Non-sibling parasites (Strepsiptera) develop together in the same paper wasp

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SUMMARY

Host discrimination by immature host-seeking endoparasites is a complex and somewhat unexplored topic. In the case of multiple infections, conflicts among conspecifics may occur to monopolize space and resources in the same host. Two or more 1st instar larvae of *Xenos vesparum* (Strepsiptera, Stylopidae) may enter into a *Polistes dominulus* (Hymenoptera, Vespidae) larva and develop together until the adult stage of both parasite and host. We carried out a screening of mitochondrial haplotypes in *X. vesparum* individuals extracted from superparasitized wasps taken in 5 naturally infected nests from different areas of Tuscany (Italy), to assess whether non-sibling parasites may infect the same colony and host. In total, we obtained 12 different haplotypes out of 122 genotyped individuals of both sexes: 17 of 34 superparasitized wasps hosted parasites that originated from females differing in their haplotypes. To date, this is the first described case of superparasitism with non-sibling host-seeking larvae infecting a single individual hymenopteran host. In addition, at least in heavily infected colonies, there is evidence of a male-biased sex-ratio and synchronous development of the parasites, regardless of their haplotypes. Finally, the distribution of haplotypes per nest is consistent with either phoretic infection or larvipositing on nests by means of superparasitized wasps.

Key words: Strepsiptera, superparasitism, mtDNA, within-host competition.

INTRODUCTION

Parasitoids and parasites (*sensu* Godfray, 1994) of social insects offer a good model for an understanding of several aspects of the parasitic life-style, due to the exceptional opportunity of finding a large number of genetically related hosts and parasites. Open nests of social wasps are suitable infection targets. Parasitoid hymenoperans, as well as host-seeking larvae of parasitic beetles and other macroparasites, focus on immobile wasp larvae, which are abundant but hidden inside cells and well defended against intruders. Superparasitism (more than 1 parasite in a single host) is common where the colony is a persistent target of infections (Schmid-Hempel, 1998). Multiple infection has been considered for a long time to be the result of a mistake, or 'the best of a bad job' scenario, because most parasitoids and parasites reject hosts that have previously been infected to avoid local resource competition or, more rarely, direct conflict (Salt, 1961; van Lenteren *et al*. 1978). Nevertheless, under some circumstances (i.e. limited availability of suitable hosts or high cost of discrimination), superparasitism is considered an adaptive reproductive strategy (van Alphen and Visser, 1990; Marris and Casperd, 1996; Outreman and Pierre, 2005; Schofield *et al*. 2005). When 'self superparasitism' occurs, the competition is among siblings, whereas 'conspecific superparasitism' implies that offspring from different females are potential competitors (van Alphen and Visser, 1990). Although theoretical models abound, empirical evidence on competition and relatedness among parasites remains scant (see Koskella *et al*. 2006).

The paper wasp *Polistes dominulus* Christ (Hymenoptera, Vespidae) is the prime host of *Xenos vesparum* (Strepsiptera, Stylopidae), the first strepsipteran described by Rossi in 1793 (see Kathirithamby, 1989 for a review of this unusual order of insects characterized by an extreme sexual dimorphism: neotenic larviparous females are permanently endoparasitic, whereas adult males are free-living and winged). The infective stage of *X. vesparum* is the triungulin (1st instar larva), which enters the host during all its larval stages (Hughes *et al*. 2003) and completes its development in the wasp abdomen. In our *Xenos*/*Polistes* system, triungulins are the ecological equivalent of female parasitoids with 1 egg to lay, low mobility and low life-expectancy (Brodeur and Boivin, 2004). Superparasitism (several strepsipterans with any combination of sexes per stylopized host, i.e. parasitized by Stylopidae) is common in

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either *P. dominulus* immatures (Hughes *et al*. 2003) or adults (about 10–20% of stylopized wasps, see Hughes *et al*. 2004*a*, *b*).

The strategy developed by immature mobile parasites – such as *Xenos* triungulins – to actively locate and select their host, is a complex and largely unexplored topic (Brodeur and Boivin, 2004). Triungulins can be dispersed by means of a foraging wasp, infected by a gravid *Xenos* female which extrudes from the host abdomen and delivers 1st instar larvae in small groups on flowers; here, they can attach to the legs/abdomen of other wasps (vectors) which carry them back to their nests (phoresy). All studies concerning hymenopterans parasitized by strepsipterans have assumed phoresy as the only mechanism of infection (Linsley and McSwain, 1957; Maeta *et al*. 2001). Nevertheless, Pardi (1946) observed that in spring stylopized *Polistes* wasps move from one nest to another. Thus, stylopized wasps – which neither build nests nor associate with foundresses (Beani, 2006) – visit several colonies, where they could directly release their triungulins. This alternative dispersal strategy has been hypothesized (Hughes *et al*. 2003; Beani, 2006; Dapporto *et al*. 2007) in the case of a massive infection of nests and a high parasite load.

In social insects very little is known about multiple infections and within-host competition by host-seeking larvae that may belong to different parasitic strains (Schmid-Hempel, 1998). In this work mitochondrial (mt) haplotypes are used to assess the genetic diversity of *X. vesparum* parasites infecting the same colony and specimen of *P. dominulus*. Because the mitochondrial genome is maternally inherited, if different individuals carry different haplotypes, it is certain that they derived from different females. On the other hand, haplotype identity between different individuals, albeit implying a close genetic affinity even between individuals from different localities, is not necessarily a proof of a sibling relationship. By sampling and analysing haplotypes of superparasitized wasps (Fig. 1) from 5 heavily infected colonies, we attempted to answer several questions. First, we investigated if non-sibling *X. vesparum* parasites may develop in the same nest and wasp (i.e. conspecific superparasitism). Second, we can assess the developmental stage of the parasites in relation to their sex and haplotypes, i.e. competition for local resources. Third, we here attempt a preliminary assessment of *X. vesparum* variability among colonies collected from different sites in Tuscany (Italy). At present, no data are available on the genetic diversity of any strepsipteran population. If an adequate assessment of genetic variation will be made, we can reasonably expect higher genetic heterogeneity after phoretic infection rather than in the case of massive release of triungulins on nests.

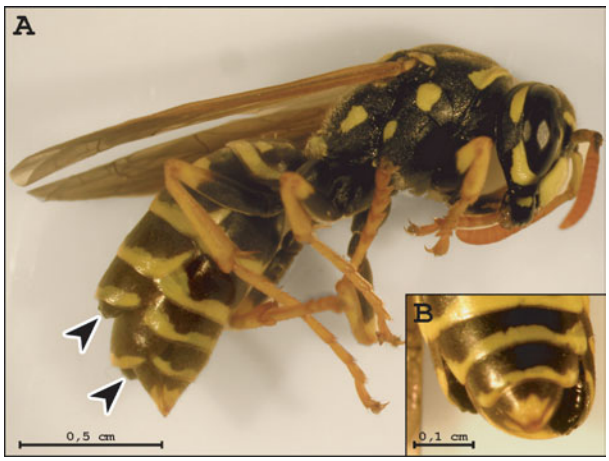

Fig. 1. A superparasitized *Polistes dominulus* wasp. (A) Arrows show 2 *Xenos vesparum* pupae. (B) Detail of superparasitized wasp's last abdominal segments.

MATERIALS AND METHODS

Parasite life-cycle and specimen collection

Strepsiptera exhibit 'the most extreme sexual dimorphism' found in insects (Grimaldi *et al*. 2005). A *Xenos* male emerges as a short-lived flying insect from the puparium, which extrudes between the tergites of the adult wasp. The male does not feed but uses its mandibles just to break its cephalotheca, actively seeks a female for reproduction, and dies after a few hours. On the other hand, female 4th instar larvae develop into neotenic larviform endoparasites, which extrude only their cephalothorax from the wasp's abdomen. *Xenos* females may survive for months inside the wasp, entering into winter diapause with their host and, once this is completed, release the infective 1st instar larvae. In spring triungulins, the only other free-living stage besides the male, emerge in small groups (20–30 individuals, *personal observation*) through the ventral canal opening in the cephalothorax, and move from the wasp to the substrate. While looking for a wasp larva to enter, they can survive for several hours, by using the yolk in the midgut lumen as a source of nutrients (Giusti *et al*. 2007).

Polistes dominulus wasps become visibly stylopized when the pupal male cephalotheca and/or the female cephalothorax of *X. vesparum* extrude from their abdominal tergites (Fig. 1), i.e. about 10 days after the adult wasp emerges from its cell (Hughes *et al*. 2004*b*). We sampled wasps (all females) which emerged from 5 naturally infected nests, collected in May (pre-emergence phase, i.e. only foundresses) in different areas of Tuscany (Italy): Cecina (CEC) and Camaiore (CAM) are on the coast, while Trespiano (TRE), Peretola (PER), and Scarperia (SCA) are inland, in the surroundings of Florence. In order to evaluate the occurrence of successful natural infections, we dissected all wasps emerging from the nest 5 weeks after its collection in the field (i.e. the

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last possible day of infection). Five weeks was chosen to allow 3 weeks for host larval and pupal development, plus 2 weeks for extrusion of cephalotheca and/or cephalothorax from the abdomen, once the wasps emerged from their cells.

Table 1 lists data from the 5 infected nests: the number of healthy and singly/multi-infected wasps in each colony; the sex-ratio of parasites (percentage of *Xenos* males); the parasite load (here, the average number of parasites per infected adult wasp); the developmental stage of the parasite. Except for 9 ambiguous cases (labelled as 'not sexed larvae' and not genotyped), 4th instar larvae were easily sexed, due to a peculiar neck-like constriction in the female prothoracic segment, whereas the male cephalic region is round-shaped (see Manfredini *et al*. 2007). In our sample of adult wasps, we adopted a few broad labels to record *Xenos* developmental stages: 'neotenic females', with fully extruded cephalothorax; 'early/late' unextruded 4th instar females, referring to less or more sclerified cephalothorax; 'early/late' male pupae, depending on their degree of sclerotization and protrusion from the wasp abdomen, whereas 4th instar males were never found.

The screening of haplotypes was accomplished in a sample of 34 multi-infected wasps, i.e. parasitized by 2 or more *Xenos* individuals (Fig. 1); thus, 19 singly-infected wasps were dissected but parasites were not genotyped. Because pupating males can be easily extracted from the wasp abdomen, our study has mostly focussed on *Xenos* pupae. In fact both unextruded and extruded 4th instar females – essentially bags of oocytes and adipocytes – are more likely destroyed at dissection than male pupae. In all, 12 *Xenos* females from 6 two-female-infected wasps and 19 from multi-infected ones were not genotyped.

DNA extraction, PCR and sequencing

In order to analyse mtDNA, total DNA was extracted from single *X. vesparum* pupae (101 in total) and female 4th instar larvae (21 in total) using the Wizard SV Genomic DNA purification system (Promega), taking care to avoid any contamination with the wasp's tissue. Two specific primer pairs were used to amplify 2 fragments that correspond to a portion of the cytochrome oxidase subunit I (*cox1*) and the NADH dehydrogenase subunit 4 (*nad4*) genes of the mitochondrial genome of *X. vesparum* (Carapelli *et al*. 2006; GenBank Accession number DG364229): COX1-1492-J (5′-GAGCCTGAGCAGGAATAGTTGGACTTTC-3′) *vs* COX1-300-N (5′-GATGGGATCTCCTCCTCCTAAAGGG-3′) and NAD4-260-J (5′-GGAGGACAAGAAAAATTAGAGGACC-3′) *vs* NAD4-865-N (5′-TTTTTCCATCTTTGTTAATACTGTTG-3′).

PCR conditions were 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min 30 s, for 35 cycles. PCR amplification was carried out in a total reaction volume of 25 μl containing 20 ng of genomic DNA, 1 mM dNTPs, 1X PCR reaction buffer (Promega), 2·5 mM Mg^{2+}, 1 μM of forward and reverse primers each, and 1 U of recombinant Taq DNA polymerase (Promega). PCR products were gel purified (Wizard SV Gel purification system, Promega) and sequenced in both directions using the original PCR primers on an automatic sequencer CEQ 8000XL (Beckman). Sequences were edited using Sequencher^{TM} (version 4.2.2; Genes Codes) and aligned using CLUSTAL X (Thompson *et al*. 1997). For each individual, *cox1* and *nad4* sequences (GenBank Accession number: EU078910-33) were concatenated and grouped manually to identify all different haplotypes. Alignments are available from the corresponding author upon request. A parsimony network was determined for the 12 haplotypes by using the TCS 1.21 software (Clement *et al*. 2000). For each nest, estimations of haplotypic diversity (h) and nucleotide diversity (π) were calculated using the Arlequin 3.1.1.1. software (Excoffier *et al*. 2005).

Statistics

The parasite sex distribution in multi-infected wasps ($N = 40$, i.e. only males *vs* only females *vs* both sexes) was tested using the Pearson Chi-square test, as well as the frequency of synchronous and asynchronous development (i.e. all early or all late *vs* mixed stages) in both *Xenos* males and females infecting the same host ($N = 29$ wasps where all parasites were sexed, i.e. 5 wasps parasitized by early instar larvae were not included). The coexistence of non-sibling male parasites in the same host was tested by Pearson Chi-square test in 34 wasps that were the source of pupae (the female sample was too small for this analysis). The probability levels for Pearson Chi-square tests were computed using a complete randomization method (permutation or exact test; P_{exact}) or by a Monte Carlo (Mehta and Patel, 1996; Good, 2000) simulation based on a 10 000 samples table ($P_{Monte\ Carlo}$) when computation was impossible. All statistical analyses were conducted using the Statistical Package for Social Sciences *ver*. 13.05 (SPSS®).

RESULTS

Sex-ratio, parasite load and developmental stage of X. vesparum

Table 1 summarizes the data concerning the population structure of the parasite across the 5 naturally infected nests. The proportion of parasitized wasps emerging in May–June was high (50–70%), regardless of monogynous or polygynous nesting and nest size: the highest rates were recorded in CAM and

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Table 1. *Polistes dominulus* colonies from which wasps (1–34), multi-infected by *Xenos vesparum* parasites, were collected and analysed, and the distribution of mt-haplotype variants (A–L) in each wasp is presented

(M, early/late male pupae; F, early/late 4th instar larvae and neotenic (neo) females; L, larvae not sexed.)

Colony data	*X. vesparum*	Distribution and stage of *Xenos* in multi-infected wasps				Males (M) and unextruded females (F) here genotyped			
			Occurrence of:						
Location, collection day, nest size, and percentage of infected workers	Sex, stage, *sex-ratio*, and parasite load	wasp	M early/late	F	L	M	haplotypes	F	haplotypes
Trespiano (TRE), 15.05.2003	2 Singly-infected (2F neo)	1	1/1	0/1		2	CH	1	C
Foundresses = 1; cells = 70	6 Multi-infected (see: 1–6)	2	2/1	0/1		3	FGI		
Infected workers: 8/17 (47·1%)	% males = 59·2; parasite load = 3·4	3	4/0	4/0		4	CCCF	4	CCFG
		4	2/0	1/0		2	CC	1	J
		5	2/1			3	CCK		
		6	2/0	0/2		2	CF	2	CL
Camaiore (CAM), 26.05.2004	3 Singly-infected (2M late, 1F neo)	7	0/2			2	AA		
Foundresses = 4; cells = 80	19 Multi-infected: 3 two-female infected	8	0/2			2	BB		
Infected workers: 22/28 (78·5%)	(1F late, 5F neo) + 16 (see: 7–22)	9	0/2			2	AB		
	% males = 70; parasite load = 2·5	10	0/2	0/1		2	AB		
		11	0/3			3	AAA		
		12	0/2			2	AA		
		13	0/2			2	AA		
		14	0/2	2/1		2	AB		
		15	0/2	1/1		2	AB		
		16	2/3	1/0		5	AAAAB		
		17	0/2			2	AA		
		18	0/2			2	AA		
		19	0/3			3	AAB		
		20	0/2			2	AA		
		21	0/2	1/0	2	2	AA		
		22	0/2	2/0	2	2	AA		
Cecina (CEC), 08.05.2005	1 Singly-infected (1F early)	23	0/3	3/0		3	BBB		
Foundresses = 1; cells = 25	5 Multi-infected: 1 two-female	24	2/3	1/0	2	5	BBBBB		
Infected workers: 6/10 (60%)	infected + 4 (see: 23–26)	25	2/4			6	BBBBBB		
	% males = 69·6; parasite load = 4·1	26	0/2			2	BB		
Scarperia (SCA), 10.05.2006	6 Singly-infected (4M late, 2F neo)	27	0/5	2/0	2	5	CDDDD		
Foundresses = 1; cells = 40	3 Multi-infected (see: 27–29)	28	0/6	0/8		6	CCCDDD	8	CCCCDDDD
Infected workers: 9/13 (62·2%)	% males = 55·9; parasite load = 4·0	29	0/4	2/3		4	CCCD	5	CCCDD
Peretola (PER), 30.05.2006	7 Singly-infected (2M late, 2M	30	3/0			3	DDE		
Foundresses = 1; cells = 60	early, 3F neo)	31	2/0			2	DD		
Infected workers: 14/23 (60·1%)	7 Multi-infected: 2 two-female	32	6/0		1	6	DDDDDE		
	infected (4F neo) + 5 (see: 30–34)	33	3/0			3	DDD		
	% males = 70; parasite load = 2·1	34	0/3	2/0		3	DDD		
TOTAL									
Infected workers: 59/91 (64·8%)	Infected: singly- = 19; multi- = 40		101	40	9	101		21	
	L = 9, M = 111 (35 early, 76 late)		33/68	22/18					
	F = 61 (23 early, 19 late, 19 neo)								

Table 2. Summary of mt-haplotype variability and distribution

(The frequency of each haplotype in each population and in the total sample is shown in parentheses; *h*: haplotype diversity; *π*: nucleotide diversity; nests are abbreviated as in Table 1.)

Haplotype	130	154	164	171	222	267	273	315	364	372	402	444	447	522	552	502	569	577	592	634	707	716	770	865	919	952	969	CAM	CEC	TRE	PER	SCA	Total
	cox1															*nad4*																	
A	T	A	T	A	C	C	G	T	G	G	T	A	A	G	C	C	C	T	A	G	A	A	G	T	T	A	C	29 (0·784)					29 (0·238)
B	·	·	·	·	·	T	·	·	·	·	·	G	·	A	·	T	·	C	·	A	T	·	·	·	·	·	·	8 (0·216)	16 (1·000)				24 (0·197)
C	·	G	·	G	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	A	·	·	A	·	C	·	·			13 (0·541)		15 (0·536)	28 (0·230)
D	A	·	C	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	G	A	·	·	A	·	C	·	·				15 (0·882)	13 (0·464)	28 (0·230)
E	·	·	·	·	·	T	A	A	·	·	·	G	T	·	·	·	·	C	·	A	T	·	A	·	·	·	·				2 (0·118)		2 (0·016)
F	·	G	·	G	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	A	·	T	A	·	C	·	·			4 (0·166)			4 (0·033)
G	·	G	·	G	·	·	·	·	·	·	·	G	·	·	·	·	T	·	·	A	·	·	A	·	C	·	·			2 (0·083)			2 (0·016)
H	·	·	·	G	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	A	·	·	A	C	C	·	·			1 (0·042)			1 (0·008)
I	·	G	·	G	·	·	·	·	·	A	·	G	·	·	·	·	·	·	·	A	·	·	A	·	C	·	·			1 (0·042)			1 (0·008)
J	·	G	·	G	·	·	·	·	·	·	C	G	·	·	·	·	·	·	·	A	·	·	A	·	C	·	·			1 (0·042)			1 (0·008)
K	·	·	·	G	·	·	·	·	A	·	·	G	·	·	·	·	·	·	·	A	·	·	A	·	C	T	T			1 (0·042)			1 (0·008)
L	·	G	·	G	·	·	·	·	·	·	·	G	·	·	T	·	·	·	·	A	·	·	A	·	C	·	·			1 (0·042)			1 (0·008)
No· of samples																												37	16	24	17	28	122
Amino acid replacement sites / No· of haplotypes	S	S						I	G							P	A				I		M			I	S	2	1	8	2	2	12
	↔	↔						↔	↔							↔	↔				↔		↔			↔	↔						
	T	G						M	S							S	G				F		V			M	F						
h																												0·3483	0	0·6920	0·2206	0·5159	0·8041
π																												0·0027	0	0·0012	0·0026	0·0035	0·0058

P A I M I S 2 1 8 2 2 12

SCA (with 4 and 1 foundresses, large and small nests, respectively). In this sample of heavily parasitized nests, 40 out of 59 wasps (67·8%) were multi-infected, although we recorded unparasitized and singly-infected adults in each colony. The sex-ratio of parasites, including both unextruded and extruded ones, was male-biased, ranging from 55·9% to 70·0% males across the 5 nests. Superparasitized wasps were mainly infected either by males or both sexes (17 by males *vs* 6 by females *vs* 17 by both sexes, $\chi^2 = 6{\cdot}250$, D.F. = 2, $P_{Exact} = 0{\cdot}05$). The parasite load was high (2·1–4·1): the most extreme case was wasp 28 from SCA, hosting 6 extruded pupae and 8 unextruded females.

We dissected parasites differing in their stage of development from all the colonies, although the development of parasites of the same sex was mostly synchronized inside the same host. In 28 out of 34 wasps that were the source of pupae (Table 1), the male parasites appeared to be at the same developmental stage (77 out of 101). Female 4th instar larvae were more scarce (40 in total) but generally synchronized: we found early and late unextruded females together only in 2 wasps. Asynchronous development in the same host was less frequent than synchronous stages both in males (5 *vs* 24 wasps, $\chi^2 = 6{\cdot}250$, D.F. = 1, $P_{Exact} = 0{\cdot}021$) and in females (3 *vs* 10 wasps, $\chi^2 = 16{\cdot}030$, D.F. = 1, $P_{Exact} < 0{\cdot}001$). Intriguingly, in multi-infected wasps we sampled 95 out of 101 pupal males with extruded cephalotheca (labelled as 'late' in Table 1) and no extruded females. Females with fully extruded cephalothorax (19, labelled as 'neotenic') were found only in singly or 2-female-infected wasps.

Haplotype variability and intra/inter-colony distribution

The analysis of mtDNA haplotypes was carried out using 2 fragments of *cox1* and *nad4* (507 and 525 bp, respectively), obtained from 122 individuals of *X. vesparum*. Overall, average A + T content of the 2 fragments was 70·9%, for *cox1* and 78·5%, for *nad4*, the latter being closer to the average value calculated for the complete mt-genome (79%: Carapelli *et al.* 2006). A detailed analysis of haplotype distribution and variability is shown in Table 2. The concatenated aligned sequences (1032 nucleotide positions) showed 27 variable sites (21 transitions and 6 transversions). Most substitutions are synonymous (leaving the encoded amino acid unchanged), while 10 substitutions are non-synonymous, therefore causing an amino acid replacement.

Twelve different haplotypes (A–L) were found, differing by as many as 12 substitutions (between haplotype D and E). Five haplotypes (H, I, L, J and K) were each found in a single individual (private haplotypes), all from TRE, while the remaining 7 haplotypes were found in more than one individual.

Three haplotypes, B, C and D, are shared by 2 populations. The 4 most frequent haplotypes, A (23·8%), B (19·7%), C (23%) and D (23%), represent the vast majority of the aligned sequences (89·5%). The only monomorphic nest was CEC, where all genotyped individuals showed haplotype B. On the other hand, the highest variation was found in TRE, with 8 haplotypes (including private ones). The other nests showed the coexistence of 2 haplotypes: A and B, in CAM; C and D, in SCA; D and E, in PER.

Haplotype and nucleotide diversity within and among colonies from coastal and inland populations are not fully concordant. With 8 different haplotypes, TRE has the highest haplotype diversity ($h = 0{\cdot}692$), but the sequences are relatively similar to each other (maximum difference = 5 substitutions), resulting in the lowest nucleotide diversity value ($\pi = 0{\cdot}0012$). On the other hand, CAM, PER and SCA have only 2 haplotypes (and h ranging from 0·3483 to 0·5159), but the number of substitutions between the haplotypes in each population is larger (8 in CAM, 12 in PER and 7 in SCA), resulting in higher values of π.

Two haplotypes (A and B) are restricted to the coastal nests (CAM and CEC); the remaining 10 haplotypes are restricted to the inland sites (Fig. 2). Haplotype E, albeit exclusive of an inland population (PER), has a lower nucleotide divergence with the coastal haplotype B (5 substitutions) than it has with all other haplotypes from the inland populations (average number of substitutions of E with C, D, F, G, H, I, J, K, L = 10·1). The network of haplotypes (Fig. 2) defines haplotype C as the ancestral sequence that is separated by 9 hypothetical substitutions from the most derived haplotype E.

Non-sibling parasites share the same host

In 34 multi-infected wasps, 17 individuals hosted parasites possessing different haplotypes, therefore originated from different females (see Table 1). In all 4 nests hosting non-sibling parasites (TRE, CAM, SCA, PER), there were 2 or more wasps carrying parasites with different haplotypes. Wasps carrying parasites with up to 3 different haplotypes (wasp 2, 3, 6) were found in the most heterogeneous nest (TRE). In a limited sample of 6 wasps – containing parasites of both sexes that were screened for their haplotype – we found non-sibling males as well as non-sibling females. Moreover, there was no evidence of any relationship between haplotype diversity and either superparasitism or developmental synchrony. Multiple infections by males with different haplotypes were not more frequent than those by parasites sharing the same haplotype ($\chi^2 = 0{\cdot}034$, D.F. = 1, $P_{Exact} > 0{\cdot}050$), and the developmental stage was synchronized in both groups ($\chi^2 = 1{\cdot}934$, D.F. = 1, $P_{Exact} > 0{\cdot}050$).

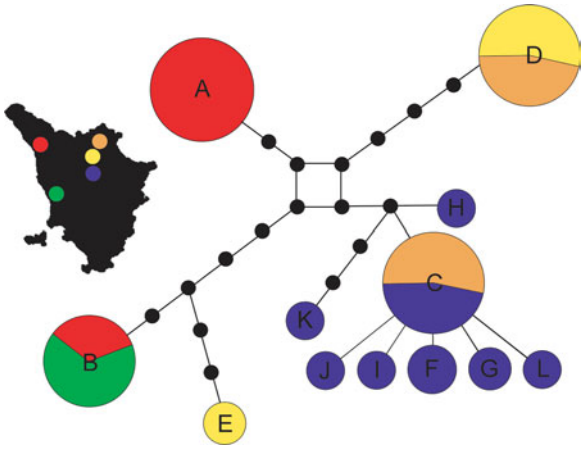

Fig. 2. Unrooted haplotype network based on 122 mtDNA sequences. Haplotypes (A–L) are coloured according to nest origin: Camaiore (red), Peretola (yellow), Trespiano (blue), Scarperia (orange) and Cecina (green). The quadrangular connection at the centre of the network depends on the homoplastic nature of 2 nucleotide substitutions: cox1-444 and nad4-919.

DISCUSSION

X. vesparum *haplotype diversity*

The clonal mechanism of replication of the mitochondrial genome, and its maternal mode of inheritance, mean that *X. vesparum* individuals bearing different mitochondrial haplotypes must have originated from different females. Our data unambiguously show that (a) *Xenos* parasites from coastal and inland collection sites in Tuscany exhibit genetic heterogeneity; (b) the same *Polistes* colony may host non-sibling parasites of both sexes; (c) these non-kin individuals have been found inside the same host in half of our superparasitized wasps. From this point of view, our results differ from studies suggesting mechanisms of competitive exclusion among diverse pathogen strains (Koskella *et al.* 2006). It should be pointed out that mt-haplotype analysis may underestimate genetic variability, because *Xenos* individuals bearing the same mtDNA sequences are not necessarily siblings; in fact they can belong to different populations. On the other hand, we found a relatively high genetic variability in our specimens. In addition to the 12 different haplotypes in our sample, a further haplotype, differing from haplotype A for a single substitution in *nad4*, was found in singly-infected wasps (thus not included in our screening): 3 from CEC and 1 from TRE nests. Indeed, parasites sharing the same haplotype in the same nest are more likely related than individuals with different haplotypes.

Nucleotide divergence between haplotypes does not correspond to their geographical distribution. In addition, while one nest (TRE) shows many genetically highly related haplotypes, other nests (CAM, PER, SCA) have fewer, highly divergent

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haplotypes. The latter condition is suggestive of multiple introductions (mediated by migrating wasps) of non-sibling *Xenos*, whereas local evolution (differentiation) of parasites from a single ancestor seems more likely for TRE. This preliminary interpretation should, however, be taken with caution, given the low number of colonies studied. The screening of additional populations, as well as collections from extra-nidal clusters of stylopized wasps (Hughes *et al*. 2004*b*) to avoid pseudo-replicates from the same colony, should help to clarify the phylogeographical picture.

Infection modality : an open question

In our sample of naturally infected nests, small as well as large colonies were the target of massive infections. Thus, the parasite load in adults was high, according to previous data from dissected immatures (Hughes *et al*. 2003). The relatively low resolution power of our genetic marker, the limited sample and the low levels of variability found, do not allow us to provide a unique answer to the question on infection modality. Nevertheless, the presence of colonies with 1 (CEC) or 2 (PER, CAM, SCA) haplotypes contrasts with the high haplotype diversity of TRE, suggesting the possibility that different nests may be infected via different modalities (direct release of triungulins or phoresy).

Given that an individual *Xenos* female is able to release thousands of triungulins (Kathirithamby, 1989), we might speculate that the low haplotype diversity of some nests might be the effect of the direct release of triungulins from 1 gravid female, infecting a wasp alighted on several nests. The 'wandering behaviour' first observed by Pardi (1946) fits with recent observations of higher inter-colony mobility by parasitized *P. dominulus* wasps – harbouring infective *Xenos* females – than unparasitized ones, at a time when nests were full of larvae (Beani and Massolo, 2007). This 'sit and wait strategy' for mature nests, previously hypothesized for healthy wasps which adopt/usurp colonies (Starks, 1998) as well as for social parasites (reviewed by Cervo and Dani, 1996), could be adopted by stylopized wasps to allow parasite's reproduction: a further example of parasitic manipulation of host behaviour (Hughes, 2005; Beani, 2006). Recent data on chemical undetectability of wasps infected by *Xenos* females support this alternative infection modality (Beani *et al*. 2005*b*; Dapporto *et al*. 2007). These 'wandering' wasps might be infected by 2 non-sibling *Xenos* females, in line with the presence of 2 haplotypes in the same nest (PER, CAM, SCA): an occurrence as likely as 2 non-sibling pupae parasitizing the same host in our sample. Most overwintering wasps are singly parasitized (Hughes *et al*. 2004*a*); nevertheless, in artificial infections 12 wasps harbouring 2 *X. vesparum* females have been used in the laboratory as useful 'containers' of triungulins (*unpublished data*).

Although larvipositing on nests via several stylopized wasps might be a source of different parasitic strains in the same colony and wasp, a phoretic infection mechanism might more parsimoniously explain intra-colony genetic diversity. The high genetic variability of the parasites from the Trespiano nest is likely to be the result of a phoretic infection by triungulins carried back from flowers by the foundress. In this area – a cemetery near Florence – the parasite prevalence is extremely high (see area E in Hughes *et al*. 2003); nesting sites – usually inside cemetery candle holders – are very close to each other and there are few foraging patches, i.e. flower-beds. On the other hand, the presence of 1–2 haplotypes in the other nests might be due to phoretic infection by several *Xenos* females, sharing the same haplotype. To date, our mt-haplotype analysis can only suggest 2 not exclusive mechanisms of infection in agreement with previous data from dissections of immatures in natural nests (Hughes *et al*. 2003).

Superparasitism and local competition for resources

Superparasitism by *Xenos* spp. is widespread in *Polistes* wasps (see Hughes *et al*. 2004*a*). Nevertheless, although host-seeking larvae face ecological constraints against host selection (Brodeur and Boivin, 2004), in the case of aggregated infection targets they could discriminate unparasitized from parasitized larvae (Royer *et al*. 1999). The synchronous emergence of healthy, singly- and multi-infected wasps from all the nests does not fit with any host selection by triungulins for unparasitized larvae, in agreement with preliminary double-choice tests (Hughes, 2003) and re-infection trials in the laboratory (Manfredini *et al*. 2007). Thus, superparasitism by *Xenos* is not simply explicable in terms of the limited availability of unparasitized hosts. Indeed, superparasitism could be promoted by the high cost of a prolonged host discrimination by triungulins, i.e. the risk of their being removed from larvae by nurse wasps (because of this behaviour adult wasps are usually removed from the nest for 30 min after artificial infections).

Interestingly, half of pluristylopized wasps are affected by parasites bearing different haplotypes, and the frequency of 'conspecific superparasitism' (van Alphen and Visser, 1990) might be underestimated (see above). To our knowledge, this is the first described case of superparasitism by non-sibling host-seeking parasite larvae infecting a single host specimen. Triungulins lack fighting mandibles, and thus may be defined as 'gregarious' parasites (Godfray, 1994). On the contrary, only 1 of 9 larvae can successfully develop into a pupa in bumblebees parasitized by conopid flies (Schmid-Hempel and

Schmid-Hempel, 1989). Immatures would be more likely to develop 'gregariously' in a host (Brodeur and Boivin, 2004), if multiple infections occur at the same time (by phoresy or – more likely – by a direct release of triungulins on nests).

In this sample, regardless of genetic diversity, we found a general synchronized development, suggesting a limited intra-sexual competition for resources. Moreover, male and female parasites develop together in about half of our samples, apparently without any sex avoidance. *Polistes* wasps with *Xenos* empty puparia and extruded females are commonly collected (Hughes *et al*. 2004*a*). Nevertheless, the male-biased sex ratio and the occurrence of fully extruded females only in wasps infected by 1 or 2 females suggest an inter-sexual competition for local resources between immatures: the cephalotheca extrudes before the cephalothorax in multi-infected wasps, in 5 of which we found undeveloped not sexed larvae. In gregarious species of parasites/parasitoids, as well as in solitary species that attack group-living hosts, males tend to emerge first and then wait for females (Godfray, 1994). In our *Xenos*/*Polistes* system, mating and auto-infection in the same nest is unlikely, because infected wasps leave the colony before the parasites are extruded (Hughes *et al*. 2004*b*), and mating occurs in extra-nidal aggregations (Beani *et al*. 2005*a*). The successful development of more than 1 parasite of both sexes in a single specimen is probably due to the apparent lack of high cost to the host during the larval stages (Hughes and Kathirithamby, 2005). Although competition for space has been hypothesized in adult stylopized wasps (Dunkle, 1979), competition among parasites during their development is absent or very limited, therefore relaxing constraints against 'self' as well as 'conspecific superparasitism'.

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