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The incidence and diversity of *Wolbachia* in gallwasps (Hymenoptera; Cynipidae) on oak

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Abstract

***Wolbachia* bacteria infect ≈ 20% of all insect species, and cause a range of alterations to host reproduction, including imposition of thelytoky. The incidence and phenotypic impact of *Wolbachia* remains to be established in many insect taxa, and considerable research effort is currently focused on its association with particular reproductive modes and the relative importance of the various pathways via which infection occurs. Gallwasps represent an attractive system for addressing these issues for two reasons. First, they show a diversity of reproductive modes (including arrhenotoky, thelytoky and cyclical parthenogenesis) in which the impact of *Wolbachia* infection can be examined. Second, they occupy two intimately linked trophic niches (gall-inducers and inquilines) between which there is potential for the horizontal exchange of *Wolbachia* infection. In the arrhenotokous gallwasp lineages screened to date (the herb-galling 'Aylacini' and the rose-galling Diplolepidini), *Wolbachia* infection always induces thelytoky. The impact of *Wolbachia* in other arrhenotokous clades, and in the cyclically parthenogenetic clades remains unknown. Here we use polymerase chain reaction (PCR) screening and sequence data for two *Wolbachia* genes (*wsp* and *ftsZ*) to examine the prevalence and incidence of *Wolbachia* infection in 64 species (a total of 609 individuals) in two further tribes: the arrhenotokous inquilines (tribe Synergini), and the cyclically parthenogenetic oak gallwasps (tribe Cynipini). We ask: (i) whether *Wolbachia* infection has any apparent impact on host reproduction in the two tribes and (ii) whether there is any correlation between *Wolbachia* infection and the apparent lack of an arrhenotokous generation in many oak gallwasp life cycles. We show: (i) that *Wolbachia* infection is rare in the Cynipini. Infected species show no deviation from cyclical parthenogenesis, and infection is no more common in species known only from a thelytokous generation; (ii) that there is a higher incidence of infection within the arrhenotokous inquilines, and generally in gallwasp tribes without cyclical parthenogensis; (iii) all *Wolbachia*-positive inquiline species are known to possess males, implying either that *Wolbachia* infection does not result in loss of sex in this tribe or, more probably, that (as for some rose gallwasps) *Wolbachia* infection leads to loss of sex in specific populations; and (iv) although we find some inquilines and gall inducers to be infected with *Wolbachia* having the same *wsp* sequence, these hosts are not members of the same gall communities, arguing against frequent horizontal transmission between these two trophic groups. We suggest that exchange may be mediated by the generalist parasitoids common in oak galls.**

Keywords: *ftsZ*, gallwasp, horizontal transmission, thelytoky, *Wolbachia*, *wsp*

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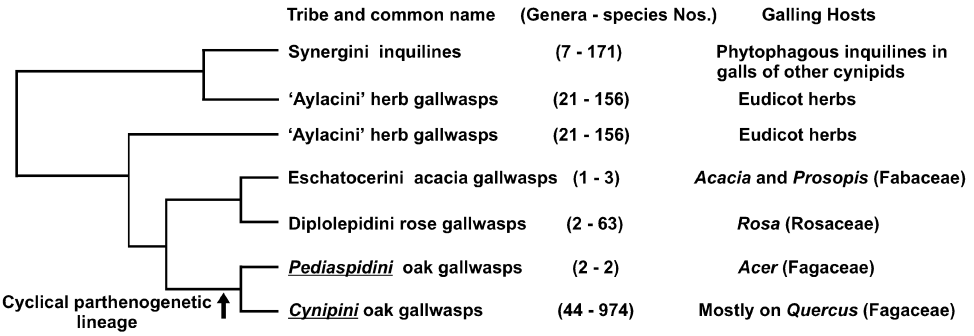

Fig. 1 Phylogenetic relationships between the different tribes within Cynipidae (data from Liljeblad & Ronquist 1998). The cyclical parthenogenetic tribe are underlined and in italics. All the other tribes are arrhenotokous. The number of genera and species within each tribe as well as information regarding their galling plant hosts are also listed.

Introduction

Alpha proteobacteria of the genus *Wolbachia* are a widespread group of maternally inherited endosymbionts of arthropod and nematode hosts (O'Neill *et al*. 1992; Sironi *et al*. 1995). *Wolbachia* infection induces a range of host phenotypes, including cytoplasmic incompatibility, male killing, feminization and thelytoky induction (Rousset *et al*. 1992; Hurst *et al*. 1999). In addition to immediate reproductive modifications, *Wolbachia* infection can have a range of longer term evolutionary impacts on host taxa such as providing a mechanism for rapid speciation and influencing the evolution of sex-determining mechanisms (e.g. O'Neill *et al*. 1997; Werren 1997, 1998; Hurst & Schilthuizen 1998; Stouthamer *et al*. 1999; Rokas 2000; Hurst & Werren 2001).

Wolbachia infects at least 16% of insect species (Werren *et al*. 1995b; West *et al*. 1998; Jeyaprakash & Hoy 2000; Werren & Windsor 2000), and levels of infection vary between genera and 'higher' taxonomic groups, between species, and between and within populations of a single species (Bourtzis *et al*. 1996; Breeuwer & Jacobs 1996; Bouchon *et al*. 1998; Hariri *et al*. 1998; Wenseleers *et al*. 1998; West *et al*. 1998; Plantard *et al*. 1998, 1999; Kittayapong *et al*. 2000; Werren & Windsor 2000; Jiggins *et al*. 2001a; Rokas *et al*. 2001). Much of this variation remains unexplained, and a number of important ecological and evolutionary questions remain unresolved. What determines the proportion of individuals that are infected with *Wolbachia* within a population/species (prevalence) or between species (incidence)? To what extent can variation in reproductive behaviour across host species be explained by *Wolbachia*? How is *Wolbachia* transmitted between species? To address these questions, we need to examine patterns of *Wolbachia* infection for taxa whose behaviour, ecology and phylogeny are well characterized.

Here we extend a developing body of work on *Wolbachia* in gallwasps, a family of wasps (Hymenoptera: Cynipidae) that induce galls on a range of plant hosts (Askew 1984; Ronquist & Liljeblad 2001; Stone *et al*. 2002). We focus specifically on: (i) the role of *Wolbachia* infection in life cycle diversity within the Cynipidae, and (ii) on the possible role of horizontal transmission of *Wolbachia* between host cynipid species.

Gallwasps are divided into six tribes (Liljeblad & Ronquist 1998; Ronquist 1999). The herb gallwasps (tribe 'Aylacini' — in inverted commas because they are a paraphyletic group), the acacia gallwasps (tribe Eschatocerini), and the rose gallwasps (tribe Diplolepidini) (Fig. 1) all reproduce by arrhenotoky (fertilized eggs give rise to females; unfertilized ones to males). Many herb and rose gallwasps are known only from a thelytokous generation. In all the species examined to date, thelytoky is automictic, occurs via gamete duplication and is associated with *Wolbachia* infection (Stille & Davring 1980; Stille 1985; Plantard *et al*. 1998, 1999). The inquiline gallwasps (tribe Synergini) are gallwasps which are unable to induce their own galls, and are obligate inhabitants of galls induced by other gallwasps (Askew 1984; Ronquist 1994). Reproduction in this tribe is also arrhenotokous.

The sycamore gallwasps (tribe Pediaspidini) and the oak gallwasps (tribe Cynipini) reproduce by cyclical parthenogenesis, their life cycle involving strict alternation between one arrhenotokous generation and one thelytokous generation (Askew 1984; Atkinson *et al*. 2002; Stone *et al*. 2002). The thelytokous generation occurs via apomixis, in which neither chromosome reduction nor fusion of nuclei takes place in the eggs developing parthenogenetically (Suomalainen *et al*. 1987). The transition to cyclical parthenogenesis within the Cynipidae has occurred only once (Fig. 1). A number of oak gallwasp species are known only from the thelytokous generation. Were *Wolbachia* able to induce thelytoky on cyclical parthenogenetic life cycles, we might expect infected species to show alternation between two thelytokous generations, one automictic (*Wolbachia* induced) and one apomictic. No life cycles of this type are known in oak gallwasps.

An alternative possibility is that *Wolbachia* infection has resulted in loss of the arrhenotokous generation altogether. Loss of sexual/arrhenotokous generations from cyclical parthenogenetic life cycles is taxonomically widespread (Hebert 1987), and such loss has long been suspected, though rarely demonstrated, in oak gallwasps (Folliot 1964; Abe 1986; Stone *et al*. 2002).

Here we use polymerase chain reaction (PCR) amplification of fragments of two *Wolbachia* genes (*ftsZ* and *wsp*) to survey the prevalence (% of individuals infected within a species) and incidence (% of species infected within tribes) of *Wolbachia* in 609 individuals of 64 gallwasp species in the Cynipini (53 species), Synergini (10 species) and 'Aylacini' (1 species) (Table 1). Of the Cynipini, 20 species are known

Table 1 List of gallwasp species screened for *Wolbachia* infections. All individual wasps screened were females. Countries and localities from which specimens were obtained are also listed. Numbers in parentheses next to localities indicate the number of specimens screened from that locality. Ratio indicates the proportion of individuals of a given species that were infected with *Wolbachia*. The LC column states the life cycle of a given species. A: arrhenotoky (for Cynipini, A denotes species known only from their arrhenotokous generation), CP: cyclical parthenogenesis, T: thelytoky (for Cynipini, T denotes species known only from their thelytokous generation)

No.	Species	Country and locality	Ratio	LC
Tribe Cynipini (oak gallwasps)				
1	*Andricus aestivalis*	Hungary: Érsekvadkert (3).	0/3	A
2	*A. amenti* (formerly known as *A. giraudianus*)	Hungary: Szentkút (1).	0/1	CP
3	*A. aries*	Hungary: Isaszeg (2).	0/2	T
4	*A. askewii*	Turkey, Çekerek (1), Tokat (1).	0/2	T
5	*A. caputmedusae*	Hungary: Balaton (5), Eger (2), Matráfüred (2), Várpalota (3), Sirok (2), Szoloske (1). Italy: Piedimonte Matese (2), Monte S. Angelo (3), Castagnola (2), Monte Vulture (2), Gildone (1), Gargáno (2). Austria: Vienna (2). Turkey: Egirdir (2), Küllüce (4), Suluova (1), Tokat (1), Kizezoglu (1).	0/38	CP
6	*A. conglomeratus*	Hungary: Matráfüred (3).	0/3	T
7	*A. conificus*	Italy: San Venanzo (1), Massa Marittima (1). Hungary: Várpalota (2).	0/4	T
8	*A. coronatus*	Hungary: Balaton (7), Szeghalom (2), Várpalota (2), Szentkút (1). Italy: Chiusi (2), Massa Marittima (2), San Venanzo (2), Bombiana (1), Greve (1), Monte S. Angelo (1). Turkey: Madenli (2), Küllüce (2), Suluova (2). Greece: Edessa (4), Pisoderi (2), Arnissa (1), Agras (1).	0/35	T
9	*A. coriarius*	Hungary: Matráfüred (5), Gödöllő (1), Tatabánya (1), Várpalota (1). Italy: Rieti (2), Molize (2), Valpiana (1), Castelli (1). Turkey: Gezende (2), Suluova (2), Tokat (1), Niksar (1), Küllüce (1), Beysehir (1). Greece: Edessa (2), Florina (1), Pisoderi (1). Slovakia: Plástovce (1). Spain: El Escorial (2), Llerida (1).	0/30	CP
10	*A. corruptrix*	Hungary: Gödöllő (5).	0/5	CP
11	*A. crispator*	Hungary: Köszeg (2).	0/2	A
12	*A. curvator*	Spain: Madrid (4). UK: Walsingham (2). Hungary: Miskolc (1).	0/7	CP
13	*A. dentimitratus* (formerly known as *A. viscosus*)	Spain: Barcelona (5). Slovenia: Idrija (2). Hungary: Köszeg (4), Devecser (1). Italy: Chiusi (2), Gargáno (5), Massa Marittima (2), Siena (1). Turkey: Beybesli (6).	0/28	CP
14	*A. fecundator*	UK: Leeds (1).	0/1	CP
15	*A. gallaetinctoriae*	Hungary: Miskolc (5). Italy: Rocca di Ruffeno (2). Turkey: Madenli (2).	0/9	CP
16	*A. glutinosus*	Hungary: Szarnalum (5), Eger (1). Austria: Vienna (2).	0/8	T
17	*A. grossulariae*	Spain: Piedralaves (2). Hungary: Devecser (3).	0/5	A

Table 1 *Continued*

No.	Species	Country and locality	Ratio	LC
18	*A. hartigi*	Italy: Lame (1), Casina (1), Massa Marittima (2), Greve (1). Hungary: Sirok (1).	0/6	T
19	*A. hungaricus*	Hungary: Karcag (4). Austria: Vienna (2).	0/6	T
20	*A. inflator*	Finland: Turku (1).	0/1	CP
21	*A. kollari*	Hungary: Hortobágy (5), Matráfüred (2).	0/7	CP
22	*A. lignicolus*	Hungary: Gödöllő (5). UK: Hampstead Heath (2).	0/7	CP
23	*A. lucidus*	Hungary: Matráfüred (5), Isaszeg (2), Szob (2), Jászberény (1), Balaton (1), Sopron (1). Italy: Casina (1), Lame (1), Piedimonte Matese (1), Gargáno (1), Chiusi (1), Radicofani (1), Vernasca (1). Czech Republic: Valtice (1). France: Nantes (2). Turkey: Antalya (5), Egirdir (3), Beysehir (3), Aglasun (1), Suluova (1).	0/35	CP
24	*A. malpighii* (formerly known as *A. nudus*)	Italy: Bibulano (1).	0/1	CP
25	*A. mayri*	Hungary: Szob (3).	0/3	T
26	*A. mediterraneae*	Turkey: Madenli (5).	0/5	T
27	*A. mitratus*	Slovenia: Idrija (5). Hungary: Matráfüred (1). Turkey: Beybesli (1).	0/7	T
28	*A. panteli*	Turkey: Madenli (5), Egirdir (4), Gezende (2), Aglasun (2). Morocco: Azrou (6). Italy: Greve (2), Chiusi (2), Poppi (2), Bombiana (1), Radicofani (1), San Venanzo (1), Monte S. Angelo (3).	0/31	CP
29	*A. polycerus*	Hungary: Isaszeg (5). Italy: Poppi (2).	0/7	T
30	*A. quercuscalicis*	Hungary: Tiszakürt (1). Ireland: Dublin (1). France: Crécy (1). Germany: Rottenbach (1).	0/4	CP
31	*A. quercusramuli*	UK: Oxford (5). Hungary: Matráfüred (2).	0/7	CP
32	*A. quercustozae*	Hungary: Sopron (5). Italy: Greve (2), Gildone (1), Felitto (1), Jelsi (1). Morocco: Azrou (2). Turkey: Gezende (2), Madenli (2), Küllüce (2), Refahiye (3), Yeniyol (1). Spain: Prado del Rey (1). Greece: Arnissa (3), Pisoderi (2).	0/28	CP
33	*A. seckendorfii*	Italy: Bra 95 (5), Massa Marittima (3), Chianti (2), Chiusi (1). Turkey: Hadim (8), Madenli (2).	0/21	CP
34	*A. solitarius*	France: St. Chinian (3).	3/3	CP
35	*A. testaceipes*	Hungary: Sopron (2).	0/2	CP
36	*A. theophrastus*	Turkey: Beysehir (3).	0/3	T
37	*A. tinctoriusnostrus*	Turkey: Madenli (2). Lebanon: Shakra (1).	0/3	T
38	*A. tomentosus*	Turkey: Madenli (1). Greece: Arnissa (1).	0/2	T
39	*Aphelonyx cerricola*	Italy: Chiusi (3), Piombino (2), Chianti (2), Rieti (1). Hungary: Várpalota (5), Kemence (1), Köszeg (1). Slovakia: Cifáre (1). Turkey: Beysehir (3).	0/19	T
40	*Biorhiza pallida*	Rokas *et al*. 2002 for countries and localities.	172/206	CP
41	*Callirhytis glandium*	Hungary: Gyöngös (2).	2/2	CP
42	*Chilaspis nitida*	Hungary: Unjuned (7).	0/7	CP
43	*Cynips cornifex*	Hungary: Matráfüred (2). Italy: Colombaro (1).	0/3	T

Table 1 *Continued*

No.	Species	Country and locality	Ratio	LC
44	*Cynips disticha*	Hungary: Kemence (2). France: B. de Mervent (1).	0/3	CP
45	*Cynips divisa*	Hungary: Gödöllő (2), Nagygyanté (1), Sopron (1).	0/4	CP
46	*Cynips longiventris*	Hungary: Devecser (3), Pácin (2), Nagygjante (2). Slovakia — Ciféra (1), Sikenica (2). Croatia: Istria (1). France: Nantes (1), St. Porquier (1). UK: Hartsholme (1).	0/14	CP
47	*Cynips quercus*	Hungary: Eger (2), Veszprém (1), Szentkút (1), Sopron (1). France: Angoulême (1), Le Barp (1). Italy: Rieti (1), Poppi (1), Anconella (2), Bibulano (1), Pianavia (1), The Matese (1). Greece: Komnina (2), Arnissa (1), Pisoderi (1), Prespa (1), Stagira (1), Edessa (1). Turkey: Tefenni (1), Aglasun (1), Guzeloluk (1), N. of Antalya (1), Sekerek (1), Yeşilyurt (1), Suluova (1), Kirezoğlu (1), Refahiye (1), Küllüce (1).	0/31	CP
48	*Cynips quercusfolii*	Hungary: Mesterszállás (5), Matráfüred (1). France: Montereau (2). UK: Oxford (2). Croatia: Petrovina (1). Greece: Olympiada (1). Turkey: Black Sea (1).	0/13	CP
49	*Neuroterus lanuginosus*	Hungary: Sirok (3).	0/3	T
50	*Neuroterus macropterus*	Hungary: Matráfüred (4), Sopron (3), Gödöllő (2), Várpalota (3), Kemence (3). Slovakia: Plástovce (2). Italy: Castelletto (1). Greece: Arnissa (1).	19/19	T
51	*Neuroterus quercusbaccarum*	Hungary: Matráfüred (5). UK: Auchtermuchty (2).	0/7	CP
52	*Plagiotrochus quercusilicis*	Spain: El Pardo (3).	3/3	T
53	*Trigonaspis synaspis*	Turkey: Beysehir (3), Suluova (2).	0/5	CP
Tribe Synergini (inquilines)				
54	*Ceroptres cerri*	Hungary: Matráfüred (2).	2/2	A
55	*Periclistus brandtii*	Sweden: Gottsunda (1).	0/1	A
56	*Synergus crassicornis*	Spain: Casa del Campo, Madrid (3).	3/3	A
57	*Synergus diaphanus*	Hungary: Sopron (1).	1/1	A
58	*Synergus gallaepomiformis*	Hungary: Szentendre (2), Karcag (1), Balaton (1), Szentkút-1 (–), Sopron (2), Szeghalom (2±), Eger (1–), Matráfüred (1–), Veszprém (1–). Slovakia: Prasnik (1). UK: Elsfield (4), Cambridge (4), Birnwood Forest (4). France: Rennes (3), Forêt Decouvres (2), Montain (1), Nantes (1). Spain: Cercedilla (2).	29/34	A
59	*Synergus hayeanus*	Hungary: Karcag (1), Gödöllő (2), Gyöngös (1), Veszprém (1).	0/5	A
60	*Synergus incrassatus*	Hungary: Matráfüred (1).	0/1	A
61	*Synergus reinhardi*	Hungary: Gödöllő (2+), Balaton (2+), Eger (1+), Felsőtárkány (1), Szentendre (1+), Veszprém (5+). France: Rennes (4), Nantes (5), Crécy (1), Castres (1). Italy: Fellizzano (1). Austria: Vienna (1+).	12/25	A
62	*Synergus umbraculus*	Hungary: Matráfüred (7±). France: Guérande (1+). UK: London (1+). Spain: Cercedilla (9).	3/18	A
63	*Synophrus politus*	Hungary: Gödöllő (1), Sopron (1), Szoloske (1), Tatabánya (1), Kemence (1). Italy: Lame (5), Greve (1), Piombino (1). Greece: Arnissa (4), Edessa (1). Spain: Madrid (2). Turkey: Madenli (4).	0/23	A
Tribe Aylacini (herb gallwasps)				
64	*Panteliella bicolor*	Sweden: Gottsunda (1).	0/1	A

only from a thelytokous generation (Melika *et al*. 2000), although we stress that purely thelytokous reproduction remains to be demonstrated for any of them. We address the following questions:

1 How widespread is *Wolbachia* infection in the Synergini and Cynipini? We combine our own results with published data on *Wolbachia* in the 'Aylacini' and Diplolepidini (Plantard *et al*. 1999) to assess whether there is any correlation between the incidence of infection and reproductive mode.
2 What are the apparent phenotypic impacts of *Wolbachia* infection in these two tribes? The expectation for the arrhenotokous Synergini is that, like the 'Aylacini' and Diplolepidini, infection will result in (automictic) thelytoky.
3 Is there any correlation between *Wolbachia* infection and the absence of a known arrhenotokous generation from oak gallwasp life cycles?

Oak galls support complex communities, consisting of the gall-inducer (tribe Cynipini), inquiline gallwasps (tribe Synergini) and chalcid parasitoids (Askew 1961, 1984; Wiebes-Rijks & Shorthouse 1992; Schönrogge *et al*. 1995, 1998; Stone *et al*. 1995, 2002). These intimate assemblages of potential hosts make galls excellent systems within which to examine evidence for horizontal transmission of *Wolbachia* among community members (e.g. West *et al*. 1998). Oak gallwasp communities have been studied in considerable detail, and in Europe the host spectra of most inquiline gallwasps are known (Askew 1984; Nieves-Aldrey & Pujade-Villar 1985, 1986; Nieves-Aldrey 1988; Stone *et al*. 2002). Here we screen 10 inquiline gallwasps, and address a further question: is there any evidence that infected inquiline gallwasps share *Wolbachia* infections with their oak gallwasp hosts, so providing strong evidence for horizontal transmission?

Finally, population-level analyses of rose gallwasps have revealed geographical variation in the prevalence of *Wolbachia* infection within species (Plantard *et al*. 1998). Here we screen samples of 18 species (4 inquilines and 14 gall inducers) over sites ranging from Morocco through Iberia, Italy, the Balkans and Turkey, and ask (i) whether any species show geographical variation in *Wolbachia* prevalence, and (ii) whether there is concordance in the regions within which species are infected. During the recent glaciations, many taxa, including oaks and associated gallwasps, were confined to refugia in southern Europe. Detailed analyses of population genetic patterns in several oak gallwasps imply that the major refugial areas, which exchanged limited gene flow, were Iberia/Morocco, Italy/the Balkans, and Turkey (Atkinson 2000; Rokas 2001; Rokas *et al*. 2001; Stone *et al*. 2001). If horizontal transmission of *Wolbachia* is frequent in oak cynipid communities, then we might expect *Wolbachia* sequence identity across infected species to reflect shared refugial history rather than species boundaries.

Materials and methods

Collection and DNA extraction

Sample locations, sample sizes and reproductive mode are shown for 64 gallwasp species in Table 1. Samples were obtained by rearing or dissection of galls. Galls from many gallwasp species are multilocular (more than one offspring emerge from a single gall). To minimize screening of siblings in such cases we used one female from each gall.

DNA was extracted from fresh female individuals as described in Rokas *et al*. (2001). With each DNA extraction three control extractions were performed using a *Nasonia Wolbachia*-positive line, a *Nasonia Wolbachia*-negative line and a no-DNA sample (Werren *et al*. 1995b; West *et al*. 1998).

Wolbachia *screening*

Screening for *Wolbachia* was performed by PCR using the *Wolbachia*-specific primers for the *ftsZ* cell-cycle gene ftsZF1 (Werren *et al*. 1995b) and WOLG-R (Rokas *et al*. 2001). Jeyaprakash & Hoy (2000) have suggested that failure of *Taq* DNA polymerase to amplify *Wolbachia* genes may have caused underestimation of the incidence of *Wolbachia* in arthropods, and that a mixture of the enzymes *Taq* and *Pwo* should be used for PCR amplification. However, here we used the *Taq*-only protocol established by Werren *et al*. (1995b) and subsequently used by many authors (e.g. Wenseleers *et al*. 1998; West *et al*. 1998; Werren & Windsor 2000) in order to allow comparison of our data with previously published studies.

All PCRs were performed in a PTC-200 DNA engine (MJ Research). Screening PCRs were attempted for sample DNA extractions at dilutions ranging from 1/10 to 1/100. Control PCRs were always performed. The PCR cycle for *ftsZ* was: one cycle of 94 °C for 3 min, 55 °C for 90 s and 72 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 90 s, 72 °C for 5 min and a final extension step at 72 °C for 5 min. The PCRs were performed in 25 μL volumes and consisted of 1 μL of DNA sample, 2.5 μL of 10× PARR Buffer (HYBAID), 1 μL of $MgCl_2$ (25 mM), 0.5 μL of dNTPs (10 mM), 0.35 μL of each primer (20 mM), 0.25 μL of *Taq* DNA polymerase (Promega) and 19.05 μL of distilled, deionized H_2O. Fifteen microlitres of each reaction were electrophoresed on a 1% ethidium bromide-stained agarose gel. To check that samples negative for *Wolbachia* were not because of: (i) failed DNA extraction; (ii) presence of PCR inhibitors; and (iii) incorrect DNA concentration, control PCRs with the general eukaryotic 28*S* ribosomal

DNA (rDNA) primers 28*S*f and 28*S*r were performed as described in Werren *et al*. (1995b). Control PCRs for a number of specimens were also performed with the mitochondrial cytochrome *b* primers CB1 and CB2 (e.g. Rokas *et al*. 2001). These primers were shown to be more accurate indicators of the DNA quality of the specimen than 28*S* primers (data not shown), perhaps because of their lower generality as universal primers (Rokas *et al*. 2002). All specimens that failed to amplify the 28*S* rDNA and/or cytochrome *b* fragments were excluded from subsequent analysis.

PCR amplification and sequencing

Wolbachia diversity in infected populations was assessed by sequencing fragments of two *Wolbachia* genes, *wsp* and *ftsZ*. *wsp* is the most polymorphic gene isolated to date from *Wolbachia* (Zhou *et al*. 1998) and hence the most likely to distinguish between two closely related *Wolbachia* groups, whereas *ftsZ* has been the most widely used *Wolbachia* gene. Two recent studies have presented evidence for recombination; Werren & Bartos (2001) convincingly demonstrated recombination in the *wsp* gene, whereas Jiggins *et al*. (2001b) provided indirect evidence for recombination in the *Wolbachia* genome. This poses a serious obstacle for phylogenetic reconstruction as the characters (nucleotides) of recombinant sequences do not share a single phylogenetic history. Both groups (Jiggins *et al*. 2001b; Werren & Bartos 2001) suggested that fine-scale phylogenies of *wsp* sequences should be treated with caution. Although we agree with these authors' conclusions, the *wsp* sequences presented here are fully concordant with the *ftsZ* results and the conclusions drawn in our study (see Results and Discussion) do not rely heavily on an accurate phylogenetic reconstruction of the *wsp* fragment.

Wolbachia-infected individuals (in most cases one from each species) were sequenced for *wsp* using the 81F and 691R primers following methods described previously (Zhou *et al*. 1998; Rokas *et al*. 2001). For *ftsZ* sequencing, PCR products were cloned using the TOPO TA Cloning kit (Invitrogen, cat. 4500-01) and 4–6 clones from each specimen (10–15 for those specimens that had more than one *wsp* product) were subsequently sequenced. Plasmids containing the fragment of interest were isolated using the QIAprep Spin Miniprep Kit (QIAGEN, cat. 27104). Plasmid DNA was subsequently quantified and sequenced. Sequencing was carried out using the Perkin-Elmer BigDye Terminator chemistry and an ABI 377 sequencer. Sequencing reactions were performed in both directions to minimize PCR artefacts, ambiguities and base-calling errors.

Analysis of sequence data

For both *wsp* and *ftsZ* datasets, representative sequences for all known *Wolbachia* groups were retrieved from GenBank and included in phylogeny reconstruction (see Fig. 2 for host species and GenBank Accession nos). Sequences were aligned using CLUSTAL W (Thompson *et al*. 1994) with the default settings. The *ftsZ* fragment was 687 bp long for all the species examined and contained no gaps. The *wsp* fragment varied in length between 540 and 594 bp, with the aligned dataset being 614 bp long. For the *wsp* dataset, nucleotide positions 74–109, 217–255 and 519–585 in the alignment submitted to TreeBASE (see below) were excluded from the analysis, because of ambiguity in the identification of positional nucleotide homology in these regions. All the sequences are available from GenBank (Accession nos: *wsp*: AF339629, AY095147–AY095158, *ftsZ*: AY095159–AY095169) and the two alignments (and their corresponding phylogenies) are available electronically from TreeBASE (http://www.herbaria.harvard.edu/treebase/, study accession number: S728, matrix Accession nos: M1159 for *ftsZ* matrix and M1160 for *wsp* matrix).

Phylogenies for *wsp* and *ftsZ* were estimated by neighbour-joining (NJ) analysis of the sequence data, using the phylogenetic analysis package PAUP*, Version 4.0b8 (Swofford 2000). Gaps were coded as missing data. Distances were calculated using maximum likelihood (ML). The model of sequence evolution and the parameter values best fitting the data for each locus was identified using likelihood ratio tests (Huelsenbeck & Rannala 1997; Lewis 1998), as implemented in MODELTEST Version 3.0 (Posada & Crandall 1998). The models with the best fit for the two datasets were rather similar. For *wsp*, the best fit model included unequal base frequencies, a transition/transversion ratio of 3.29 and rate heterogeneity among sites (gamma distribution shape parameter $\alpha = 0.3548$). The best-fit model for *ftsZ* included unequal base frequencies, a different rate between transitions and transversions (but with the two types of transitions, A $\leftrightarrow$ G and T $\leftrightarrow$ C, showing ratios of 7.41 and 18.7, respectively, relative to transversions) and rate heterogeneity among sites (gamma distribution shape parameter $\alpha = 0.13$). The parameter values suggested by MODELTEST for each dataset were used to specify the distance matrix for neighbour-joining analysis on 1000 bootstrap replicates.

Analysis of infection patterns within and among gallwasp tribes

For statistical comparison of the incidence of *Wolbachia* in different gallwasp tribes, we compare incidence for the Synergini and Cynipini from our survey with published data for the 'Aylacini' and Diplolepidini (Plantard *et al*. 1999). We assumed that infected species represent independent datapoints, allowing application of chi-squared tests. The underlying assumption is then that gallwasp species acquire infections independent of each other,

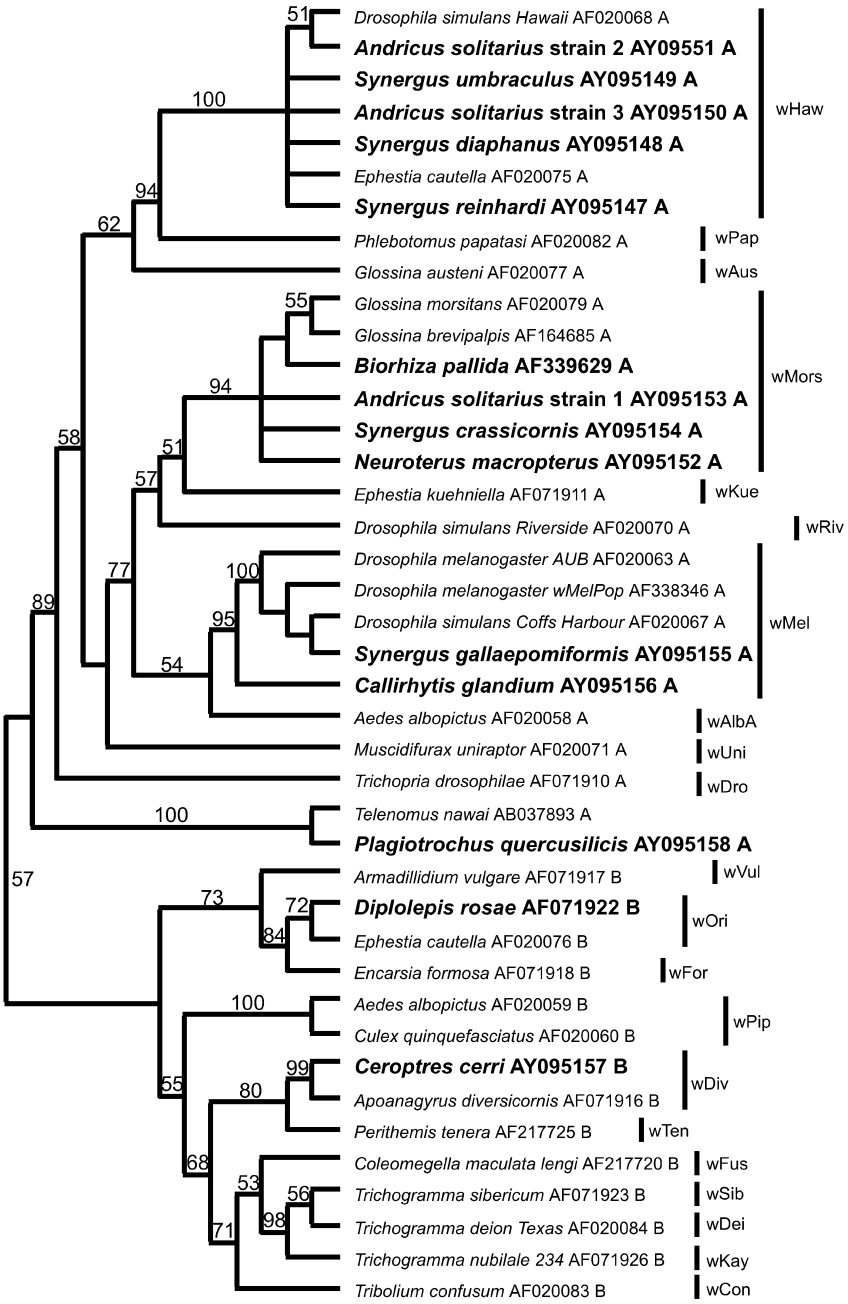

Fig. 2 The phylogenetic relationships between the different *Wolbachia wsp* sequences as reconstructed using a neighbour-joining algorithm on a maximum likelihood estimated distance matrix. Name of the host arthropod species is followed by GenBank Accession no. and clade designation. The *Wolbachia* groups to which sequences belong to are also shown. The tree shown is rooted on the clade A–clade B split for easy visualization. Values above branches indicate bootstrap support (all compatible groupings with bootstrap values < 50% are shown, but without the bootstrap values). Sequences isolated from gallwasp species are shown in larger font and bold. The *Telenomus nawai–Plagiotrochus quercusilicis Wolbachia* clade has not been assigned a group name.

rather than through common ancestry. This assumption is justified by current knowledge on transmission of *Wolbachia* between different arthropod hosts (Werren *et al*. 1995a; Schilthuizen & Stouthamer 1997; Huigens *et al*. 2000), has been employed in many published studies (e.g. Wenseleers *et al*. 1998; Kittayapong *et al*. 2000; Werren & Windsor 2000), and is supported by the observed pattern of *Wolbachia* infection in our dataset (see below). Were *Wolbachia* to be inherited among daughter species of a shared common ancestor (vertical transmission), infected species could not be treated as independent data, and comparative methods controlling for phylogeny would have to be applied (Grafen 1989; Harvey & Pagel 1991). The statistical power of these methods is determined by the number of evolutionarily independent transitions in the variable of interest (in this case, reproductive strategy). Within the gallwasps, the transition from purely arrhenotokous reproduction to cyclical parthenogenesis has happened only once (in the shared common ancestor of the Pediaspidini and the Cynipini: Fig. 1), and no phylogeny-based method could refute the null hypothesis of no association between reproductive mode and *Wolbachia* incidence.

Table 2 Numerical ratio and percentage of *Wolbachia* incidence within the various tribes and reproductive modes of Cynipidae. The ancestral reproductive mode in each tribe is indicated in parentheses (see Table 1 for abbreviations)

	Wolbachia-infected species/total no. species screened	% Incidence of *Wolbachia*/Total	Study
Tribe			
Cynipini (CP)	5/53	9.4	This study.
'Aylacini' (A)	4/9	44.4	Plantard *et al*. (1999); this study.
Synergini (A)	6/10	60.0	This study.
Diplolepidini (A)	11/19	57.9	Plantard *et al*. (1999).
Reproductive mode			
Arrhenotoky	21/38	55.3	Plantard *et al*. (1999); this study.
Cyclical parthenogenesis	3/31	9.7	This study.
Thelytoky	2/20	10.0	This study.

Results

The distribution of Wolbachia *infection in the Cynipidae*

Eleven of the 64 species screened (17.2%) were infected with *Wolbachia*: 6 are inquiline gallwasps (tribe Synergini; *Ceroptres cerri*, *Synergus crassicornis*, *S. diaphanus*, *S. gallaepomiformis*, *S. reinhardi*, *S. umbraculus*) and 5 are gall-inducers (tribe Cynipini; *Andricus solitarius*, *Biorhiza pallida*, *Neuroterus macropterus*, *Callirhytis glandium*, *Plagiotrochus quercusilicis*). Among the gall-inducers, only single species in 5 genera are infected, and these genera are widely distributed in the gallwasp phylogeny (Stone & Cook 1998; Rokas 2001). It is thus highly unlikely that the infected species have acquired their *Wolbachia* through descent from an infected shared common ancestor, and far more likely that the infected species represent independent infection events.

All species harboured clade A *Wolbachia*, with the exception of *C. cerri* which was infected with a clade B *Wolbachia*. This higher incidence of clade A *Wolbachia* in gallwasps is in agreement with previous surveys in the Hymenoptera (West *et al*. 1998; Werren & Windsor 2000). One specimen of *A. solitarius* yielded three different *wsp* sequences (two of them differed only by a 3 bp indel). Variability of the *ftsZ* gene in clade A *Wolbachia* clade is generally very low, and it is probable that this *A. solitarius* specimen harboured *Wolbachia* bacteria from two or possibly three clade A groups. All the other gallwasp species were infected with bacteria from a single group. In 7 of the 11 infected species, all individuals screened showed the presence of *Wolbachia*. The remaining four species are discussed below.

Intraspecific variation in Wolbachia *prevalence*

Multiple individuals from a wide geographical range of localities were screened for 18 of the 64 gallwasp species. Thirteen of these species (the inquiline *Synophrus politus* and 12 gall-inducing species) were not infected with *Wolbachia*. Of the five infected species only one (*N. macropterus*) was found to be infected for all individuals and locations. The other four species showed varying levels of *Wolbachia* prevalence (see Table 1) (percentage prevalence over all samples is given in parentheses after each species). In the gall-inducer *Biorhiza pallida* (85.4%) and the inquiline *Synergus reinhardi* (48%), *Wolbachia* prevalence levels did not vary within populations (i.e. infection with *Wolbachia* was either fixed or absent in individual populations), but varied among sample sites. *B. pallida* is free of *Wolbachia* in central Spain, but is infected in southern and northern Spain and in the rest of Europe. *S. reinhardi* is infected in Hungary and Austria and uninfected in France and Italy. The other two infected *Synergus* species show variation in *Wolbachia* infection within individual populations. *S. gallaepomiformis* (85.3%) is infected throughout Europe, only a few individuals in certain Hungarian sites are not infected, whereas *S. umbraculus* (16.7%) is infected in Hungary, France and the UK but not in Spain.

Wolbachia *incidence and life cycle variation within the Cynipidae*

Patterns in the cyclically parthenogenetic tribe Cynipini. The limited distribution of *Wolbachia* in the Cynipini shows that this symbiont can play no fundamental role in gallwasp cyclical parthenogenesis. Infection with *Wolbachia* is also not correlated with the absence of a known arrhenotokous generation in this tribe: only 2 of the 20 sampled oak gallwasp species known only from a thelytokous generation are infected with *Wolbachia* (see Table 2). This ratio does not differ significantly from the ratio (3/31) of *Wolbachia* infection observed in cyclical parthenogenetic species ($\chi^2 = 0.20$, df = 1, $P > 0.05$).

Patterns across gallwasp tribes. The incidence of *Wolbachia* in other gallwasp tribes reported in Plantard *et al*. (1999) is

Table 3 Per cent incidence of *Wolbachia* infection in various lineages. The group studied, its taxonomic status, the percentage of *Wolbachia* incidence as well as the source study are indicated

Group	Taxonomic status	Incidence of *Wolbachia* (%)	Study
Drosophila fruit-flies	Genus	8/48 (16.7)	Bourtzis *et al*. (1996)
Diopsidae stalk-eyed flies	Family	4/17 (23.5)	Hariri *et al*. (1998)
Formicidae ants	Family	Whole family: 25/50 (50.0) Dependently founding species: 11/22 (50) Independently founding species: 1/8 (13)	Wenseleers *et al*. (1998)
Aphidoidea aphids	Various	Aphids: 0/4 (0.0)	West *et al*. (1998)
Hymenopteran parasitoids and hyperparasitoids of aphids		Aphid parasitoids: 0/19 (0.0)	
Gracillariidae leaf-mining moths		Aphid hyperparasitoids: 1/8 (12.5)	
Hymenopteran parasitoids of leaf-miners		Leaf-miners: 8/21 (38.1)	
Various Lepidoptera		Leaf-miner parasitoids: 5/18 (27.8) Various Lepidoptera: 4/13 (30.8)	
Phytoseiidae predatory mites and Tetranychidae spider mites	2 families	Both families: 10/27 (37) Spider mites: 6/16 (37.5) Predatory mites: 4/11 (36.4)	Breeuwer & Jacobs (1996)
Culicidae mosquitoes	13 genera	25/89 (28.1) Frequency of infected species differed significantly among genera	Kittayapong *et al*. (2000)
Acraea butterflies	Genus	7/24 (29.2)	Jiggins *et al*. (2001a)
Crustaceans	5 orders	Isopoda: 22/63 (34.9) Amphipoda: 0/12 (0) Tanaidacea: 0/1 (0) Cumacea: 0/3 (0) Decapoda: 0/4 (0)	Bouchon *et al*. (1998)
Diplolepidini rose gallwasps	Tribe	11/19 (57.9)	Plantard *et al*. (1999)
Cynipini oak gallwasps	Tribe	5/53 (9.4)	This study

shown in Table 2. If we assume that *Wolbachia* infections in all tribes represent independent infection events, then *Wolbachia* incidence is unevenly spread between tribes with different reproductive modes; species in the tribes (combined) that reproduce purely by arrhenotoky are more likely to be infected than cyclically parthenogenetic Cynipini ($\chi^2 = 20.58$, df = 1, $P < 0.001$) (Table 2). There is no significant difference among the purely arrhenotokous tribes ('Aylacini' vs. Synergini, $\chi^2 = 0.05$, df = 1, $P > 0.05$/ 'Aylacini' vs. Diplolepidini, $\chi^2 = 0.07$, df = 1, $P > 0.05$/ Synergini vs. Diplolepidini, $\chi^2 = 0.08$, df = 1, $P > 0.05$).

Molecular phylogenetic analysis of two Wolbachia *genes (*ftsZ *and* wsp*)*

In accordance with previous studies (e.g. Werren *et al*. 1995a), the phylogenetic tree for *ftsZ*, was unresolved within clade A *Wolbachia* groups (tree not shown). Five of the 11 infected gallwasp species have identical *ftsZ* sequences (the inquilines *S. diaphanus*, *S. reinhardi*, *S. umbraculus*, and the gall-inducers *N. macropterus* and *A. solitarius*), with two more differing only in one nucleotide position (*S. crassicornis* and *S. gallaepomiformis*).

In contrast, the *wsp* tree is very structured (Fig. 2) and provides a much clearer picture of the different *Wolbachia* groups infecting gallwasps. A major conclusion based on the *wsp* sequence data (detailed below) is that species from different cynipid tribes (Cynipini and Synergini) are commonly infected with the same group of *Wolbachia* (groups wHaw, wMors and wMel), and in some cases have identical sequences. Two groups of sequences can be identified. Sequences isolated from four gallwasp species (the inquilines *S. reinhardi*, *S. diaphanus*, *S. umbraculus* and sequence 3 in the gall-inducer *A. solitarius*) have identical *wsp* sequences and together with sequence 2 in *A. solitarius* belong to the wHaw group. *Wolbachia* sequences from three other species (the inquiline *S. crassicornis*, sequence 1 in the gall-inducer *A. solitarius* and the gall-inducer *N. macropterus*) are also identical and together with the sequence amplified from *B. pallida* belong to the wMors group. Sequences isolated from *S. gallaepomiformis* and the gall-inducer *C. glandium* belong to the wMel group, the sequence isolated from the gall-inducer *P. quercusilicis* is closely related to the *Telenomus nawai Wolbachia* (not assigned to any group, Arakaki *et al*. 2000) and the sequence amplified from the inquiline *C. cerri* lies within the clade B *Wolbachia* wDiv group.

Discussion

The incidence of Wolbachia *in oak gallwasps*

Although *Wolbachia* infection is widespread in arthropods (Table 3), induction of thelytoky by *Wolbachia* is known only from two haplodiploid insect orders — the Hymenoptera and Thysanoptera (Stouthamer 1997; Arakaki *et al*. 2001). Within the Hymenoptera, *Wolbachia*-induced thelytoky is limited to two super-families of parasitoid wasps — the Chalcidoidea and the Cynipoidea (Stouthamer 1997). Within the Cynipoidea, the only taxa to have been widely screened for *Wolbachia* infection are the herb gallwasps (tribe 'Aylacini') and the rose gallwasps (tribe Diplolepidini). In both taxa, reproduction is arrhenotokous, and *Wolbachia* infection leads to the imposition of automictic thelytoky (Plantard *et al*. 1998, 1999).

A major aim of this survey was to reveal the incidence and phenotypic consequences of *Wolbachia* infection in the cyclically parthenogenetic oak cynipids (the sister group tribes Pediaspidini and Cynipini). Not only does this clade represent a rare case of cyclical parthenogensis within the Metazoa (Hebert 1987), the Cynipini are also the most species-rich clade within the gallwasps (Ronquist & Liljeblad 2001; Stone *et al*. 2002). Finally, in common with other cyclically parthenogenetic taxa, a range of oak gallwasps are known only from thelytokous generations (Folliot 1964; Abe 1986; Melika *et al*. 2000). A second aim was to establish the incidence of *Wolbachia* infection within the Cynipini, and the extent of any correlation between life cycle structure and infection status.

Wolbachia infection is rare in European oak gallwasps. Only five infected species were identified in our survey, in each case represented by a single species in a genus. These genera are themselves widely distributed through the oak gallwasp phylogeny (Stone & Cook 1998; Rokas 2001). This pattern strongly suggests that these *Wolbachia* infections are the outcome of independent transmission events, and not the result of vertical transmission from a *Wolbachia*-infected shared common ancestor.

Those species which are infected show no phenotypic alteration in their life cycle. This is in dramatic contrast to the thelytoky induced in rose gallwasps (tribe Diplolepidini) and herb gallwasps (tribe 'Aylacini'). Were *Wolbachia* infection to have a similar impact in cyclically parthenogenetic life cycles, we might expect to see oak gallwasp species with two thelytokous generations each year — one occurring by *Wolbachia*-induced automixis, and the other occurring by the apomixis characteristic of the thelytokous generation in cyclical parthenogenetic gallwasp life cycles (Atkinson *et al*. 2002). However, no life cycles of this type are known. It is possible that the cytological environment associated with cyclical parthenogenesis in the Cynipini precludes the induction of thelytoky by *Wolbachia*, although the reproductive cytology of these life cycles remains little studied (Sanderson 1988; Atkinson 2000; Atkinson *et al*. 2002).

An alternative potential impact of *Wolbachia* infection (though without an obvious a *priori* mechanism) could be suppression of the arrhenotokous generation, resulting in collapse of the life cycle to a single thelytokous generation each year. Many oak gallwasps are known only from a thelytokous generation, but our data show there to be no correlation between this state and the incidence of *Wolbachia*. The extent to which arrhenotokous generations have been lost from ancestrally cyclical parthenogenetic species remains an issue of debate in cynipid biology. Ongoing population genetic work suggests that a number of oak gallwasps currently known only from a thelytokous generation do in fact possess cryptic arrhenotokous generations (Cook *et al*. 1998; Atkinson 2000; Rokas 2001; Stone *et al*. 2001; Atkinson *et al*. 2002). Genuine loss of the arrhenotokous generation has been shown experimentally in two *Andricus* species — in a proportion of females of *Andricus quadrilineatus* (Folliot 1964), and in all females of *A. targionii* (Abe 1986). Detailed screening of *A. targionii* for *Wolbachia* has shown that this species is not infected (Abe & Miura 2002). Taken together, these results suggest that *Wolbachia* infection of oak gallwasps is unlikely to be a causative agent in loss of arrhenotokous reproduction in this group.

Incidence of Wolbachia *infection in the inquiline tribe Synergini*

Six inquiline species were found to be infected with *Wolbachia*. In the absence of infection, the Synergini have purely arrhenotokous life cycles, so, as for the herb and rose gallwasps, *Wolbachia* infection is predicted to result in thelytoky. However, all of the infected inquiline species are known to possess males (Nieves-Aldrey & Pujade-Villar 1985; Nieves-Aldrey 1985). If *Wolbachia* does induce thelytoky in the Synergini, then the presence of males in these species suggests that they show variation in the prevalence of infection within and among populations, as demonstrated for the rose gallwasp *Diplolepis spinosissimae* by Plantard *et al*. (1998). Population level surveys of sex ratios and *Wolbachia* prevalence are ongoing in these species (J.L. Nieves-Aldrey & G.N. Stone, unpublished data).

Variation in the incidence of Wolbachia *among tribes*

Gallwasp species in tribes with purely arrhenotokous reproduction are much more frequently infected with *Wolbachia* than cyclically parthenogenetic oak gallwasps (55.3 vs. 9.7%; Table 2). It is tempting to suggest, as above, that the mechanisms involved in cyclical parthenogenesis may cause the lower incidence of *Wolbachia* in oak gallwasps (see also Table 3). However, the single evolution of

this more complex life cycle within the Cynipidae prevents a phylogeny-based test of this hypothesis. Furthermore, as the cynipid life cycle is unique among insects (Stone *et al*. 2002), the hypothesis is probably untestable using correlational evidence. Nevertheless, the scattered occurrence of *Wolbachia* infection within the oak gallwasp phylogeny strongly suggests that infections are not long-lived enough to persist through speciation events. Possible limits to *Wolbachia* transmission may be revealed when more is learned about the cytological mechanisms involved in cynipid cyclical parthenogenesis.

Horizontal transmission of Wolbachia *between gall-inducers and their associated inquilines*

Wolbachia interspecies transmission pathways have been analysed in a few assemblages, but the route by which transmission occurs is less clear (Werren *et al*. 1995a; Schilthuizen & Stouthamer 1997; West *et al*. 1998; Vavre *et al*. 1999; Huigens *et al*. 2000). Ten species from the tribe Synergini were screened for *Wolbachia* infection to assess the evidence for horizontal transmission of *Wolbachia* between inquilines and their gall-inducing hosts. Inquilines and gall-inducers develop in close physical proximity within the gall structure, and the relationships among these two cynipid groups are well known for the European fauna (Askew 1984; Nieves-Aldrey & Pujade-Villar 1985, 1986; Nieves-Aldrey 1988; Schönrogge *et al*. 1995). Were horizontal transmission to be a major feature of the ecology of *Wolbachia* in oak cynipid galls, we would expect the infected inquilines to be associated with the communities centred on infected gall-inducers. As explained below, this is not the case in our survey.

Based on the 100% similarity shown in their *wsp* sequences, there are two potential cases of horizontal transmission in our dataset. The first involves *Wolbachia* sequences for three inquiline species (*S. reinhardi*, *S. diaphanus*, *S. umbraculus*) and the gall-inducer *Andricus solitarius* (whose *Wolbachia* sequence 3 is identical to the *Synergus* species). The second case involves *Wolbachia* sequences for one inquiline (*S. crassicornis*) and two gall-inducers — *A. solitarius* (sequence 1) and *N. macropterus*.

None of these inquilines are known from the communities of either gall-inducer, and the majority of them attack galls that are structurally different from either potential host. On the basis of known host spectra, only *S. crassicornis* attacks galls (such as the thelytokous generation of *A. fecundator*) that are structurally similar to the thelytokous generation galls of *A. solitarius*, and could possibly be found in association with *A. solitarius* with more extensive sampling. The most abundant inquiline in *A. solitarius* galls is usually *S. gallaepomiformis* (Nieves-Aldrey & Pujade-Villar 1986; J.-L. Nieves-Aldrey, unpublished data). This inquiline was found to be infected with *Wolbachia*, but (i) its *wsp* sequence differed from those found in *A. solitarius*, and (ii) this inquiline was free of infection at French sites close to those where the *A. solitarius* were sampled (Table 1).

We can look at the issue of possible horizontal transmission from another perspective by asking whether hosts known to be attacked by *Wolbacia*-infected inquilines were sampled in this survey. *S. gallaepomiformis* is an extremely widespread species, and attacks one or both of the generations of the many of the gall-inducers screened. *S. umbraculus* is also widespread in the larger thelytokous generation galls of many *Andricus* species (Nieves-Aldrey & Pujade-Villar 1985, 1986; Nieves-Aldrey 1988). Were horizontal transmission of *Wolbachia* common between inquilines and their hosts, we would expect to find a higher incidence of infection in the gall inducers.

Why do inquilines and gall-inducers that do not commonly occur in the same gall community share *Wolbachia* infections? A possible exchange route is via attack of galls harbouring the two species by generalist parasitoids. Oak galls commonly harbour rich communities of chalcid parasitoids, most of which are specialists in oak galls, but many of which attack a very wide range of host cynipids, both gall-inducers and inquilines (Askew 1961, 1984; Stone *et al*. 2002). Parasitoids of oak galls do include species that are infected with *Wolbachia* (R. James and M. Jervis, personal communication), but the extent to which the *Wolbachia* bacteria match those found in gall inducers and inquilines remains unknown.

Prevalence of Wolbachia *infection within species*

If *Wolbachia* infects only a small percentage of a population (or species), screening a few individuals may lead to an underestimation of the real percentage of species in which it occurs (Werren *et al*. 1995b; Jiggins *et al*. 2001a). In our analysis we have included rare species represented by only one or a few specimens and therefore, our results must provide a lower limit estimate of infection levels. Of the 18 species represented by larger sample sizes from multiple populations, only four showed geographical variation in infection status. There was no consensus in the geographical location of infected populations across species, and no evidence of horizontal exchange of *Wolbachia* among populations sharing the same glacial refuge. Although absence of such a pattern could be ascribed to inadequate sampling, it is compatible with a general conclusion of low rates of horizontal transmission in European oak cynipid communities.

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This project is part of a larger long-term study being carried out in Graham Stone's laboratory on various aspects of the population biology, phylogeography and phylogeny of gallwasps. Antonis Rokas' main interest has been the application of 'tree-thinking' in a variety of evolutionary ecological questions (including *Wolbachia*-related questions) relating to gallwasps at various taxonomic levels. Rachel Atkinson is interested in the application of molecular population genetics to address small- and large-scale ecological questions regarding gallwasps, ranging from the oviposition behaviour of a given gallwasp species to its postglacial recolonization route into northern Europe. Jose Luis Nieves-Aldrey has published many taxonomic papers on gallwasps and their associated communities and has a special interest in gallwasp inquilines. Stuart West's main research interest is the evolution of reproductive strategies.
