



Original article

## Pathogenic ability and saline stress tolerance of two *Fusarium* isolates from *Odontesthes bonariensis* eggs



Suani G. Pacheco Marino<sup>a,b,\*</sup>, Marta N. Cabello<sup>a,c</sup>, María I. Dinolfo<sup>b,d</sup>, Sebastián A. Stenglein<sup>b,d,e</sup>, Mario C.N. Saparrat<sup>b,f</sup>, Alfredo Salibián<sup>c,g</sup>

<sup>a</sup> Instituto de Botánica Spegazzini, FCNyM, UNLP, La Plata, Argentina

<sup>b</sup> Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

<sup>c</sup> Comisión de Investigaciones Científicas (CIC), La Plata, Buenos Aires, Argentina

<sup>d</sup> Laboratorio de Biología Funcional y Biotecnología (BIOLAB-CICBA-INBIOTEC), Buenos Aires, Argentina

<sup>e</sup> Facultad de Agronomía, Cátedra de Microbiología, UNCPBA, Buenos Aires, Argentina

<sup>f</sup> Instituto de Fisiología Vegetal (INFIVE), CCT-La Plata-CONICET, Universidad Nacional de La Plata (UNLP), La Plata, Argentina

<sup>g</sup> PRODEA-INEDES Departamento de Ciencias Básicas, Universidad Nacional de Luján, Luján, Buenos Aires, Argentina

### ARTICLE INFO

#### Article history:

Received 17 February 2014

Accepted 25 February 2015

Available online 27 May 2015

#### Keywords:

Fish pathogens

FIESC

FSSC

Pathogenicity

*Fusarium*

Saline stress

### ABSTRACT

**Background:** Several fungal species represent a potential risk to embryos of *Odontesthes bonariensis* (Cuvier and Valenciennes, 1835), a euryhaline freshwater fish that lives in the Pampean inland waters and has potential economic relevance.

**Aims:** To identify two fungi isolated from *O. bonariensis* eggs exposed to saline conditions and to characterize their pathogenicity and tolerance to sodium chloride solutions.

**Methods:** The isolates were identified by morphological features, and a preliminar phylogenetic analysis using sequences of translation elongation factor 1- $\alpha$  (EF-1 $\alpha$ ) and calmodulin (CAM) was performed. Koch's postulates were tested to identify the causative agent of fungal infection. The influence of NaCl on the fungal growth was evaluated in in vitro assays.

**Results:** The isolates LPSC 1001 and 1002 were identified as representatives of the genus *Fusarium*, and belonging to the *Fusarium incarnatum-Fusarium equiseti* species complex (FIESC) and the *Fusarium solani* species complex (FSSC), respectively. Histological observations on eggs exposed in vitro to both isolates in infectivity assays confirmed the ability of the fungal isolates to penetrate to egg's chorionic membrane, leading to the death of embryos. Increasing NaCl concentration in the culture medium reduced the growth of the isolates LPSC 1001 and 1002, being completely inhibited at 160 and 120 g/l NaCl respectively.

**Conclusions:** The isolates LPSC 1001 (FIESC) and 1002 (FSSC) were identified as fungal pathogens to *O. bonariensis* eggs. The use of NaCl solutions as antifungal treatment was not effective to control the infection with these strains.

© 2014 Asociación Española de Micología. Published by Elsevier España, S.L.U. All rights reserved.

## Patogenicidad y tolerancia al estrés salino de dos aislamientos de *Fusarium* aislados de huevos de *Odontesthes bonariensis*

### RESUMEN

**Antecedentes:** Diversas especies de hongos pueden representar un riesgo importante para los embriones de *Odontesthes bonariensis* (Cuvier & Valenciennes 1835), un pez de agua dulce eurihalino que vive en las aguas interiores pampeanas y tiene una relevancia económica potencial.

**Objetivos:** Identificar dos especies de hongos de huevos de *O. bonariensis* expuestos a condiciones salinas y caracterizar su patogenicidad y tolerancia a las soluciones de cloruro de sodio.

**Métodos:** Se identificaron los aislamientos por sus características morfológicas, y se realizó un análisis filogenético preliminar utilizando secuencias de traslación del factor 1 alfa elongación (EF- 1 $\alpha$ ) y

#### Palabras clave:

Patógenos de los peces

FIESC

FSSC

Patogenicidad

*Fusarium*

Estrés salino

\* Corresponding author.

E-mail addresses: [suani.pm@gmail.com](mailto:suani.pm@gmail.com), [spacheco@fcv.unlp.edu.ar](mailto:spacheco@fcv.unlp.edu.ar) (S.G. Pacheco Marino).

calmodulina (CAM). Se llevaron a cabo los postulados de Koch para identificar el agente causante de la infección fúngica. La influencia de NaCl sobre el crecimiento fúngico se evaluó en ensayos in vitro.

**Resultados:** Se identificaron los aislamientos LPSC 1001 y 1002 como representantes del género *Fusarium*, y pertenecientes al complejo de especies *Fusarium incarnatum-Fusarium equiseti* (FIESC) y al complejo de especies de *Fusarium solani* (FSSC), respectivamente. Las observaciones histológicas en los huevos expuestos in vitro a ambos aislamientos en los ensayos de infectividad confirmaron la capacidad de estos para penetrar en la membrana coriónica, lo que condujo a la muerte de los embriones. El aumento de la concentración de NaCl en el medio de cultivo redujo el crecimiento de los aislamientos LPSC 1001 y 1002, quedando completamente inhibidos a 160 y 120 g/l de NaCl, respectivamente.

**Conclusiones:** Se identificaron los aislamientos LPSC 1001 (FIESC) y 1002 (FSSC) como hongos patógenos para los huevos de *O. bonariensis*; el uso de soluciones de NaCl como tratamiento antifúngico no resultó eficaz para el control de la infección con estas cepas.

© 2014 Asociación Española de Micología. Publicado por Elsevier España, S.L.U. Todos los derechos reservados.

The Argentinian silverside, or “pejerrey”, *Odontesthes bonariensis* (Cuvier & Valenciennes 1835) is an atherinid fish that inhabits rivers and lakes located in the southern sector of La Plata basin. The silverside is considered one of the most promising fish species of Argentina for the development of its intensive culture.<sup>40</sup> The great potential of this species leads to the need of having a better knowledge of its biology, as well as of its pathogens, for implementing an adequate management of intensive culture systems, which includes a control diseases program.

Fungal diseases can be a serious threat to culture fish species. Several fungal species and water molds can pose a substantial risk to fish embryos.<sup>35,45</sup> In combination with environmental stressors, the pathogens can cause severe declines in fish populations. Infected eggs and larvae act as a dissemination source, and the infection spreads quickly. Spread of the diseases can occur via spores that are released to the aquatic environment adhering to healthy individuals, or by mycelial extension from infected to contiguous healthy embryos.<sup>35</sup>

Although several authors<sup>21,45</sup> point to the order of the Saprolegniales (Oomycetes) as the most important pathogens in freshwater fish and their eggs, Rand<sup>35</sup> highlights the role of anamorphic fungi, due to their wide distribution and adaptability to different environmental conditions. Fungal fish pathogens list include species of the genera *Aureobasidium*, *Cladosporium*, *Exophiala*, *Hormoconis*, *Ochroconis*, *Paecilomyces*, *Penicillium*, *Phoma* and *Verticillium*, which have been reported to cause mortality in fish of the orders Salmoniformes, Perciformes, Siluriformes, Squaliformes and Pleuronectiformes.<sup>9,11,13,20,39,42</sup>

*Fusarium* is a wide and taxonomically complex genus. Recent molecular phylogenetic studies have revealed that the most commonly reported pathogens are part of several species complexes.<sup>24,48</sup> The wide distribution and diverse ecology of the *Fusarium* species reflect its metabolic diversity, since these fungi are abundant in soil, as saprophytes, mutualists, and parasites on plants and animals, in different habitats.<sup>2,18,36,38</sup> In fish, *Fusarium culmorum*,<sup>14</sup> *Fusarium oxysporum*,<sup>13</sup> *Fusarium solani*<sup>5,29,39</sup> and other *Fusarium* species<sup>2</sup> have been found as cutaneous or visceral pathogens, capable of causing sometimes lethal infections.

The aim of the present work was to isolate and characterize by traditional morphology and molecular methodologies the fungal biota belonging to genus *Fusarium* associated with *O. bonariensis* eggs exposed to saline conditions. Furthermore, we prove the pathogenicity of the isolates by following Koch's postulates and examine the etiological agents' tolerance to NaCl as potential control measure.

## Materials and methods

### Fungal isolation and identification

Eggs of *O. bonariensis* were obtained from the Chascomús Fisheries Station of the Ministerio de Agricultura de la Provincia de Buenos Aires, Argentina and incubated as described in Pacheco Marino & Salibián<sup>31</sup> with the addition of NaCl (Anedra) in a range of 10–30 g/l. During the incubation, the infected eggs were collected aseptically and washed with an antibiotic solution (chloramphenicol and streptomycin (Sigma) at a concentration, of 2.5 and 5% (w/v), respectively). Eggs were rinsed with sterile distilled water and then used as fungal inoculum in Petri plates containing 20 ml of potato glucose agar (PGA),<sup>17</sup> at a final concentration of 0.006 and 0.012% (w/v) of chloramphenicol and streptomycin.

The identification of the isolates was based on cultural and morphological features following the original taxonomic papers,<sup>2,7,18</sup> including the culture on potato sucrose agar (PSA), carnation leaf agar (CLA) and oatmeal agar (OMA),<sup>16</sup> and synthetic low nutrient agar (SNA),<sup>23</sup> at 25 °C and darkness during 2–5 weeks.

### DNA isolation, sequencing and phylogenetic analysis

For the molecular identification genomic DNA was extracted using the CTAB method described by Stenglein and Balatti.<sup>41</sup> PCRs were performed to amplify partial sequences of the elongation factor 1-alpha (EF-1 $\alpha$ ) and calmodulin (CAM) genes according to O'Donnell et al.<sup>26,27</sup> Amplifications were carried out in a XP thermal cycler (Bioer Technology Co.). The successful amplifications were confirmed by gel electrophoresis. PCR products were purified with the aid of a PureLink PCR purification kit (Invitrogen). DNA sequencing from both the sense and antisense ends of the fragments was carried out using Big Dye Terminator version 3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) in an Applied Biosystems Sequencer (ABI/Hitachi Genetic Analyzer 3130). The sequences generated in this study were deposited into GenBank under the following accession numbers: JN614903.1, JN614904.1, HQ201752.1 and HQ201753.1.

For the phylogenetic reconstruction we used the dataset of O'Donnell et al.,<sup>27,28</sup> though the *Fusarium tricinctum* (FTSC) and the *Fusarium sambucinum* (FSAMSC) complexes were excluded due to non-availability of sequences of calmoduline gene for these fungi (see Supplementary S1 Table 1: Data of sequences used in the phylogenetic analysis in Appendix A). All the dataset sequences for each single molecular marker were aligned using MAFFT v.6 (<http://mafft.cbrc.jp/alignment/server/>).<sup>15</sup> The nexus files used in

this analysis were uploaded to TreeBASE (study accession number 14120).

Phylogenetic analysis was conducted based on the aligned sequences for each of the data set using PAUP v. 4.0b10.<sup>44</sup> All the alignment gaps were treated as missing data. Each data set was initially analyzed based on different optimality criteria: maximum parsimony (MP), neighbor-joining (NJ) and Bayesian Inference (BI). In the MP analysis the starting trees for branch swapping were obtained via a stepwise addition procedure. A heuristic search, with random sequence addition of 1000 replicates and tree-bisection-reconnection branch-swapping algorithm, was performed. The bootstrap consensus tree is inferred from 1000 replicates; branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Models of nucleotide substitution and parameters for phylogenetic reconstruction were selected by jModelTest version 0.1.1<sup>12,33</sup> and used to perform NJ and BI analysis (see models of nucleotide substitution and parameter related in Supplementary material S1B: Models of nucleotide substitution in Appendix A). Clade stability was assessed by 1000 bootstrap replicates with random sequence addition. Consistency index (CI) and retention index (RI) were calculated for the indication of the amount of homoplasy present in both MP and NJ analysis. Bayesian MCMC phylogenetic analysis using MrBayes 3.2.3<sup>37</sup> was performed using two simultaneous chains of  $1.5 \times 10^7$  generations and a sample frequency of 300, for a total of 50,000 sample trees, and the prior parameters of the corresponding substitution model and distribution rate. To assess whether the tree topologies inferred from MP and BI analysis were more similar than expected by chance, the congruence index (*I*cong index) was used.<sup>6</sup>

#### Pathogenicity assays

A conidial suspension of each isolate was made according to Toledo et al.<sup>46</sup> Silverside eggs were exposed for 96 h to 20 ml of conidial suspensions of  $1 \times 10^6$  to  $1 \times 10^9$  conidia/ml of each fungal strain separately. Eggs not exposed to conidial suspensions were used as a control. Three trials with 10 individuals by replicate were carried out for each fungal isolate. Survival, mortality and infection rate (IR) were recorded at the end of the test, and median lethal concentration (LC-50) was computed using the Trimmed Spearman-Kärber 1.5 software program.

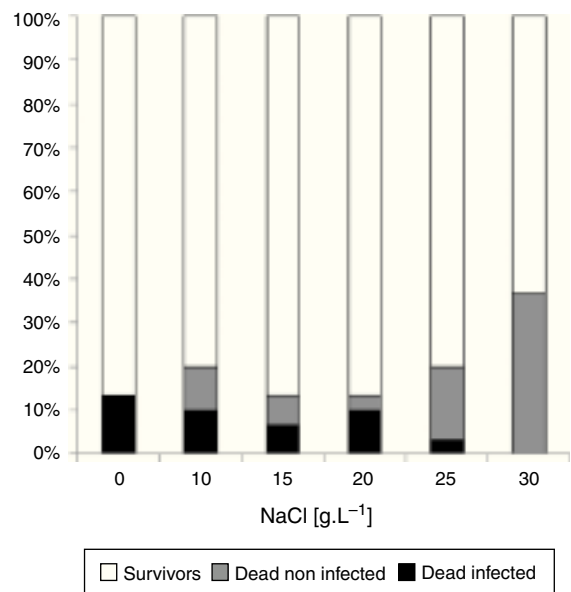
#### Histopathological examination

Infected and uninfected eggs were fixed by immersion in 10% phosphate-saline buffered formalin and were processed according to standard procedures. Sections (5  $\mu$ m) of each egg were stained with hematoxylin eosin (HE) and a silver methenamine staining (Grocott), as described by Prophet et al.,<sup>34</sup> and examined by light microscopy.

#### Influence of NaCl on fungal growth

Three sets of Petri dishes containing 20 ml of Czapek's Dox medium<sup>17</sup> with 6 different NaCl concentrations (0 – control-, 40, 80, 120, 160 and 200 g/l) were used for each fungal strain. Plates were inoculated at the center, with a 3 mm diameter plug cut from axenic and monospore culture plates, and incubated at 25 °C in darkness. Three days after the inoculation, the diameter of the colonies was measured every 24 h in the same direction for two weeks, using a vernier caliper (accuracy = 0.1 mm); colonies were observed for an additional week to detect possible changes in the growth rate.

The inhibition rate was expressed as the percentage reduction in colony diameter compared with the control group



**Fig. 1.** *Odontesthes bonariensis*. Survivorship of eggs infected by *Fusarium* spp. and exposed to various concentrations of NaCl for 96 h. Mean percentages are shown. Concentrations are given in g/l.

(Czapek's Dox). The percentage of reduction was calculated as follows:

$$\text{Inhibition \%} = 100 \times \left[ 1 - \left( \frac{\text{Diameter on media with NaCl}}{\text{Diameter on Control}} \right) \right]$$

#### Statistical analysis

In the pathogenicity assays, the differences in survival and infection rates between treatments and the control group were analyzed by a Mann-Whitney *U* test. Growth rate was calculated from the linear regressions. Pearson's correlation coefficient was used to determine the linear equation. Variance was analyzed using Kruskal Wallis non-parametric tests to determine differences between growth rates of each species under different treatments. Comparisons between experimental and control values were performed by a posteriori Tukey multiple comparisons test. Statistical calculations were performed using the software program XL STAT7.5.

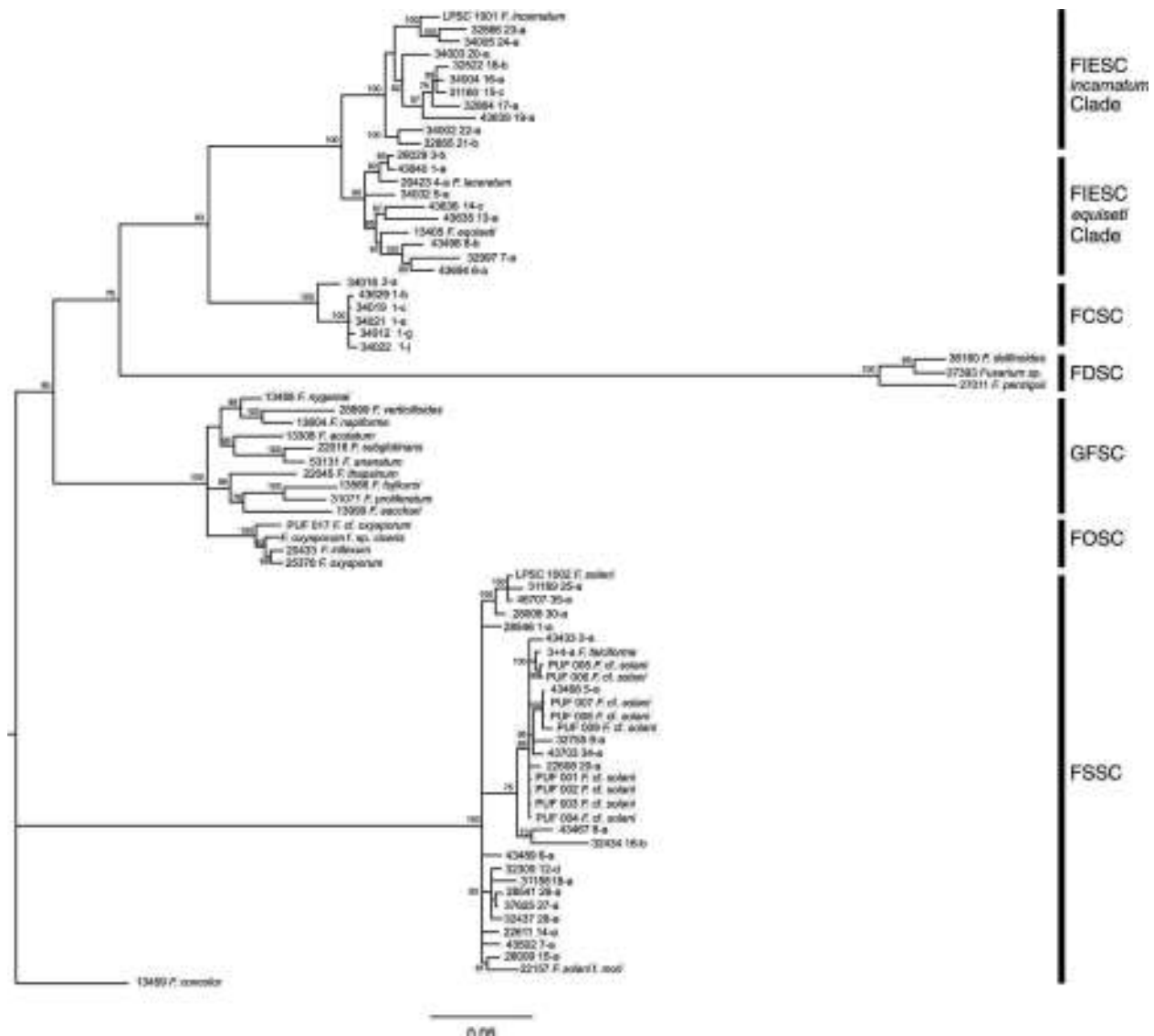
## Results

#### Fungal isolation and morphological identification

A 13.3% of the non-exposed to NaCl eggs died due to fungal infections, and a mortality rate of 3.3–10% was found in eggs exposed to NaCl at a range of concentrations between 10 and 25 g/l (Fig. 1). Two morphotypes isolated were morphologically identified as *Fusarium incarnatum* (Desm.) Sacc. (1986) and *Fusarium solani* (Mart.) Sacc. (see Supplementary S2A: Taxonomic descriptions, and S3: Illustrations in Appendix A) and were deposited in the strain-culture collection of Spegazzini Institute Herbarium (LPSC) with accession codes LPSC 1001 and 1002, respectively.

#### Molecular identification and phylogenetic analysis

The amplified products of isolates LPSC 1001 and 1002 had 699 and 653 bp for the elongation factor 1-alpha (GenBank Accession Number: HQ201752-3), and 710 and 775 bp for calmodulin (GenBank Accession Number: JN614903-4), respectively. A phylogenetic analysis based on MP, NJ and BI criteria was performed using



**Fig. 2.** Bayesian MCMC analysis of the single data set from *EF1-α*. Only Bayesian posterior-probabilities (100×) above 50 are shown. *Fusarium concolor* isolate (13459) was used as an outgroup for the phylogram. FIESC: *Fusarium equiseti-incarnatum* species complex, FCSC *Fusarium chlamydosporum* species complex, FDSC: *Fusarium dimerum* species complex, FSSC: *Fusarium fujikuroi* species complex, FOSC: *Fusarium oxysporum* species complex, FSSC: *Fusarium solani* species complex.

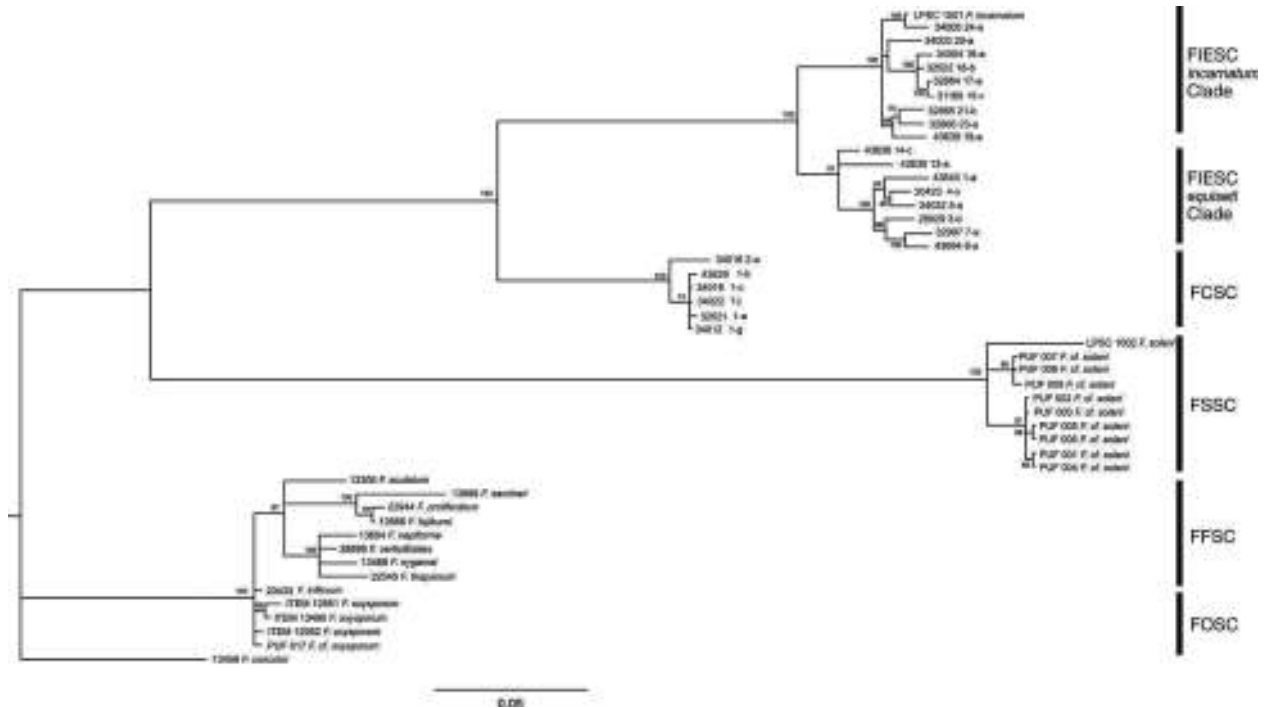
separate data sets of the two loci and the *Fusarium concolor* strain 13459 as an outgroup. Bayesian analysis of *EF1-α* locus resolved six well supported clades (with a support value of 100%), and the distribution of the monophyletic clades was according to the scheme proposed by O'Donnell et al.<sup>27</sup> (Fig. 2). On the latter, the isolate LPSC 1001, morphologically identified as *Fusarium incarnatum*, was indeed located within the FIESC clade and specifically within the *F. incarnatum* subclade, with the haplotypes 23 and 24 (a support value of 100%). The isolate LPSC 1002 falls within FSSC clade, and was closely related to the haplotypes FSSC 25-a, 30-a, and 35-a (support value of 100%). We found similar topologies in the trees generated when this locus was analyzed under MP and NJ criteria (see MP and NJ trees of *EF1-α* locus in Supplementary S4 and S6, respectively, in Appendix A). The statistical support of this observation is provided by the Congruence Index (*I* cong) obtained from the comparative analysis of the tree topologies inferred from BI and MP analysis (4.04,  $p = 2.96 \times 10^{-28}$ ). Additionally, the MP analysis showed that 335 were parsimony informative on a total of 781.

When the phylogenetic relationship using a BI analysis was based on available data from CAM locus, five clades well supported (a support value of 100%) were resolved (Fig. 3). In this case,

LPSC 1001 was closely associated with haplotype 24 of *Fusarium incarnatum-equiseti* species complexes (FIESC) clade (*incarnatum* subclade; with a support value of 100%), and LPSC 1002 was related with the strains PUF 001 to 009 belonging to *Fusarium solani* species complex (FSSC) clade (a support value of 100%). Similar tree topologies were also found in the analysis performed under MP and NJ criteria (see MP and NJ trees of CAM locus in Supplementary S5 and S7, respectively, in Appendix A). The MP analysis revealed 301 out of 841 characters were parsimony informative, and the statistical comparison of the tree topologies obtained with MP and BI criteria revealed that these were more similar than expected by chance (*I* cong: 4.24;  $p = 3.15 \times 10^{-26}$ ). Finally, based on the rate between parsimony informative characters (PIC) and bp, the *EF1-α* locus was the most phylogenetically informative since it showed the greatest nucleotide diversity (PIC/bp = 0.4289) in relation to that obtained for CAM locus (0.3579).

#### Pathogenicity

Both isolates were able to infect silverside eggs. Not fungal infections were recorded in the control groups, and mortality rates of



**Fig. 3.** Bayesian MCMC analysis of the single data set from CAM. Only Bayesian posterior-probabilities (100×) above 50 are shown. *Fusarium concolor* isolate (13459) was used as an outgroup for the phylogram. FIESC: *Fusarium equiseti-incarnatum* species complex, FCSC *Fusarium chlamydosporum* species complex, FSSC: *Fusarium solani* species complex, FFSC: *Fusarium fujikuroi* species complex, FOISC: *Fusarium oxysporum* species complex.

non-infected eggs were lower than 3.3% for both strains under treatment as well as in the control groups. In the eggs exposed to  $1 \times 10^7$  and  $1 \times 10^6$  conidia/ml of LPSC 1001, and  $1 \times 10^7$  conidia/ml of LPSC 1002 the infection rate (IR) was lower than 50% (Fig. 4).

Only when the eggs were exposed to  $1 \times 10^9$  conidia/ml, the IR of the isolate LPSC 1002 (96.7% of IR) was higher than LPSC 1001 (56.7%) ( $p=0.043$ ). No significant differences were found. When comparing the IR of different doses of isolate LPSC 1001; however, the IR obtained in eggs exposed to  $1 \times 10^9$  conidia/ml of the isolate LPSC 1002 was significantly higher than the ones recorded in  $1 \times 10^8$  ( $p=0.002$ )  $1 \times 10^7$  ( $p=0.043$ ) and  $1 \times 10^6$  ( $p=0.002$ ) conidia/ml. For both isolates (LPSC 1001 and 1002), the IR of the eggs exposed to  $1 \times 10^8$  was higher than in those exposed to  $1 \times 10^7$  ( $p=0.008$  and  $0.012$ ). On the other hand, the mean lethal concentrations were  $1.25 \times 10^7$  (CL 95%=  $3.98 \times 10^6$ – $3.95 \times 10^7$ ) and

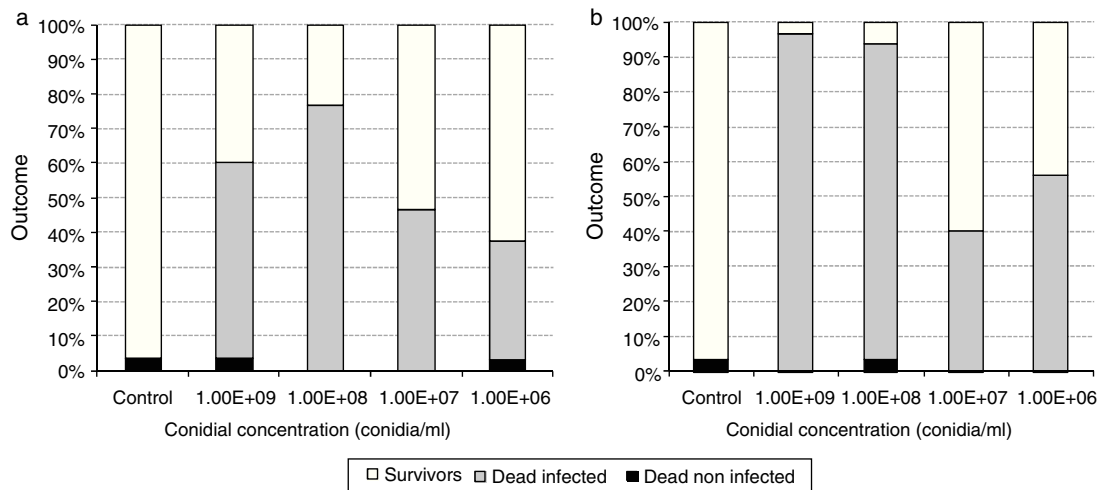
$1.09 \times 10^7$  (CL 95%=  $5.50 \times 10^6$ – $2.16 \times 10^7$ ) to LPSC 1001 and 1002 respectively.

*Histopathological study*

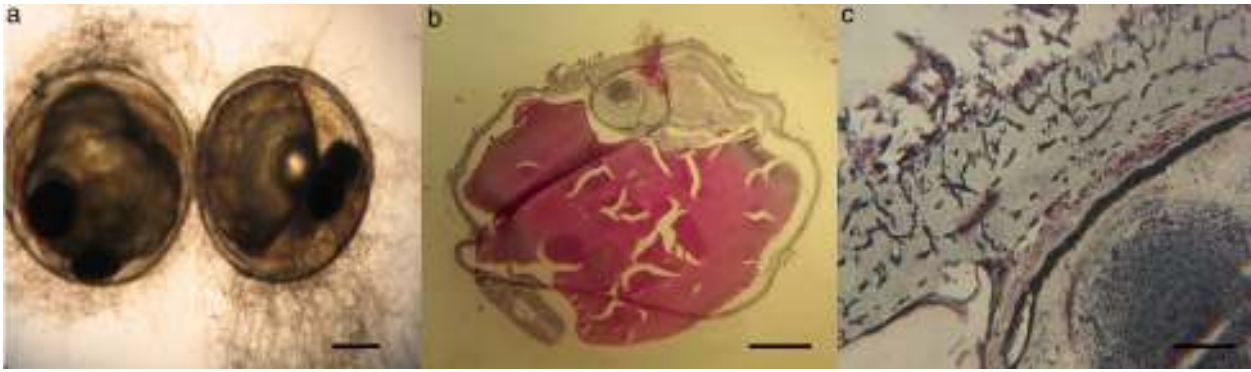
Microscopical observations of infected eggs from pathogenicity tests were done. A tiny and inconspicuous mycelial development was found on infected eggs (Fig. 5a). Using Grocott-stained histological sections of these eggs, septate hyphae in the corionic membrane were also detected (Figs. 5b and c).

*Influence of NaCl on the in vitro fungal growth*

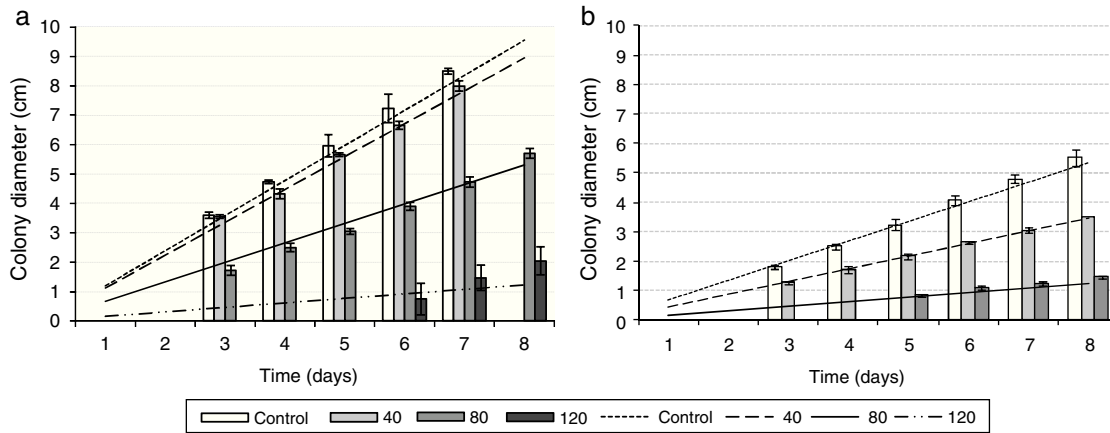
The effect of NaCl on the growth of both isolates is shown in Fig. 6. The growth rate of LPSC 1001 is greater than that of LPSC



**Fig. 4.** *Odontesthes bonariensis* eggs. Survival, infection, and mortality rates of silverside eggs exposed to different conidial suspensions of isolates LPSC 1001 (a) and LPSC 1002 (b). Concentrations are given in conidia/ml.



**Fig. 5.** (a–c) *Odontesthes bonariensis*. (a) Eggs infected by *Fusarium* spp. Scale bar = 0.25 mm. (b) Transversal section of eggs stained with Grocott technique, with hyphae. Scale bar = 200  $\mu$ m. (c) Detail of hyphae penetrating the chorion. Scale bar = 25  $\mu$ m.



**Fig. 6.** Effect of NaCl on the growth rate in Czapeck Dox of the isolates LPSC 1001 (a) and LPSC 1002 (b). Concentrations are given in g/l.

1002 on basal medium, while the latter is more susceptible to NaCl. Growth was completely inhibited at 120 g/l and 160 g/l for both LPSC 1001 and LPSC 1002, respectively.

The slope values obtained in the linear regression, the corresponding Pearson's coefficient, and the inhibition rate for *Fusarium* isolates are shown in Supplementary material S8 in Appendix A (Table 1). In general, the higher NaCl concentrations exhibited inhibitory effect on the growth rates. The analysis of variance showed, compared with the control, significant differences in the growth rate of the isolate LPSC 1001 when exposed to 80 and 120 g/l NaCl. Significant differences were also found in the isolate LPSC 1002 growth rate between treated and control groups.

## Discussion

Two *Fusarium* isolates obtained from *O. bonariensis* eggs (LPSC 1001 and 1002) were characterized morphologically, physiologically and with molecular biology. The morphology of isolates LPSC 1001 and 1002 was similar to that of *F. incarnatum* (formerly *F. semitectum*) and *F. solani* respectively, as described by Booth,<sup>2</sup> Leslie and Summerell,<sup>18</sup> Nelson et al.,<sup>22</sup> and Sutton et al.<sup>43</sup> Traditionally the systematic of the *Fusarium* genus was based on morphological species concept,<sup>49</sup> but in recent years molecular phylogenetic analyses have been applied to examine the taxonomy of this genus and have led to a new taxonomic system based on the phylogenetic species concept.<sup>24,48</sup> Studies based on genealogical concordance of multilocus DNA sequence data revealed an extensive cryptic speciation across the phylogenetic breadth of the *Fusarium* genus, especially within the *F. solani* species complex (FSSC) and *F. incarnatum*-*F. equiseti* species

complexes (FIESC).<sup>27,28</sup> Recently, O'Donnell et al.<sup>28</sup> published an Internet-accessible database for identifying novel etiologic agents of fusarioses within a precise phylogenetic framework. This is based on the multilocus analysis of partial sequences from three nuclear genes: translation elongation factor (EF-1 $\alpha$ ), the largest subunit of RNA polymerase (RPB1), and the second largest subunit of RNA polymerase (RPB2). Their use for identifying isolates to the species level using a single DNA marker was anticipated by their authors.<sup>28</sup>

Since molecular identification of LPSC 1001 and 1002 was based in the phylogenetic analysis of two genetic markers, EF-1 $\alpha$  and CAM, our analysis was done using additional sequences of *Fusarium* species not included in O'Donnell's dataset. The phylogenetic analysis of EF-1 $\alpha$  sequences under NJ, MP and BI criteria resolved six well supported clades with a similar topology to that reported by O'Donnell et al.<sup>28</sup> In MP analysis the statistics values of more parsimonious tree were similar to that reported by O'Donnell et al.,<sup>28</sup> being the MP tree length of 1205 and 1183 steps; the rate PIC/bp = 0.45 and 0.43; the consistency index CI = 0.54 and 0.47; and the retention indexes 0.91 and 0.87, respectively. Finally, all phylogenetic criteria showed that the isolates LPSC 1001 and 1002 are placed in the monophyletic groups FIESC and FSSC respectively. The phylogenetic analysis of CAM sequences resolved five monophyletic clades well supported, with a similar distribution than that observed in EF-1 $\alpha$  analysis. The most parsimonious tree was 1183 steps in length, other parameters as rate PIC/bp = 0.36, IC = 0.70 and IR = 0.94 indicated that the data set used in this analysis were more informative than those used by O'Donnell et al.<sup>27</sup> Finally, the topological comparison of trees obtained in BI and MP phylogenetic analyses of each single data set exhibited high level of topological concordance with similar bootstrapping levels of clade support.

**Table 1**

Slope of the linear regression of the colony diameters (growth rate), Pearson correlation coefficient values and growth inhibition rate for the isolates LPSC 1001 and LPSC 1002 exposed to different concentrations of NaCl for 8 days. Data correspond to the mean values of Fig. 6.

NaCl concentration (g/l)	LPSC 1001			LPSC 1002		
	Slope	Pearson coefficient	Inhibition rate	Slope	Pearson coefficient	Inhibition rate
Control	1.194	0.995	–	0.670	0.999	–
40	1.118	0.993	5.88	0.434	0.996	36.46
80	0.663	0.998	44.41	0.154	0.993	73.96
120	0.153	0.857	82.65	0	0	100
160	0	–	100	0	–	100

Considering that the phylogenetic analysis shows that isolates LPSC 1001 and 1002 shared a strong relationship with clinical isolates belonging to FIESC and FSSC respectively, and since both are considered the two main groups of pathogenic representatives of *Fusarium* genus, a potential risk of zoonoses cannot be discarded.<sup>25,43</sup>

Previously, *Fusarium* strains have been described as etiological agents of infections in carp (*Cyprinus carpio*) and red sea bream (*Pagrus sp.*).<sup>18,19</sup> Ferguson<sup>10</sup> isolated a *F. solani* strain from a mycetoma of desert pupfish (*Cyprinodon macularius*). Furthermore, fusariosis in sharks in marine habitats has been reported by Rand,<sup>2</sup> Smith et al.,<sup>39</sup> Crow et al.<sup>5</sup> and Marancik et al.<sup>20</sup> One case of infection by *F. incarnatum* was reported by Khoa et al.<sup>16</sup> in a black tiger shrimp (*Penaeus monodon*). Although these reports show the diversity of hosts and environments from which they were isolated, these data only correspond to diseases in adult hosts; therefore, our data extend the host range of fusariosis to teleost fish eggs and gives evidence that pathogenic ability of some fungi might be not suppressed only by increasing osmotic pressure. As *Fusarium* species are commonly observed in soil and in organic matter from fresh and brackish water, it is highly probable that the isolates LPSC 1001 and 1002 are part of the aquatic mycobiota associated to culture systems of *O. bonariensis*. Environmental conditions such as the presence of suspended organic matter, the increase of temperature, and overcrowding can contribute to fungal proliferation and therefore increase the infective burden in aquatic environment, making possible the colonization of a susceptible host. However, such events could be overlooked because the infections caused by these fungi generally have moderate mortality rates and their growth on eggs is inconspicuous compared to that caused by Saprolegniales.<sup>30</sup>

Sodium chloride is widely used in aquaculture as a therapeutic chemical to control infectious diseases. Application of salt baths (1–3%) in freshwater fish and amphibians is extensively used to control and treat ectoparasites and other pathogens as *Ichthyophthirius multifiliis*, *Edwardsiella* spp., and the causative agent of saprolegniasis.<sup>47</sup> Since *O. bonariensis* is a euryhaline species able to live in habitat with up to approximately 21 g/l salinity,<sup>40</sup> the use of sodium chloride as an efficient microbicide is feasible; however, we found that *O. bonariensis* eggs exposed to a range of NaCl concentrations of 10–25 g/l were infected by two strains of the *Fusarium* genus. A decrease in the mortality by the fungal infection was observed when increasing the NaCl concentration, until the mortality observed was only due to a deleterious effect by NaCl on eggs in concentrations up 20 g/l; similar findings were reported by Pacheco Marino and Salibián.<sup>31</sup>

Many fungi isolated from extreme environments possess diverse mechanisms that allow them to counterbalance the effects of NaCl and other salts, though their growth may also be reduced when the salt concentration in the medium increases, reason why the NaCl may be applied as a fungistatic agent.<sup>3,47</sup> Deleterious effects of the increase in the salinity can have a direct response in mycelia, causing osmotic stress or acting as a toxic component interfering with membrane stability and/or enzymatic activity.<sup>1</sup> Although only a few fungi are considered as obligate halophiles,

some others may grow better in the presence of salt, thus having a competitive advantage over less salt-tolerant species.<sup>50</sup> The capacity to adapt metabolically to environments with low osmotic potentials has been observed in some members of *Fusarium* genus isolated from saline environments.<sup>32</sup> Other studies on *Fusarium* spp. showed that conidial germination in *F. solani* and *F. equiseti* was positively affected by the decrease of osmotic potential of the aqueous medium.<sup>32</sup> El-Abyad et al.<sup>8</sup> also found that the addition of salt to the media increased mycelial growth and/or conidial germination of *F. avenaceum*, *F. culmorum*, *F. diversisporum*, *F. equiseti* and *F. oxysporum* f. sp. *betae*, but reduced also the growth rate in *F. verticillioides*. In contrast to our results, typical water molds that commonly infect eggs and adults of fish such as the members of the Saprolegniaceae family are not tolerant to saline conditions.<sup>4</sup>

In order to corroborate the halotolerance of the isolates LPSC 1001 and LPSC 1002, their response to a range of 40–160 g/l NaCl was evaluated. The inhibiting NaCl concentrations for the *in vitro* growth of isolates LPSC 1001 and LPSC 1002 was 160 and 120 g/l NaCl, respectively. The mean percentage of colony diameter reduction for LPSC 1001 was 82.65% with 120 g/l of NaCl; in the case of LPSC 1002, with 80 g/l of NaCl, this value was 73.96%. Similarly *F. solani* and other species of the *Fusarium* genus, recovered from hypersaline habitats (up to 5% NaCl), exhibited an increase (up 25%) of their *in vitro* tolerance to NaCl.<sup>19</sup> This might be due, at least in part, to the availability of osmolytes synthesized by the fungus that alleviate the saline stress and, therefore, increases its osmotolerance.<sup>4</sup> *In vitro* assays performed by Palmero et al.<sup>32</sup> showed that the colony diameter of *F. equiseti* exposed to NaCl concentrations was similar to that observed in LPSC 1002, but lesser than that from LPSC1001. In higher NaCl concentrations the growth of LPSC 1002 was higher than those reported for *F. equiseti*.

In conclusion, isolates LPSC 1001 and LPSC 1002 that were recovered from infected eggs of *O. bonariensis* were identified as representatives of the genus *Fusarium* based on classical (morphological) and molecular taxonomy. In this sense, our phylogenetic analysis confirmed the morphological identification, clustering the isolate LPSC 1001 in FIESC clade and the isolate LPSC 1002 in FSSC clade, belonging to the two main groups pathogens involved in human infections, within the *Fusarium* genus, according to O'Donnell et al.<sup>28</sup> Pathogenicity assays showed that both isolates are capable to infect *O. bonariensis* eggs, while LPSC 1002 is more pathogenic than LPSC 1001. Furthermore, the response of the isolates LPSC 1001 and 1002 to NaCl, a chemical agent that could be used to control the occurrence of fungal infections, showed that a concentration of 160 and 120 g/l of NaCl inhibits *in vitro* growth of each isolate respectively. Such information is key for the prevention and control of fusariosis in fish farms, although further studies are necessary to understand the physiological mechanisms of salt tolerance in both isolates and how their pathogenicity might be affected by NaCl concentration.

#### Conflict of interest

The authors declare no conflict of interest.

## Acknowledgements

We thank the Fisheries Office of the Ministry of Production of the Buenos Aires Province for supplying test organisms. This research was partially supported by grants from Agencia Nacional de Promoción Científica y Técnica (PRH32/PICT 110) and from Comisión de Investigación Científicas de la Provincia de Buenos Aires (CICPBA). Stenglein S.A. and Saparrat M.C.N. are researchers from CONICET; Cabello M.N. and Salibián A. are researchers from CICPBA. Dinolfo M.I. and Pacheco Marino are fellows from CONICET.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.riam.2015.02.005>.

## References

- Adler L. Fungi and salt stress. In: Frankland JC, Magan N, Gadd GM, editors. *Fungi and environmental change: symposium of the British Mycological Society*. Cambridge: Cambridge University Press; 1996. p. 217–34.
- Booth C. The genus *Fusarium*. Commonwealth Mycological Institute: Kew Surrey; 1971.
- Cantrell SA, Casillas-Martínez L, Molina M. Characterization of fungi from hypersaline environments of solar salterns using morphological and molecular techniques. *Mycol Res*. 2006;110:962–70.
- Cooke RC, Whipps JM. *Ecophysiology of fungi*. London: Blackwell Scientific Publications; 2001.
- Crow GL, Brock JA, Kaiser S. *Fusarium solani* fungal infection of the lateral line canal system in captive scalloped hammerhead sharks (*Sphyrna lewini*) in Hawaii. *J Wildlife Dis* 1993;31:562–5.
- De Vienne DM, Giraud T, Martin OC. A congruence index for testing topological similarity between trees. *Bioinformatics*. 2007;23:3119–24.
- Domsch KH, Gams W, Anderson TA. *Compendium of soil fungi*, vol. 1. IHW Verlag: Germany; 1993.
- El-Abyad MS, Hindorf H, Rizk MA. Impact of salinity stress on soil-borne fungi of sugarbeet II. Growth activities in vitro. *Plant Soil*. 1988;110:33–7.
- Faisal M, Elsayed E, Fitzgerald SD, Silva V, Mendoza L. Outbreaks of phaeophomycosis in the chinook salmon (*Oncorhynchus tshawytscha*) caused by *Phoma herbarum*. *Mycopathologia*. 2007;163:41–8.
- Ferguson HW. *Systemic pathology of fish*. Iowa: State University Press; 1989.
- Gozlan RE, Marshall WL, Lilje O, Jessop CN, Gleason FH, Andreou D. Current ecological understanding of fungal-like pathogens of fish: what lies beneath? *Front Microbiol*. 2014;19:62.
- Guindon S, Gascuel O. A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. *Syst Biol*. 2003;52:696–704.
- Hatai K, Kubota S, Kida N, Ugawa S. *Fusarium oxysporum* in Red Sea Bream (*Pagrus* sp.). *J Wildlife Dis*. 1986;22:570–1.
- Hörter R. *Fusarium* als Erreger einer Hautmykose bei Karpfen. *Z Parasitenk*. 1960;40:355–8.
- Katoh K, Toh H. Recent developments in the MAFFT multiple sequence alignment program. *Brief Bioinform*. 2008;9:286–98.
- Khoa LV, Hatai K, Aoki T. *Fusarium incarnatum* isolated from black tiger shrimp, *Penaeus monodon* Fabricius, with black gill diseases culture in Vietnam. *J Fish Dis*. 2004;27:507–15.
- Kirk PH, Cannon PF, David JC, Stalpers JA. *Dictionary of the fungi*. 9th ed. Oxon: CAB International; 2001.
- Leslie JF, Summerell BA. *The Fusarium laboratory manual*. Iowa: Blackwell Publishing; 2006.
- Mandeel QA. Biodiversity of the genus *Fusarium* in saline soil habitats. *J Basic Microbiol*. 2006;46:480–94.
- Marancik DP, Berliner AL, Cavin JM, Clauss TM, Dove ADM, Sutton DA, et al. Disseminated fungal infection in two species of captive sharks. *J Zoo Wildl Med*. 2011;42:686–93.
- Neish GA, Hughes GC. *Fungal diseases of fishes*, Book 6. In: Snieszko SF, Axelrod HR, editors. *Diseases of Fishes*, Book 6. New Jersey: T.W.F. Publications; 1980.
- Nelson PE, Toussoun TA, Marasas WFO. *Fusarium* species. An illustrated manual for identification. Pennsylvania: University Press; 1983.
- Nirenberg H. Untersuchungen über die morphologische differenzierung in der *Fusarium* sektion-Liseola. *Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft Berlin-Dahlem*. 1976;169:1–117.
- O'Donnell K. Molecular phylogeny of the *Nectria haematococca-Fusarium solani* species complex. *Mycologia*. 2000;92:919–38.
- O'Donnell K, Nirenberg HI, Aoki T, Cigelnik E. A multigene phylogeny of the *Gibberella fujikuroi* species complex: detection of additional phylogenetically distinct species. *Mycoscience*. 2000;41:61–78.
- O'Donnell K, Sutton DA, Fothergill A, McCarthy D, Rinaldi MG, Brandt ME, et al. Molecular phylogenetic diversity, multilocus haplotype nomenclature, and *in vitro* antifungal resistance within the *Fusarium solani* species complex. *J Clin Microbiol*. 2008;46:2477–90.
- O'Donnell K, Sutton D, Rinaldi M, Gueidan C, Crous P, Geiser D. Novel multilocus sequence typing scheme reveals high genetic diversity of human pathogenic members of the *Fusarium incarnatum-F. equiseti* and *F. chlamydosporum* species complexes within the United States. *J Clin Microbiol*. 2009;47:3851–61.
- O'Donnell K, Sutton DA, Rinaldi MG, Sarver BA, Balajee SA, Schroers HJ, et al. Internet-accessible DNA sequence database for identifying fusaria from human and animal infections. *J Clin Microbiol*. 2010;48:3708–18.
- Ostland VE, Ferguson HW, Armstrong RD, Asselin A, Hall R. Granulomatous peritonitis in fish associated with *Fusarium solani*. *Vet Rec* 2006. 1987;121:595–6.
- Pacheco Marino SG, Steciow MM, Barbeito C. First report of saprolegniasis on eggs and a juvenile of Argentinian silverside (*Odonthestes bonariensis*). *Bull Eur Ass Fish Pathol*. 2009;29:10–5.
- Pacheco Marino SG, Salibián A. Acute toxicity of three antifungal chemicals on silverside *Odonthestes bonariensis* (Valenciennes 1835) eggs. *Int J Environ Health*. 2010;4:333–41.
- Palmero D, de Cara M, Iglesias C, Gálvez L, Tello JC. Comparative study of the pathogenicity of seabed isolates of *Fusarium equiseti* and the effect of the composition of the mineral salt medium and temperature on mycelial growth. *Braz J Microbiol*. 2011;42:948–53.
- Posada D. jModelTest: phylogenetic model averaging. *Mol Biol Evol*. 2008;25:1253–6.
- Prophet EB, Mills B, Arrington JB, Sobin LH. *Laboratory Methods in Histotechnology*. Washington: Armed Forces Institute of Pathology, American Registry of Pathology; 1992.
- Rand TG. Fungal diseases of fish and shellfish. In: Howard DH, Miller JD, editors. *The Mycota VI: a comprehensive treatise on fungi as experimental systems for basic and applied research*. Berlin: Springer-Verlag; 1996. p. 297–313.
- Refai MK, Mohamed LA, Kenawy AM, Shimaa El-SMA. The assessment of mycotic settlement of freshwater fishes in Egypt. *J Am Sci*. 2010;6:595–602.
- Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*. 2003;19:1572–4.
- Short DPG, O'Donnell K, Zhang N, Juba JH, Geiser DM. Widespread occurrence of diverse human pathogenic types of the fungus *Fusarium* detected in plumbing drains. *J Clin Microbiol*. 2011;49:4264–72.
- Smith AG, Muhvich AG, Muhvich KH, Wood C. Fatal *Fusarium solani* infections in baby sharks. *J Med Vet Mycol*. 1989;27:83–91.
- Somoza GM, Miranda LA, Berasain GE, Colautti D, RemesLenicov M, Strüssmann CA. Historical aspects, current status and prospects of pejerrey aquaculture in South America. *Aquacult Res*. 2008;39:784–93.
- Stenglein SA, Balatti PA. Genetic diversity of *Phaeoisariopsis griseola* in Argentina as revealed by pathogenic and molecular markers. *Physiol Mol Plant Pathol*. 2006;68:158–67.
- Strongman DB, Morrison CM, Mc Clelland G. Lesions in the musculature of captive American plaice *Hippoglossoides platessoides* caused by the fungus *Hormoconis resiniae* (Deuteromycetes). *Dis Aquatic Org*. 1997;28:107–13.
- Sutton DA, Fothergill AW, Rinaldi MG. *Guide to clinically significant Fungi*. Baltimore: Williams and Wilking; 1998.
- Swofford DL. PAUP and other methods. *Phylogenetic analysis using parsimony*, version 4. MA: Sinauer Associates; 1998.
- Thoen E, Evensen Ø, Skaar I. Pathogenicity of *Saprolegnia* spp. to Atlantic salmon, *Salmo salar* L., eggs. *J Fish Dis*. 2011;34:601–8.
- Toledo AV, Marino de Remes Lenicov AM, López Lastra CC. Pathogenicity of fungal isolates (Ascomycota: Hypocreales) against *Peregrinus maidis*, *Delphacodes kuscheli* (Hemiptera: Delphacidae), and *Dalbulus maidis* (Hemiptera: Cicadellidae), vectors of corn diseases. *Mycopathologia*. 2007;163:225–32.
- Treves-Brown KM. *Applied fish pharmacology*. Dordrecht: Kluwer Academic Publishers; 2001.
- Wang H, Xiao M, Kong F, Chen S, Dou H-T, Sorrell T, et al. Accurate and practical identification of 20 *Fusarium* species by seven-locus sequence analysis and reverse line blot hybridization, and an *in vitro* antifungal susceptibility study. *J Clin Microbiol*. 2011;49:1890–8.
- Watanabe M, Yonezawa T, Lee KI, Kumagai S, Sugita-Konishi Y, Goto K, et al. Molecular phylogeny of the higher and lower taxonomy of the *Fusarium* genus and differences in the evolutionary histories of multiple genes. *BMC Evol Biol*. 2011;11:322–38.
- Zak JC, Wildman HG. *Fungi in stressful environments*. In: Mueller GM, Bills GF, Foster MS, editors. *Biodiversity of Fungi*. 1st ed. Amsterdam: Elsevier; 2004. p. 303–16.