tadpoles.

Species	Prevalence in different areas (95% CI)			
Kaloula verrucosa	0.0% (0-33.63%) Chuxiong	40.0% (12.16-73.76%) Kunming		
Kaloula verrucosa tadpoles	100% (82.35-100%) Honghe	92.0% (84.24-96.71%) Kunming		
Nidirana pleuraden	0.0% (0-36.94%) Chuxiong	20.7% (11.18-33.36%) Kunming		
Amolops mantzorum	0.0% (0-45.93%) Liangshan	100% (59.04-100%) Deyang	0.0% (0 -84.19%) Chengdu	
Rana chaochiaoensis	90.9% (58.71-99.77%) Liangshan	100% (66.37%-100%) Mianzhu	50.0% (11.81-88.19%) Deyang	
Rana catesbeiana	0.0% (0 - 97.50%) Chuxiong	100% (59.04%-100%) Dali	0.0% (0%-21.80%) Kunming	0.0% (0-84.19%) Shanghai

Table 5. Prevalence of *G. agilis* in the same species from different areas.

Altitude	Prevalence	95% CI
Higher than 870m (including)	46.53%	41.67 - 51.44%
Lower than 870m	0.00%	0.00 – 8.04%

Table 6. Total prevalence of *G. agilis* in different altitudes.

Figures

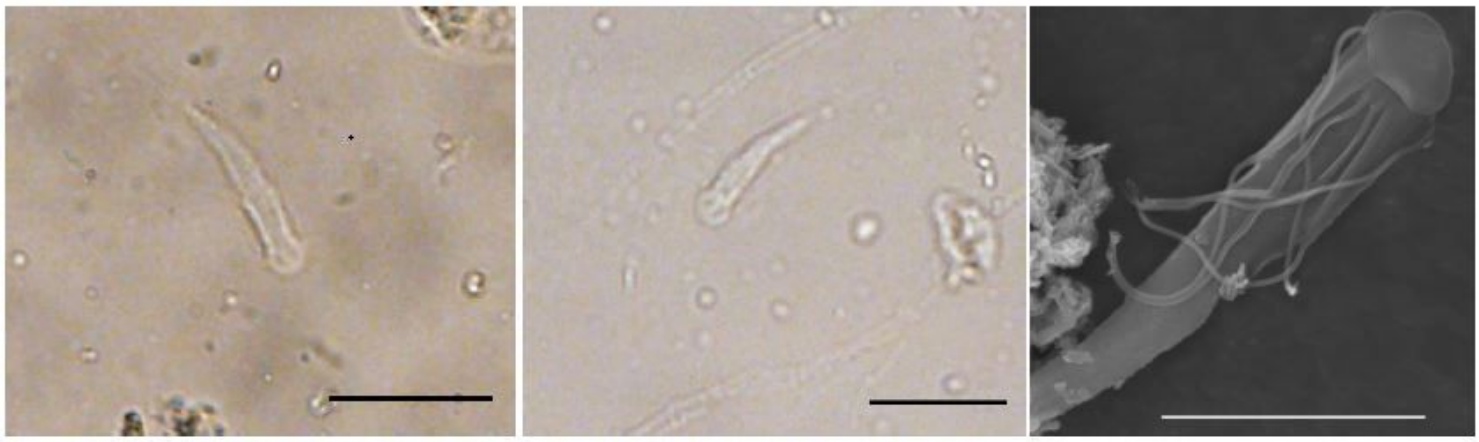

Figure 1

Trophozoites of G. agilis A, B. The trophozoites of G. agilis under bright-field microscope have a narrow and elongated body. Bar=20□m. C. Trophozoites of G. agilis under scanning electron microscope. Bar=10□m.

Figure 2

Distribution of sampling positions All samples were collected from these 14 places of 5 provinces in China. The sizes of circles represent the sample sizes. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

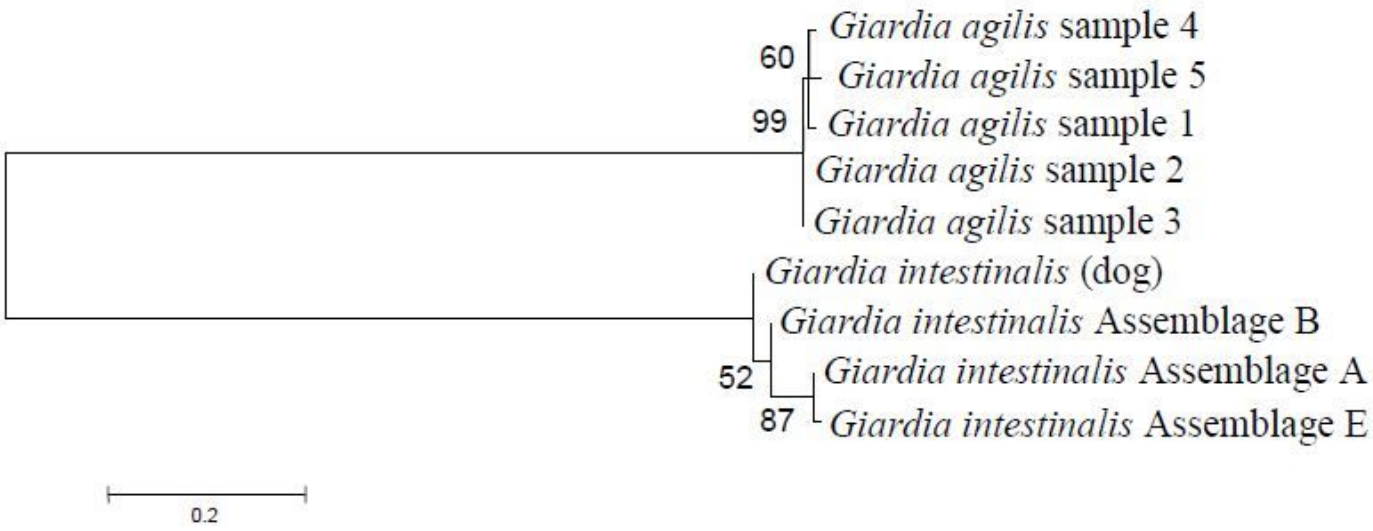

Figure 3

Phylogenetic tree of SSU rRNA Phylogenetic tree of SSU rRNA, indicating that all G. agilis we detected were from the same species.

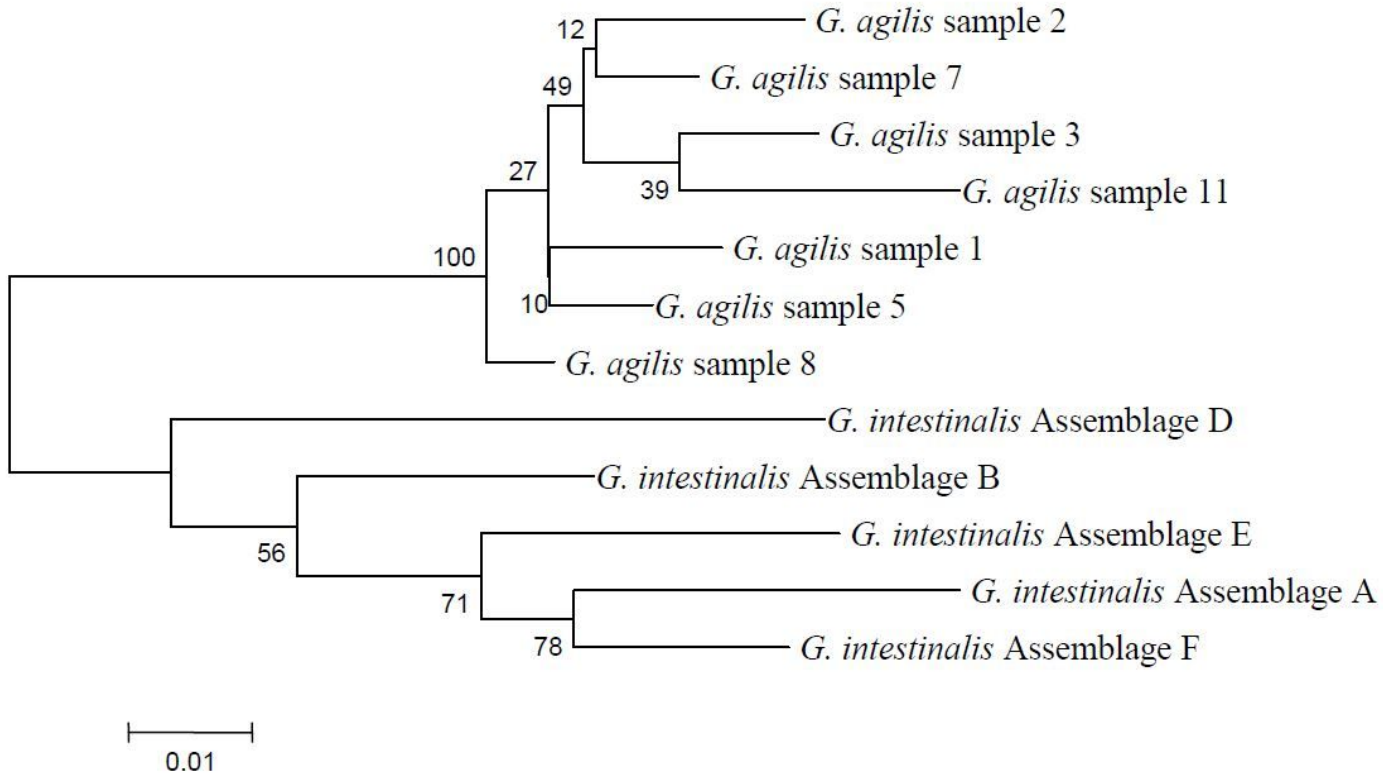

Figure 4

Phylogenetic tree of -giardin Phylogenetic tree of -giardin, showing that all G. agilis we detected were from the same species.

Supplementary Files

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- additionalfile4.pdf
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