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Few parasites, and no evidence for *Wolbachia* infections, in a freshwater ostracod inhabiting temporary ponds

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Biological systems with asexual reproduction have often attracted research on parasites and host immune defence, because parasites are expected to be better able to exploit genetically less diverse populations. In addition, maternally inherited parasitic microorganisms such as *Wolbachia* can directly alter the reproductive systems of their hosts and induce parthenogenesis. In the freshwater ostracod *Eucypris virens*, both sexual and asexual reproduction is known, and we speculated that parasite pressures might help to explain their co-existence. This species complex inhabits shallow, often eutrophic temporary water bodies, conditions that should provide ample opportunities for parasite infections. We surveyed natural populations of *E. virens* throughout its Europe-wide range for natural parasites, and particularly tested for the presence of intracellular *Wolbachia* bacteria. Surprisingly, the results indicate that very few *E. virens* populations support parasite infections. We also found no evidence for the presence of *Wolbachia* in the populations screened. The results therefore show that parasitic infections do not play a role in the maintenance of sex in this system. *Biological Journal of the Linnean Society*, 2010, ••, ••–••.

ADDITIONAL KEYWORDS: alphaproteobacteria – Cyprididae – maintenance of sex – microcrustaceans – parthenogenesis – Red Queen – temporary pools.

INTRODUCTION

Parasites are potentially important in explaining the maintenance of sexual and asexual reproduction, and perhaps also the pattern of geographic parthenogenesis (Vandel, 1928). Co-evolutionary dynamics between hosts and their parasites are expected to facilitate the maintenance of sexual populations in areas that might otherwise be occupied by asexual lineages, and/or have large impacts on the genetic

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structure and clonal diversity of asexual populations (the 'Red Queen' mechanism; Hamilton, 1980; Lively *et al*., 2004; Jokela, Dybdahl & Lively, 2009).

Surprisingly, very few studies so far have specifically focused on parasites in freshwater ostracods (Crustacea, Ostracoda), even though this animal group shows frequent transitions between reproductive modes (Bell, 1982; Butlin, Schön & Martens, 1998). *Eucypris virens* (Jurine, 1820) is a freshwater ostracod typically found in temporary ponds. It is a classical example of geographic parthenogenesis: in most of Central and Northern Europe, asexual (female-only) populations occur, whereas around the Mediterranean, sexual as well as mixed (consisting of both sexual and asexual females) populations can be found (Horne, Baltanás & Paris, 1998). Different sexual and mixed populations are characterized by different sex ratios, which can range from 1 : 1 to only a very few males being present (Vandekerkhove *et al*., 2007). This system has been addressed in a range of studies to elucidate patterns of distribution, interactions between the reproductive modes, and molecular evolution of clonal lineages (Butlin *et al*., 1998; Schön *et al*., 2000, 2003; Martins, Vandekerkhove & Namiotko, 2008; Adolfsson *et al*., 2010; Bode *et al*., 2010; Martins *et al*., 2010). Here, we report the results of a screen for parasites across the European range of the species.

Apart from the search for any fitness-reducing parasites, in host systems with alterations in reproductive modes there are good reasons for a special focus on infections by intracellular parasites such as *Wolbachia* (Werren, Baldo & Clark, 2008). *Wolbachia* are alphaproteobacteria, estimated to infect around 66% of species of arthropods (Hilgenboecker *et al*., 2008). Being mainly transmitted via the female germline, *Wolbachia* can maximize their own fitness by favouring reproduction of infected females. In arthropods, they are associated with cytoplasmic incompatibility, the induction of parthenogenesis, the killing of male hosts, and the feminization of genetic males (reviewed in Werren *et al*., 2008). Among other animal groups, *Wolbachia* are common in filarial nematodes, where they have most often evolved into mutualistic symbionts (Fenn & Blaxter, 2004).

In contrast to terrestrial arthropods, aquatic crustaceans seem to be less frequently involved as *Wolbachia* hosts (e.g. Bouchon, Rigaud & Juchault, 1998; Fitzsimmons & Innes, 2005). The lack of published studies on *Wolbachia* in aquatic crustaceans might have arisen because: (1) there have been very few attempts to find *Wolbachia* in this group; or (2) there has been a failure to report negative results. Interestingly, Baltanás *et al*. (2007) recently reported the presence of *Wolbachia* in three European non-marine ostracods, including two populations of *E. virens* from Spain. In their study, a polymerase chain reaction (PCR)-based approach was chosen, using *Wolbachia 16S* ribosomal DNA (rDNA) primers.

In our current study, we first present our findings following a general screen for potentially harmful parasites in *E. virens*. The results indicate very few parasite infections in natural populations. We followed this up by molecular-based tests for the presence of *Wolbachia*: here, four standard *Wolbachia*-specific PCR primer pairs were tested on 34 populations of *E. virens* in its European range. We found no evidence for *Wolbachia* infections in the *E. virens* populations examined.

MATERIAL AND METHODS

General parasite screening

In total, 534 *E. virens* were sampled from 11 sites (range, 20–90 individuals per site), representing both putative asexual females from sites without males, and females and males from sites with males present, which indicates at least partial sexual reproduction (Table 1). Following transport to the laboratory, specimens were kept alive in culture for a maximum of 7 days before screening. The ostracods were thoroughly dissected and searched for parasitic infections (e.g. cestodes, trematodes, and cysts) by removing both valves, examining the valves, soft tissues, and water, and finally by examining tissue smear preparations. Samples were analysed under a Leica MZ16 stereomicroscope equipped with a transmitted light attachment to enhance contrast in unstained samples. The maximum total magnification used was 230×. Similar screening procedures have previously been successfully applied in other taxa, for example in freshwater snails (Jokela & Lively, 1995) and amphipod crustaceans (Moret *et al*., 2007).

After initial screening and finding one infected population, specific screening for the presence of this newly encountered parasite was performed on an additional 1286 *E. virens* from two sites included in the general survey, and from ten additional sites (range, 7–280 individuals per site; Table 1). Here, the aforementioned procedure was followed, but without analysis of smear preparations. Using this approach, any other prominent parasites would also have been detected.

Wolbachia PCR screening

Wolbachia were screened for in DNA samples from 34 different populations of *E. virens*, as well as in two populations of other freshwater ostracods from the same family, namely *Heterocypris incongruens* (Ramdohr, 1808) and *Eucypris pigra* (Fischer, 1851) (Cyprididae). The *E. virens* populations tested were

Table 1. Name, location and male absence/presence for *Eucypris virens* sites screened for parasites

Country, region	Name	GPS (Longitude E°/Latitude N°)	Males	ES	SS
France, Languedoc Roussillon	FIO	2.9908/42.9242	No	–	250
France, Languedoc Roussillon	FI6	2.9417/42.8866	Yes	–	250
France, Languedoc Roussillon	ROU	3.844/43.8131	No	–	225
France, Provence	ARL	4.8515/43.4806	No	–	50
France, Provence	AR2	4.8515/43.4806	No	–	8
France, Provence	AR3	4.8515/43.4806	No	–	7
France, Provence	CAM	4.6747/43.4921	No	–	280
France, Provence	CA2	4.6747/43.4940	No	–	50
Greece, Corfu	COB	19.7856/39.6978	Yes	40	–
Malta, Ghallis	GHI	14.4436/35.9514	No	–	40
Malta, Mosta	MO2	14.4325/35.9172	No	30	–
Poland, Kartuzy	STA	18.1772/54.2272	No	–	8
Poland, Gdańsk	JAB	20.7839/53.0208	No	26	67
Spain, Castilla La Mancha	MF3	–6.0634/39.9108	Yes	20	–
Spain, Castilla La Mancha	CC5	–4.0644/38.8244	Yes	74	–
Spain, Castilla La Mancha	MFZ	–6.0605/39.9061	Yes	23	–
Spain, Castilla La Mancha	CL1	–4.0646/38.8224	No	41	–
Spain, Valencia	VA3	–0.3083/39.3208	No	90	–
Spain, Valencia	MA3	–0.3050/39.3243	No	46	–
UK, Yorkshire	RIF	–1.6214/53.9136	No	31 + 48	–
UK, Yorkshire	NEW	–2.1344/54.0150	No	65	51

ES, number of individuals extensively screened; SS, number of specimens on which specific screening for parasite found in MO2 was performed. Specimens from RIF were collected twice approximately 6 weeks apart.

represented by both genders and reproductive modes, and covered a wide range of geographic localities (Table 2).

Ostracod DNA was extracted with the DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A quality check of DNA extractions in *E. virens* was conducted using specific primers for *E. virens* mitochondrial DNA (mtDNA) *cytochrome oxidase I* (*COI*) and/or *16S* loci (following the method described in Bode *et al*., 2010). Three different *Wolbachia* loci were targeted in PCR-based tests of infection status: *Wolbachia* cell surface protein (*wsp*; Braig *et al*., 1998), *FtsZ* (a protein involved in bacterial cell division; Holden, Brookfield & Jones, 1993), and *16S* rDNA (the small ribosomal subunit). For the 16S rDNA locus, we used three different primer sets: *16SWolbF*/*16SWolbR3* by Casiraghi *et al*. (2001) [here referred to as '*16SWolb*(*1*)'], as well as primers according to Van Borm *et al*. (2001), where a general forward primer, *16SWolbF1* is used in combination with either *16SWolbRA* or *16SWolbRB*, to target *Wolbachia* subgroups A and B [here, we refer to these sets as '*16SWolb*(*2A*)' and '*16SWolb*(*2B*)']. All primer pairs have been used in previous studies for detecting a wide range of *Wolbachia* strains (e.g. Casiraghi *et al*., 2001; Cordaux, Michel-Salzat & Bouchon, 2001; Van Borm *et al*., 2001; Baltanás *et al*., 2007). PCR conditions were the same as described previously (Werren, Zhang & Guo, 1995; Braig *et al*., 1998; Casiraghi *et al*., 2001; Baltanás *et al*., 2007). From a total of 40 samples, 39 were tested with *wsp*, 39 with *FtsZ*, and 34 with *16SWolb*(*1*), whereas a smaller subset was additionally tested with *16SWolb*(*2A*) (31 samples) and (*2B*) (18 samples) (Table 2). As positive controls, we used DNA from *Wolbachia*-infected fig wasps *Pleistodontes imperialis* Saunders, 1882 (Hymenoptera: Agaonidae), *Ephestia kuehniella* Zeller, 1879 (Lepidoptera: Pyralidae) moth larvae, and/or bed bugs *Cimex lectularius* L. (Hemiptera: Cimicidae). A 5-µl volume of PCR products was electrophoresed on ethidium bromide (EtBr)-stained agarose gels, along with a size standard (100 bp and/or 1 kb, GeneRuler).

The PCR products obtained by *wsp* 81F/*wsp* 691R primers were cloned with pGem T Easy Vector System (Promega, Madison, WI, USA) and transformed into chemically competent DH5α cells (Invitrogen, Carlsbad, CA, USA). Insert sequencing was performed with BigDye Terminator v3.1 Cycle Sequencing Kit and analysed on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). For *16SWolb*(*2A*) the initial steps of the procedure

Table 2. Summary of PCR-screening for the presence of *Wolbachia*

Country, region	Name	GPS (Longitude E°/Latitude N°)	Gender	Males	DNA pooled	*COI/16S* ostr.	Results				
							wsp	*Ftsz*	*16Swolb(1)*	*16Swolb(2A)*	*16Swolb(2B)*
Germany, Gauting	*Epig*	11.4758/48.1597	F	N.a.	2	–	0	0	0	–	–
Germany, Gauting	*Hei*	11.4758/48.1597	F	N.a.	2	–	0	0	0	–	–
Belgium, Flanders	DR1	3.6653/51.0449	F	No	43	1	> 1 kb	0	0	Band	Unspec.
Croatia, North Adriatic	KRK	14.5944/45.0306	F	No	3	1	0	0	–	0	0
Croatia, Mid–Adriatic	OMI	16.6958/43.4508	F	No	2	1	0	0	0	0	0
Croatia, South Adriatic	MET	17.6331/43.0244	F	No	2	1	0	0	0	0	–
France, Corsica	COU	9.4480/42.0609	F	?	2	1	0	0	0	0	0
France, Languedoc Roussillon	DUR	3.9867/43.9908	F	No	3	1	0	0	0	0	0
France, Languedoc Roussillon	GAB	3.0689/43.0689	F	?	2	1	0	0	–	0	–
France, Normandy	NOR	0.1205/49.1897	F	No	3	1	0	0	0	–	–
Greece, Corfu	COA	19.7856/39.6978	F	yes	1	0/1	0	0	0	–	–
Greece, Corfu	COB	19.7856/39.6978	M	Yes	3	0/1	0	0	0	0	–
Greece, Corfu	COB	19.7856/39.6978	F	yes	1	1	0	0	0	–	–
Greece, Corfu	COD	19.7986/39.6256	F	Yes	1	1	0	0	0	0	0
Italy, Emilia Romagna	VDS	10.2381/44.6253	F	No	4	1	> 1 kb	0	0	Unspec.	–
Italy, Emilia Romagna	RVL	10.139/44.819	F	No	3	1	0	0	0	–	–
Italy, Emilia Romagna	MFR	10.232/44.635	F	No	1	1	> 1 kb	0	0	–	–
Italy, Sicily	URI	14.381/37.891	F	Yes	3	1	0	0	0	Unspec.	–
Italy, Sicily	URI	14.381/37.891	M	Yes	3	1	> 1 kb	0	0	Unspec.	–
Italy, Sardegna	SA2	8.8199/39.8508	F	No	1	1	0	0	0	0	0
Italy, Parma	COL	10.2139/44.9492	F	No	1	1	> 1 kb	0	0	Unspec.	–
Italy, Toscana	PIS	10.330/43.731	F	No	1	1	> 1 kb	0	0	–	–
Latvia, Gulbene	GUL	26.895/57.1906	F	No	1	1	0	0	0	0	0
Morocco, Chemaia	ML1	–8.3764/31.9794	F	Yes	2	1	0	0	0	0	–
Poland, Gdańsk	JAB	20.7839/53.0208	F	No	2	1	0	0	0	0	0
Poland, Chojnowo	CHO	21.2281/52.6558	F	No	1	1	–	–	–	0	0
Portugal, Alto Alentejo	VEN	–7.3667/38.4647	F	No	1	1	0	0	0	0	0
Portugal, Alto Alentejo	MAN	–7.7336/38.4436	F	No	2	1	0	0	0	0	–
Spain, Castilla La Mancha	CC9	–4.0644/38.8244	F	Yes	2	1	0	0	0	0	0
Spain, Castilla La Mancha	CAR	–4.0648/39.8224	F	Yes	1	1	0	0	–	0	0
Spain, Castilla La Mancha	ALF	–4.1531/38.7375	F	No	4	1	> 1 kb	0	0	0	–
Spain, Extremadura	MF6	–6.0689/39.9247	F	Yes	1	1	0	0	0	0	0
Spain, Extremadura	MFZ	–6.060/39.906	F	Yes	4	1	> 1 kb	0	0	Band	–
Spain, Extremadura	MFZ	–6.060/39.906	M	Yes	3	1	> 1 kb	0	0	Unspec.	–
Spain, Valencia	RF4	–0.3447/39.295	F	No	1	1	0	0	0	Unspec.	–
Spain, Valencia	VA4	–0.3108/39.3433	F	No	1	1	0	0	–	–	–
Tunisia, Raoued	TUN	10.2303/36.9544	F	No	4	0/1	0	0	–	0	0
Turkey, Northcentral	KAS	35.7239/40.9917	F	No	4	1	0	0	0	0	0
UK, Yorkshire	NEW	–2.1344/54.0150	F	No	3	1	0	0	0	0	Unspec.

All samples are *Eucypris virens*, except those coded *Epig* (= *Eucypris pigra*) and *Hei* (= *Heterocypris incongruens*). Males, presence/absence of males; DNA pooled, number of individuals and/or DNA samples pooled for PCR tests; *COI/16S* ostr., amplification of *E. virens* mtDNA *COI* or *16S* as control (results for amplification of *E. virens 16S* ostracod locus are reported only for cases where *COI* amplification was not performed, or failed). In columns with results for specific primer pairs: –, test not done' 0, no product; Band, product of expected size; > 1 kb, large product in *wsp* PCR; Unspec., presence of multiple weak bands, indicating unspecific amplification. Primers for *Wolbachia 16S* RNA labelled as (*1*) are according to Casiraghi *et al*. (2001), whereas (*2A*) and (*2B*) are primers according to Van Borm *et al*. (2001).

were the same, but sequencing was performed by Macrogen (Seoul, Korea). Sequences were aligned with CodonCode Aligner (Dedham, MA, USA), and compared with the database using NCBI BlastP search (Altschul *et al*., 1990).

RESULTS

General parasite screening

Of the first 534 individuals tested, only one type of parasite was found, present exclusively in one asexual population from Malta (MO2). According to its morphology, this parasite was preliminarily identified as a microsporidian (Y. Qiu & J. Smith, University of Leeds, pers. comm.). The initial prevalence of infection was 0.167 ($N = 30$, see Table 1). The remaining specimens from the same population were kept alive in culture and a 100% infection rate was observed after 7 days. However, repeated attempts to horizontally infect *E. virens* from other populations failed. We did not find the same or similar infection in any of the other populations screened.

Wolbachia PCR screening

Table 2 provides an overview of the PCR screening for the presence of *Wolbachia*. In all PCRs, positive controls amplified well, showing clear bands for the expected product size. In contrast, reactions using ostracod DNA as the template generally failed to amplify, with certain exceptions. The first exception was found using the *wsp* primer pair, which yielded PCR products in nine *E. virens* samples. However, these bands were much larger (> 1 kb) than the expected 500–600 bp of a *wsp* product found in the positive controls. Sequencing of these large products gave BLAST matches that showed no similarity to *Wolbachia* sequences (data not shown; GenBank accession nos HM032906–HM032913).

Secondly, using the primer pair *16SWolb*(*2A*) also resulted in bands on agarose gels in eight of the ostracod samples tested; however, only in two of them (DR1 and MFZ-Fem) were the signals clear and of approximately the correct product size. Sequencing of these products also did not confirm the presence of *Wolbachia*: the BLAST database search indicated only very weak similarities to sequences that were neither related to crustaceans nor to bacteria (GenBank accession nos HM032899–HM032905). Finally, tests with *16SWolb*(*2B*) primers only resulted in non-specific amplification in two of the samples.

DISCUSSION

The parasite hypothesis for the maintenance of sexual reproduction predicts that asexuals thrive in parasite-free habitats (Lively & Jokela, 2002). In *E. virens*, sexuals are rare and many asexual populations have a relatively high genetic diversity (Rossi *et al*., 2008; Adolfsson *et al*., 2010). Under these conditions, asexuals, unburdened with the cost of sex, are expected to outcompete the sexuals. However, parasites are expected to be everywhere, and hence asexuals should also be targets for parasite adaptation. Could the key to the success of asexuality in *E. virens* lie in their ability to avoid parasites?

Despite the wide geographic range of the *E. virens* samples covered in this study, a surprisingly low prevalence of parasites was found compared with the infections previously described in other pond-inhabiting taxa, including host species that often coexist with *E. virens* (Green, 1974; Thomas *et al*., 1997). These results, clearly showing the rarity of infections in *E. virens*, therefore call for more systematic parasite screening in other ostracod species to determine if such a phenomenon is widespread, and for studies focusing on characteristics of ostracod habitats that might promote low infection rates.

We were expecting to find parasites with complex life cycles, which might use the ostracod as an intermediate host, and where vertebrates or arthropods serve as the final host. Such parasites are otherwise common in freshwater invertebrates like copepods, isopods, amphipods, and snails. Some earlier studies have also presented evidence for freshwater ostracods being intermediate hosts for cestodes (Grytner-Zięcina, 1995; Haukos & Neaville, 2003), acanthocephalans (Dezfuli, 1996), trematodes (Zelmer & Esch, 1998a, b), and nematodes (Moravec, Nagasawa & Miyakawa, 2005). That such infections were not present in our survey suggests either a lack of final hosts, or some other, as yet unknown, characteristics of *E. virens* that enable them to evade such parasites (see below).

Interestingly, only one ostracod population was found to be infected, and the parasite in question was probably a microsporidian. The intensity of infection suggests a rather strong selective pressure on this Maltese *E. virens* population. However, the attempts to transmit it to other populations failed, which indicates local adaptation, but might also result from the need for an additional host species to complete the life cycle. Microsporidian infections are otherwise common in freshwater crustaceans (Terry *et al*., 2004), and have also been reported in other species of ostracods (Bronnvall & Larsson, 1995). Vertically transmitted microsporidians are often responsible for feminization and sex-ratio distortion (e.g. Haine, Motreuil & Rigaud, 2007; Mautner *et al*., 2007). Whether or not such a mechanism operates in *E. virens* is unclear, although it is worth noting that if parasitic sex-ratio distorters were present, and

generally relevant in *E. virens*, then such infections should have been observed in many more populations.

Of course, our results do not necessarily imply that *E. virens* are never infected by any other parasites. Unpublished observations of parasites, symbionts, and epibionts of *E. virens* are common: for example ciliates, diatoms, and rotifers have been observed either on the valve surface or inside the body cavity (F. Mezquita, R. Symonova, D. J. Horne, M. J. F. Martins & R. Bruvo, pers. observ.; see also Fernandez-Leborans & Tato-Porto 2000). Even without direct parasitism, epibionts may be harmful, for instance if they occur in large numbers and impede host movement (Griffiths & Evans, 1994). In our screening we did not encounter any ostracod population with naturally occurring high numbers of epibionts, so that their negative effects are probably low in natural populations.

Low infection rates in *E. virens* could be the result of efficient immune systems or ecological and environmental factors that reduce the likelihood of parasite transmission. As at this point there is little information on ostracod immunity, or on the general parasitism risk in temporary ponds, these factors remain to be investigated experimentally.

We suspect that the temporal and spatial dynamics of the system might make it more difficult for parasites. According to the model by Ladle, Johnstone & Judson (1993), asexuals temporarily escape from parasites, provided that their dispersal within a metapopulation is higher than the parasite mobility. This is explained by the inability of parasites to locally adapt to highly dispersing host populations (Gandon *et al*., 1998). *Eucypris virens* inhabit small temporary ponds that undergo strong seasonal fluctuations, and the local populations often become extinct. As an adaptation to life in such environments, *E. virens* produce diapausing eggs, which can undergo long-distance transport by wind. The model by Ladle *et al*. (1993) requires that the host migration occur in an essentially parasite-free phase: that is, wind-transported ostracod eggs should be uninfected. In addition, sexual populations are expected to have more parasites because of being more persistent in terms of habitat stability. As only a few sexual populations were screened, this last prediction cannot be directly confirmed. In fact, because habitat types are not necessarily different for sexuals and asexuals, and are influenced by many factors, including hydroperiod length, predation pressure, and intra- and interspecific competition, it is difficult to predict the role of parasites in this context. We suggest that metapopulation dynamics and environmental fluctuations may partially explain the observed low frequency of parasites in *E. virens*. Even so, it remains unclear whether the 'spatial escape from parasites' theory also plays a role in the dynamics of the reproductive modes in this system.

Overall, the *Wolbachia* PCR primers tested on ostracod DNA yielded only products unrelated to *Wolbachia* sequences. Therefore, we conclude that intact *Wolbachia* are either not present at all, or, less likely, are present at undetectable levels.

One possible explanation for such PCR amplification failure is that the parasite DNA, even if present, was of too low quantity or quality. This is unlikely to be the case, however, especially because *E. virens* are approximately of the same size or even larger than many *Wolbachia*-infected insects. DNA was extracted using the same methods from ostracods and positive controls. The parasites, if present, are most abundant in reproductive tissue, which takes up a considerable proportion of ostracod body volume. Therefore, if ostracods were infected, their DNA samples should have contained sufficient quantities of parasite DNA. Furthermore, the DNA extractions were shown to be of good quality, as they amplified well in PCRs with ostracod *COI* or *16S* primers.

Although our present survey covered a wide geographical range of European *E. virens* populations, none of which seemingly hosted *Wolbachia*, this does not exclude the possibility that some other populations do harbour infections. For instance, Baltanás *et al*. (2007) reported the positive amplification of the *Wolbachia 16S* rDNA locus in *E. virens* samples from Valdecarpinteros (Salamanca) and La Berzosa (Madrid), two localities not screened by us. From among our seven Spanish sampling sites, Extremadura is the closest to Salamanca (~180 km distance), and Caracuel is at a similar distance from Madrid. Such distances imply isolation between localities, which could explain the discrepancy between studies. Nevertheless, it remains puzzling that so many other samples were apparently free of *Wolbachia*. In light of this, it would also be instructive to test the *E. virens* samples from Valdecarpinteros and La Berzosa with different primer pairs, targeting other *Wolbachia* loci.

Interestingly, *Wolbachia* are almost exclusively found in only two host types: terrestrial arthropods and filarial nematodes. One reason for this might be that cross-species horizontal transfer mostly occurs between closely related hosts (Jiggins *et al*., 2002; Werren *et al*., 2008). Apart from the study on ostracods by Baltanás *et al*. (2007), the only other aquatic crustaceans previously found to harbour *Wolbachia* are six species of isopods and amphipods (Bouchon *et al*., 1998; Cordaux, Michel-Salzat & Bouchon, 2001). In addition, previous studies suggest an absence of *Wolbachia* in other freshwater organisms such as molluscs (Schilthuizen & Gittenberger, 1998) and *Daphnia* (Cladocera) (Fitzsimmons & Innes, 2005). That *Wolbachia* seems to be generally less

successful in aquatic hosts could result from there being fewer opportunities for transmission between terrestrial and aquatic environments, for instance, or from specific physiological adaptations to terrestrial systems.

Based on our current results we conclude that the evolutionary dynamics of the reproductive types in *E. virens* are not governed by either *Wolbachia* or any common (extracellular) parasite infection. Consequently, the maintenance of (rare) *E. virens* sexual populations in competition with asexuals is most likely to be the result of factors unrelated to Red Queen dynamics. On the other hand, the very maintenance and widespread persistence of *E. virens* asexuals might be a lucky side-effect of not having to encounter and combat diseases.

Nevertheless, it remains possible that differently targeted screening for other parasites might reveal different patterns. For instance, several recent studies, including our preliminary finding of one microsporidian infection, highlight the importance of testing for other intracellular microorganisms like *Cardinium*, *Spiroplasma*, and microsporidia, which may also manipulate arthropod reproductive systems (Gotoh, Noda & Ito, 2007; Duron *et al*., 2008). Future research in these directions might well reveal interesting new findings for ostracods (R. Symonova, unpubl. data).

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