Vol. 22: 39–43, 1995 | DISEASES OF AQUATIC ORGANISMS
Dis aquat Org | Published May 4

Taxonomic affinities of *Cryptocaryon irritans* and *Ichthyophthirius multifiliis* inferred from ribosomal RNA sequence data

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ABSTRACT: Comparison of partial sequences of the 18S rRNA gene of the parasitic ciliates *Cryptocaryon irritans* and *Ichthyophthirius multifiliis* confirmed that these taxa are not as closely related as was first thought. Phylogenetic trees generated from sequence data grouped *I. multifiliis* with 3 species of *Tetrahymena*, supporting the existing taxonomic classification of these 2 genera together in the Order Hymenostomatida, Class Oligohymenophora. In contrast, *C. irritans* was grouped with *Colpoda inflata* (Class Colpodea) supporting the theory that the life cycle and morphological similarities evident between *I. multifiliis* and *C. irritans* are an example of convergent evolution.

KEY WORDS: *Cryptocaryon irritans* · *Ichthyophthirius multifiliis* · 18S rRNA gene · Molecular taxonomy

INTRODUCTION

The ciliates *Cryptocaryon irritans* and *Ichthyophthirius multifiliis* are important parasites of marine and freshwater fishes, respectively. Neither exhibit strict host specificity, but both may be highly pathogenic, cause severe epizootics and contribute to major losses of cultured and ornamental fishes. *I. multifiliis*, the causative agent of 'Ich', is a serious pathogen of virtually all freshwater fish in aquaria, aquaculture (Nigrelli et al. 1976) and even in the wild (Wurtsbaugh & Tapia 1988). *C. irritans* causes significant problems in marine aquaria (Nigrelli & Ruggeri 1966, Wilkie & Gordin 1969), and in the culture of many species of marine fish (Huff & Burns 1981, Colorni 1985, Kaige & Miyazaki 1985, Rasheed 1989).

Cryptocaryon irritans has generally been regarded in the past as a closely related marine counterpart to *Ichthyophthirius multifiliis*, due mainly to the many similarities in their superficial morphologies and life cycles. In fact, *C. irritans* was first observed in marine aquaria by Sikama (1937), who later (Sikama 1961) described the 'new' ciliate parasite of marine fishes as *Ichthyophthirius marinus* due to its close resemblance to *I. multifiliis*, not knowing the parasite had been previously described as *C. irritans* by Brown (1951). Both *C. irritans* and *I. multifiliis* are parasites of the body surface and gill epithelium of fishes. Both species also exhibit a direct life cycle involving palintomic division within the cyst stage (tomont) which bursts, liberating infective stages (theronts) which mature into adult parasites (trophonts) on the host, these appearing as visible 'white-spots' on the surfaces of infected fish. Because of these similarities, both species have been placed together in the family Ichthyophthiriidae by some authors (Nigrelli & Ruggeri 1966, Corliss 1975, 1979, de Puytorac 1994) whereas other authors regard *C. irritans* as a holotrich ciliate *incertae sedis* (Cannella 1972, Lom & Dykova 1992).

Many authors (e.g. Sogin & Elwood 1986, Lynn & Sogin 1988, Greenwood et al. 1991) have suggested that too much emphasis has previously been placed on the classification of ciliates on the basis of morphological criteria, particularly with regard to the characterisation of ciliature and infraciliature, and that more effective methods of resolving taxonomic relationships among ciliates may be through the use of molecular techniques to acquire genomic information, such as 18S rRNA gene sequences. The present investigation was conducted to determine and compare partial sequences of the 18S rRNA gene of *Ichthyophthirius multifiliis* and *Cryptocaryon irritans*. These sequences were then compared with those of 6 other ciliate species to study their phylogenetic relationships.

MATERIALS AND METHODS

Nucleotide sequences were determined for 3 isolates of *Cryptocaryon irritans* and 2 isolates of *Ichthyophthirius multifiliis*. Isolates of *C. irritans* tomonts were obtained from bream *Acanthopagrus australis* caught in the Brisbane River, Moreton Bay, Queensland, iodine bream *Gymnocranius audleyi* from Heron Island, Queensland, and gilt-head sea bream *Sparus aurata* from Eilat, Israel. Isolates of *I. multifiliis* were obtained from comet goldfish *Carassius auratus* from local fish distributors, and black moor goldfish *C. auratus* imported from Hong Kong and obtained during quarantine.

Tomonts of each *Cryptocaryon irritans* isolate were collected with a fine paint brush from the bottom of tanks containing infected fish, fixed in 70% ethanol and stored in 100% ethanol. DNA was subsequently extracted from single tomonts using a phenol-chloroform method (Sambrook et al. 1989). Each *Ichthyophthirius multifiliis* isolate consisted of 6 to 10 trophonts collected by pipette as they dropped off infected goldfish placed in 3 l aquaria against a black background. These were fixed and processed as for *C. irritans* isolates. DNA extracts were amplified by polymerase chain reaction (PCR), and sequenced by the dideoxy chain termination method as described previously (Adlard et al. 1993). The first internal transcribed spacer (ITS1) of the ribosomal RNA gene and the flanking 3' end of the 18S region was amplified by PCR using oligonucleotide primers 1 (forward primer – 5' GTT CCC CTT GAA CGA GGA ATT C) and 2 (reverse primer – 5' CGC ATT TCG CTG CGT TCT TC). Primer 1 was located approximately 230 bp upstream of the 3' end of the 18S/ITS1 boundary, while primer 2 was located in the 5.8S region. Primers were designed for conserved regions from published sequences (Hillis & Dixon 1991).

Primer 2 and 2 other primers located within the amplified fragment were used to determine nucleotide sequences. The sequencing primers were primer 3 (forward primer – 5' GTC CCT GCC CTT TGT ACA CA) and primer 4 (reverse primer – 5' GAT CCT TCT

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C.irr.  CTTGAACGAGGAATTCCTAGTAAGTGCAAGTCATCAGCTTGTACTGATTACGTCCCTGCCCTTTGTACACACCGC 75
I.mul.  CTTGAACGAGGAATTCCTAGTAAGTGCAAGTCATCAGCTTGCGTTGATTATGTCCCTGCCGTTTGTACACACCGC
T.pyr.  CTTGAACGAGGAATTTCTAGTAAGTGCAAGTCATCAGCTTGCGTTGATTATGTCCCTGCCGTTTGTACACACCGC
T.aus.  CTTGAACGAGGAATTTCTAGTAAGTGCAAGTCATCAGCTTGCGTTGATTATGTCCCTGCCGTTTGTACACACCGC
T.can.  CTTGAACGAGGAATTTCTAGTAAGTGCAAGTCATCAGCTTGCGTTGATTATGTCCCTGCCGTTTGTACACACCGC
Co.in.  CTTGAACAAGGAATTCCTAGTAAGCATAAGTCATCAGCTTGTGCTGATTACGTCCCTGCCCTTTGTACACACCGC
O.gra.  CTTGAACGAGGAATTCCTAGTAAGCGCAAGTCATTACCTTGCGCTGATTAAGTCCCTGCCCTTTGTACACACCGC
On.qu.  CTTGAACGAGGAATTCCTAGTAAGCGCAAGTCATTAGCTTGCGCTGATTAAGTCCCTGCCCTTTGTACACACCGC
        ******* ******* ********   ******* * ****   ****** ********* **************

C.irr.  CCGTCGCTCCTACCGATTTCGAGTGATCCGGTGAACCTTCTGGACTGCGCTAACAXTAG---TTAGTGCGGGAAG 150
I.mul.  CCGTCGCTTGTAGTAA---CGAATGGTCTGGTGAACCTTCTGGACCGAGGTCG----CAAGGC---TTTGGGAAG
T.pyr.  CCGTCGCTTGTAGTAA---CGAATGGTCTGGTGAACCTTCTGGACTGCGGTAG----CAATAC---TGCGGGAAA
T.aus.  CCGTCGCTTGTAGTAA---CGAATGGTCTGGTGAACCTTCTGGACTGTGACAG----CAATGT---TACGGAAAA
T.can.  CCGTCGCTTGTAGTAA---CGAATGGTCTGGTGAACCTTCTGGACTGCGGTAG----CAATAC---TGCGGAAAA
Co.in.  CCGTCGCTCCTACCGATTTTGAGTGATCCGGTGAACCTTCTGGACTGTGGTCAGGCTTGACCTGATTGTGGGAAG
O.gra.  CCGTCGCTCCTACCGATTTCGAGTGGTCCGGTGAACCTTTTGGACTGCGCGAGGCCCCGAGCCTTGTGCGGAAAA
On.qu.  CCGTCGCTCCTACCGATTTCGAGTGATCCGGTGAACCATTTGGACTGCG-AGGTCCTCGTGACTT-TGTGGAAAA
        ********  **   *    ** ** ** ******** * ***** * *                 *  ** **

C.irr.  TTAAGTAAACCACTTCACTTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  224
I.mul.  TTAAGTAAACCCTACCATTTGGAACAACAAGAAGTCGTAACAAGGTATCTGTAGGTGAACCTGCAGATGGATCA
T.pyr.  ATAAGTAAACCCTACCATTTGGAACAACAAGAAGTCGTAACAAGGTATCTGTAGGTGAACCTGCAGATGGATCA
T.aus.  ATAAGTAAACCCTACCATTTGGAACAACAAGAAGTCGTAACAAGGTATCTGTAGGTGAACCTGCAGATGGATCA
T.can.  ATAAGTAAACCCTACCATTTGGAACAACAAGAAGTCGTAACAAGGTATCTGTAGGTGAACCTGCAGATGGATCA
Co.in.  TTAAGTAAACCTTATCACTTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA
O.gra.  TCAAGTAAACCATATCACTTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA
On.qu.  TCTAGTAAACCATATCACTTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA
           ********    ** ** **  **  ****************** ** ************** ** ******
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Fig. 1 Clustal V alignment of nucleotide sequences beginning at the 5' end of the amplified fragment. Base number 224 corresponds with the 3' end of the 18S region. Dashes (-) indicate alignment gaps, asterisks (*) indicate where bases are identical throughout the alignment. X = variable base. *C. irr.* = *Cryptocaryon irritans*, *I. mul.* = *Ichthyophthirius multifiliis*, *T. pyr.* = *Tetrahymena pyriformis*, *T aus.* = *T. australis*, *T can.* = *T canadensis*, *Co. in.* = *Colpoda inflata*, *O. gra.* = *Oxytricha granulifera*, *On. qu.* = *Onychodromus quadricornutus*

GCA GGT TCA CCT AC). For all isolates, at least 1 replicate isolate was extracted, amplified and sequenced. Both the sense and non-sense strands were sequenced to further validate results.

Sequences of *Ichthyophthirius multifiliis* and *Cryptocaryon irritans* were compared to known sequences stored in GenBank using the Basic Local Alignment Search Tool (BLAST) routine (Altschul et al. 1990) available through the Australian National Genomic Information System (ANGIS). The 3 closest sequences to each were then used for comparison after alignment. The sequences obtained from GenBank were from the ciliates *Colpoda inflata* (from Greenwood et al. 1991), *Oxytricha granulifera* and *Onychodromus quadricornutus* (from Schlegel et al. 1991), and *Tetrahymena pyriformis*, *T. canadensis* and *T. australis* (from Sogin et al. 1986). Alignments were made by eye using the sequence editor ESEE (Cabot & Bekenbach 1989) and verified using the alignment program Clustal V (Higgins et al. 1992). Alignment gaps were treated as missing bases.

Trees were constructed using maximum parsimony methods in PAUP ver. 3.0s (Swofford 1991). Bootstrap resampling of the data was used to indicate the degree of support for each branch of the tree.

RESULTS

Sequences were obtained for 224 bases from the 3' end of the 18S region. In this region 66 bases were variable, of which 47 were informative. The sequence alignments used for analysis (Fig. 1) generated by Clustal V were identical to those alignments adjusted by eye. Parsimony analyses using PAUP generated 2 most-parsimonious trees each 102 steps long (branch and bound search, consistency index 0.840 excluding uninformative characters) which differed only in the arrangement of the *Tetrahymena* spp. (Figs. 2 & 3). The hypotrichous ciliate *Onychodromus quadricornutus* was the designated outgroup in these analyses. Two major groupings of taxa were evident in both trees generated. One group consisted of *Cryptocaryon irritans* and *Colpoda inflata*, whilst the other contained *Ichthyophthirius multifiliis* and *Tetrahymena pyriformis*, *T. canadensis* and *T. australis*. Bootstrap resampling, which consisted of 1000 heuristic bootstrap replicate samples of the sequence data, indicated that support for these 2 separate groups was high (81%), with 77% support for the *C. irritans*/*Co. inflata* group and 100% support for the *I. multifiliis*/*Tetrahymena* spp. group (Fig. 4). There was one variable base (position number 131 in Fig. 1) found between all 3 isolates of *C. irritans* sequenced, and no variation between the sequences of the 2 isolates of *I. multifiliis*.

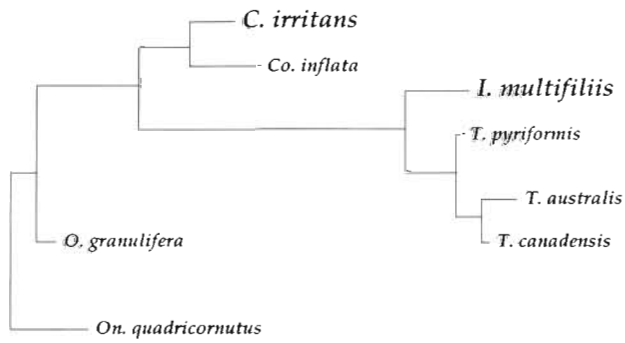

Fig. 2. The first of 2 most-parsimonious trees generated by the maximum parsimony analysis program PAUP. *Onychodromus quadricornutus* is the designated outgroup

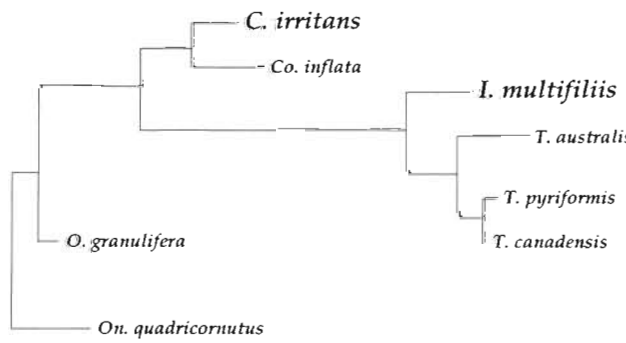

Fig. 3. The second of the most-parsimonious trees, differing from Fig. 2 only in the positioning of the *Tetrahymena* species *T. pyriformis* and *T. australis*

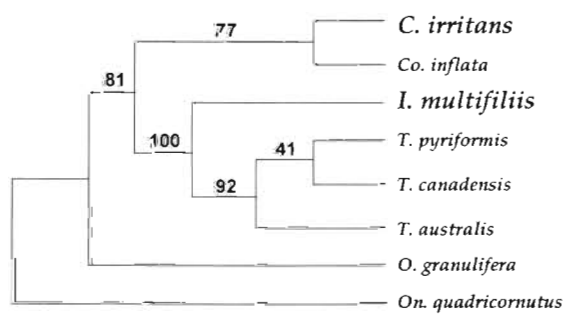

Fig. 4. Percentage support for each branch derived from 1000 heuristic bootstrap replicate samples of the sequence data

DISCUSSION

There was strong support for the grouping of *Ichthyophthirius multifiliis* with the 3 species of *Tetrahymena* to the exclusion of *Cryptocaryon irritans*. This rRNA gene sequence data supports the present taxonomic placing by Corliss (1979) and Small & Lynn (1985) of *I. multifiliis* (Suborder Ophyroglenina, Family Ichthyophthiriidae) within the Order Hymenostom-

atida, Class Oligohymenophorea along with the Suborder Tetrahymenina (containing the 3 *Tetrahymena* species), but refutes the placing of *C. irritans* within the Family Ichthyophthiriidae. From this data, the Family Ichthyophthiriidae containing *C. irritans* is not seen to form a monophyletic group.

The placement of *Cryptocaryon irritans* in the Order Hymenostomatida has previously been questioned on morphological grounds (Cheung et al. 1981) due to the simplicity of its buccal apparatus which consists of a cytostome surrounded by a ring of cirri-like structures and lacks the distinct buccal cavity and oral membranelles of typical hymenostome ciliates. Its placement in the Suborder Ophyroglenina is also questionable as it does not possess an organelle of Lieberkühn ('watch-glass organelle') at any stage of its life history (Brown 1951), which contradicts the view of Lynn et al. (1991) who regarded the organelle of Lieberkühn as a synapomorphy of the Ophyroglenina.

Recent ultrastructural studies have revealed differences between the 2 species which also indicates that they are not as closely related as first thought. Colorni & Diamant (1993) examined the development of trophonts, tomonts and theronts of *Cryptocaryon irritans* utilising TEM and found that the trophont possesses monokinetid somatic ciliature and an electron-dense 'foamy' substance in the pellicular alveoli. The origin and function of the foamy substance was not clear, but this substance is not present in *Ichthyophthirius multifiliis*. They concluded that the development of *C. irritans* and *I. multifiliis* differed significantly. Matthews et al. (1993) found a similar electron-dense material in the pellicular alveoli of trophonts, tomites and theronts of *C. irritans*, and found that its mucocysts differed from those of *I. multifiliis* in size, shape and distribution. These mucocysts also appeared not to be directly involved in encystment as found for *I. multifiliis* (Ewing *et al.* 1983).

Our results, along with the morphological and ultrastructural evidence described above lend further support to the theory of Colorni & Diamant (1993), who suggest that the superficial similarities between *Ichthyophthirius multifiliis* and *Cryptocaryon irritans* are due to an adaptive convergence of life histories rather than phylogenetic proximity.

From the sequence data of the species available to us, *Cryptocaryon irritans* was consistently grouped in these analyses with *Colpoda inflata*. The genus *Colpoda* was recently placed in the new Class Colpodea (O. Colpodida) by Small & Lynn (1985). Data obtained by analysis of 18S rRNA (Lynn & Sogin 1988) supported the Class status of the Colpodea and indicated that the colpodids may be a deeply split sister group of the Oligohymenophorea (which includes *Tetrahymena* and *Ichthyophthirius multifiliis*). The grouping of *C. irritans* with a colpodid in this study (although as a consequence of the close grouping of *Ichthyophthirius* and the *Tetrahymena* spp.) is nonetheless interesting given the similarities between the life histories of these taxa, with both *C. irritans* and many colpodids exhibiting encystment and reproduction by palintomy within the resting cyst. The simple buccal apparatus of *C. irritans* is also similar in appearance to the cytostomes found in some colpodids (for example *Rostrophyra camerounensis*; see Njine 1979). However, the fine structure of the somatic kinetid of colpodids is of a unique dikinetid construction (Lynn & Small 1990) and presently little is known about the structure of the somatic kinetid of *C. irritans* except that it is of monokinetid construction (Colorni & Diamant 1993).

Both the ultrastructural evidence and our sequence data suggest that the taxonomic position of *Cryptocaryon irritans* is distinct from that of *Ichthyophthirius multifiliis*. The association with the Colpodea suggested by the sequence data is interesting and has some support from morphological and life history viewpoints. However, no authoritative taxonomic placement of *C. irritans* can be performed until more information is available on the fine ultrastructure of its somatic kinetid. Important taxonomic information may also be revealed by silver staining techniques, as these methods have been underutilized in past studies of *C. irritans*. Also, the future availability of complete 18S sequence data for *C. irritans* and an increased range of species of key ciliates (such as other colpodids and *Ophryoglena*) will provide for a clearer understanding of the taxonomic affinities of *C. irritans*.

Acknowledgements. The authors thank Dr Angelo Colorni for his prompt assistance in sending samples of fixed *Cryptocaryon* from Israel; also Dr Bob Lester and Dr Peter O'Donoghue for their comments on the manuscript and Dr Steve Barker for his assistance with the phylogenetic analysis. This work was supported in part by an Australian Postgraduate Research Award, and forms part of the doctoral thesis of B.K.D.

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Responsible Subject Editor: W. Körting, Hannover, Germany

Manuscript first received: July 12, 1994
Revised version accepted: December 23, 1994