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Cryptic microsporidian parasites differentially affect invasive and native *Artemia* spp. ☆

Nicolas O. Rode *, Eva J.P. Lievens, Adeline Segard, Elodie Flaven [1], Roula Jabbour-Zahab, Thomas Lenormand

Centre d'Ecologie Fonctionnelle et Evolutive, UMR CNRS 5175, 1, 1919 route de Mende, 34293 Montpellier cedex 5, France

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ABSTRACT

We investigated the host specificity of two cryptic microsporidian species (*Anostracospora rigaudi* and *Enterocytospora artemiae*) infecting invasive (*Artemia franciscana*) and native (*Artemia parthenogenetica*) hosts in sympatry. *Anostracospora rigaudi* was on average four times more prevalent in the native host, whereas *E. artemiae* was three times more prevalent in the invasive host. Infection with *An. rigaudi* strongly reduced female reproduction in both host species, whereas infection with *E. artemiae* had weaker effects on female reproduction. We contrasted microsporidian prevalence in native *A. franciscana* populations (New World) and in both invaded and non-invaded *Artemia* populations (Old World). At a community level, microsporidian prevalence was twice as high in native compared with invasive hosts, due to the contrasting host-specificity of *An. rigaudi* and *E. artemiae*. At a higher biogeographical level, microsporidian prevalence in *A. franciscana* did not differ between the invaded populations and the native populations used for the introduction. Although *E. artemiae* was the only species found both in New and Old World populations, no evidence of its co-introduction with the invasive host was found in our experimental and phylogeographic tests. These results suggest that the success of *A. franciscana* invasion is probably due to a lower susceptibility to virulent microsporidian parasites rather than to decreased microsporidian prevalence compared with *A. parthenogenetica* or to lower microsporidian virulence in introduced areas.

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1. Introduction

Parasites have been shown to play a prominent role in determining the success and extent of biological invasions (Dunn, 2009). Studies investigating the importance of parasites in mediating invasion success usually rely on 'community' studies which contrast parasite prevalence in native and invasive species co-occurring within the same habitat, or on biogeographical studies which compare parasite prevalences in native versus introduced populations (Colautti and MacIsaac, 2004; Colautti et al., 2004). However, both approaches have rarely been conducted simultaneously using the same model system (Colautti et al., 2004; Liu and Stiling, 2006).

In recent decades, New World brine shrimps, *Artemia franciscana*, have been repeatedly introduced to Old World salterns, which has led to a severe decline in native populations of *Artemia parthenogenetica* and *Artemia salina* (Amat et al., 2005, 2007; Mura et al., 2006; Scalone and Rabet, 2013). In southern France, dormant eggs, also called cysts, were intentionally and massively introduced from 1970 to 1983 from two American populations (San Francisco Bay and The Great Salt Lake, D. Facca, personal communication). *Artemia* are infected by several parasites, among which the microsporidia form an important group (Ovcharenko and Wita, 2005). Microsporidian parasites can have a strong impact on host fitness in natural populations (e.g. Stirnadel and Ebert, 1997; Otti and Schmid-Hempel, 2008; Ryan and Kohler, 2010). In addition, they are relatively host-specific compared with other parasites (Solter and Maddox, 1998; Shaw et al., 2000; Ebert, 2005; Saito and Bjornson, 2008). Hence, they are likely to affect the fitness of invasive and native hosts differentially and to play a role in the outcome of host competition. Surprisingly, only a handful of studies have investigated the potential role of microsporidia in host invasions (Slothouber Galbreath et al., 2004, 2010; Wattier et al., 2007; Yang et al., 2010). More generally, little is known about the importance of microsporidia in mediating invasion success.

The goal of the current study is to examine the impact of two gut microsporidian parasites, *Anostracospora rigaudi* and

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* Corresponding author. Tel.: +33 467 613 259; fax: +33 467 613 336.
E-mail address: nicolas.rode@ens-lyon.org (N.O. Rode).
[1] Current address: Institut des Sciences de l'Evolution (UM2-CNRS), Université Montpellier 2, Montpellier, France.

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Enterocytospora artemiae, infecting native and invasive *Artemia* hosts in sympatry (Rode et al., 2013). These two cryptic parasites are both difficult to detect (spore size ~1 μm) and difficult to distinguish from other small gut microorganisms (e.g. bacteria, yeast, unicellular algae), which makes them difficult to identify without molecular methods (Rode et al., 2013). Using field, phylogeographic and experimental approaches, we (i) investigated microsporidian host-specificity by comparing prevalence in the invasive *A. franciscana* and in the native *A. parthenogenetica*, (ii) quantified the impact of microsporidian parasites on the reproduction of sympatric females, (iii) compared microsporidian prevalence at the community and biogeographical scales and (iv) tested for the potential introduction of a microsporidian parasite with the invasive host.

2. Materials and methods

2.1. Anostracospora rigaudi and E. artemiae prevalence, phenotypic effects and effect on brooding probability of sympatric females from Aigues-Mortes, France

Juvenile and adult *Artemia* were sampled with a plankton net in three shallow salterns (depth <30 cm) in Aigues-Mortes (France) in May 2011. Two samples were taken from Site 1 and Site 2, whereas one sample was taken from Site 3 (Table 1). Samples were kept in 10 L tanks (salinity 50 g/L, Thalasea, Camargue-Pêche, Grau du Roi, France) and fed ad libitum with an algal solution (*Dunaliella tertiolecta*). Individuals were randomly selected from each sample and their total length was measured to the nearest 0.05 mm. Sex, species, the number of infecting cestodes (mostly *Flamingolepis liguloides*) and the reproductive status of females (empty brood pouch versus presence of ovules/embryos) were observed using a binocular microscope. *Artemia parthenogenetica* females with atrophied or absent brood pouch due to *F. liguloides* infection were considered to be mature and non-reproducing when they measured more than 0.7 mm (which was 0.1 mm longer than the smallest reproducing female). The sample size of *A. parthenogenetica* was increased in order to obtain a higher number of *A. parthenogenetica* females not infected with *F. liguloides*, and thus increase our statistical power to independently disentangle the effects of single infections versus co-infections by several parasites on female brooding probability. The final sample size was 243 *A. franciscana* and 987 *A. parthenogenetica* individuals. Upon observation, individuals were killed and placed in ethanol in 96-well plates and PCR was performed using species-specific microsporidian primers (Msp1p2f/Msp1p3r and Msp2p1f/Msp2p3r, see Rode et al., 2013 for the complete protocol). Microsporidian infection was judged based on the presence of bands on electrophoretic gels. We investigated the differential prevalence of *An. rigaudi* and *E. artemiae* in *A. franciscana* and *A. parthenogenetica* from Aigues-Mortes using χ^2 tests for independence ('stats' package in R 2.14.2).

Analysis of female brooding probability was performed using a generalised linear model with a Bernouilli error distribution ('stats' package in R 2.14.2). Models included host species, presence of *An. rigaudi*, presence of *E. artemiae*, presence of *F. liguloides*, sample identity (five in total), length, the square of length, the interaction between the three parasite species together with each interaction between length and each of the other effects tested. In addition, we tested for differential phenotypic effects on invasive and native species by including an interaction between host species and the presence of *An. rigaudi* or *E. artemiae*.

The effects of length (as a proxy for age) on the probabilities of infection by *An. rigaudi* and *E. artemiae* were analysed similarly. Models included host species, length and their interaction. All model selections were based on the corrected Akaike's information criterion (AICc), which was computed independently for the different models (Hurvich and Tsai, 1989). We discuss the effects of the best models (i.e. all models whose AICc differs by less than two from the lowest AICc; Burnham and Anderson, 2002).

2.2. Microsporidian prevalence, diversity and polymorphism in New and Old World populations

Average microsporidian prevalence is usually low among *Artemia* populations, but generally high within infected populations (Codreanu, 1957; Martinez et al., 1992), a pattern commonly found in microsporidian parasites infecting other crustacean hosts (e.g. Ebert et al., 2001; Terry et al., 2004). To accurately estimate microsporidian prevalence despite this large between-population variation, we sampled a few individuals at a large number of sites.

In order to investigate differential microsporidian prevalence between *A. franciscana*-invaded and native populations, we examined the presence of microsporidian parasites in 27 invaded and eight native salterns ($n = 437$ and $n = 70$, respectively; Tables 1 and 2). To test for parasite spillback in invaded populations and for *E. artemiae* introduction along with *A. franciscana* (see below and Section 2.3), microsporidian parasite prevalence was investigated in *A. parthenogenetica* and *A. salina* from eight invaded and nine non-invaded salterns from northern Africa, Europe and central Asia ($n = 1040$ and 67, respectively, Tables 1 and 2). All individuals were killed and preserved in 96% ethanol. DNA from each individual was screened for microsporidian infection using V1f/530r primers and *An. rigaudi* and *E. artemiae* species-specific primer sets (*A. salina* DNA quality was checked with the COI_Fol-F/COI_Fol-R primers; see Muñoz et al., 2008; Rode et al., 2013 for complete protocols). Importantly, such universal primers might fail to detect microsporidian species that have accumulated mutations in one of the primer sites. However, we did not expect any bias in the geographical distribution of these potentially undetected species, so our results should not be biased. Sample sizes were lower in non-French samples, resulting in a less accurate estimation of microsporidian prevalence within each population (Tables 1 and 2). We used χ^2 tests that account for the actual number of individuals sampled to compare microsporidian prevalence either between *A. franciscana* native and invaded populations or between *A. parthenogenetica* populations invaded by *A. franciscana* and non-invaded *A. parthenogenetica* populations. Hence, our results should be robust despite the low size of some population samples.

To investigate the phylogeography of microsporidian parasites, we sequenced eight and 12 positive V1f/530r-PCR products from Old and New World populations, respectively, on an ABI prism 3130xl genetic analyser (Applied Biosystems, Foster City, California, USA) with an ABI Prism Big Dye Terminator cycle sequencing kit. Sequences were aligned using the ClustalW algorithm in BioEdit v7.0.9 (Hall, 2001, North Carolina State University, Raleigh, North Carolina, USA). A BLAST database search in GenBank was used to identify and select the closest matches together with already characterised clade-specific microsporidian sequences (Supplementary Table S1, Vossbrinck and Debrunner-Vossbrinck, 2005; Wang et al., 2005). Phylogenetic analyses were performed only on those portions of the sequences that could be unambiguously aligned. The selection of the best model for the base frequencies and substitution rates was based on AICc calculated using jModelTest 0.1, (TPM3 model with a gamma variation rate among sites; Posada and Crandall, 1998; Posada, 2008). Maximum likelihood analyses were carried out using Phyml v3.0 (Guindon et al., 2010) and robustness of nodes was assessed with 100 bootstrap replications. Representative sequences of newly characterised lineages (*Microsporidium* sp. 3, *Microsporidium* sp. 4) have been deposited in GenBank (Accession Nos.: JX839890, JX839891).