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Genetic and morphologic differentiation of *Bolbophorus confusus* and *B. levantinus* (Digenea: Diplostomatidae), based on rDNA SSU polymorphism and SEM

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ABSTRACT: Metacercariae of *Bolbophorus* species are serious pathogens of farmed fish. Molecular diagnostic tools, capable of identifying and differentiating these parasites, may assist in the development of rationale control strategies. The rDNA 18S (small sub-unit: SSU) genes of adult *B. confusus* and *B. levantinus* obtained from a pelican, *Pelecanus onocrotalus*, and a night heron, *Nycticorax nycticorax*, respectively, were amplified, sequenced, and aligned. Based on this alignment, we developed a genetic differentiation assay between *B. confusus* and *B. levantinus*. These 2 species were compared genetically with the North American species *B. damnificus* and *Bolbophorus* sp. ('Type 2'). The relationship between species is outlined and discussed. In addition to the molecular study, specimens of *B. confusus* and *B. levantinus* were compared morphologically, using scanning electron microscopy. Morphologic analysis revealed interspecific differences in details of the holdfast organ and the position of the acetabulum.

KEY WORDS: Digenea · Diplostomatidae · *Bolbophorus confusus* · *Bolobphorus levantinus* · 18S (SSU) rDNA gene · SEM

INTRODUCTION

Metacercariae of *Bolbophorus* species induce massive infections in the muscles of fish, and consequent malformations and mortalities in fish from natural habitats and cultured fish ponds in both Israel and southern USA (Paperna 1996, Pasnik 1999, Veneble et al. 2000, Levy et al. 2002, Overstreet et al. 2002). The definitive host of *B. confusus* (Diplostomatidae) is the Eurasian white pelican *Pelecanus onocrotalus* (see Dubois 1938). In Europe, fish from diverse families (including Cyprinidae) are intermediate hosts for *B. confusus* (Bykhovskaya-Pavlovskaya et al. 1964, Dubois 1970), but the snail host remains unknown.

Paperna & Lengy (1963) described the life cycle of *Bolbophorus confusus levantinus* from Israel, later designated by Dubois (1970) as a new species: *B. levantinus*. Its definitive hosts are herons, and metacercariae develop in cichlid fishes (Paperna 1996). A purple heron, *Ardea purpurea*, was experimentally infected by feeding it on naturally infected *Tilapia zillii* with metacercariae. Obtained trematode eggs successfully developed to cercaria stage in the snail *Bulinus truncatus*.

Overstreet et al. (2002) recently described a new species, *Bolbophorus damnificus*, from the North American white pelican *P. erythrorhynchos*. *B. damnificus* had previously been erroneously identified as *B.*

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confusus, but it differs from *B. confusus* by having larger eggs, and the vitellarium not reaching anterior to the holdfast organ. Furthermore, there is morphological and molecular evidence for the existence in North America of another species of *Bolbophorus* ('Type 2'), presently known only from immature specimens found in pelicans (Levy et al. 2002, Overstreet et al. 2002). Both recently discovered species were found to develop in the ram's horn snail *Planorbella trivolis*. Since the discovery of *B. damnificus*, the common pathogenic prodiplostomulum found in the flesh of the channel catfish *Ictalurus punctatus* has been identified as this species (Overstreet et al. 2002, Levy et al. 2002); the natural piscine host of *Bolbophorus* sp. 'Type 2' remains unknown.

Species-specific DNA probes have been developed for the North American species *Bolbophorus damnificus* and *Bolboporus* sp. 'Type 2', and have provided an important diagnostic tool for all stages of these trematodes, which are pathogenic as metacercariae to fish (Levy et al. 2002); experimental infections with *Bolbophorus* sp. 'Type 2' were highly pathogenic to several fish species (Levy et al. 2002).

The purpose of the present study was to develop a genetic differentiation assay between *Bolbophorus confusus* and *B. levantinus*, as well as to genetically compare the Old World with the New World species of the *Bolbophorus* genus.

MATERIALS AND METHODS

Sources of parasites and morphological characterization. Nine *Bolbophorus levantinus* Dubois 1970, were collected from mortally injured night heron *Nycticorax nycticorax* found in a fish farm on the Mediterranean coast of Israel. Hundreds of *B. confusus* (Krause 1914) Dubois 1935, were collected from the guts of a fall migrant Eurasian white pelican, *Pelecantus onocrotalus*, that had died of exhaustion in north Israel. Both species were collected by sedimentation and washing of scrapings from the anterior portion of the intestine. Collected trematodes were washed several times in physiological saline and preserved in 70% ethanol. Additional specimens of each species were fixed in 70% alcohol overnight, under light pressure between 2 cover glasses, to prepare them as stained whole mounts. Whole mounts were then stained in aceto-carmine, followed by differentiation in acid alcohol. For scanning electron microscopy (SEM), trematodes fixed in 70% ethanol were dehydrated in ascending alcohols, critical-point dried in liquid carbon dioxide in a 'Polaron E-3000' critical-point drying apparatus, and sputter-coated with gold in a 'Polaron E-5100' sputter-coater. Specimens were examined and photographed using a Joel JSM 35C SEM.

Gene amplification. Individual worms of each species were washed overnight in buffer TE (10 mM), followed by 2 more 1 h washes prior to DNA extractions. DNA extraction, PCR amplification of the 18S and internal transcribed spacer (ITS) genes, purification of PCR products and sequence determination and analysis were done following the same procedures described previously (Levy et al. 2002).

Species-specific oligonucleotide primers. Alignments of the rDNA small sub-unit (SSU) genes revealed polymorphic regions to which species-specific oligonucleotide primers were designed. The *Bolbophorus confusus*-specific primer set was composed of the forward primer Bcon 650F 5′ GATTTCGGTTAGTTCAGG 3′, and the reverse primer Bcon 1470R 5′ GGTCTACGGCCCAATC 3′. The *B. levantinus*-specific primer set was composed of the forward primer Pap180F 5′ GGAGCGGCTTCGGCTGT 3′, and the reverse primer Pap 600R 5′ ATCATCGCCCGGAACTGA 3′. These oligonucleotides were used as both singleplex and multiplex configurations. PCRs were carried out in 50 µl volumes using 10× buffer, 2.5 U *Taq* polymerase (HotStarTaq; Qiagen) 10 mM of each deoxynucleoside triphosphate, and 100 ng of each primer. The cycling conditions were as follows: 94°C for 15 min once, then 35 cycles of 94°C for 1 min, 58°C for 45 s, and 72°C for 1 min followed by a final 5 min extension at 72°C.

PCR amplicons were electrophoresed in 1.5% agarose gels, stained with ethidium bromide and visualized under ultraviolet light.

Phylogenesis analysis. The rDNA SSU ~1800 bp nucleotide sequences of *Bolbophorus confusus* and *B. levantinus* were aligned with other available strigeid trematode sequences, including *B. damnificus* Overstreet, Curran, Pote, King, Blend, Grater 2002, and *Bolbophorus* sp. 'Type 2', *Clinostomum complanatum* and *C. marginatum* were utilized as out groups. The origin of sequences was from both the authors' material and GenBank. This was conducted using the aligning tool supplied by the ARB phylogenetic program package (Strunk & Ludwig 1998[1]). Phylogenetic trees were generated with the neighbor-joining and maximum-likelihood methods with the ARB program package, using the Felsenstein correction method applying a 50% cut-off filter. The topologies of the resulting trees were compared. Branching order was supported by both methods.

[1]Strunk O, Ludwig W (1998) ARB: a software environment for sequence data. Department of Microbiology, Technische Universität Munchen, Munich, available at http://mikro.biologie.tu-muenchen.de

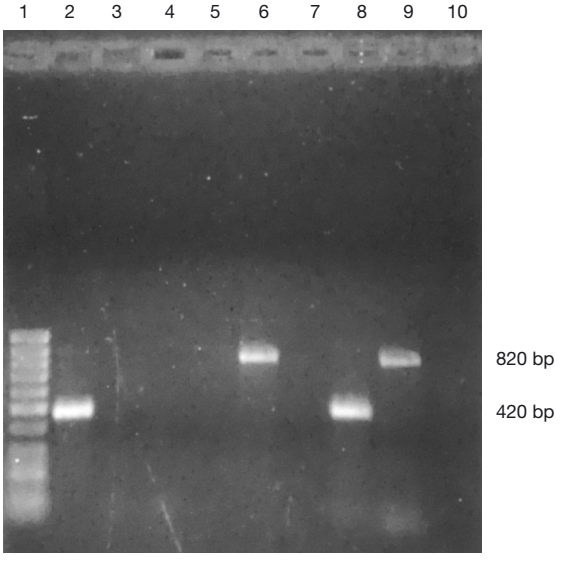

Fig. 1. *Bolbophorus confusus* and *B. levantinus*. PCR products of adult trematodes using species-specific PCR reactions with either single-species oligonucleotide primer sets (Lanes 2 to 4 *B. levantinus*, Lanes 5 to 7 *B. confusus*), or a multiplex configuration (Lanes 8 to 10). Lanes: 1 = 50 kb DNA marker; 2, 5 and 8 = *B. levantinus*; 3, 6 and 9 = *B. confusus*; 4, 7 and 10 = H_2O

RESULTS

The rDNA SSU (18S) and ITS gene sequences were obtained from adult specimens of *Bolbophorus confusus* collected from the Eurasian white pelican, and compared to the sequence of *B. levantinus* from the night heron from Israel. Gene sequences were submitted to GenBank (accession numbers: *B. levantinus* = AF490576; *B. confusus* = AY242851).

PCR assay using singleplex and multiplex oligonucleotide primers designed for the polymorphic regions of the rDNA SSU of each species were developed and optimized. Under the conditions described above, *B. confusus*-specific singleplex yielded an 820 bp amplicon, while *B. levantinus*-specific singleplex yielded a 420 bp amplicon. PCR reactions containing the multiplex of all 4 oligonucleotide primers have been able to differentiate between the 2 species by producing the specific amplicon sizes for DNA of each species (Fig. 1). There was no cross-reaction of either the singleplexes or the multiplexes between the 2 species. There was also no cross-reaction of these sets with DNA from the pelican, the night heron, the freshwater snail *Bulinus truncatus* and the fish *Tilapia zillii*. Amplification of DNA from 2 North American *Bolbophorus* spp., *B. damnificus* and *Bolbophorus* sp. 'Type 2' (Levy et al. 2002, Overstreet et al. 2002), as well as DNA from other available trematode species infecting birds and fish in Israel, have failed to yield amplicons.

The species-specific singleplex/multiplex designed to differentiate between the 2 North American species (Levy et al. 2002) did not react with DNA of either *Bolbophorus confusus* or *B. levantinus*.

Phylogenetic analysis of 4 *Bolbophorus* spp., and their relationship to other trematode species of the order Strigeidida, based on SSU analysis, indicated that these species form a separate cluster for the genus *Bolbophorus* within the order. The genetic relatedness of these species, as demonstrated by this analysis, indicates significant relatedness between *B. confusus* and *Bolbophorus* sp. 'Type 2', and between *B. levantinus* and *B. damnificus* (Fig. 2).

Measurements and other morphological characteristics of *Bolbophorus levantinus* and *B. confusus*, obtained from whole mounts and SEM, were in agreement with previous reports (Paperna & Lengy 1963, Dubois 1970). Of all *B. confusus*, only one contained any (4) eggs at all (110–112 × 50–53 µm in size).

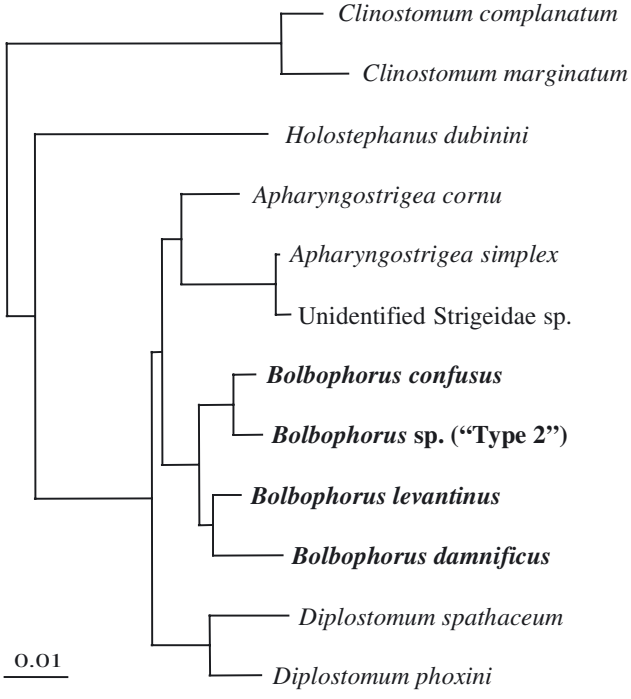

Fig. 2. Neighbor-joining phylogenetic analysis of 4 *Bolbophorus* species (bold) and their relation to other Strigeidae species, based on the rDNA small sub-unit (SSU) alignment. Scale bar = 1% estimate difference in nucleotide sequence positions. GenBank accession numbers: *Clinostomum complanatum* = AY245701; *C. marginatum* = AY25760; *Holostephanus dubinini* = AY245707; *Apharyngostrigea cornu* = AY245756; *A. simplex* = AY245757; unidentified Strigeidae sp. = AY245711; *B. confusus* = AY242851; *Bolbophorus* sp. ('Type 2') = AF490575; *B. levantinus* = AF490576; *B. damnificus* = AF490574; *Diplostomum spathaceum* = AY245761; *D. phoxini* = AJ287503

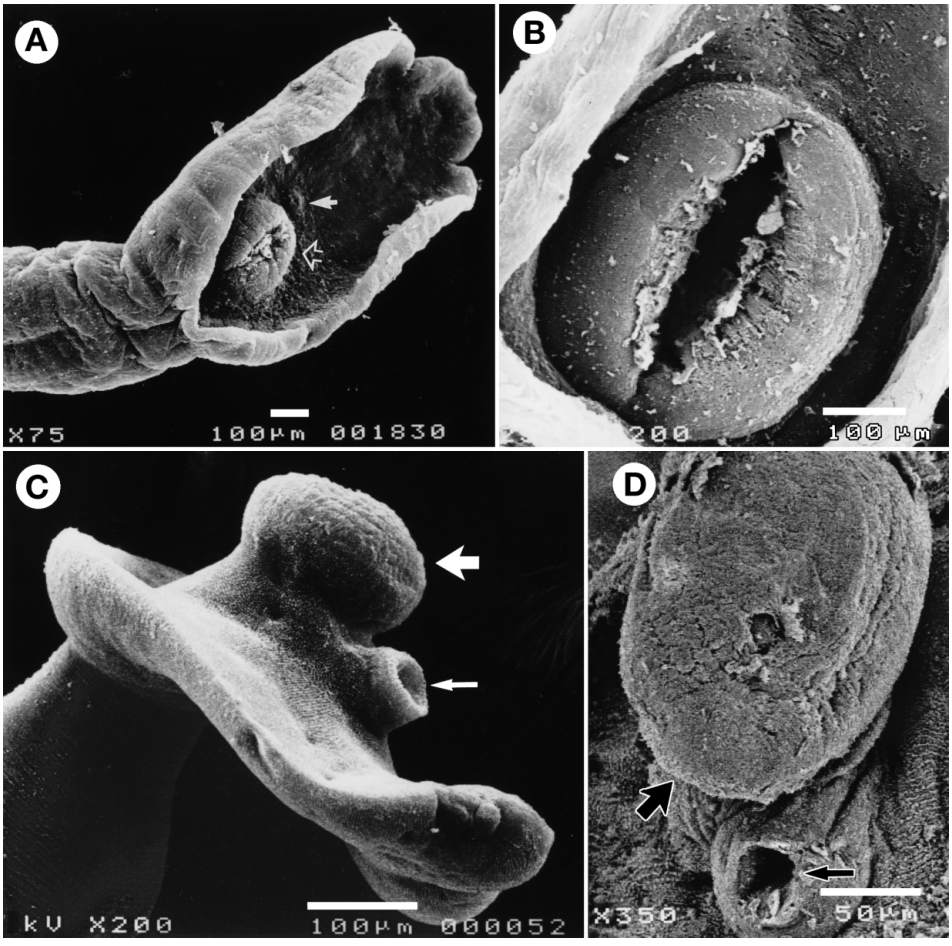

Fig. 3. *Bolbophorus confusus* and *B. levantinus*. Scanning electron micrograph of (A) *B. confusus*: lateral view of the whole worm: acetabulum (white arrow) and holdfast organ (hollow arrow); (B) *B. confusus*: lateral view of the holdfast organ; (C) *B. levantinus*: lateral view of the anterior part of the body: acetabulum (thin arrow) and holdfast organ (thick arrow); (D) *B. levantinus*: ventral view of the acetabulum (thin arrow) and holdfast organ (thick arrow)

Specimens of *B. levantinus* did not contain eggs. The distance between the posterior part of the holdfast organ and the anterior part of the acetabulum was 132 to 140 µm in *B. confusus* and only 30 to 35 µm in *B. levantinus*. The holdfast organ of *B. confusus* protruded slightly from the surface, with a medial longitudinal slit (Fig. 3A,B); however, the holdfast organ of *B. levantinus* protruded robustly from the surface, and the medial longitudinal slit was absent (Fig. 3C,D). The SEM image of *B. levantinus*' body surface shows it to be covered with spines, which are absent in *B. confusus* (Fig. 3).

DISCUSSION

The genetic divergence in the sequence of the rDNA SSU between *Bolbophorus confusus* and *B. levantinus* validates past classification by morphological criteria (Dubois 1970). The SEM study of these species further highlights structural differences between the species, particularly in the details of the holdfast organ and the position of the acetabulum (lack of spine cover in *B. confusus* may be attributable to a postmortem artifact). Polymorphisms in the SSU allowed us to develop a sensitive, species-specific assay for these 2 trematodes. The applications of this assay are not only for taxonomical purposes, but also for the study of transmission aspects of these parasites, which are serious pathogens to fish (Paperna 1996). The methodology we developed allows us to identify infection in molluscan and piscine hosts. So far, the molluscan host of *B. confusus* is unknown. A similar molecular methodology has recently been applied to provide a link between the adult and juvenile stages of the North American species *B. damnificus* and *Bolbophorus* sp. 'Type 2' (Levy et al. 2002).

Overstreet et al. (2002) have shown that *Bolbophorus* sp. found in North American hosts (pelicans), and

previously regarded as conspecific with *B. confusus* found in Old World pelicans, are in fact a distinct species (*B. damnificus*). Fox (1965) identified the planorbid snail *Planorbella* (syn. *Helisoma*) *trivolis* as *B. damnificus*' first intermediate host. Pasnik (1999) and Venable et al. (2000) reported metacercarial infection in channel catfish *Ictalurus punctatus*. In the present study, we provided genetic evidence that *B. damnificus* found in American pelicans is also a species distinct from *B. confusus*, which infects the Eurasian white pelican.

Genetic relatedness between the 4 studied species of *Bolbophorus*, based on molecular data obtained from the SSU rDNA gene sequences, indicated closest similarity between *B. confusus* and *Bolbophorus* sp. 'Type 2', and similarly between *B. levantinus* and *B. damnificus*. This implies that the species from a pelican (*B. damnificus*) is closer to the species from a heron (*B. levantinus*) from the other side of the Atlantic than to that from the pelican (*Bolbophorus* sp. 'Type 2') from the same continent. The reasons for closer genetic similarity of the rDNA SSU between *Bolbophorus* spp. from 2 different continents, compared with species from the same continent, remains a challenging question for further investigations. Genetic comparison with further specimens documented from other parts of the world (Australia, Philippines, Vietnam, India, the Nile River Valley and central Africa; Dubois 1970) may alter our taxonomical concept of this group, and provide further clues to the understanding of the evolution of the *Bolbophorus* species.

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