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Morphological and molecular phylogenetic analysis of two *Saprolegnia* sp. (*Oomycetes*) isolated from silver crucian carp and zebra fish

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ABSTRACT

Two *Saprolegnia* isolates, JY isolated from silver crucian carp (*Carassius auratus gibelio* Bloch) and BMY isolated from zebra fish (*Brachydanio rerio* Hamilton) came from infections occurring concurrently in different locations in China. To confirm whether the two isolates were from the same *Saprolegnia* clone, comparative studies have been carried out based on their morphological, physiological and molecular characteristics. Observations showed that morphologically (both asexual and sexual organs) the two isolates were broadly similar and both isolates underwent repeated zoospore emergence. Comparing 704 base pairs of internal transcribed spacer (ITS) region and the 5.8S rDNA, we found isolates JY and BMY shared an identical ITS sequence with a minor variation (99.6 % similarity). Forty available sequences for representatives *Saprolegnia* spp. belonged to four phylogenetically separate clades. The two studied isolates fell within clade I that comprised a group of isolates which showed almost an identical ITS sequence but had been identified as a number of different morphological species. Our findings suggest that isolates JY and BMY appear to belong to the *S. ferax* clade and this clade (I) contains a number of closely related phylogenetic species. This is distinct from the more common fish pathogenic isolates, which belong to the *S. parasitica* clade (III) and are characterized by having cysts decorated by bundles of long hooked hairs and two further clades (II and IV) containing largely saprotrophic or soil born species.

Introduction

Saprolegnia is one of the main genera of water molds responsible for 'fungal infections' of freshwater fish and their eggs. These infections are usually termed ''saprolegniasis'' (Roberts 1989; Beakes *et al.* 1994), which can cause severe losses of freshwater fish in both nature and commercial fish farms. However, identification of the causative agent is often difficult, especially for isolates taken from fish lesions and infested eggs. Traditional classification is mainly based on the precise descriptions of sexual reproductive organs including oogonia, antheridia, antheridial branch origin, oogonium ornamentation, oospore and lipid droplet position in the oospore (Dick 1969; Seymour 1970; Willoughby 1978; Neish & Hughes 1980; Leclerc *et al.* 2000). Unfortunately, many isolates obtained from animals often fail to produce sexual structures in *vitro* (Hatai *et al.* 1990; Stueland *et al.* 2005; Diéguez-Uribeondo *et al.* 2007) or form these structures

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only after a long developmental time (Leclerc *et al.* 2000) or immature oogonia are aborted (Seymour 1970; Johnson *et al.* 2002). Therefore, using traditional morphological criteria *Saprolegnia* isolates taken from fish lesions are often difficult or even impossible to name.

Alternative approaches to identifying and defining *Saprolegnia* species are required, such as using DNA fingerprinting used in true fungi to distinguish different clonal lineages within populations (McDonald & Martinez 1991; Goodwin *et al.* 1992; Milgroom *et al.* 1992). Such an approach has been applied to *Saprolegnia* isolates from salmonid fish by Whisler (1996). Random amplification of polymorphic DNA polymerase chain reaction (RAPD-PCR) has also been applied to the analysis different species and isolates of fish pathogenic *Saprolegnia* spp. (Diéguez-Uribeondo *et al.* 1996; Bangyeekhun *et al.* 2001). This method is fast and economic for screening large numbers of samples and can offer the possibility of detecting polymorphisms without any prior knowledge of the DNA sequences of the organism investigated. Nevertheless, some researchers are critical of the sometimes poor reproducibility of RAPD patterns (Penner *et al.* 1993).

In 2007, silver crucian carp (Caidian fish farm, Wuhan, Hubei province, China) and zebra fish (used for model organisms; Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei province, China) both suffered from *Saprolegnia* infections. Representative isolates from silver crucian carp and zebra fish, labeled 'JY' and 'BMY' respectively, were obtained but could not be satisfactorily identified using traditional morphological criteria. In order to identify and clarify their taxonomic positions, we have studied their morphological traits, their ability to undergo repeated zoospore emergence (RZE) and sequenced the internal transcribed spacer (ITS) including 5.8S rDNA to analyse their molecular and phylogenetic relationships.

Materials and methods

Strains

Ten silver crucian carp (*Carassius auratus gibelio*) and thirteen zebra fish (*Brachydanio rerio*) suffering from fungal infections were respectively collected alive from Caidian fish farm and the Institute of Hydrobiology for further study in October and November 2007. Isolate JY from silver crucian carp and isolate BMY from zebra fish were most frequently obtained. Isolations were made by depositing a small portion of excised mycelium or infected tissue which had been washed in 70 % alcohol for 3 seconds onto Petri dishes containing Corn Meal Agar (CMA) and penicillin-streptomycin (100 $\mu g\,ml^{-1}$). Pure cultures were subsequently obtained using the single spore culture method described by Inaba & Tokumasu (2002). These isolates were preserved in the Institute of Hydrobiology, CAS, Wuhan, Hubei province, China.

Asexual reproduction and repeated zoospore emergence (RZE)

The two studied isolates were grown on CMA with several autoclaved hemp seeds (*Cannabis sativa*) at 25 °C. After four days, the fully infested hemp seeds of each isolate were transferred to sterile lake water and incubated at 23 °C. Observations were respectively carried out at 5 h later, 10 h later, 14 h later, 18 h later, 19 h later and 20 h later under an inverted microscope (Zeiss Axiovert 200). Zoospore discharge time was recorded and then measured and photographed under a light microscope (Zeiss Axioplan 2 imaging system and Axiophot 2). Secondary cysts were obtained as described in Fregeneda-Grandes *et al.* (2000). The presence of bundles of long hairs on the secondary cyst was checked for under a phase contrast microscope (Olympus, Japan).

Assessment of repeated zoospore emergence (RZE) was checked for using the protocol described by Diéguez-Uribeondo *et al.* (1994). Mycelia were grown on hemp seeds for 4 d at 25 °C. To trigger sporulation, the hemp seeds were transferred to Petri dishes containing lake water for 14 h at 23 °C. The swimming zoospores were encysted by agitation with a vortex mixer. The release of new zoospores was observed under the microscope after incubating the cyst suspension at 23 °C for 180 min. Percentage of secondary zoospores was estimated by counting empty cysts under the dark field microscopy. Three separate replicates were made for each isolate and at least 50 cysts were observed on each occasion.

Sexual reproduction

Three fully infested hemp seeds of each isolates were transferred to Petri dishes containing sterile lake water and incubated in the dark respectively at 7 °C, 15 °C, 18 °C and 23 °C. Three separate replicates were prepared of each temperature for each isolate. Examination of these hemp seed colonies for sexual structures was made periodically using an inverted microscope and was continued for 4 weeks at each incubation temperature.

DNA extraction and polymerase chain reaction (PCR)

As described before (Ke *et al.* 2008), ten milligrams (net weight) of freshly subcultured fungi was transferred to a 1.5 ml centrifuge tube containing 20 μl TE buffer (10 mM Tris-Hcl, pH 8.0; 10 mM EDTA, pH 8.0) and frozen at −70 °C for 30 min, then grinded using a vitreous sharpener which exactly match the tube. This process was repeated once. The mycelial trituration was diluted with 400 μl steriled double distilled water. Then, 100 μl of the dilution was suspended in 500 μl CTAB digestion buffer [100 mM Tris-Hcl, pH 8.0; 20 mM EDTA, pH 8.0; 1.4 M NaCl; 3 % CTAB; 2 % β-Mercaptoethanol (adding just before use); 4 % (w/v) PVP (Polyvinylpyrrolidon-e)] and incubated at 65 °C for 2 h. The DNA was extracted successively with phenol–chloroform-isoamyl alcohol. Primers ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) (White *et al.* 1990) were used to amplify the two internal transcribed spacers (ITS1 and ITS2) region including the 5.8S rDNA in the PTC-100 Peltier Thermal Cycler. The reaction profile included one initial cycle, 94 °C, 5 min; followed by 35 cycles, 94 °C, 30 s; 58 °C, 30 s and 72 °C, 1 min. A final cycle at 72 °C for 5 min completed the amplification. PCR products were isolated using 1 % agarose gel electrophoresis and purified using the Gel Extraction Kit (OMEGA). Amplified fragments were cloned into pMD-1 8T vetor [TaKaRa Biotechnology (Dalian)] and four clones with inserted products were selected for sequencing in both directions using M13

forward and reverse primers on ABI PRISM®3730 DNA sequencer (Applied Biosystems, USA).

Phylogenetic analysis

The ITS sequences of isolates JY and BMY were deposited in GenBank under the accession numbers: EU163405 and EU163406. Other thirty-nine nucleotide sequences used in phylogenetic analysis were available in the GenBank/EMBL databases under the following accession numbers: *Saprolegnia mixta*[1], EF126339, *Saprolegnia mixta*[2] AB219390, *Saprolegnia ferax*[1] AM228790, *Saprolegnia ferax*[2] AM228788, *Saprolegnia ferax*[3] AM228845, *Saprolegnia ferax*[4] EU124763, *Saprolegnia ferax*[5] AF036543, *Saprolegnia ferax*[6] AB219387, *Saprolegnia longicaulis* AF270032, *Saprolegnia bulbosa*[1] AY267011, *Saprolegnia bulbosa*[2] EF126324, *Saprolegnia oliviae* AY270031, *Saprolegnia litoralis* AY310503, *Saprolegnia* sp. UNCW253 DQ393519, *Saprolegnia* sp. UNCW254 DQ393520, *Saprolegnia diclina*[1] AM228850, *Saprolegnia* sp. GD18A EU240070, *Saprolegnia anomalies*[1] EF064134, *Saprolegnia anomalies*[2] DQ322632, *Saprolegnia* sp. UNCW266 DQ393529, *Saprolegnia* sp. UNCW176 DQ393511, *Saprolegnia* sp. UNCW172 DQ393510, *Saprolegnia* sp. UNCW286 DQ393536, *Saprolegnia hypogyna* AB219389, *Saprolegnia salmonis* AY647193, *Saprolegnia parasitica*[1] AM228804, *Saprolegnia parasitica*[2] AY455776, *Saprolegnia parasitica*[3] AM228840, *Saprolegnia parasitica*[4] AM228755, *Saprolegnia parasitica*[5] AM228839, *Saprolegnia parasitica*[6] AM228838, *Saprolegnia polymorpha* AB219394, *Saprolegnia australis* AY647195, *Saprolegnia diclina*[2] AY455775, *Saprolegnia* sp. UNCW375 DQ393559, *Saprolegnia monilifera* AB219377, *Saprolegnia torulosa* AB219379, *Saprolegnia terrestris* AB219396. *Achlya flagellate* AF218143 were used as outgroup.

All sequences were globally aligned using the Clustal-X (Thompson *et al.* 1997) with default settings and then examined by eye. The postulated gaps and ambiguously aligned regions were excluded from phylogenetic analyses. Phylogenetic trees were constructed by the Neighbour Joining method, the Maximum Parsimony method and the Bayesian method, which implemented with the MEGA3 computer package (Kumar *et al.* 2004), PAUP* computer package (Swofford 2003) and MrBayes version 3.0b4 (Huelsenbeck & Ronquist 2001), respectively. The NJ tree was constructed using the Kimura-two-parameter distance and PAUP trees were generated using a heuristic search. To estimate the relative branch support of NJ and MP trees, bootstrap analysis with 1000 replicates was conducted. The Bayesian analysis was performed using the general time reversible model and four chains were run for 1,000,000 generations with trees sampled every 1000 generations, and 500 sampled trees were used for inferring the Bayesian tree. Priors were left at default settings.

Results

Asexual reproduction and repeated zoospore emergence (RZE)

Saprolegnia isolates JY and BMY grew luxuriantly on hemp seeds in water at 23 °C. After 5 h, zoosporangia had formed abundantly and zoospores were discharged between 10 and 14 h. In both isolates the hyphae were stout, sparingly branched and measured between 25–60 μm in diameter. Zoosporangia were fusiform, clavate, straight or bent and usually terminal (Fig 1A), and proliferated internally (Fig 1B). They typically measured 120–490 × 25–60 μm in isolate JY and 125–500 × 31–60 μm in isolate BMY. Primary zoospore discharge in both was typically saprolegnoid (Fig 1C). The primary zoospores were pyriform, biflagellate and quickly encysted to form spherical primary cysts, 9–11 μm in diameter (Fig 1E). The secondary zoospores were reniform and laterally biflagellate (Fig 1F). Both isolates produced plentiful gemmae which were spherical, pyriform, or irregular in shape and born either terminally or laterally (Fig 1D).

RZE experiments showed both isolates could form new generations of secondary cysts (Fig 1G). The percentage of secondary cysts releasing swimming 'tertiary' zoospores was 21 % in isolate JY and 19 % in isolate BMY. No bundles of long hairs were observed on the secondary cysts in either isolate when examined at ×600 magnification under phase contrast optics (Fig 1H–I). The retracted pattern of germination with cross walls which is a characteristic feature of *S. parasitica* (Beakes *et al.* 1994), occurred respectively less than 1 % and 2 % in germinating cysts from isolates JY and BMY.

Sexual reproduction

At 7 °C and 23 °C both isolates had not formed oogonia, even after 4 weeks incubation. At 15 °C isolate JY developed oogonia and antheridia whereas at 18 °C antheridium formation was suppressed. Isolate BMY produced oogonia at 15 and 18 °C, but lacked antheridia at both temperatures. The oogonia of isolate JY were lateral, terminal, or intercalary; spherical, pyriform or ellipsoidal and ranged between 80–115 μm in diameter. Oogonial walls were smooth or rough, pitted and stalks were short and stout (Fig 2A–B). The small number of antheridiate oogonia observed in isolate JY showed variable antheridium attachment (monoclinous, androgynous and diclinous, Fig 2A–C). Isolate BMY produced oogonia that were rough, pitted; lateral, terminal, or frequently intercalary and spherical or pyriform and measured 70–121 μm in diameter and stalks were short and stout (Fig 2D). The oogonia of isolate JY produced between 1 and 30 spores per oogonium, whereas those of isolate BMY could contain up to 50 atypical small spores (Fig 2E–F) which did not appear to mature. Even in isolate JY most oospores did not mature although a few centric/subcentric mature oospores were occasionally observed. Oospore germination was never observed in either isolate.

Sequences comparisons and phylogenetic analysis

About 704 bp of the ITS rDNA complex were obtained from isolates JY and BMY. The sequence of isolate JY differed from that of BMY due to three nucleotides in the sites 127, 349 and 554 (Similarity 99.6 %). The phylogenetic trees constructed by three different methods (NJ/MP/Bayes) were similar in the main topology node especially those of in the MP and MrBayes trees. Fig 3 shows the three kinds of trees where the bootstrap value are indicated under the branches. All *Saprolegnia* species fell into four clades.

Clade I comprises sequences from isolates named as *S. ferax*, *S. mixta*, *S. longicaulis*, *S. bulbosa*, *S. oliviae*, *S. litoralis* and *S. anomalies*. It also contains one strain described as

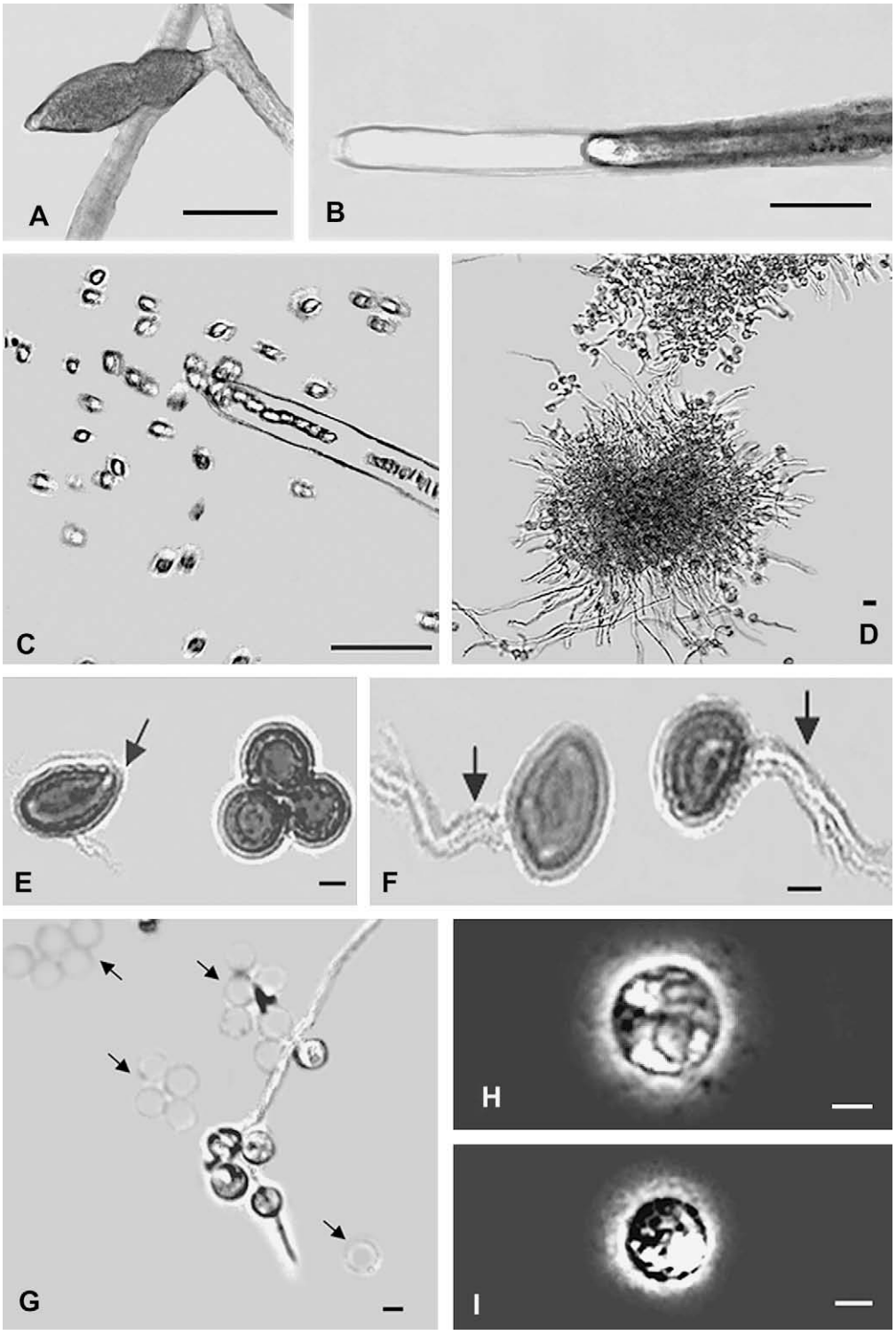

Fig 1 – Identical asexual reproduction of isolates JY and BMY. (A) Mature zoosporangia. Bar = 100 μm (B) Sporangial renewal by internal proliferation. Bar = 100 μm (C) Saprolegnoid discharge of zoospores. Bar = 100 μm (D) Germinating zoospores. Bar = 10 μm. (E) Primary and encysted zoospores. Arrowheads pointing to acrogenous flagellum of primary zoospores. Bar = 3 μm. (F) Secondary zoospores. Arrowheads pointing to lateral flagellum. Bar = 3 μm. (G) Germinating zoospores and the empty cysts which have undergone repeated zoospore emergence. Arrowheads pointing to empty cysts. Bar = 2 μm. (H) Phase contrast montage image of secondary cyst from isolates JY not showing bundles of hairs. Bar = 4 μm. (I) Phase contrast montage image of secondary cyst from isolates BMY not showing bundles of long hairs. Bar = 5.0 μm.

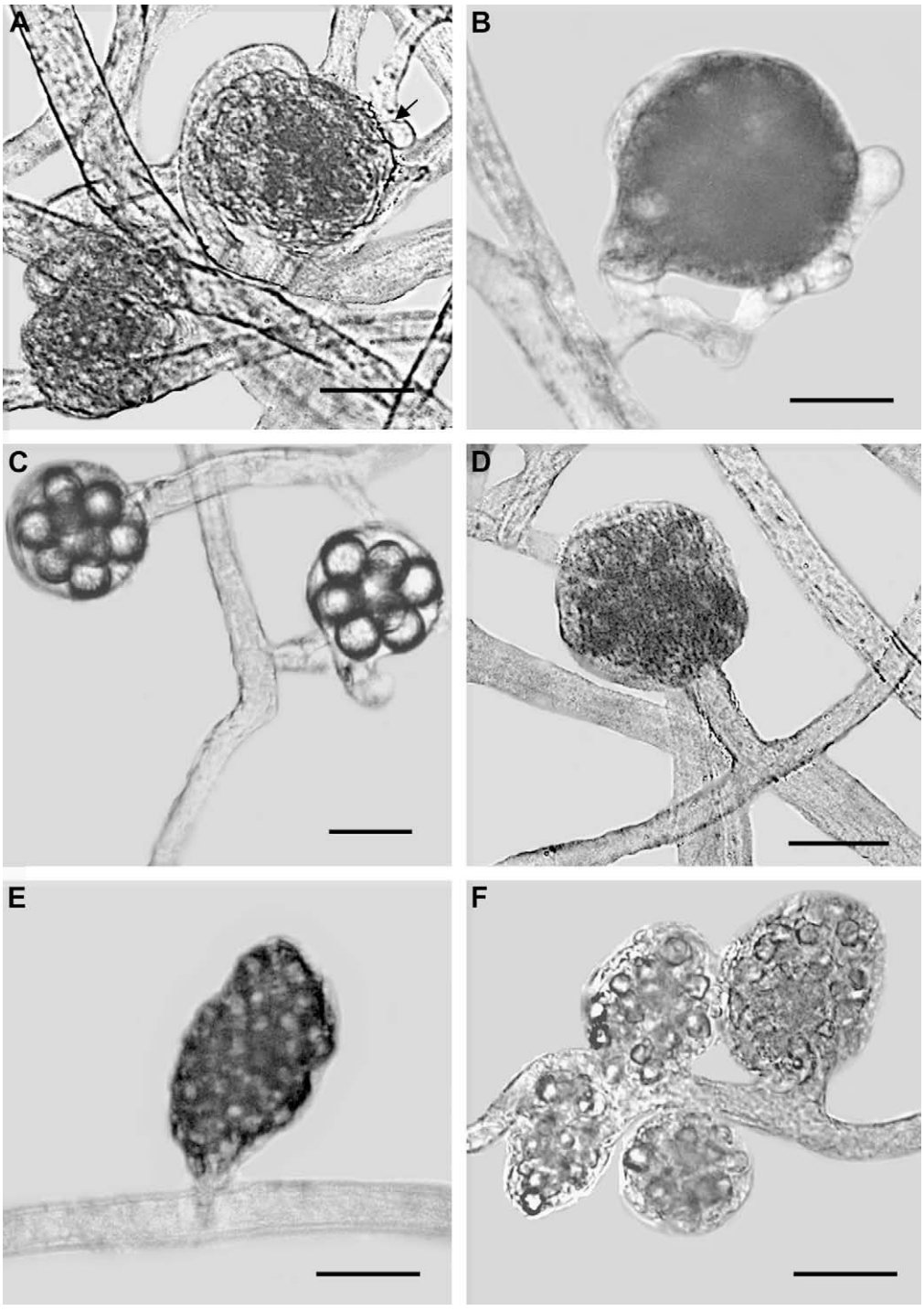

Fig 2 – Sexual reproduction. Isolate JY: (A) Terminal oogonia with diclinous antheridia. (B) Lateral oogonia with androgynous antheridia. (C) Terminal and intercalary oogonia with centric oospores. Isolate BMY: (D) Terminal oogonia. (E) Oospores filling the lateral oogonium. (F) The glomerulate oogonia with immature oospores. (A–F) bar = 50 μm.

S. diclina and a group of unidentified species. These isolates are from a wide geographical range, i.e. Indian, Japan, Poland, Switzerland, Spain, Finland, Argentina, Australia, USA and Germany, and from different hosts and habitats, i.e. *Nematalosa erebi*, crayfish, amphibian and fish eggs, water and soil. Isolates JY and BMY are also clustered into this clade.

Clade II comprises sequences from *S. polymorpha*, *S. diclina* and *S. australis*. They are all from Japan and the latter two are respectively obtained from coho salmon and its eggs.

Clade III contains the sequences from *S. salmonis* and from all the isolates named as *S. parasitica*. They are obtained from different hosts and habitats of diverse geographical origin, i.e.

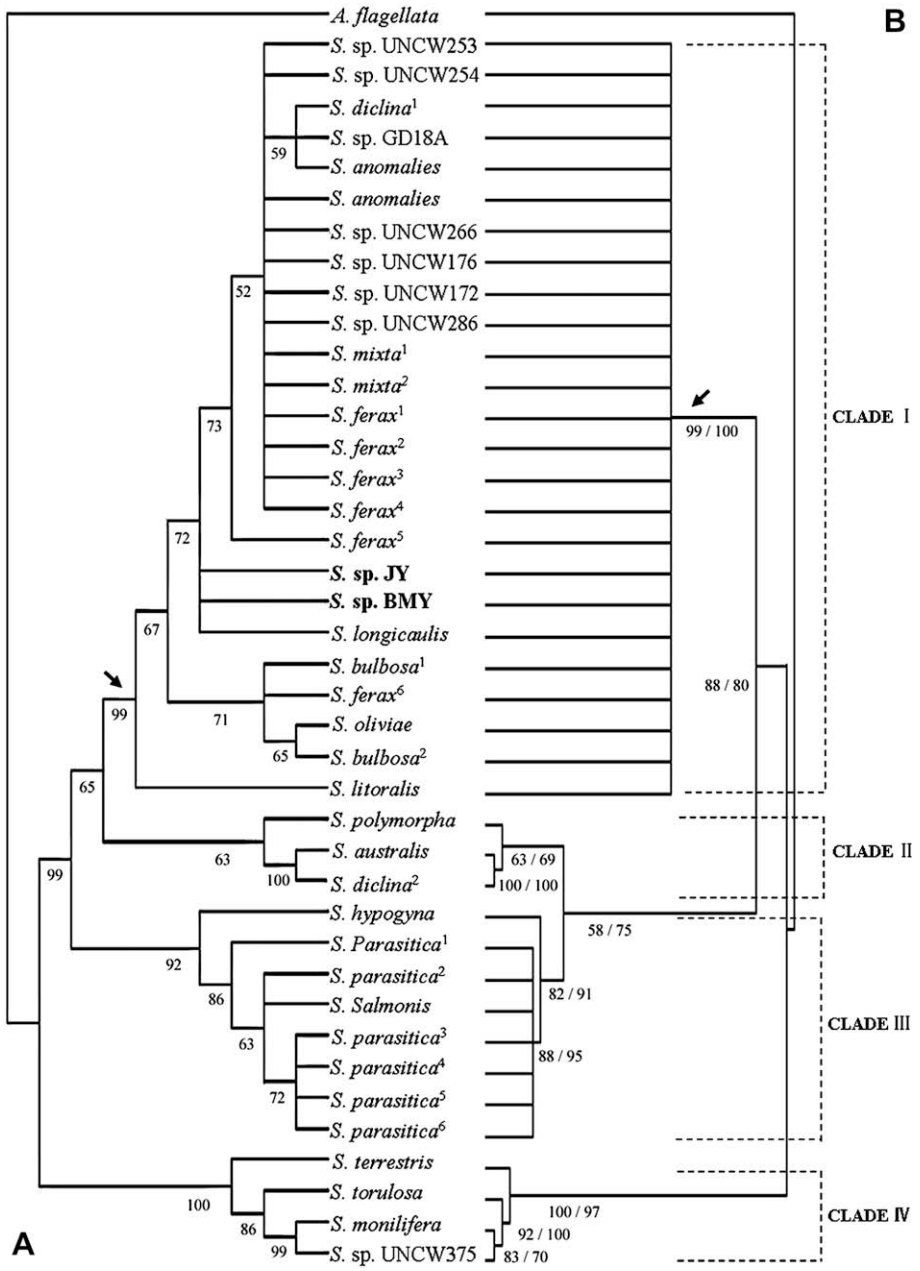

Fig 3 – Phylogenetic positions of isolates JY and BMY in relation to other sequenced *Saprolegnia* species based on ITS rDNA region. (A) It showed Neighbour-joining tree. (B) It showed the Maximum Parsimony tree (MP) and Bayes tree. Bootstrap values under the branches are displayed by MP/ Bayes. All bootstrap values are indicated at 1000 repetitions (values smaller than 50 % not shown).

the *corhynchus mykiss* fish of Chile, the water of Spain, the sockeye salmon fish or the *salmo trutta* fish of Finland. This clade also comprises the sequence of *S. hypogyna* from Japan.

Clade IV includes the sequences from S. sp. UNCW375 and three isolates originally obtained from soil of Japan which characterized as *S. monilifera*, *S. torulosa* and *S. terrestris*.

Discussion

Morphologically, isolate JY and isolate BMY are very difficult to distinguish from each other. The oogonia from both are smooth or unsmooth; spherical or pyriform and their size ranges overlap. In both isolates oogonia typically contained 30 or more spores per oogonium, although few of these appeared to fully mature. The few mature oospores were observed centric or subcentric. Sequence comparisons indicated that the ITS regions of isolate JY exhibited 99.6 % similarity to that of isolate BMY. All these results show that isolates JY and BMY may be conspecific. According to the classical taxonomic criteria (Dick 1969; Seymour 1970; Johnson *et al.* 2002), the two isolates were morphologically closest to *S. australis* and *S. ferax*, in having smooth or unsmooth oogonia, variably present antheridia and up to 30 or more, centric or subcentric

oospores. It should be noted that relatively few mature oospores are produced and in isolate BMY many oogonia contain 50 or so spores which are abnormally small for oospores. However, comparing the ITS sequences of these four isolates, we found isolates JY and BMY both respectively show about 96 % similarity to *S. australis* and above 99 % similarity to *S. ferax*. Based on these morphological and molecular evidences, isolates JY and BMY can be identified as belonging to the *S. ferax* clade.

A large number of currently described species, i.e. *S. mixta* from soil, *S. longicaulis* from water, *S. bulbosa* from soil and water, *S. oliviae* from water, *S. litoralis* from crayfish, *S. anomalies* from water and one strain *S. diclina* from *Nematalosa erebi* also fall into this clade. These isolates form a sister clade to our studied isolates JY and BMY with high support by MP and MrBayes analyses (99 %, 100 %). The ITS sequences from all these isolates of clade I exhibit high similarity (above 99 %). However the morphological characteristics of the oognia of species such as *S. mixta*, *S. longicaulis*, *S. bulbosa, S. oliviae* and *S. litoralis* are described as being different (Steciow 2001; Johnson *et al.* 2002; Steciow 2003; Steciow *et al.* 2007), which shows how unsatisfactory oogonium morphology appears to be as a predictor of genetic relatedness. More interestingly, according to classical descriptions and figures of Johnson *et al.* (2002), it seems that the distinct shapes of oogonia from currently described species, i.e. *S. mixta, S. longicaulis, S. bulbosa, S. oliviae, S. litoralis*, all can be found in the *S. ferax* clade, although they differed in their size ranges. These suggest the isolates in the clade may be the members of the same phylogenetic species. In the Neighbor-Joining tree, some isolates constitute small distinct groups within this clade, but the bootstrap supports are not high (52 %–73 %).

Many isolates of this clade were isolated from water, but some were obtained from fish and others had been specifically identified as the pathogens to amphibians or fish at their embryonic or larval stages, i.e. *S. ferax*, *S. diclina* (Blaustein *et al.* 1994; Kiesecker *et al.* 2001; Fernández-Benéitez *et al.* 2008). Kitancharoen *et al.* (1997) and Fregeneda-Grandes *et al.* (2007) also reported a group of *Saprolegnia* isolates mostly isolated from fish eggs assigned to *S. diclina*, on the basis of their morphological characteristics. Isolates assigned to *S. diclina*, which have cysts decorated by short spines (Hatai *et al.* 1990) appear to be distributed in different clades with high support as shown in this present study and that of Diéguez-Uribeondo *et al.* (2007). This is probably due to misidentification of some isolates. The strain of *S. diclina* which Fernández-Benéitez *et al.* (2008) showed could infect amphibian eggs as the primary pathogen, was the one which showed above 99 % similarity in its ITS sequence to the isolates of Clade I, in which our two isolates fell. Preliminary experiments have shown that both JY and BMY isolates can infect healthy eggs of silver crucian cap (unpublished observations). Studies show the isolates obtained from infected eggs and alevins were different to those from infected live fish and *S. parasitica* was rarely isolated from infected eggs (Kitancharoen *et al.* 1997; Fregeneda-Grandes *et al.* 2007). These observations suggest that although long-hooked hairs characterize the taxon of *S. parasitica* sensu stricta (Diéguez-Uribeondo *et al.* 2007), there may be other 'short-spined' *Saprolegnia* species that are able to infect amphibian and fish eggs. The evidence from the present study and other recent studies seems to suggest that isolates in clade I may be important primary pathogens of amphibian and fish eggs as well as being more general saprotrophs.

Clade II is a group of saprobiotic species which is relatively far from clade I and clade III as discussed by Diéguez-Uribeondo *et al.* (2007). However, in the present study clade II is more related to clade I in the NJ phylogenetic analyses and it show more closely to Clade III in the analyses of MP/MrBayes, though the bootstrap values are not high (63 %, 63 %, 69 %). These differences may due to the small number of investigated isolates. The actual relationships among clade II isolates and other clades need to be studied further, especially to determine whether this is exclusively a saprotrophic clade.

Clade III and clade IV are both well supported in all the phylogenetic analyses. Clade III isolates are a group of pathogenic species, most of which are obtained from fish lesions and have been the subject of an in depth analysis by Diéguez-Uribeondo *et al.* (2007). All the isolates of this clade, except for *S. hypogyna*, are characterized by having bundles of long-hooked hairs on their secondary cysts and the septate cyst germination (Beakes *et al.* 1994), although they do show significant differences in their pathogenecity (Diéguez-Uribeondo *et al.* 2007; Fregeneda-Grandes *et al.* 2000, 2001). Based on the high bootstrap support we conclude that the species in clade IV (*S. terrestris*, *S. torulosa*, *S. monilifera* and *S.* sp. UNCW375) form a discrete group, clearly separated from other three clades. All of these species were isolated from the soil rather than freshwater and more work on this clade needs to be done.

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