Molecular Characteristics of *Camallanus* Spp. (Spirurida: Camallanidae) in Fishes from China Based On its rDNA Sequences

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Source: Journal of Parasitology, 94(3):731-736. 2008.
Published By: American Society of Parasitologists
DOI: 10.1645/GE-1219.1
URL: http://www.bioone.org/doi/full/10.1645/GE-1219.1

J. Parasitol., 94(3), 2008, pp. 731–736

MOLECULAR CHARACTERISTICS OF *CAMALLANUS* SPP. (SPIRURIDA: CAMALLANIDAE) IN FISHES FROM CHINA BASED ON ITS rDNA SEQUENCES

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ABSTRACT: In the paper, we explored the intra- and interspecific evolutionary variation among species of *Camallanus* collected from different fish species in various regions of China. We determined the internal transcribed spacers of ribosomal DNA (ITS rDNA) sequences of these nematodes. The divergence (uncorrected p-distance) of ITS1, ITS2, and ITS rDNA data sets confirmed 2 valid species of *Camallanus* in China, i.e., *C. cotti* and *C. hypophthalmichthys*. The 2 species were distinguished not only by their different morphologies and host ranges but also by a tetranucleotide microsatellite (TTGC)n present in the ITS1 region of *C. cotti*. Phylogenetic analyses of the nematodes disclosed 2 main clades, corresponding to different individuals of *C. cotti* and *C. hypophthalmichthys* from different fish species in various geographical locations, although the interior nodes of each clade received poor support.

Species of *Camallanus* (Spiruridea: Camallanidae) occur almost globally in freshwater and marine fishes and even in amphibians (Chabaud and Bain, 1994; Levsen and Berland, 2002; Moravec et al., 2003). In China, 3 species, i.e., *C. cotti*, *C. hypophthalmichthys*, and *C. zacconis*, have been recorded from various freshwater fish. Among the 3 species, *C. cotti* is a potential pathogen that can affect host behavior and even cause death (McMinn, 1990; Kim et al., 2002). As a generalist parasite, it is frequently found in many species of the Cypriniformes, Siluriformes, and Perciformes. Recent surveys of helminths reveal that the host range and geographic distribution of this species are increasing and that the worm is a potential danger to endemic fishes (Font and Tate, 1994; Wu et al., 2007). Of the remaining species, *C. hypophthalmichthys* is a relative specialist, recorded in only 2 species of fish in the Hypophthalmichthyinae, whereas *C. zacconis* shares some hosts with *C. cotti*. So far, there have been no reports of pathogenicity of *C. hypophthalmichthys* or *C. zacconis* for their hosts.

The 3 species have been traditionally distinguished by their morphological characters. However, there are only a few differences among them. *Camallanus hypophthalmichthys* is distinguished from the other 2 by the presence of 3 small, but prominent, caudal processes (Moravec et al., 2004). There is almost no conspicuous morphological distinction between *C. cotti* and *C. zacconis*, and they share many common host species and similar geographic distributions. Therefore, it was proposed that *C. zacconis* is a junior synonym of *C. cotti* (Moravec, 1973; Moravec et al., 2004), leaving just 2 *Camallanus* species, i.e., *C. cotti* and *C. hypophthalmichthys*, in China.

In recent decades, several genetic markers, such as internal transcribed spacer of ribosomal DNA (ITS rDNA), have proved to be valuable for determining the phylogenetic relationships of closely related species of nematodes (Hoste et al., 1998; Perlman et al., 2003; Otranto and Traversa, 2004). In the present study, we determined the ITS rDNA sequences of the *Camallanus* spp. These sequence data were used to explore the intra- and interspecific evolutionary variation among species of *Camallanus* collected from different fish species in various regions of China.

Received 11 February 2007; revised 3 September 2007, 8 November 2007; accepted 21 November 2007.

MATERIALS AND METHODS

Sample collection, identification, and DNA extraction

The camallanids were sampled from fishes in the Yangtze River from 2002 to 2005. They were removed from the fish intestine, and then they were identified using their respective hosts, as well as morphological characters via dissecting microscopy. Preliminary examination showed that the camallanid from fishes of the Hypophthalmichthyinae was easily identified (as *C. hypophthalmichthys*) and compared favorably with those found in other fishes. The other camallanids were difficult to distinguish morphologically. Therefore, they were tentatively identified as *Camallanus cotti*. Information on the respective hosts, geographic localities, and sample codes are listed in Table I and Figure 1. *Procamallanus fulvidraconis* (accession number DQ076698) was used as the outgroup for the phylogenetic analyses.

All nematodes were washed in 0.6% saline before being stored in 85% alcohol. Worms were then soaked in TE buffer (pH 8.0) for 2 days to remove ethanol before DNA was released. Total nematode genomic DNA was extracted from 1 specimen in the respective fish host species using standard proteinase K, phenol/chloroform extraction (Sambrook, 1989). The extraction was then eluted into 25 μl of TE, pH 8.0, and stored at −20 C until use.

Polymerase chain reaction (PCR) amplification and DNA sequencing

The forward primer TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and the reverse primer AB28 (5′-ATATGCTTAAGTTCAGCGG GT-3′), as used by Subbotin et al. (2001), were used to amplify the fragment corresponding to the 18S gene in part, ITS1 rDNA, 5.8S gene, ITS2 rDNA, and 28S gene in part. PCR mixtures consisted of 20 ng of worm genomic DNA, 2 μl of each of the 2 primers at 20 mM, 2.4 U of Takara Ex Taq DNA polymerase (TaKaRa Biotechnology Co. Ltd., Dalian, China), 8.0 μl of 2.5 mM dNTPs solution, 10 μl of 10× PCR reaction buffer with 20 mM $MgCl_2$, and double-distilled water to a final volume of 100 μl. The PCR profile consisted of an initial denaturation step of 5 min at 94 C, followed by 35 cycles of denaturation at 94 C for 1 min, annealing at 55 C for 45 sec, elongation at 72 C for 1 min 10 sec, and a final extension at 72 C for 10 min in a PTC-100TM programmable thermal controller (MJ Research, Watertown, Massachusetts). A negative control was included in each PCR reaction. PCR amplification products were detected on ethidium bromide-stained 1.0% agarose-Tris-acetate-EDTA gels under UV light, and then purified over spin columns (Wizard PCR Prep, Promega, Gardner, Massachusetts). The purified products were cloned into pMD18-T vector following the manufacturer's protocol. Flanking sequence primers M13(+)/M13(−) were used to determine the plasmid DNA on an automatic DNA sequencer (model 3730, ABI Applied Biosystems, Foster City, California) in both directions. The obtained sequences have been deposited in GenBank database under accession numbers DQ403203~DQ403233.

TABLE I. Host and geographical origins of the *Camallanus* samples included in the present study.

Parasite species	Host fish species	Sample code	Geographical origin
Camallanus cotti	*Culter erythropterus*	CE	Danjiangkou Reservoir, Hubei
	C. mongolicus	CM	Danjiangkou Reservoir, Hubei
	C. ilishaeformis	CIL	Danjiangkou Reservoir, Hubei
	Hemiculter bleekeri	HB	Danjiangkou Reservoir, Hubei
	Pseudolaubuca sinensis	PS	Danjiangkou Reservoir, Hubei
	Acanthobrama simoni	AS	Danjiangkou Reservoir, Hubei
	Mylopharyngodon piceus	MP	Danjiangkou Reservoir, Hubei
	Ctenopharyngodon idella	CI	Danjiangkou Reservoir, Hubei
	Squaliobarbus curriculus	SC	Danjiangkou Reservoir, Hubei
	Zacco platypus	ZP	Danjiangkou Reservoir, Hubei
	Opsariichthys bidens	OB	Danjiangkou Reservoir, Hubei
	Gnathopogon imberbis	GI	Danjiangkou Reservoir, Hubei
	G. argentatus	GA	Danjiangkou Reservoir, Hubei
	Saurogobio dabryi	SD	Danjiangkou Reservoir, Hubei
	Pelteobagrus fulvidraco	PF	Danjiangkou Reservoir, Hubei
	Silurus asotus	SA	Danjiangkou Reservoir, Hubei
	Liobagrus marginatoides	LM	Danjiangkou Reservoir, Hubei
	Siniperca chuatsi	SCH	Danjiangkou Reservoir, Hubei
	Ophiocephalus argus	OA	Danjiangkou Reservoir, Hubei
	Hypseleotris swinhonis	HS	Danjiangkou Reservoir, Hubei
	Ctenogobius shennongensis	CS	Danjiangkou Reservoir, Hubei
	Odontobutis obscurus	OO	Niushan Lake, Hubei
	Mystus macropterus	MM	Jialingjiang River, Chongqing
C. hypophthalmichthys	*Hypophthalmichthys molitrix*	HM	Danjiangkou Reservoir, Hubei
	H. molitrix	HM2	Niushan Lake, Hubei
	H. molitrix	HM3	Tangxun Lake, Hubei
	H. molitrix	HM4	Jialingjiang River, Chongqing
	Aristichthys nobilis	AN1	Danjiangkou Reservoir, Hubei
	A. nobilis	AN2	Niushan Lake, Hubei
	A. nobilis	AN3	Tangxun Lake, Hubei
	A. nobilis	AN4	Jialingjiang River, Chongqing

Sequence alignment and analyses

The sequences were aligned initially using Clustal X (Thompson et al., 1997), with the following parameters: gap opening penalty = 10.0 and gap extension penalty = 5.0. Upon completion, the alignments were visually inspected in Seaview (Galtier et al., 1996), and slight modifications by eye were made to improve their accuracy. Alignment files for ITS1 rDNAs, ITS2 rDNAs and the combined ITS (ITS1 and ITS2) rDNAs are available by anonymous FTP from ftp.ebi.ac.uk in directory/pub/databases/embl/align or via the EMBLALIGN database via SRS at http://www3.ebi.ac.uk/Services/webin/help/webin-align/align_SRS_help.html; under accessions numbers ALIGN_001095, ALIGN_001096, and ALIGN_001097. Sequence divergence of the ITS1 rDNAs and ITS2 rDNAs, and the combined ITS rDNAs were implemented by Mega 2.1 (Kumar et al., 2001) after alignments, and the frequency of nucleotide bases of the ITS rDNA was also performed in the program.

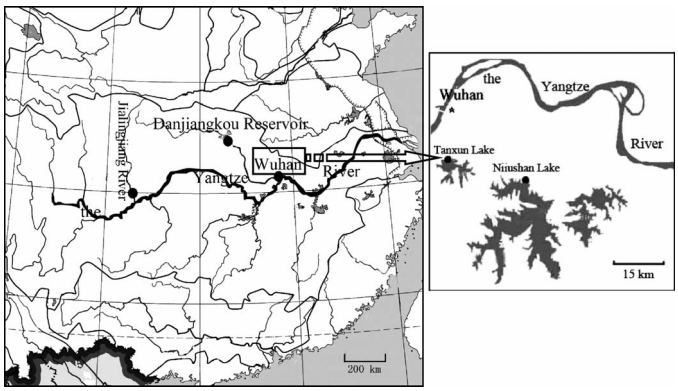

FIGURE 1. Geographic localities for *Camallanus* spp. sampling.

Transitions and transversions were plotted against sequence divergence in DAMBE 4.0.59 (Xia and Xie, 2001) to evaluate the possibility of sequence saturation. Sequence saturation was inferred from the shape of the trend line, with a linear relationship indicating the sequences were unsaturated and an asymptotic relationship showing the presence of saturation. Different nucleotide sequences have variant DNA evolution substitution patterns. The best-fit model helps to resolve phylogenetic relationships accurately. Modeltest 3.6 (Posada and Crandall, 1998) was used to find the best-fit nucleotide evolutionary model. According to the hierarchical likelihood ratio tests (hLRT), the HKY+G model was selected based on the ITS rDNA sequences, which was then used in the model-based phylogenetic methods (NJ and Bayesian analyses).

Three methods, i.e., maximum parsimony (MP), neighbor-joining (NJ), and the Bayesian approach, were used for the phylogenetic analyses to gauge the robustness of our resulting hypotheses. MP and NJ analyses were implemented in PAUP*4.0b (Swofford, 2002), and the Bayesian approach was performed in MrBayes 3.6 (Huelsenbeck and Ronquist, 2001). Equally weighted MP analyses were computed. The heuristic search setting was 100 replicates of random taxon addition tree bisection-reconnection branch swapping, multiple trees retained, no steepest descent, and accelerated transformation. Gaps were treated as missing data. Bootstrap analysis with 1,000 replicates was performed to assess the support for each branch on the corresponding tree. The NJ algorithm was performed by application of the DNA substitution model generated from Modeltest 3.6, and 1,000 replicates were also used for the bootstrap analysis. The Bayesian approach was used to construct a maximum likelihood tree. Four independent Markov chains were simultaneously run for 1,000,000 replicates by sampling 1 tree per 100

TABLE II. Uncorrected p-distance between different individuals and species of *Camallanus* parasites with different molecular markers.

	Cc*	Ch*	Between Cc* and Ch*	Cc* and outgroup	Ch* and outgroup	Ingroup and outgroup
ITS1	0–2.5	0–0.3	13.5–14.6%	†	†	53.8–56.6%
ITS2	0–0.6	0–0.4	21.2–22.1%	†	†	54.9–59.2%
ITS	0–1.5	0–0.4	19.1–20.3%	56.1–57.1	55.4–55.8	55.4–57.1%

* Cc, *Camallanus cotti*; Ch, *C. hypophthalmichthys*.
† Data not calculated.

replicates with the Bayesian procedure. The first 1,000 trees were discarded as part of a burn-in procedure, and the remaining 9,000 sampling trees were used to construct a 50% majority rule consensus tree. The combined ITS rDNA sequences were used in the phylogenetic analyses.

RESULTS

Characteristics of ITS1 and ITS2 rDNA sequences

In total, 31 sequences of the *Camallanus* nematodes, *Camallanus cotti*, and *C. hypophthalmichthys*, were obtained from different fish host species collected from different localities in China. The sequences were compared with the rDNA sequence of *Onchocerca volvulus* in GenBank (Morales-Hojas et al., 2001) to determine the boundaries of code and spacer regions. The 33-base pair (bp) 18S rDNA 3′ end, 156-bp 5.8S rDNA, and 43-bp 28S rDNA 5′ end were determined, in addition to the ITS1 and ITS2 rDNA.

Among the different individuals, of *C. cotti* from 23 fish species and individuals of *C. hypophthalmichthys* from 2 fish species collected at different localities, the length of ITS1 rDNA sequences ranged from 667 to 690 bp and from 637 to 645 bp, respectively. The ITS2 rDNA sequences were consistently 501 bp in the *C. cotti*, but varied from 462 to 469 bp in *C. hypophthalmichthys*. The variations resulted mainly from deletion/insertion nucleotides. In ITS rDNA, the G+C contents varied from 33.2 to 34.4% in the ingroup. In total, 1,305 characters were analyzed, of which 543 were variable and 164 were phylogenetically informative. The divergence (uncorrected p-distance) of various data sets is shown in Table II. When the outgroup taxon was excluded, the saturation plots of uncorrected sequence divergence (K80) against transitions and transversions revealed unsaturated relationships among the sequences (plots not shown). Furthermore, in the ITS1 rDNA region of *C. cotti*, a simple sequence repeated (SSR) polymorphism, a tetranucleotide (TTGC)n was detected with n ranging from 4 (Danjiangkou Reservoir, Hubei; host *Opsariichthys bidens*) to 10 (Jialingjiang River, Chongqing; host *Mystus macropterus*), but commonly 6 or 7 were present. However, this SSR did not occur in *C. hypophthalmichthys*.

Phylogenetic analyses

Two major clades, A and B, within *Camallanus* were identified distinctively by all 3 methods of analysis (Fig. 2). Clade A contained individuals of *C. cotti* from different fish species from 3 localities, whereas clade B only included strains of *C. hypophthalmichthys* in *Hypophthalmichthys molitrix* and *Aristichthys nobilis* from 4 localities, both with high bootstrap values or posterior probabilities. Although major clades were well resolved, the interior nodes of each clade received only poor support by the 3 methods. In the Bayesian tree, however, 4 significant support sub-clades in interior nodes of clade A were determined. The first sub-clade included the parasites from ZP (=*Zacco platypus*) and CIL (=*Culter ilishaeformis*) (100%); the second from PF (=*Pelteobagrus fulvidraco*) and HS (=*Hypseleotris swinhonis*) (100%); the third from GI (=*Gnathopogon imberbis*), SD (=*Saurogobio dabryi*), and AS (=*Acanthobrama simoni*) (95%); and the fourth from CI (=*Ctenopharyngodon idella*) and MP (=*Mylopharyngodon piceus*) (100%).

DISCUSSION

Analysis of ITS rDNA sequence of *Camallanus* spp.

Limited sequence data have been available for the ITS rDNA of parasitic nematodes, especially in fish hosts. The data presented herein represent the first report of the ITS rDNA sequences from *Camallanus* spp. The ITS1 region is longer than the ITS2 region, in agreement with similar findings for *Trichostronglus* spp. (Hoste et al., 1998) and the cyst-forming nematodes of the Heteroderidae (Subbotin et al., 2001). Analogous to the ITS region in many other parasitic nematodes, the G+C content of *Camallanus* spp. is smaller than the A+T content (33.2 to 34.4% vs. 65.6 to 66.8%) (Hoste et al., 1998; Subbotin et al., 2001; Otranto and Traversa, 2004).

Despite the great divergence seen in the ITS region of the genus, there were still several conserved domains (data not shown). Similarly conserved regions are also detected in the ITS1 region of the *Thelazia* species (Hoste et al., 1998) and the ITS region of *Trichostrongylus* species (Otranto and Traversa, 2004). It is generally believed that conserved regions are important to maintain the secondary structure of the pre-rRNA of the spacer(s) and may help to mediate cleavages in the ITS region that occur during rRNA transcript procession (Mai and Coleman, 1997; Hoste et al., 1998).

Species validity within *Camallanus* spp.

The ITS rDNA region has been successfully used for phylogenetic study and identification of closely related species of nematodes. Generally, species are regarded as valid if all of the mean variation values of the interspecific ITS sequences are much higher than those of the intraspecies. However, the literature does not suggest how much higher the values need to be to validate species differences. For example, Otranto and Traversa (2004) stated that for species of the spirurid *Thelazia*, the intraspecific variation of the ITS1 region varied from 0.3 to 2.5% and interspecific ranged from 35 to 77%. In contrast, Hoste et al. (1998) found that divergences of the ITS1 region among 3 closely related species of *Trichostrongylus* ranged from 1.3 to 5.7%. Newton regarded *Cooperia oncophora* and *C. surnabada* as synonyms, because the difference between the

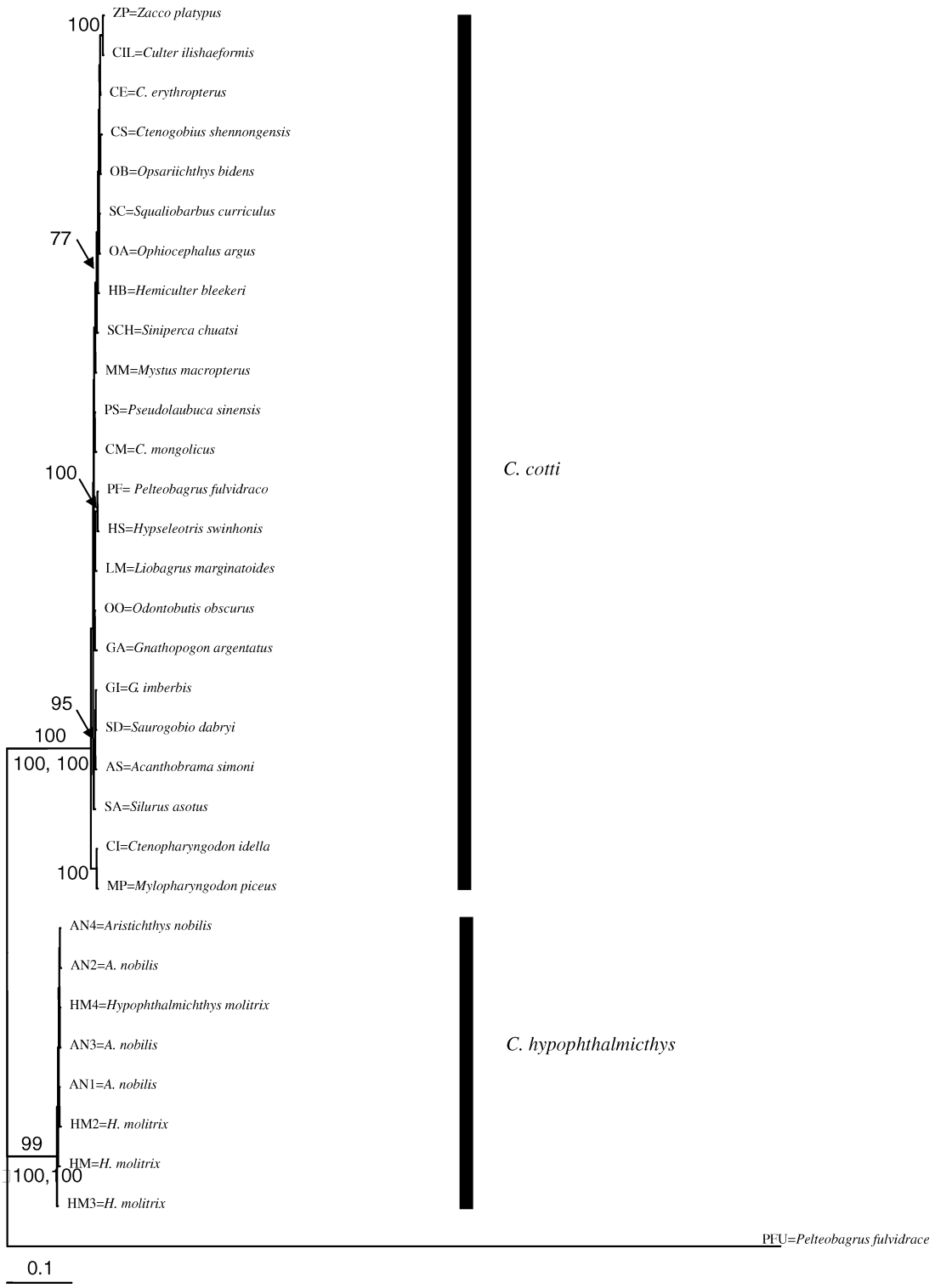

FIGURE 2. Phylogenetic relationships of Chinese *Camallanus* spp. inferred by Bayesian approach based on ITS rDNA sequences. The tree is rooted with *Procamallanus fulvidraconis*. Numbers represent node supports derived from Bayesian posterior probability (only values above 50% are shown). Values of 2 main clades obtained with MP and NJ methods are shown in parentheses.

ITS2 fragments was no greater than 1.7%. The threshold of 1.7% was established using as standard the difference between valid species in the genus. Recently, research on 9 species of philometrids collected in China revealed that the interspecific divergence was more than 7.32% in the ITS region (Wu et al., 2005). All these studies, and many others, indicated that for parasitic nematodes, there was a significant difference between the variations of intra- and interspecies whether the ITS1, ITS2,

or combined rDNA sequences were used. In the present study, the sequence variations for *C. cotti* from different localities and hosts are 2.5% (ITS1), 0.6% (ITS2), and 1.5% (ITS), whereas within *C. hypophthalmichthys*, the differences are only 0.3% (ITS1), 0.4% (ITS2), and 0.4% (ITS). However, the divergences between the 2 groups are as high as 14.6% (in ITS1), 22.1% (in ITS2), and 20.3% (in ITS). This suggests that *C. cotti* and *C. hypophthalmichthys* are most likely different species.

Three *Camallanus* species have been reported in China from different fish species. Among them, *C. zacconis* was first recorded in *Zacco temmincki* from the Jialingjiang River, a branch of the upper reaches of the Yangtze River (Li, 1941). Subsequently, *C. zacconis* was reported by Wang and Ling (1975) and Wang et al. (1979) from *Hemiculter leucisculus*, *Elopichthys bambusa*, *Siniperca chuatsi*, *Megalobrama terminalis*, and *Erythroculter ilishaeformis* in Fujian Province and *Silurus asotus* in Poyang Lake, Jiangxi Province. Sun (1988) also recovered *C. zacconis* from *H. leucisculus*, *Culter erythropterus*, *Mastacembelus mastacembelus*, *S. chuatsi*, *S. asotus*, and *Pelteobagrus fulvidraco* in Wuhan City and Honghu Lake. In the present study, although its typical host *Z. temmincki* was not examined, *C. cotti* from the congeneric host *Z. platypus* was found and included. Furthermore, *C. cotti* was also found in another fish, *Mystus macropterus*, from the Jialingjiang River, where *C. zacconis* is typically found. Additionally, many *C. cotti* individuals were collected from several species of fishes that have been reported as hosts of *C. zacconis*. In the present molecular phylogenetic tree, *C. cotti* from different fish hosts from different localities form a single clade with a high bootstrap value, and molecular divergences remained at an intraspecific level, thus supporting the hypothesis, based on morphological characteristics, that *C. zacconis* is a junior synonym of *C. cotti* (Moravec, 1973; Moravec et al., 2004). We agree that, so far, only 2 *Camallanus* species (*C. cotti* and *C. hypophthalmichthys*) have been validated as parasites of various freshwater fishes in China.

Similar to the finding by Otranto and Traversa (2004) with respect to *Thelazia* spp. in the ITS1 region, there was also a microsatellite found in *C. cotti*. The microsatellite makes it easy to distinguish *C. cotti* from *C. hypophthalmichthys*. The minimum repeated number of the microsatellite comes from the strain found in *O. bidens* in Danjiangkou Reservoir, and the maximum repeated number comes from an individual in *M. macropterus* in Jialingjiang River. This suggests genetic differentiation between the populations. Microsatellites have been widely used as important genetic markers for epidemiology and population structure study on isolates from different geographic areas (McCoy et al., 2001; Otranto and Traversa, 2004). Thus, it is possible to use the microsatellite to examine the population genetics and epidemiology of *C. cotti*.

In summary, 2 *Camallanus* nematodes, *C. cotti* and *C. hypophthalmichthys*, from different fish host species collected from different locations in China are recognized in this study. However, further study should include more species of *Camallanus*, as well as other genes, to clarify the evolutionary relationships in this genus. Population genetics and phylogeography of *C. cotti* should also be examined.

ACKNOWLEDGMENTS

We are grateful to Dr. Y. Song and Q. Y. Tang for kind suggestions in preparing the paper. We sincerely acknowledge W. J. Yao for assistance in collecting samples. Our thanks are also given to Dr. F. Moravec of the Institute of Parasitology, Academy of Sciences of the Czech Republic, for generous presentation of many useful publications. The research was supported by the National Natural Science Foundation of China through the projects 30571413 and 30371102 and by the National Basic Research Program through the project 2002CB412308.

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