Occurrence and Transmission of *Wolbachia* Endosymbionts in the Oak Gall Wasp Community: Application of Denaturing Gradient Gel Electrophoresis

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by

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Abbreviations

Ac – *Andricus curvator*

Aq – *Andricus quadralineatus*

Asp. – *Aulogymnus* species

A / T – Adenine / Thymidine

B – sampling site B (Bute Park)

BLAST – basic local alignment search tool

bp – base pairs

Bp – *Biorhiza pallida*

Bsp. – braconid species

C – sampling site C (Cosmeston Park)

Csp. – *Cecidostiba* species

CI – cytoplasmic incompatibility

D – sampling site D (Forest of Dean)

dATP – 2'-deoxyadenosine 5'-triphosphate

dCTP – 2'-deoxycytidine 5'-triphosphate

DGGE – denaturing gradient gel electrophoresis

dGTP – 2′-deoxyguanosine 5′-triphosphate

dNTP – deoxyribose nucleotide triphosphate

dsDNA – double stranded DNA

dTTP – 2'-deoxythymidine 5'-triphosphate

EDTA – ethylenediaminetetraacetic acid

F – feminisation

F1 – sampling site France 1

G / C – guanine / cytosine

H – sampling site H (Heath Park)

Abbreviations

H1 – sampling site Hungary 1

HCl – hydrochloric acid

IPTG – isopropyl β-D-thiogalactopyranoside

Kb – kilo base pairs

L – sampling site L (Llanishen)

Mb – mega base pairs

Mf – *Mesopolobus fasciiventris*

Md – *Megastigmus dorsalis*

MK – male killing

MLST – Multilocus sequence typing

Ms – *Mesopolobus sericeus*

Mt – *Mesopolobus tibialis*

NaCl – sodium chloride

Nn – *Neuroterus numismalis*

Nq – *Neuroterus quercusbaccarum*

PAGE – polyacrylamide gel electrophoresis

PCR – polymerase chain reaction

PI – parthenogenesis induction

P-symbiont – primary symbiont

RFLP – restriction fragment length polymorphism

S – sampling site S (Sirhowy Park)

S1 – sampling site Spain 1

Sal – *Synergus albipes*

Sap – *Synergus apicalis*

SDS – sodium dodecyl sulphate

Abbreviations

Sg – *Synergus gallaepomiformis*

Sn – *Synergus nervosus*

SOPE - *Sitophilus oryzae* primary symbiont

sp. – species

spp. – species (plural)

SSCP – single strand conformational polymorphism

ssDNA – single stranded DNA

S-symbiont – secondary symbiont

TAE – tris-acetate-EDTA

TBE – tris-borate-EDTA

Ta – *Torymus auratus*

TCA - tricarboxylic acid cycle

Tf - *Torymus flavipes*

Tg - *Torymus geranii*

Tm – melting temperature

TRFLP – terminal restriction fragment length polymorphism

Tris – 2-Amino-2-(hydroxymethyl)-1,3-propanediol

U – sampling site U (Usk)

U1 – sampling site UK 1

wMel – *Wolbachia* from *Drosophila melanogaster*

X-Gal – 5-bromo-4-chloro-3-indolyl-β-D-galactoside

Abstract

The *Wolbachia* genus is a complex of bacterial endosymbionts from the *Alphaproteobacteria* that have been found in arthropods and nematodes, and are capable of manipulating the reproduction of their arthropod hosts to ensure their own transmission. A rapid screening method involving denaturing gradient gel electrophoresis (DGGE) was developed for the discrimination of *Wolbachia wsp* sequence variants. This was compared with the established 16S rRNA gene and *Wolbachia* specific *wsp* gene, cloning and DNA sequencing screening approaches to detect the diversity of *Wolbachia* infection in members of the oak gall wasp community. DGGE was found to be sensitive and reproducible, and significantly reduced the need for cloning and sequencing.

The oak gall wasp (Hymenoptera: Cynipidae: Cynipini) community represents an attractive system for the study of *Wolbachia* because each wasp-induced gall supports a characteristic, species rich and ecologically closed community of gall-causer, inquiline and parasitoid wasp species. The wasp assemblages (total of 19 species) associated with 5 species of oak gall wasp; *Andricus curvator*, *A. quadralineatus*, *Biorhiza pallida*, *Neuroterus numismalis* and *N. quercusbaccarum*, were screened for the presence or absence of *Wolbachia*, and the diversity of infection was determined using DGGE.

Nineteen species of wasp were reared and identified from 253 galls collected from South Wales, and 10 species (53%) were found to be infected with *Wolbachia*. DGGE was optimised to allow discrimination of amplimers (~600 bp) of the marker gene *wsp* differing by as little as 1 bp (0.17%). A- and B-clade *Wolbachia* variants were clearly separated, and double and triple infections were easily detected.

Eight *Wolbachia* variants were identified in the wasp community, including two double infections. Use of DGGE facilitated screening of large numbers (256) of infected samples, resulting in the detection of rare infection types. Identical *Wolbachia wsp* sequence variants were identified in inquiline and parasitoid wasp species suggesting that horizontal transmission of *Wolbachia* occurs in this community.

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Chapter 1

An introduction to *Wolbachia*, obligate endosymbionts of insects, and studying *Wolbachia* transmission in the oak gall wasp feeding community

1.1 Wolbachia

The genus *Wolbachia* is a complex of intracellular bacteria that form a monophyletic group in the *Alphaproteobacteria*. They are closely related phylogenetically to species in the *Cowdria*, *Ehrlichia*, *Anaplasma* and *Neorickettsia* genera (family *Anaplasmataceae*, order *Rickettsiales*) (Dumler *et al.*, 2001; Garitty *et al.*, 2002; Uilenberg *et al.*, 2004). According to Bergey's Manual of Systematic Bacteriology (Garrity *et al.*, 2002), the *Wolbachia* genus is comprised of three species: *W. pipientis*, *W. persica* and *W. melphagi*. *W. pipientis*, which was first seen and subsequently identified in the mosquito *Culex pipiens* (Hertig & Wolbach, 1924; Hertig 1936), is the type species for this genus and shows significant ecological and genetic differences to the other two species. It has therefore been suggested that *W. persica* and *W. melophagi* should be removed from the genus (Dumler *et al.*, 2001; O'Neill *et al.*, 1997a). For the purpose of this study, the term *Wolbachia* refers to the group of species / strains that comprise the *Wolbachia pipientis* genus (see below).

Wolbachia are obligate endosymbionts of arthropods and filarial nematodes. According to 16S rRNA gene sequence analysis, they are intermediately related to both the tick-transmitted groups from *Ehrlichia* and *Anaplasma* and the helminth-borne *Neorickettsia*, but unlike these close relatives, *Wolbachia* have not been detected in vertebrates. Members of the *Anaplasma* and *Ehrlichia* have not been cultivated in cell-free media or chicken embryos, but some species are cultivatable in mammalian and tick cell lines, and *Wolbachia* have recently been cultivated in mammalian cell lines, revealing a broader potential host range than previously thought (Fenollar *et al.*, 2003; Noda *et al.*, 2002).

Wolbachia are gram-negative, rod-shaped to coccoid and have three enveloping layers, including an outer membrane of host origin (Oh *et al.*, 2000), much like other members of the *Anaplasmataceae.* Members of the family *Rickettsiaceae* (*Rickettsia, Orientia*, order *Rickettsiales*) occupy intra-cytoplasmic compartments and *Rickettsia* have been cultured *in vitro*.

The genus *Wolbachia* is currently divided into 6 supergroups based on 16S rRNA (Fig. 1.1) and *ftsZ* (cell division protein; Werren *et al.*, 1995b) gene sequences, but as

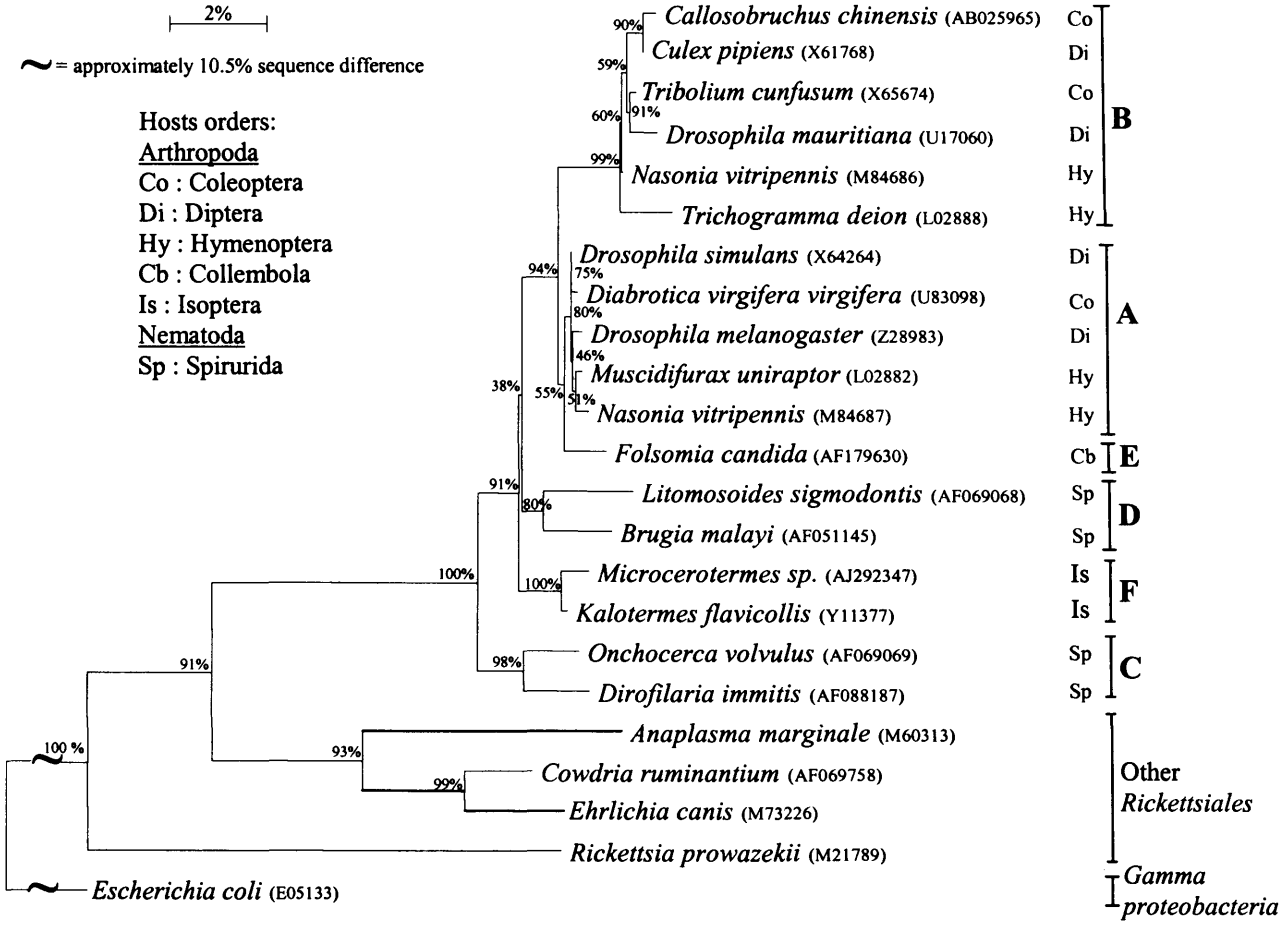

FIG. 1.1 *Wolbachia* phylogeny. Phylogenetic tree based on analysis of 16S rRNA gene sequences from Genbank, constructed using Jukes-Cantor to compare ClustalX aligned sequences (850 bp) followed by Neighbour Joining. Bootstrapping was carried out at 1000 replicates. *Wolbachia* strains are characterized by their host species names and GenBank accession numbers are given in parenthesis. Group designations are according to literature (Lo *et al.*, 2002; O'Neil *et al.*, 1992: Rousset *et al.*, 1992; Stouthamer *et al.*, 1993).

stated above, these may eventually be reclassified as more than one species (Werren, 1997). Supergroups A, B, E and F include variants from crustaceans, arachnids and insects (O'Neill *et al.*, 1992; Vandekerckhove *et al.*, 1999; Werren *et al.*, 1995b). The A- and B-clade variants are extremely widespread among insects and have been detected in several of the major groups, including the Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera and Orthoptera (Jeyaprakash & Hoy, 2000; Werren *et al.*, 1995a; 1995b; Werren & Windsor, 2000; West *et al.*, 1998). Variants from clades C and D are found only in filarial nematodes, and E- and F-clade variants have been found only in springtails and termites, respectively (Bandi *et al.*, 1998; Lo *et al.*, 2002; Vandekerchove *et al.*, 1999).

The faster evolving *wsp* (*Wolbachia* surface protein; Braig *et al.*, 1998) is the standard gene for strain identification and phylogenetic reconstruction in *Wolbachia*. *Wolbachia* that differ in *wsp* nucleotide sequence have been designated as different strains, and strains showing ≥97.5% nucleotide sequence similarity have been grouped together in 'subgroups' (Zhou *et al.*, 1998). Where *Wolbachia* with identical *wsp* sequences have been shown to induce different phenotypic effects in different hosts, unique strain designations have also been given.

However, there is increasing evidence to suggest that this system in inappropriate. The *wsp* gene is now believed to be under positive selection and to undergo recombination, and differences in substitution rates between *Wolbachia* lineages have been detected (Jiggins, 2002; Jiggins *et al.*, 2001c; 2002b; von der Schulenberg *et al.*, 2000). Therefore, for the purpose of this review, *Wolbachia* that have been distinguished based only upon single, or few gene sequences will be referred to as variants or sequence variants (seqvar) (Boutzis, 2003).

1.2 Bacterial symbionts of insects

'Symbiosis is the acquisition and maintenance of one or more organisms by another that results in novel structures and metabolism. Some symbiotic evolution may involve partner genetic exchanges' (Zook, 1998). Bacterial endosymbionts are extremely common among insects and *Wolbachia* are one of the most prevalent species, having been detected in 16-76% of the species tested (Jeyaprakash & Hoy, 2000; Kikuchi & Fukatsu, 2003; Kittayapong *et al.*, 2000; Reuter & Keller, 2003; Ricci *et al.*, 2002; Rokas *et al.*, 2001; Werren *et al.*, 1995a; West *et al.*, 1998). Interactions between the host and its bacterial symbiont range from casual to obligatory and can be broadly classified as mutualistic

(beneficial), commensal (neutral) or pathogenic (harmful). The association may be complex, involving alteration from one classification to another during the life of the host (Ishikawa, 2003; O'Neill *et al.*, 1997a; Rio *et al.*, 2003; Werren & O'Neill, 1997).

Many insect species harbor bacteria within the gut lumen that are important for the breakdown, mineralization and cycling of organic compounds. These associations are highly variable, ranging from facultative to mutually obligate (Dillon & Dillon, 2004; Reeson *et al.*, 2003).

Intracellular symbionts (endosymbionts) are vertically transmitted through host generations and associations range from mutualistic (primary endosymbionts), to facultative (secondary endosymbionts), to parasitic (reproductive parasites). Members of diverse bacterial groups have evolved intimate associations with eukaryotes. As shown in Table 1.1, most endosymbionts of insects belong to the *Proteobacteria*, primarily the gamma division, but some have also been identified in the alpha and beta divisions. Endosymbionts from the *Bacteroidetes* have been identified in cockroaches and wasps, and *Spiroplasma* symbionts are associated with several insect groups (Clark & Kambhampati, 2003; Fukatsu *et al.*, 2001; Williamson *et al.*, 1998).

Primary endosymbionts (P-symbionts) are restricted to specialized cells called bacteriocytes (or mycteocytes), which are assembled into organs called bacteriomes (mycetomes). These bacteria provide essential nutrients that are absent from the host diet. They are obligately associated with their hosts and strictly vertically transmitted, resulting in congruence between the host and symbiont phylogenies (Akman *et al.*, 2001; Chen *et al.*, 1999; Clark *et al.*, 1992; Munson *et al.*, 1991; Sauer *et al.*, 2000). Examples of P-symbionts are shown in Table 1.1.

In contrast to primary symbionts, secondary symbionts (S-symbionts) are thought to have been acquired by their hosts more recently (Table 1.1). Infections are more sporadic and less stable, and there is evidence to suggest that horizontal transmission has occurred (Akman *et al.*, 2001; Hypsa & Askoy, 1997; Moran & Telang, 1998). S-symbionts have been found in bacteriocytes, midgut cells, and many other tissues (Ishikawa, 2003). Most appear to contribute to the host's nutrition but they are not essential for the host's survival and some S-symbionts have been cultivated *in vitro* (Akman *et al.*, 2001).

Some bacterial symbionts are more parasitic in their nature, manipulating the reproduction of their host to promote their own transmission and may produce negative effects on their host. These reproductive distorters, which include *Wolbachia*, are

TABLE 1.1 Endosymbionts of insects

Host	Symbiont	Taxonomic division / subdivision	Location in host[a]	References
Aphids (Hemiptera)	*Buchnera aphidicola* P-symbiont	*Gamma-proteobacteria*	Primary-bacteriocyte	Baumann *et al.*, 1995
	R-type symbiont[b]	*Gamma-proteobacteria*	Sheath cells	Fukatsu *et al.*, 2000
			S-bacteriocyte	Chen & Purcell, 1997
	T-type symbiont[c]	"	& other	Sandström *et al.*, 2001
	U-type symbiont	"	locations	Unterman *et al.*, 1989
	Ars-symbiont (*Arsenophonus*)	*Gamma-proteobacteria*	-	Russell *et al.*, 2003
	So-So (like SOPE)	"		
	V-type symbiont	"		
	PAR-Pea aphid *Rickettsia*	*Alpha-proteobacteria*	Haemolymph	Chen *et al.*, 1996
	Spiroplasma	*Mollicutes*	Haemolymph	Fukatsu *et al.*, 2001
Whiteflies (Hemiptera)	P-symbiont *Candidatus* Portiera aleyrodidarum	*Gamma-proteobacteria*	Bacteriocyte	Clark *et al.*, 1992 Zchori-Fein & Brown, 2002 Baumann *et al.*, 2004
	S-symbiont	*Gamma-proteobacteria*	-	Clark *et al.*, 1992 Zchori-Fein & Brown, 2002
	S-symbiont *Arsenophonus*	*Gamma-proteobacteria*	-	Thao & Baumann, 2004 Spaulding & von Dohlen, 2001
	Rickettsia	*Alpha-proteobacteria*	-	Spaulding & von Dohlen, 2001
Mealybugs (Hemiptera)	P-symbiont *Candidatus* Tremblaya princes	*Beta-proteobacteria*	Bacteriocyte	Munson *et al.*, 1992
Planthoppers (Hemiptera)	Yeast-like symbionts	*Ascomycetes*	Fat body	Noda *et al.*, 1995
Leafhoppers (Hemiptera)	P-symbiont *Candidatus* Baumannia cicadellinicola	*Gamma-proteobacteria*	Bacteriocyte	Moran *et al.*, 2003
	S-symbiont	*Gamma-proteobacteria*		Campbell & Purcell, 1993
Pseudococcids (Hemiptera)	P-symbiont	*Beta-proteobacteria*	Bacteriocytes	Fukatsu & Nikoh, 2000
	S-symbiont	*Gamma-proteobacteria*		
	Spiroplasma	*Mollicutes*		
Psyllids (Hemiptera)	P (X)-symbionts – *Candidatus* Carsonella (gen. nov.)	*Gamma-proteobacteria*	Bacteriocytes	Thao *et al.*, 2000a; 2000b Spaulding & Dohlen, 1998
	S (Y)-symbionts Arsenophonus	*Gamma-proteobacteria*	Syncytial cytoplasm	Fukatsu & Nikoh, 1998
Kissing bugs (Hemiptera)	*Arsenophonus*	*Gamma-proteobacteria*		Hypsa & Dale, 1997

TABLE 1.1 Continued

Host	Symbiont	Taxonomic division / subdivision	Location in host[a]	References
Bedbugs (Hemiptera)	P-symbiont S-symbiont	*Gamma-proteobacteria*	Ovaries	Hypsa & Aksoy, 1997
Fruitflies (Diptera)	*Spiroplasma*	*Mollicutes*	-	Hurst & Jiggins, 2000
Tsetse flies (Diptera)	P-symbiont *Wigglesworthia glossinidia* S-symbiont - *Sodalis glossinidius*	*Gamma-proteobacteria* *Gamma-proteobacteria*	Bacteriocytes in gut Midgut cells	Aksoy, 1995 Chen *et al.*, 1999 Beard, 1993; Aksoy, 1995
Butterflies (Lepidoptera)	*Rickettsia*	*Alpha-proteobacteria*	-	Jiggins *et al.*, 1998
		Bacteroidetes	-	Hurst *et al.*, 1997
Beetles (Coleoptera)	Yeast-like symbionts	*Ascomycetes*	Myceteocytes	Noda & Kodama, 1996
	Rickettsia	*Alpha-proteobacteria*		Hurst *et al.*, 1999
Weevils (Coleoptera)	SOPE[d]	*Gamma-proteobacteria*	Bacteriocytes	Campbell *et al.*, 1992 Charles *et al.*, 1997
Parasitic wasps (Hymenoptera)	S-symbiont *Arsenophonus*	*Gamma-proteobacteria*	-	Gherna *et al.*, 1991
	Candidatus Cardinium hertigii	*Bacteroidetes*	Ovaries	Hunter *et al.*, 2003; Weeks *et al.*, 2001; Zchori-Fein *et al.*, 2001; 2004
Carpenter ants (Hymenoptera)	P-symbiont - *Candidatus* Blochmannia camponotis	*Gamma-proteobacteria*	Bacteriocytes	Schröder *et al.*, 1996 Wernegreen *et al.*, 2002 Sauer *et al.*, 2000
Cockroaches (Blattaria)	*Blattabacterium cuenoti*	*Bacteroidetes*	Bacteriocytes	Bandi *et al.*, 1994; 1995
Termites[e] (Isoptera)	*Blattabacterium cuenoti*	*Bacteroidetes*	Bacteriocytes	Bandi *et al.*, 1995
Sucking Lice (Anoplura)	Many bacterial spp	-	Bacteriocytes	Wernegreen, 2002
Fleas (Siphonaptera)	*Rickettsia* sp.	*Alpha-proteobacteria*	Haemolymph	Azad & Beard, 1998
Ticks (Ixodida)	*Rickettsia* sp.	*Alpha-proteobacteria*	Haemolymph	Azad & Beard, 1998
Arthropods	*Spiroplasma*	*Mollicutes*	-	Williamson *et al.*, 1998
Arthropods from all genera	*Wolbachia*	*Alpha-proteobacteria*	Most tissues	O'Neill *et al.*, 1992 Werren *et al.*, 1997

[a] - : location undetermined. [b] R-type: Also known as S-sym & pea aphid secondary symbiont (PASS). [C] T-type: Also known as pea aphid Bemisia-like symbiont (PABS).[d] SOPE: *Sitophilus oryzae* primary symbiont [e]Single species: *Mastotermes darwiniensis.*

vertically inherited but are not restricted to the host's reproductive tissues (Aksoy, 1995, 1997; Chen *et al.*, 1996; Fukatsu *et al.*, 2000).

Although *Wolbachia* behave more like parasites in their arthropod hosts, the association is obligate for the endosymbionts and *Wolbachia* have not been cultured *in vitro*. In their filarial nematode hosts the association is more mutualistic, both the bacteria and the worm are dependent on each other for survival, and elimination of *Wolbachia* using antibiotics leads ultimately to the death of the adult nematode (Bandi *et al.*, 2001; Taylor, 2002).

1.2.1 Genome evolution in bacterial endosymbionts

Due to the difficulty of working with obligate intracellular bacteria, little is currently known about the molecular mechanisms of *Wolbachia*-host interactions but with the completion of the genome sequence of several strains of *Wolbachia*, new insights may be gained (Foster *et al.*, 2004; Wu *et al.*, 2004; see Section 5.0).

The number of bacterial endosymbionts for which genome sequence information is available is increasing and comparative genome analysis has provided some interesting insights into the evolution of endosymbiont genomes. Mutualistic intracellular bacteria and some pathogenic intracellular bacteria show evidence of an increased rate of sequence change and a greater rate of substitution in functional genes than seen in their free-living relatives. This is thought to be a result of relaxed purifying selection and accumulation of deleterious mutations due to the small population size of bacteria transmitted to the progeny, and changes in the functional requirements of genes. Also, an increased rate of mutation may result from the loss of DNA repair genes in endosymbionts (Itoh *et al.*, 2002; Moran, 1996). As a result, a reduction of the G / C content of the genome may also be a feature of intracellular lifestyle (Table 1.2) (Heddi *et al.*, 1998), but the relationship is unequivocal as both intracellular and free-living bacteria show extremes of G / C content; e.g. *Candidatus* Tremblaya princeps, the primary endosymbionts of mealybugs have a G / C content of 57.1%, and SOPE from *Sitophilus oryzae* have a 55% G / C content, which is close to many free-living bacteria (Baumann *et al.*, 2002; Blattner *et al.*, 1997; Herbeck *et al.*, 2003; Thao *et al.*, 2002).

Some obligate intracellular bacteria obtain metabolic precursors from their hosts and have lost many of the genes from biosynthetic pathways. These bacteria often have greatly reduced genomes in comparison to their free living relatives (Table 1.2) but maintain a basic set of essential genes. Mutualistic endosymbionts often provide their

TABLE 1.2 Genomic features of bacterial endosymbionts of insects. Information about closely related intra- and extracellular bacteria is included for comparison.

Organism	Host	Taxonomic division/ subdivision	Relationship	Tissue location	Mode of Transmission	Genome size (Mb)[a]	GC content	Coding capacity	rRNA operon copy number	Reference
Buchnera aphidicola	Aphid species (Hemiptera)	*Gamma-proteobacteria*	P-symbiont Mutualistic Uncultivated[d]	Bacteriocyte	Vertical	0.45 – 0.67	26%	88%	1	Shigenobu *et al.*, 2000 van Ham *et al.*, 2003; Charles & Ishikawa, 1999 Wernegreen *et al.*, 2000 Gil *et al.*, 2002
Wigglesworthia glossinidia	*Glossina brevipalpis* (Diptera)	*Gamma-proteobacteria*	Mutualistic Uncultivated	Bacteriocyte	Vertical	0.75	22%	89%	2	Akman *et al.*, 2004 Akman & Aksoy, 2001
Candidatus Blochmannia camponotus	Carpenter ants (Hymenoptera)	*Gamma-proteobacteria*	P-symbiont Mutualistic	Bacteriocyte	Vertical	0.7	27.38%	83.2%	1	Sauer *et al.*, 2000; 2002 Gil *et al.*, 2003 Wernegreen *et al.*, 2002
SOPE[b]	*Sitophilus oryzae* (Coleoptera)	*Gamma-proteobacteria*	P-symbiont Mutualistic	Bacteriocyte	Vertical	3	54%	-	-	Akman, 2001 Heddi *et al.*, 1998 Charles *et al.*, 1997
Candidatus Baumannia cicadellinicola (sp. nov)	Leafhoppers, sharpshooters (Bugs)	*Gamma-proteobacteria*	P-symbiont Uncultivated	Bacteriocyte	Vertical	0.68	36.7%[c]	-	-	Moran *et al.*, 2003
Candidatus Portiera aleyrodidarum	*Bemisia tabaci* (Diptera)	*Gamma-proteobacteria*	P-symbiont Mutualistic	Bacteriocyte	Vertical	33 Kb segment	30-2%	71.2%	1	Baumann *et al.*, 2004
Candidatus Tremblaya princeps (sp. nov)	Mealybugs (Hemiptera)	*Beta-proteobacteria*	P-symbiont Mutualistic	Bacteriocyte	Vertical	65 Kb segment	57.1%	81.6%	2	Baumann *et al.*, 2002 Thao *et al.*, 2002
Blattabacterium sp.	*Cryptocercus relictus* (Blattaria)	*Bacteroidetes*	Unknown	Bacteriocyte	Vertical	-	-	-	-	Clark & Kambhampati, 2003; Bandi *et al.*, 1995

TABLE 1.2 continued

Organism	Host	Taxon	Relationship	Tissue location	Mode of Transmission	Genome size (Mb)	GC content	Coding capacity	rRNA operon copy number	Reference
Candidatus Carsonella ruddi	Psyllids (Hemiptera)	*Gamma-proteobacteria*	P-symbiont Uncultivated	Bacteriocyte	Vertical	37 Kb segment	19.9%	>99.9%	1	Thao *et al.*, 2000a; 2000b; 2001; Spaulding & von Dohlen, 2001 Clark *et al.*, 2001
Wolbachia pipientis	*Brugia malayi*	*Alpha-proteobacteria*	Mutualistic Uncultivated	Reproductive tissues	Vertical	1.1	35%	-	-	Foster *et al.*, 2004 Ware *et al.*, 2002 Sun *et al.*, 2001
Wolbachia pipientis	*Drosophila melanogaster* (Diptera)	*Alpha-proteobacteria*	Reproductive parasite Uncultivated	Reproductive & somatic tissues	Vertical & Horizontal	1.26	35%	85.4%	1	Wu *et al.*, 2004 Sun *et al.*, 2001 Fenollar *et al.*, 2003 Noda *et al.*, 2002
Sodalis glossinidis	*Glossina brevipalpis* (Diptera)	*Gamma-proteobacteria*	S-symbiont Unknown role Cultivated	Multiple somatic and reproductive tissues	Vertical & Horizontal	2	55%	-	-	Akman *et al.*, 2001 Dale *et al.*, 2001 Dale & Maudlin, 1999
Anaplasma marginale	Rodents &Ticks	*Alpha-proteobacteria*	Obligate Intracellular pathogen Uncultivated	Somatic tissues	Horizontal	1.2	56%	-	-	Alleman *et al.*, 1993 Dumler *et al.*, 2001 Moreno, 1998
Rickettsia prowazekii	Arthropods	*Alpha-proteobacteria*	Obligate Intracellular pathogen Uncultivated	Somatic tissues	Horizontal	1.1	29.1%	76%	1	Andersson *et al.*, 1998
Esherichia coli	Animals	*Gamma-proteobacteria*	Extracellular Cultivated	Gut	Horizontal	4.5-5.5	50.8%	85%	7	Bergthorsson & Ochman, 1998 Blattner, 1997

[a] Where the size of whole genome has not been determined, the size of the characterised segment is given. [b] SOPE: *Sitophilus oryzae* primary symbiont. [c] GC content measured in 2.9 Kb fragment of *rpoBC* gene. [d] In cell-free media

hosts with small metabolites such as amino acids, supplementing nutrient poor diets, and may have multiple copies of genes for the biosynthesis of these compounds. For example, 10% of the *Buchnera* genome is dedicated to the biosynthesis of amino acids (Tamas & Andersson, 2003). As shown by Table 1.2, most obligate endosymbionts have only 1 copy of the rRNA operon, reflecting the relatively stable host environment. Free-living bacteria such as *Escherichia coli* have multiple operon copies, allowing them to respond to environmental change more rapidly.

Sequencing of the 1.26 Mb genome of the wMel strain of *Wolbachia*, originally isolated from *Drosophila melanogaster*, has been completed (Fig. 1.2; Wu *et al.*, 2004). The genome sequence of the wPip strain from the mosquito *Culex quinquefasciatus* and *Wolbachia* from the filarial nematodes *Onchocerca volvulus* and *Brugia malayi* are currently underway (Foster *et al.*, 2004), details can be found at the Sanger Institute and New England Biolabs websites. Their estimated genome sizes are 1.5 Mb, 1.1 Mb and 1.1 Mb, respectively. In addition, Sun *et al.* (2001) estimated the genome size of *Wolbachia* strains wMelPop (*D. melanogaster*), wRi (*D. simulans* Riverside), wMelCS (*D. melanogaster* Canton-S), wDim (*Dinofilaria immitis)* and wAlbB (*Aedes aplbopictus*) by pulse field gel electrophoresis, indicating that genome size varied between 1.66 - 1.36 Mb in arthropod-associated strains, and was 1.1 Mb and 0.96 Mb in the nematode-associated strains. Studies like these may provide valuable information about the genome changes associated with the evolution of different *Wolbachia*-host associations.

1.3 Wolbachia induced reproductive manipulations and infection dynamics

Reproductive distorters interfere with the sexuality and reproduction of their hosts to promote their own transmission by increasing the proportion or fitness of the female hosts, i.e. the sex through which cytoplasmically inherited microorganisms are transmitted. The manipulations induced by *Wolbachia* are summarised in Box 1.1, and include induction of Cytoplasmic Incompatibility (CI), Parthenogenesis (PI), Feminisation (F) and Male Killing (MK).

The connection between CI and *Wolbachia* was not made until 1973 (Yen & Barr, 1973) but CI has since been shown to be the most widely distributed of the *Wolbachia*-induced phenotypes, occurring in the Diptera (Dobson *et al.*, 2002a; James & Ballard, 2000; Jamnongluk *et al.*, 2002; Kittayapong *et al.*, 2002; Merçot *et al.*, 1995; Werren & Jaenike, 1995), Coleoptera (Hsiao & Hsiao, 1985; Wade & Stevens, 1985), Hemiptera (Noda *et al.*, 2001; Rousset *et al.*, 1992a), Hymenoptera (Perrot-Minnot & Werren, 1999;

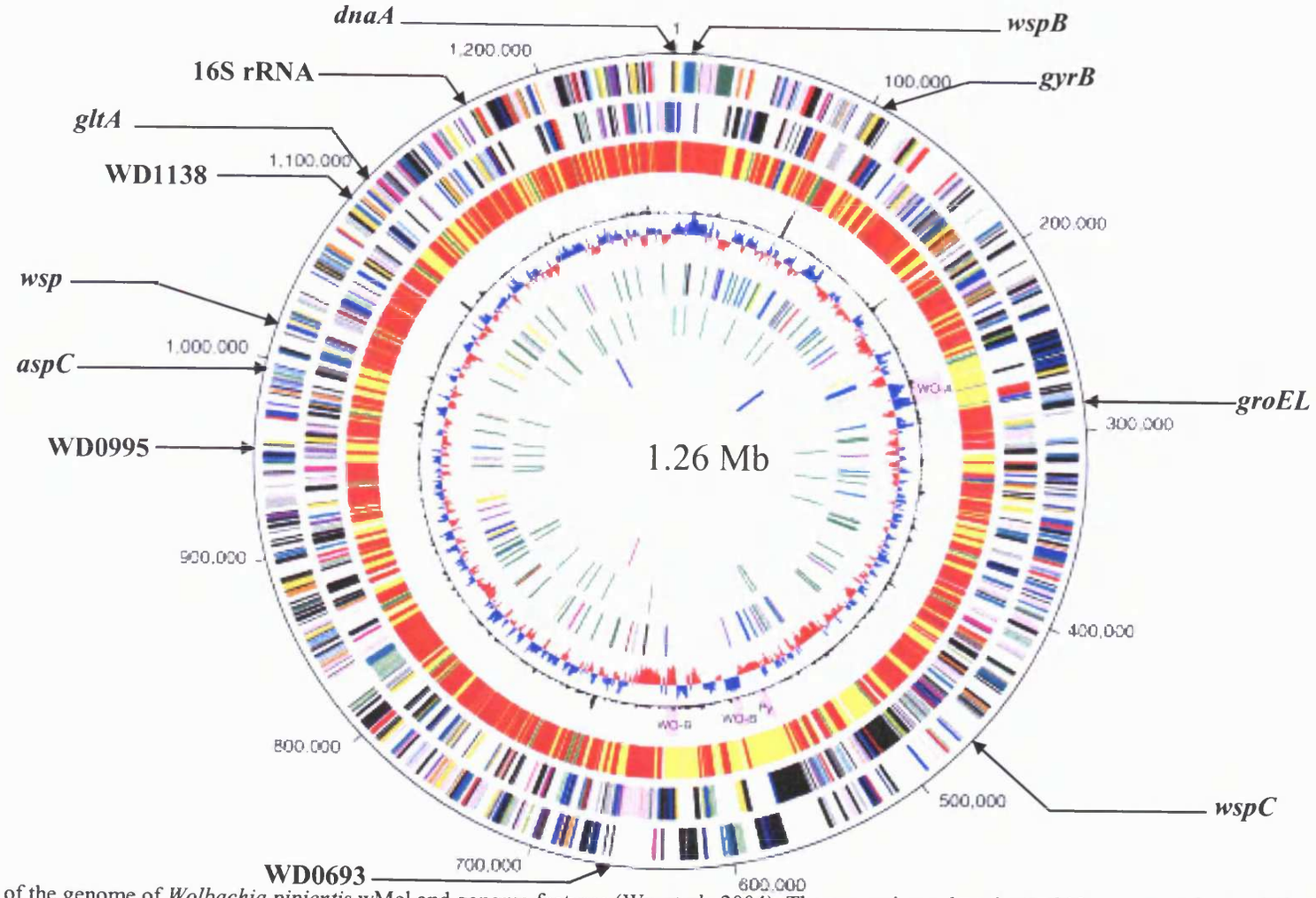

Fig. 1.2 Circular map of the genome of *Wolbachia pipientis* wMel and genome features (Wu *et al.*, 2004). The approximate locations of 12 genes are shown. 16S rRNA, *wsp* and *ftsZ* genes have been used for phylogenetic reconstruction of *Wolbachia* (section 1.1). Two paralogs of the *wsp* gene are shown (*wspB* & *wspC*) and *wspB* could be useful for future phylogenetic reconstruction (section 5.0). The *gyrB*, *gltA*, *dnaA*, *groEL*, *aspC* (*aspAT*), *wsp*, *ftsZ* and 16s rRNA genes may be used in an MLST-based approach for future strain typing (Bourtzis, 2003; section 5.0). The WD0693, WD0995 and WD1138 genes encode putative reverse transcriptases (section 4.3.5).Circles correspond to the following: (1) forward strand genes; (2) reverse strand genes, (3) in red, genes with likely orthologs in both *Rickettsia conorii* and *R. prowazekii*; in blue, genes with likely orthologs in *R. prowazekii*, but absent from *R. conorii*; in green, genes with likely orthologs in *R. conorii* but absent from *R. prowazekii*; in yellow, genes without orthologs in either *Rickettsia*; (4) plot is of $\chi 2$ analysis of nucleotide composition; phage regions are in pink; (5) plot of GC skew (G–C)/(G+C); (6) repeats over 200 bp in length, colored by category; (7) in green, transfer RNAs; (8) in blue, ribosomal RNAs; in red, structural RNA.

Box 1.1 *Wolbachia* induced phenotypes

Cytoplasmic incompatibility (CI)

(Reviewed in Bourtzis *et al.*, 2003; Hoffmann & Turelli, 1997)

Wolbachia-induced CI has been detected in Coleoptera, Diptera, Hemiptera, Hymenoptera, Orthoptera, Lepidoptera, Arachnida and Isopoda (Bourtzis *et al.*, 2003). CI results in reproductive incompatibility between males and females with different *Wolbachia* infections. The modification-rescue model proposed by Werren (1997) is widely accepted: sperm modified by *Wolbachia* during spermatogenesis (mature sperm do not contain *Wolbachia*) must be rescued by a compatible *Wolbachia* CI-type in the female. Unidirectional incompatibility occurs between infected males and uninfected females, and the reciprocal cross is compatible. Bidirectional incompatibility occurs between infected males and females carrying different CI-types. Incompatibility results in the loss of mitotic synchrony, producing haploid embryos (Tram & Sullivan, 2002). This results in embryo mortality in diploid host species and in the production of infected haploid males in haplodiploid host species. Embryo mortality has been seen in one haplodiploid species (Bordenstein & Werren, 1998). In all host species, the selective fitness of infected females is increased relative to uninfected females because they can mate successfully with both infected and uninfected males.

Thelytokous parthenogenesis (PI)

(Reviewed in Huigens & Stouthamer, 2003; Stouthamer, 1997)

Wolbachia induced parthenogenesis appears to be restricted to haplodiploid host species. PI-*Wolbachia* cause infected females to produce diploid offspring (female) without fertilisation. Unfertilised haploid eggs normally produce males. There is evidence that PI occurs by more than one cytological mechanism in haplodiploids, producing both homozygous and heterozygous diploid females (Stouthamer, 1997; Weeks & Breeuwer, 2001).

Male killing (MK)

MK is expressed by a wide range of Eubacteria. *Wolbachia* induced MK has been detected in *Adalia*, *Acraea*, *Ephestia* and *Drosophila* species (Fujii *et al.*, 2001; Hurst *et al.*, 1999; 2000; Sasaki *et al.*, 2002). The mechanism of MK is currently unknown (Hurst *et al.*, 2003). *Wolbachia* induced MK increases the survival and reproductive success of female siblings due to reduced inbreeding, and its associated fitness costs, reduced competition for resources and reduced cannibalism of females.

Feminisation (F)

F-inducing *Wolbachia* have been detected in isopod crustaceans and a moth species (Bouchon *et al.*, 1998; Kageyama *et al.*, 2002; Rousset *et al.*, 1991). In *Armadillidium vulgare*, MK-*Wolbachia* inhibit the response of the host to the male hormone androgen, resulting in the development of functional females from genetic males (Stouthamer *et al.*, 1999).

Van Borm *et al.*, 2001; Vavre *et al.*, 2000), Orthoptera (Kamoda *et al.*, 2000; Mandel *et al.*, 2001), Lepidoptera (Sasaki & Ishikawa, 1999), Arachnida (Egas *et al.*, 2002; Gotoh *et al.*, 1999; Johanowicz & Hoy, 1998; Vala *et al.*, 2000) and Crustacea (Moret *et al.*, 2001).

Thelytokous parthenogenesis induction by *Wolbachia* was first described in *Trichogramma* wasps (Rousset *et al.*, 1992a; Stouthamer & Werren, 1993), and is thought to be restricted to host species with haplodiploid sex determination, in which males normally develop from unfertilised haploid eggs, and females from fertilised diploid eggs.

The crustacean *Armadillium vulgare* was the first host species in which *Wolbachia* induced feminisation was identified, and to date, F-inducing *Wolbachia* have only been found in one insect species, the moth *Ostrinia furnacalis* (Kageyama *et al.*, 2002). The first incidence of *Wolbachia*-induced male-killing was detected in the butterfly *Acraea encedon* and the ladybird *Adalia bipunctata* (Hurst *et al.*, 1999), and fly and wasp species have since been shown to also be affected by this type of *Wolbachia* (Fujii *et al.*, 2001; Hurst *et al.*, 1999; 2000; Sasaki *et al.*, 2002).

Wolbachia are not unique in their ability to manipulate the reproduction of their hosts. CI, PI, MK and F are also induced by members of the *Spiroplasma*, *Rickettsia*, *Arsenophonus* and *Bacteroidetes* (Bandi *et al.*, 1994; Hurst & Jiggins, 2000; Weeks *et al.*, 2001; Werren *et al.*, 1994; Zchori-Fein *et al.*, 2001), but *Wolbachia* have attracted particular attention because they are capable of inducing all four of the manipulations listed above. In addition, these reproductive manipulations can directly affect compatibility between host populations / species, and *Wolbachia* may have contributed to the reproductive isolation and speciation of some hosts (Bordenstein, 2003; Bordenstein *et al.*, 2001; Boutzis & O'Neill, 1998; Jamnongluk *et al.*, 2002; O'Neill *et al.*, 1997a; Pannebakker *et al.*, 2004; Weeks *et al.*, 2002; Weeks & Breeuwer, 2001; Werren, 1997). Recently however, the endosymbiont *Candidatus* Cardinium hertigii, (previously referred to as '*Encarsia* Bacterium' or 'Cytophaga-like organism'), originally described in *Encarsia* wasps, has been shown to induce CI and PI in wasp hosts, and F in the mite species *Brevipalpus phoenicis* (Hunter *et al.*, 2003; Weeks *et al.*, 2001; Zchori-Fein *et al.*, 2001; 2004). This endosymbiont has also been found to occur in 6% of 99 insect species (Zchori-Fein & Perlman, 2004).

CI-, PI- and MK-inducing *Wolbachia* are spread throughout clades A and B, but F-type *Wolbachia* have only been found in clade-B (Bourtzis & O'Neill, 1998). This suggests that the ability to manipulate the host has evolved more than once in the *Wolbachia* genus or perhaps that the phenotype is a host response to infection.

Experimental transfer of *Wolbachia* and introgression studies show that the phenotype induced can be significantly influenced by the host species. Transfer of a CI-inducing strain from *Cadra cautella* (Lepidoptera), or an F-inducing strain from the adzuki bean borer *Ostrinia scapulalis*, to the Mediterranean flour moth *Ephestia kuehniella*, results in expression of MK, suggesting that the MK phenotype is a function of the host *E. kuehniella* (Fujii *et al.*, 2001; Sasaki *et al.*, 2002). Conversely, CI-type *Wolbachia* have been transferred into embryos of both closely related and distantly related host species, and the resulting infection continued to induce CI, suggesting the phenotype is a function of the bacteria (Braig *et al.*, 1994).

It is now known that *Wolbachia* variants can recombine, so independent evolution of the phenotypes can no longer be assumed (Jiggins *et al.*, 2001c). Although information about the mechanism of each phenotype is limited, both CI and PI are known to target the first mitotic divisions (Stouthamer, 1997; Tram & Sullivan, 2002; Weeks & Breeuwer, 2001), suggesting they could have evolved from an ancestral strain / phenotype.

1.3.1 Infection dynamics

Mutualistic symbionts such as *Buchnera* spp. provide their hosts with clear nutritional benefits. *Wolbachia* however, use these reproductive manipulations to ensure they continue to be inherited and spread through the host population. The dynamics of infections have been extensively modelled for CI-*Wolbachia*, though less so for sex-ratio distorting strains. In an isolated population, infection frequency is predicted to increase gradually due to the increased fitness of females infected with CI-type *Wolbachia* (Hoffmann & Turelli, 1997; Turelli, 1994). The infection frequency will not increase if there is a decrease in fecundity associated with the infection or if maternal transmission is less than perfect. Maternal transmission is often imperfect due to segregation during oogenesis, environmental curing (natural antibiotics or elevated temperature), or host age (Charlat *et al.*, 2004; Hoffmann & Turelli, 1997; Hurst *et al.*, 2001), and reduced host fecundity or fitness has been detected in some species (Fleury *et al.*, 2000; Hoffmann & Turelli, 1997; Tagami *et al.*, 2001). As a result, most of the species populations sampled have been found to be polymorphic for *Wolbachia* infection (Jiggins *et al.*, 2002a; Vala *et al.*, 2004)

1.3.2 Evolution of mutualistic *Wolbachia*-arthropod associations

Wolbachia are mutualistic endosymbionts in nematodes and reproductive parasites in arthropods. This is thought to be related to the mode of transmission in the different hosts. In nematodes, bacterial transfer is strictly vertical and bacteria-host associations are long term and stable, resulting in co-evolution and co-adaptation. In arthropods the association is less stable and horizontal transfer (see Section 1.5) is thought to have occurred frequently in the past. The bacteria must balance efficiency of vertical transmission (high replication rates and bacterial load), with any negative effects that a reproductive phenotype has on the host. However, strains in which virulence is attenuated in favour of more efficient vertical transmission cannot compete effectively with more virulent strains that invade by horizontal transfer (Bandi *et al.*, 2001; Dedeine *et al.*, 2003). Selection favours strains that increase the fitness of transmitting individuals (i.e. females), and reduce the fitness of non-transmitting individuals (i.e. males).

However, theoretical models predict that as CI-*Wolbachia* reach high prevalence, the frequency of incompatible matings will decrease and as a result the CI phenotype will no longer be selected for. Therefore, *Wolbachia* could be expected to become more mutualistic due to selection pressures. Some strains that provide direct fitness benefits have been identified (Dedeine *et al.*, 2003; Wade & Chang, 1995). For example, uninfected individuals show lower fecundity in *Trichogramma* wasps and the mosquito *Aedes albopictus* (Dobson *et al.*, 2002b; Grenier et al 2002; Vavre *et al.*, 1999b). Interestingly, *Candidatus* Cardinium hertigii has recently been found to increase the fecundity of the mite *Metaseiulus occidentalis* (Weeks & Stouthamer, 2004), which means that this endosymbiont almost equals *Wolbachia* in the host effects it induces. In addition, several apparently neutral *Wolbachia* variants have been identified (Charlat *et al.*, 2004; Hoffmann *et al.*, 1996; Vavre *et al.*, 2002), including non-CI-*Wolbachia* that are thought to have derived from CI-*Wolbachia* (Charlat *et al.*, 2003; Turelli, 1994). Such strains are predicted to be lost from the population unless maternal transmission is perfect or there is some fitness benefit associated with the infection (Charlat *et al.*, 2004; Hoffmann *et al.*, 1996).

1.4 *Wolbachia* as a method of biocontrol

Wolbachia naturally infect several agricultural pest and disease carrying insect species, and therefore represent an attractive system for use in biocontrol, and because they

naturally infect so many different insect species, a strategy developed to control one pest may be adaptable to several other pest species.

A CI-inducing *Wolbachia* strain will sweep through a host population if the initial infection frequency is high enough, and the cost to fecundity is low enough, eventually replacing the native host population (Section 1.3). It may be possible to exploit this ability, and to use *Wolbachia* to combat pest / disease causing insect species, by means one of several mechanisms: 1) To replace a disease causing host population with a population of harmless counterparts. For instance, mosquito populations carrying the malarial parasite could be replaced with *Plasmodium*-free mosquitoes. 2) To promote the spread of a mortality-inducing *Wolbachia* variant that will shorten the adult lifespan of a disease transmitting host. The 'popcorn' variant from *D. melanogaster* causes massive tissue damage due to over-replication of the endosymbiont (McGraw *et al.*, 2001; Min & Benzer, 1997). 3) To promote the spread of a second cytoplasmic element, or useful nuclear genes introduced into the host genome. 4) It may be possible to engineer transgenic *Wolbachia* to express genes that inhibit the pest species. This has already been attempted in the secondary symbiont of the tsetse fly, which has initially been transformed to express the green fluorescent protein (GFP), showing that the transgenic symbiont can be successfully reintroduced into the host (Cheng & Aksoy, 1999). In addition, Durvasula *et al.* (1997) successfully transformed the *Rhodococcus rhodnii* extracellular symbiont of the heteropteran bug species *Rhodnius prolixus*, to express a protein that is lethal to the parasitic cause of Chaga's disease, *Trypanosoma cruzi*.

Due to phylogenetic and transfection studies, it is already known that the same strain of *Wolbachia* can infect more than one species of host, therefore it may be possible to create an infection with whichever strain of *Wolbachia* is most suitable. If the strain induces CI in its natural host, it may also do so in the pest host. The advantage of this strategy is that there is no reduction of fitness of the released population, and repeated populations sweeps using different strains could be carried out.

In practice it may not be so simple. Not all strains will become established in novel hosts (Rigaud *et al.*, 2001) and several transfection studies have also shown that a CI-inducing strain from one host may in fact induce a completely different phenotype in another host (Fujii *et al.*, 2001; Sasaki *et al.*, 2002; Section 1.3). Also, the environment will have a significant effect on the spread and maintenance of the infection: several strains have been characterised from laboratory stocks but the infection dynamics are often very different in nature (Hoffman, 1990; 1998; Hoffmann & Turelli, 1995; Olsen *et*

al., 2001; Rasgon & Scott, 2003). Temperature changes or host diapause has been seen to lead to total loss of infection or loss of one half of a double infection due to different tolerance levels of dissimilar strains (Keller *et al.*, 2004).

Detailed information about the infection status of the target population would be needed, including the possibility of horizontal transmission of *Wolbachia* between the target and other host species. This would involve large scale screening of the pest population and trials to ensure complete incompatibility. There are also cost considerations and the added concern associated with releasing large numbers of host insects that are natural disease carriers, into the population.

1.5 Horizontal transfer of *Wolbachia*

Horizontal transmission of these endosymbionts is believed to have occurred frequently in the past for several reasons. The phylogenies of arthropod host and bacterial endosymbiont are incongruent. Distantly related host species may be infected with closely related or identical *Wolbachia* variants and several host species have been found to carry more than one, distantly related infection, often occurring as multiple infections. For example, the wasp species *Leptopilina heterotoma* carries a variant identical to that found in the fly *Drosophila simulans* (Riverside strain) and the wMors subgroup contains *Wolbachia* variants found in species from the Diptera, Hemiptera and Hymenoptera (Kikuchi & Fukatsu, 2003; Rokas *et al.*, 2002a; Vavre *et al.*, 1999; Zhou *et al.*, 1998). Multiple infections have been found in insects from several different orders (Baurdry *et al.*, 2003; Breeuwer *et al.*, 1992; Ijichi *et al.*, 2002; Jamnongluk *et al.*, 2002; Kikuchi & Fukatsu, 2003; Kondo *et al.*, 2002; Mercot *et al.*, 1995; Mitsuhashi *et al.*, 2002; Nirgianaki *et al.*, 2003; Riegler & Stauffer, 2002; Van Borm *et al.*, 2003; Vavre *et al.*, 1999; Wenseleers *et al.*, 1998; Werren & Windsor, 2000) and co-infection of variants from different clades is common (Kikuchi & Fukatsu, 2003; Perrot-Minnot *et al.*, 1996; Wenseleers *et al.*, 1998; Werren *et al.*, 1995b; West *et al.*, 1998)

Furthermore, experimental transfection studies have shown conclusively that some *Wolbachia* variants can be transmitted and stably maintained in new, closely or distantly related host species (Heath *et al.*, 1999; Huigens *et al.*, 2000; Pintreau *et al.*, 2000; Rigaud & Jachualt, 1995; van Meer & Stouthamer, 1999), though as stated in Section 1.4, others cannot.

There are several possible routes through which horizontal transfer could occur; for example, injury, ingestion and parasitism. Rigaud & Jachualt (1995) showed that F-

Wolbachia could be transferred between injured woodlice via infected haemolymph and lead to expression of the F phenotype in the new host. Mitsuhashi *et al.* (2002) found two leafhopper species that shared *Wolbachia* infection types and also acted as vectors for the causative agent of Mulberry dwarf-disease, *Phytoplasma*. *Phytoplasma* are transmitted from plant to plant through the leafhopper saliva during feeding on plant sap. The *Wolbachia* strains in question were also found in the salivary glands of the leafhoppers and it was hypothesised by the author that the shared infection was due to the transfer of *Wolbachia* between leafhopper species via the plant sap, though this was not proven. Schilthuizen & Stouthamer (1997) suggested that *Wolbachia* could be horizontally transmitted between *Trichogramma* species parasitising the same host egg and *R. rickettsii* is known to be transmitted between co-feeding ticks (Neibylski *et al.*, 1999).

The most widely accepted hypothesis is that transfer of *Wolbachia* is mediated by parasitoid vectors: insect species that parasitise and eventually kill the host larva. Transfer of the bacteria could occur as a result of ingestion of the infected larva, or by contamination of wasp parasitoid ovipositors (tubular egg laying structure) during egg-laying. Similar *Wolbachia* variants have been identified in fly species such as *Protocalliphora* and *Drosophila*, and their respective parasitoids *Nasonia giraulti* and *Asobara tabida* (Werren *et al.*, 1995; Vavre *et al.*, 1999), and between the moth *Ephestia kuehneilla* and its *Trichoogramma* spp. parasitoids (van Meer *et al.*, 1999). Heath *et al.* (1999) showed that *Wolbachia* could be transferred experimentally between *D. simulans* (Riverside) and its wasp parasitoid *Leptopilina boulardi*, though the infection was gradually lost.

Horizontal transmission is not seen in mutualistic bacteria such as bacteriocyte associated *Buchnera* and the nematode associated clades of *Wolbachia* (Bandi *et al.*, 1998; Casirhagi *et al.*, 2001; Funk *et al.*, 2000), but the *Wolbachia*-arthropod association is less restricted and arthropod associated *Wolbachia* are intermediate in their nature between insect secondary symbionts and arthropod associated pathogens (see Section 1.2.1; Table 1.2). The secondary symbionts of tsetse flies, whiteflies and aphids are known to undergo horizontal transmission (Akman *et al.*, 2001; Russel *et al.*, 2003; Thao *et al.*, 2004) and members of the *Wolbachia*-related *Anaplasma* and *Ehrlichia* are horizontally transmitted by tick bites, through the tick saliva as it feeds on its blood-meal (Dumler *et al.*, 2001). It therefore seems logical that *Wolbachia* could have evolved a mechanism for horizontal transmission. In several host species *Wolbachia* are distributed in somatic as well as reproductive tissues, including the salivary glands (Dobson *et al.*,

1999; Mitsuhashi *et al.*, 2002; Oh *et al.*, 2000). As already stated (Section 1.3), recombination has been detected in *Wolbachia*, suggesting not only that dissimilar strains can come in contact with each other through horizontal transmission, but also that recombination could create new *Wolbachia* variants, better able to invade and spread through a host population (Jiggins *et al.*, 2001c). The rate of recombination in *Wolbachia* is similar to that of other horizontally transmitted pathogens (Jiggins, 2002)

For horizontal transmission to occur, an ecological interaction between different host species is required. Feeding communities, in which several insect species interact on different trophic levels, may be the most suitable study system for investigating horizontal transmission in *Wolbachia.* Previous studies have provided phylogenetic evidence both for and against the hypothesis of horizontal transmission via parasitoid vectors (Schilthuizen & Stouthamer, 1998; West *et al.*, 1998; Shoemaker *et al.*, 2002; Kittayapong *et al.*, 2003).

1.6 How *Wolbachia* are studied: non-molecular approaches

Wolbachia cannot be cultivated by conventional microbiological methods. Therefore, most of the studies described in the literature are based on the detection of *Wolbachia* by treatment with antibiotics, genetic crossing experiments, microscopy and more recently, amplification of *Wolbachia* specific marker genes (Werren & O'Neill, 1997).

Wolbachia are sensitive to antibiotics including tetracycline and rifampicin, and insects harbouring the bacteria can be 'cured' of the infection if these compounds are mixed with their food source (Stouthamer *et al.*, 1999). If this results in a significant change in the sex ratio of the offspring, directly or following genetic crossing experiments, it indicates the presence of a reproductive parasite. For example, Jiggins *et al.* (2000, 2001b) fed *Acraea* butterflies leaves covered with aqueous tetracycline hydrochloride which resulted in an increase in male progeny, confirming that this species is infected by a male-killing bacterium. However this method is not diagnostic for *Wolbachia* infection, as other reproductive parasites may also be sensitive to the aforementioned antibiotics. Nor can it discriminate between *Wolbachia* variants, therefore cannot be used to identify multiple infections. It is also restricted to use with host species that can be cultured and strains of *Wolbachia* inducing more obvious host effects.

Light and electron microscopy have been used to detect bacteria with *Wolbachia*-like morphology in hosts such as the ladybird *Adalia bipunctata*, springtails (Collembola), the estuarine isopod *Sphaeroma rugicauda* and members of the *Drosophila simulans*

complex (Hurst *et al.*, 1996; Martin *et al.*, 1994; Rousset *et al.*, 1992; Vanderkerckhove *et al.*, 1999). DNA staining with diamidinophenylindole (DAPI) has been employed for determining *Wolbachia* tissue localisation (Rousset & Solignac, 1995) and Hsiao (1996) detected *Wolbachia* in weevils using giemsa staining. Dot blot hybridisation using *Wolbachia* specific probes, ^{32}P labelled PCR amplimers of the *Wolbachia dnaA* gene for example, have been used to identify *Wolbachia* and establish their tissue distribution (Dobson *et al.*, 1999). Anti-*Wolbachia* antibodies have also been used to analyse the expression of the *wsp* gene, showing that the protein (and therefore the bacteria) is present in almost all host tissues (Dobson *et al.*, 1999).

1.6.1 Molecular approaches

The most widely used method for the detection of *Wolbachia* is PCR-amplification of *Wolbachia*-specific marker genes, which coupled with cloning and DNA sequencing confirms the infection status of the studied host and facilitates strain identification. To date, the following gene sequences have been employed: 16S rRNA gene (O'Neill *et al.*, 1992), 23S rRNA gene, spacer-2 region and 5S rRNA gene (van Meer *et al.*, 1999a), *dnaA* (encoding a replication initiator protein; Bourtzis *et al.*, 1996), *groE* (encoding a bacterial heat shock protein; Masui *et al.*, 1997), *ftsZ* (Werren *et al.*, 1995b) and *wsp* (Braig *et al.*, 1998).

In almost all studies the 16S rRNA gene has been used to detect, and/or confirm detection of *Wolbachia*, but functional genes such as the cell cycle gene *ftsZ* and *wsp*, which show greater sequence divergence, have been more useful for the differentiation of *Wolbachia* sequence variants (Bourtzis & Braig, 1999; Zhou *et al.*, 1998). Sequencing of the genome of several *Wolbachia* variants is underway, the wMel genome sequence is complete (Section 1.2.1) and these efforts may reveal more suitable genes for strain typing (Foster *et al.*, 2004; Sun *et al.*, 2003; Wu *et al.*, 2004; see Section 5.0). PCR-amplimers are often cloned and typically 5 or 6 clones (or up to 15 for multiply infected individuals) for each specimen must be sequenced to obtain a good estimate of sequence diversity, which is both time-consuming and expensive (Jiggins *et al.*, 2002a; Rokas *et al.*, 2002a; Shoemaker *et al.*, 2002).

Few studies have exploited the potential of molecular typing methods (Section 1.7) to reduce the time and effort required to characterise *Wolbachia* infections in larger sample numbers. Rousset *et al.* (1999) found heteroduplex analysis (Section 1.7) was able to distinguish between three strains in different double infections of *Drosophila* samples

resulting from microinjection experiments. However the study involved detecting only three, previously characterised variants, and the ability to detect *Wolbachia* variants from other infections has not been tested. Only 300 bp of the *wsp* gene was analysed in this study and it has been shown that the mutation detection rate of heteroduplex analysis falls to only 80 % over larger fragments (Nollau *et al.*, 1997).

Restriction fragment length polymorphism (RFLP; Section 1.7) analysis uses restriction endonucleases to cut the marker gene amplimer at specific sites, the position of which is not random and allows some degree of phylogenetic inference. This has been used by some groups to pre-screen amplimers of the 16S rRNA, *ftsZ* or *wsp* genes and to reduce the need for sequencing. The difficulty with this technique is establishing which restriction enzymes are most suitable. Kikuchi & Fukatsu (2003) used the enzymes *Cla*I and *Dra*I to identify *Wolbachia* variants infecting heteropteran bugs and found that RFLP failed to detect all variants that were later identified using cloning and sequencing of *wsp*. In other studies the enzymes *Eco*RV, *Rsa*I, *Hin*dIII, *Nla*IV, *Bfa*I, *Hin*fI, *Dde*I, *Eco*47III, *Pac*I have been used in various combinations to produce simple, strain specific *wsp* restriction patterns (Jamnongluk *et al.*, 2002; Jiggins *et al.*, 2002a; Kondo *et al.*, 2002; Mitsuhashi *et al.*, 2002; Riegler & Stauffer, 2002; Reuter & Keller, 2003). However, RFLP has most frequently been used to screen members of a population known to be infected with a small number of variants (Kondo *et al.*, 2002) and may not be suitable for distinguishing larger numbers of sequence variants, as this would undoubtedly require combinations of multiple endonucleases and produce more complex restriction profiles.

What is needed is a high-throughput technique that will allow large numbers of samples to be screened rapidly, to gain information about the incidence and prevalence of infection in different species populations, and measure changes over time and space. This will provide a greater understanding of population dynamics and infection transmission in insect species that may be targeted for biocontrol.

1.7 Molecular typing methods

Natural bacterial communities often consist of large numbers of species or strains, which makes preparation of clone libraries and identification of each species / strain through clone sequencing time consuming, expensive and impractical. Genetic fingerprinting techniques have been developed that help to screen multiple samples rapidly with a greatly reduced need for cloning and sequencing.

These molecular typing methods provide a pattern or profile of a microbial community based on the physical separation of unique nucleic acid sequences and enable comparison of microbial communities from different environments, or characterisation of a changing community over time (Muyzer, 1999). Typing methods vary in complexity and the method of choice depends on the type of community under investigation and the molecular markers in use.

Examples include Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis (TGGE), Constant Denaturant Gel Electrophoresis (CDGE), Restriction Fragment Length Polymorphism (RFLP), Terminal Restriction Fragment Length Polymorphism (TRFLP), Single Strand Conformational Polymorphism (SSCP) and derivatives thereof, Chemical or enzyme mismatch cleavage analysis, Amplified rDNA Restriction Analysis (ARDRA), ribosomal intergenic spacer analysis (using SSCP to look at 16S-23S spacer region), Randomly Amplified Polymorphic DNA (RAPD). The advantages and disadvantages of each technique are given in Table 1.3. These methods are generally more cost-effective than sequencing and are very useful as prescreening techniques.

DGGE, RFLP, TRFLP and SSCP are the most commonly employed profiling techniques applied to the investigation of endosymbiont diversity (Table 1.4). For example, Reeson *et al.* (2003) used DGGE to establish that the social wasp *Vespula germanica* does not appear to be dependent on specific mutualistic gut microbes. Simon, *et al.* (2003) measured the genetic diversity of *Buchnera* using SSCP as an indication of genetic divergence of aphid populations feeding on clover, alfalfa and pea, and Fukatsu & Nikoh (2000) examined the diversity of endosymbionts associated with the pseudococcid (mealybug) *Antonuba crawii* using TRFLP, identifying three symbionts from the *Beta-* and *Gammaproteobacteria*, and a *Spiroplasma* species.

1.7.1 Profiling microbial populations using denaturing gradient gel electrophoresis

DGGE was developed by Fischer & Lerman (1983), originally for the identification of differences in a single gene, for which nucleotide sequence information was available, and it was especially important for examining human genes (Hofstra *et al.*, 2004; van Orsouw *et al.*, 1998). In the last decade applications have extended to the study

TABLE 1.3 DNA profiling techniques

Method	Method principle	Advantages	Disadvantages	Reference
SSCP[a]	Difference in electrophoretic mobility of single stranded conformers resulting from sequence differences.	Simple to execute.	Optimum limited to 200 bp.	Hayashi *et al.*, 1999 Sheffield *et al.*, 1993
PLACE-SSCP[b]	Fluorescently labeled single stranded conformers are separated on automated sequencing gels or capillaries.	Greater resolution and automation.	Optimum limited to 200 bp.	Hayashi *et al.*, 1999
REF-SSCP[c]	Mixture of restriction digested fragments analysed using SSCP.	Facilitates whole gene analysis.	Use of multiple running conditions required for maximum sensitivity. Size limitation and dependent on choice of restriction endonuclease.	Lui & Sommers, 1995
RISA[d]	Variability in length of intergenic spacer regions of rRNA genes.	Simple to execute.	ITS regions can be of equal length. Requires good reference library.	Ranjard *et al.*, 2000
ARDRA[e]	Variation in restriction fragment size of 16S rRNA gene.	Simple to execute.	Multiple products for each sample.	Ranjard *et al.*, 2000
TRFLP[f]	Variation in length of fluorescently labeled terminal restriction fragment.	High accuracy of sizing.	Requires large reference library. Mutations in rest of molecule missed.	Marsh, 1999 Dahllof, 2002
DGGE/TGGE[g]	Separation of equal length fragments based on sequence dependent *Tm* in denaturant gradient (chemical or temperature).	100% detection rate if fully optimised. Rapid, reproducible, inexpensive.	Size limited to 1000 bp Co-migration of sequence variants and different conformers of same amplimer possible.	Muyzer *et al.*, 1993 Muyzer & Smalla, 1998
CDGE[h]	Applies single optimum chemical denaturant concentration between *Tm* of DNA fragment and clamp.	Specific to detection of one sequence variant.	Useful for G/C rich sequences.	Wu *et al.*, 1999

TABLE 1.3 continued

CSGE[i]	Homo and heteroduplexes are detected using DGGE.	High detection rate of single nucleotide polymorphisms in human genes.	More than one band produced for each variant.	Korko *et al.*, 1998
TDGS[j]	Combines DGGE in first dimension with size separation in second.	Multiple exons can be examined in single gel.	Complex profile produced. Special apparatus required.	Orsouw *et al.*, 1998 McGrath *et al.*, 2001
RAPD[k]	Amplification of genomic DNA with short (10 bp) primers to produce profile of random fragment lengths unique to species.	Simple to execute.	Complex output. No phylogenetic inference. Very sensitive to PCR-bias.	Ranjard *et al.*, 2002
Heteroduplex Anlaysis	Difference in electrophoretic mobility of heteroduplexes relative to homoduplexs in non-denaturing polyacrylamide gel.	Running conditions require minimal optimization.	Limited to products ~900 bp. 80 % detection rate.	Nollau *et al.*, 1997
Enzyme/ chemical cleavage	Mismatches in heterduplexes cut by enzyme / chemical to produce unique profile of fragment lengths.	Large products (2 kb) can be analysed. Chemical cleavage gives 100% detection of point mutations.	Not all mismatches are cleaved by enzymes with equal efficiency. Toxic chemicals required. Multiple products.	Nollau *et al.*, 1997
CFLP[l]	Cleavase I endonuclease cuts hairpin loops of ssDNA formed by denaturation to produce unique profile of fragment lengths.	Large products (2 kb) can be analysed.	Multiple products.	Nollau *et al.*, 1997

[a] Single stranded conformational polymorphism. [b] Post labeling automated capillary electrophoresis – SSCP. [c] Restriction endonuclease fingerprinting – SSCP. [d] Ribosomal intergenic spacer analysis. [e] Amplified ribosomal DNA restriction analysis. [f] Terminal restriction fragment length polymorphism. [g] Denaturing / temperature gradient gel electrophoresis. [h] Constant denaturant gel electrophoresis. [i] Conformation sensitive gel electrophoresis. [j] Two dimensional gene scanning. [k] Random amplified polymorphic DNA. [l] Cleavage fragment length polymorphism.

TABLE 1.4 Application of genetic profiling techniques to the study of microbial endosymbionts

Method	Purpose of study	Marker Gene	Reference
SSCP	Examining the genetic diversity of *Buchnera* endosymbionts in aphid host races	3 genome segments [a]	Simon *et al.*, 2003
TRFLP	Examining the diversity of symbiotic archael communities in marine sponges	16S rRNA	Lee *et al.*, 2003
RFLP	Detection of two endosymbionts in the mulberry psyllid *Anomoneura mori* (Insecta: Hemiptera).	16S rRNA	Fukatsu & Nikoh, 1998
RFLP	Examining the diversity of endosymbionts associated with the bamboo pseudococcid *Antonuba crawii* (Insecta: Hemiptera)	16S rRNA	Fukatsu & Nikoh, 2000
RFLP & DGGE	Profiling symbiotic dinoflagellate populations from the sea anemone *Anthopleura elegantissima* along a latitudinal gradient.	16S rRNA & ITS2	La Jeunesse & Trench, 2000
DGGE	Profiling microbial symbionts of the benthic dinoflagellate *Ostreopsis lenticulars.*	16S rRNA	Ashton *et al.*, 2003
DGGE	Analysis of tick-infecting bacterial communities	16S rRNA	Schabereiter-Gurtner *et al.*, 2003
DGGE	Profiling microbial endosymbionts associated with the social wasp *Vespula germanica*	16S rRNA	Reeson *et al.*, 2003
DGGE	Assessing diversity of bacteria associated with natural aphid populations	16S rRNA	Haynes *et al.*, 2003

[a] *aroQ-PheA, GroEL. Ffh-rpsP*

of natural microbial communities (Hayes *et al.*, 1999; Muyzer, 1999; Muyzer & Smalla, 1998). DGGE is a reproducible, relatively inexpensive technique that facilitates the simultaneous analysis of multiple samples, allowing comparison over time and space.

The DGGE technique has great potential as a rapid, simple discriminatory method for screening *Wolbachia*-infected host specimens. DGGE facilitates the separation of PCR-amplified gene fragments of equal size, based on altered electrophoretic mobility resulting from nucleotide sequence differences. As with techniques such as SSCP and T-RFLP, DGGE is relatively straightforward to execute, but this technique also has the potential to allow larger gene fragments to be distinguished using a single set of conditions, producing relatively simple profiles of results.

During DGGE, regional variation in guanine and cytosine nucleotide content in the dsDNA fragment creates melting domains - stretches of base-pairs with the same melting temperature (*Tm*). As the DNA molecule migrates through the chemical denaturant or temperature gradient, the domain with the lowest *Tm* melts, altering the conformation of the DNA and causing migration to halt. Any change in base composition of this region will result in an alteration in *Tm*, and will be detected as a change in the banding position on the DGGE gel. This is significant because if the dsDNA molecule contains several melting domains, mutations located in melting domains with a higher *Tm* might not be detected. This would limit the range of gene fragments to which DGGE could be applied to those with a single melting domain. To combat this, Myers *et al.* (1985) developed the GC-clamp, which is attached to one end of the dsDNA molecule during PCR with a primer carrying a stretch of G / C nucleotides at its 5'-end. This becomes the highest melting domain; all other domains have significantly lower melting temperatures relative to the clamp and melt as one domain. The clamp facilitates the use of higher concentrations of denaturants to cause the whole molecule to melt, whilst the double stranded clamp prevents it unzipping into ssDNA and running off the end of the gel. This has been shown to improve the ability to detect mutations from 50% to 100% detection rate over 500 bp (Muyzer & Smalla, 1998; Myers *et al.*, 1985; Sheffield, 1989). A major advantage of the DGGE technique is the ability to excise sequence-variants from the gel and use the resuspended DNA as a template for PCR and DNA sequencing. This would allow multiple *Wolbachia* infections to be characterised without the need for cloning and should facilitate rapid screening of *Wolbachia* infected species on a much larger scale.

In the study of bacterial communities, DGGE has most frequently been applied to the 16S rRNA gene. The marker gene is amplified from the chosen environment such as soil, to produce a mixture of PCR products that when separated on a DGGE gel, produce a profile in which every band theoretically represents a different sequence variant and therefore a distinct bacterial strain. The representation of a specific bacterial group within a community can be determined using nested PCR with general and group specific primers, or using taxon-specific oligonucleotide probes to analyse DGGE profiles (Muyzer, 1999; Muyzer & Smalla, 1998).

Recent literature shows an increase in the number of studies exploiting functional genes such as enzyme encoding genes, which generally have more sequence variation and are more useful as molecular markers of bacterial strains / variants rather than bacterial species (Hein *et al.*, 2003; Henckel *et al.*, 1999; Wawer *et al.*, 1995; 1997; Webster *et al.*, 2002). It should therefore be possible to analyse fragments of the *wsp* gene using DGGE to distinguish between *Wolbachia* sequence-variants.

1.8 Oak gall wasps, tribe Cynipini

The oak gall wasp (Hymenoptera: Cynipidae: Cynipini) community is a model system in the study of community structure (Csóka *et al.*, 2004; Stone *et al.*, 2002) and represents an attractive system for the study of *Wolbachia* transmission because each wasp-induced gall supports a characteristic, species rich and ecologically closed community of gall causers, inquilines and parasitoid wasps (Askew, 1984; Csóka *et al.*, 2004; Stone *et al.*, 2002). In addition, it is thought that horizontal transmission may be more likely to occur between more closely related species that provide similar physiological backgrounds for infection (Huigens *et al.*, 2004; Russel *et al.*, 2003). Therefore the oak gall wasp community provides a good chance of detecting horizontal transmission and determining the frequency at which it occurs.

1.8.1 Cynipid gall wasp phylogeny

The family Cynipidae consists of phytophagous gall-inducing or gall-associated wasps and is divided into six tribes (Table 1.5) based on morphology (Liljebald & Ronquist, 1998; Ronquist, 1999b).

The tribe Cynipini includes gall-inducers of oaks (genus *Quercus*) and is the most species rich of the six tribes with *c.* 1000 described species. The herb gall wasp tribe Aylacini includes 133 species, while the remaining three gall-inducing tribes, the

TABLE 1.5 The Cynipidae:
the number of genera and species belonging to the six tribes of Cynipidae and the host plants which they exploit[a]

Tribe	Genera	Species	Host
Aylacini	21	133	Asteraceae, Rosaceae, Lamiaceae, Papaveraceae, Apiaceae, Valerianaceae, Brassicaceae
Cynipini	27	*c.* 1000	Fagaceae (mostly *Quercus*)
Eschatocerini	1	3	*Acacia*, *Prosopis* (Fabaceae)
Pediaspidini	3	3	*Acer* (Aceraceae)
Diplolepidini	2	62	*Rosa* (Rosaceae)
Synergini	7	145	Inquilines in galls induced by *Diastrophus* (Aylacini), *Diplolepis* and Cynipini

[a] Csóka *et al.*, 2004; Ronquist, 1999a.

Diplolepidini (rose gall wasps), Pediaspidini (gallers of maple trees) and the Eschatocerini (gallers of *Acacia* and *Prosopis* plants) have much lower species richness. The Synergini tribe includes 145 species of inquiline gall wasps that can not induce galls themselves, so live in, and can influence the development of galls induced by other cynipids (Csóka *et al.*, 2004; Stone *et al.*, 2002).

The Cynipini mainly exploit oaks of the genus *Quercus* (family *Fagaceae*), which includes over 500 species worldwide. The subgenus *Quercus* contains four sections: *Cerris*, *Quercus sensu stricto* (white oaks), *Lobatae* (red oaks) and *Protobalanus* (golden cup oaks). Most Cynipini species attack a group of closely related oak species from a single oak section, with similar plant chemistry (Abrahamson *et al.*, 2003) and are specific in their choice of host-plant organ. There are exceptions however: some species undergo host alternation (heteroecy), for example the sexual generation of some *Andricus* species develop on oaks of the section *Q. sensu stricto* but the asexual generation is found on section *Cerris* oaks (Askew, 1984). The sexual generation gall of *Neuroterus quercusbaccarum* develops on both catkins and leaves (Redfern & Askew, 1992).

1.8.2 Mode of reproduction in the Cynipini

Reproduction in most Hymenoptera, including many cynipid species, occurs by arrhenotokous parthenogenesis. Males develop from unfertilised (haploid) eggs, and females from fertilised (diploid) eggs (haplodiploidy). In the Cynipinae, *Wolbachia*-induced thelytokous parthenogenesis also occurs (Section 1.3) and is thought to result from a doubling of the chromosome complement, producing homozygous, diploid females (Plantard *et al.*, 1998; 1999).

Reproduction in oak gall wasps and their sister group Pediaspidini, occurs by cyclical parthenogenesis (heterogony). This is the strict alternation between sexual and asexual generations and in most cynipids a single generation of each reproductive mode occurs each year (Askew, 1984). The genetic mechanism of cyclical parthenogenesis is not known but the life cycle is highly variable across the Cynipini (Askew, 1984; Atkinson *et al.*, 2003; Csóka *et al.*, 2004; Stone *et al.*, 2002). The sexual and asexual generation wasps often differ in size and this has lead to confused estimates of species richness within the Cynipini (Cook *et al.*, 2002; Rokas *et al.*, 2002b; Stone *et al.*, 2002).

1.8.3 Cynipini-induced galls

Cynipid-induced galls provide food, shelter and protection for the gall causer and gall community members. Gall development is initiated from meristematic cells in response to oviposition by the female cynipid gall wasp, and subsequent tissue differentiation is controlled by the larva (or larvae), though the exact mechanism by which this occurs is unknown (Stone & Schonrögge, 2003). Galls induced by different wasp species occur on roots, stems, fine branches or twigs, buds or flowering parts of the host plant (Askew, 1984; Csóka *et al.*, 2004; Stone *et al.*, 2002).

Each gall consists of one (monolocular) or many (multilocular) inner chambers surrounded by a layer of nutritive tissue on which the larvae feed, and a thin protective shell of sclerenchyma. The outer layer consists of parenchyma cells, which create the highly varied morphology seen in cynipid galls (Fig 1.3). Each gall structure is highly characteristic of the gall-causer species and can be used to identify the causer with a high degree of certainty (Csóka *et al.*, 2004).

In most oak gall wasp species, galls containing the sexual generation wasps develop in spring or early summer. The sexual generation females emerge, lay their eggs, and the induced galls (containing the asexual generation) develop over the summer and autumn. The asexual generation females emerge in the autumn to lay their eggs, and gall induction either occurs at this time, the eggs overwintering in the protective gall, or the eggs lie dormant over the winter, ready for gall induction the following spring. Some species deviate from this pattern, for instance the development of the asexual generation of *Biorhiza pallida* may be delayed until the following year (Askew, 1984). The galls of each generation may be produced on different host plant organs and are structurally different. Due to the variation in gall structure and position, galls induced by different wasp species or by the sexual and asexual generations of the same species, are associated with their own characteristic community of insects.

1.8.4 Insect communities associated with cynipid galls

Cynipid galls support species-rich communities of insects including the gall-causer, obligate phytophagous inquilines, parasitoids, hyperparasitoids and opportunistic predators and scavengers.

Cynipid galls may be attacked by hymenopteran, dipteran, lepidopteran or coleopteran inquilines. All the wasp inquilines belong to the Cynipoidea and are members of the cynipid tribe Synergini or the family Figitidae. The Synergini are closely related to

Neuroterus numismalis (sexual generation)
Leaf blister gall (size: 0.3cm)

Andricus curvator (sexual generation)
Swollen leaf gall (size: 0.6-1.2cm)

Andricus quadralineatus (asexual generation)
Catkin gall (size: 0.4-0.6cm)

Biorhiza pallida (sexual generation)
Apple galls in early summer and late summer, after the wasps have emerged. (size: 2-6cm diameter)

Neuroterus quercusbaccarum (sexual generation)
Currant galls on leaf and catkin (size: 0.4-0.8cm)

FIG. 1.3 Summer generation galls of 5 oak gall wasp species (Cynipidae: Cynipini), demonstrating the variation in morphological structure and position of cynipid-induced galls on oak trees (Redfern & Askew, 1992; http://www.hainaultforest.co.uk/3Oak%20galls.htm).

the gall-causers (Cynipini) and are quite similar in appearance (Ronquist, 1999; Ronquist & Liljeblad, 2001). Inquiline cynipids have lost the ability to induce their own galls, but are able to induce the development of larval chambers in, and feed on, the nutritive tissue of galls induced by the gall-causer (Csóka *et al.*, 2004). Many species attack a wide range of oak cynipid galls (Askew, 1984). Most species do not harm the gall-causer but others kill the gall-causer with their ovipositor during egg laying, and others accidentally crush the causer larva as the inquiline larva develops (Askew, 1984; Stone *et al.*, 2002).

Most of the parasitoids that attack cynipid hosts belong to the superfamily Chalcidoidea but members of the families Ichneumonidae and Braconidae may also attack cynipid galls. Most are specific to cynipid communities, often to only one cynipid tribe, but few parasitoids that attack the Cynipini are restricted to only one type of oak gall (Stone *et al.*, 2002). Parasitoids feed on the other members of the gall community including inquilines and other parasitoids (hyperparasitoids).

Parasitoid-host associations are highly varied. Most parasitoids are solitary idiobionts, which feed externally on the host larvae, the development of which is arrested prior to oviposition by stinging by the parasitoid mother (Csóka *et al.*, 2004). Others allow the host to continue developing to provide a more substantial source of food for the parasitoid larva (koinobionts). A few species are endoparasitoids, the eggs of which are laid inside the host larva and a few feed on both host and gall tissue (Askew, 1984; Csóka *et al.*, 2004). Some species are very specific in their host choice, others are not; the parasitoid *Torymus auratus* for example, will attack any larva they encounter (Askew, 1961b). Galls that develop on same plant organ at the same time and are similar in morphology, tend to have similar parasitoid communities (Askew, 1984). Little is known about how parasitoids locate their hosts (Stone & Schönrogge, 2003). Galls are also attacked by other insects, birds and mice which feed on the gall tissue and/ or wasp larva, and endophytic fungi also contribute to gall wasp mortality (Csóka *et al.*, 2004; Stone *et al.*, 2002).

1.9 Project aims

It is implicit that horizontal transmission of endosymbionts is more likely to occur between insect species in prolonged intimate contact. A major aim of this study was to determine the strength of evidence of horizontal transmission of *Wolbachia* endosymbionts in natural host populations of closely related host species. Members of the well characterised oak gall wasp feeding community, in which numerous wasp species are

intimately associated, were screened to test the hypothesis that horizontal transmission occurs via parasitoid vectors.

Screening of lab stocks of gall community member species was precluded by the obligate nature of the association between the parasitoid, inquiline and gall wasp species, and between the gall causer and the host oak tree. Therefore a purely molecular-phylogenetic approach was employed during the investigation.

A second aim was to determine whether the technique of DGGE could be successfully applied to the *Wolbachia* research field. The objective was to optimise the technique to allow full length fragments of the *wsp* strain marker gene, amplified with the most widely used primer set (Braig *et al.*, 1998), to be distinguished from each other visually. The DGGE screen was required to provide maximum information about the *wsp* sequence variants present in the insect community, and display this information directly on the DGGE profile, reducing the need for subsequent cloning and sequencing.

In order to realise these aims, members of the parasitoid and inquiline communities associated with the spring generation galls of 5 gall-causer species, were collected, reared and screened for the presence or absence of *Wolbachia.* Initially, the traditional PCR, cloning and sequencing based approach was used to screen the wasp species reared from *Biorhiza pallida*-induced galls (Chapter 2). This provided characterised *wsp* amplimers that were used to develop the DGGE screen (Chapter 3). DGGE was then used to analyse the *Wolbachia* infected members of the wasp communities from *Andricus curvator*, *A. quadrilineatus*, *B. pallida*, *Neuroterus numismalis* and *N. quercusbaccarum*-induced galls and the productivity of the two screening strategies was compared (Chapter 4).

Chapter 2

Investigation of *Wolbachia* prevalence in the oak gall wasp community using PCR, cloning and sequencing

2.1 Introduction

Wolbachia are considered to be one of the most prevalent species of bacterial endosymbiont found in insects; approximately 20% of all insect species are estimated to be infected (Werren *et al.*, 1995a; Reuter & Keller, 2003; Kikuchi & Fukatsu, 2003; Kittayapong *et al.*, 2000; Ricci *et al.*, 2002; Rokas *et al.*, 2001; West *et al.*, 1998) and in one study an infection rate of 76% was detected (47 infected species; Jeyaprakash & Hoy, 2000). These obligate endosymbionts have received much attention due to their ability to manipulate the reproduction of their hosts in order to promote their own transmission.

Wolbachia are primarily maternally transmitted but incongruence between host and bacterial phylogenies, and the occurrence of multiple infections, suggests that *Wolbachia* also undergo occasional horizontal transmission. For this to occur, an ecological interaction between different host species is required. Potential routes include blood-blood contact (Rigaud & Juchault, 1995), feeding (Huigens *et al.*, 2000, Mitsuhashi *et al.*, 2002) and parasitism (Heath *et al.*, 1999; Vavre *et al.*, 1999a).

Studies of *Wolbachia* infections in feeding communities have provided phylogenetic evidence both for and against the hypothesis of horizontal transmission (Kittayapong *et al.*, 2003; Schilthuizen & Stouthamer, 1998; Shoemaker *et al.*, 2002; West *et al.*, 1998). Many investigations have focused on parasitoid community members due to the close developmental association between parasitoids and their hosts, which could allow parasitoid wasps to act as vectors of *Wolbachia.* Related intracellular parasites from the *Rickettsia* and *Ehrlichia* genera are transmitted by arthropod vectors (Azad & Beard, 1998; Hackstadt, 1998). Evidence in support of parasitoid vectors of *Wolbachia* has been obtained experimentally (Heath *et al.*, 1999; Huigens *et al.*, 2000; van Meer & Stouthamer, 1999) and similar or identical *Wolbachia* variants have been identified in several host species and their corresponding parasitoids (Noda *et al.*, 2001; van Meer *et al.*, 1999; Vavre *et al* 1999a; Werren *et al.*, 1995a).

Wolbachia infections have been detected in several gall wasp species (Hymenoptera: Cynipidae) and phylogenetic estimations indicate that multiple independent infections have occurred (Abe & Muira, 2002; Plantard *et al.*, 1999; Rokas *et al.*, 2001; 2002a; Schilthuizen & Stouthamer, 1998). Oak gall wasps (Hymenoptera:

Cynipidae: Cynipini) induce complex gall structures on oaks and other Fagaceae. Each gall supports a characteristic, closed community of wasps, composed of gall-causer, inquiline and parasitoid species (Askew, 1961; Stone *et al.*, 2002). This creates a web of interacting species through which *Wolbachia* could be transmitted within, and between different gall communities.

Rokas *et al.* (2002a) screened 64 gall wasp and inquiline species from the Cynipini and Synergini tribes and identified several inquiline species infected with *Wolbachia.* Some inquiline and gall-causer species shared identical *Wolbachia* variants, based on the *Wolbachia* surface protein (*wsp*) marker gene sequences (Braig *et al.*, 1998), suggesting that horizontal transmission had occurred within the community. However, the inquiline and gall wasp species in question are not known to belong to the same gall communities, therefore the infection could not have been transmitted directly between these species. The author hypothesised that horizontal transmission could have been mediated by generalist parasitoids, which attack several different gall communities. Schilthuizen & Stouthamer, (1998) tested all wasp species associated with galls induced by the rose gall wasp *Diplolepis rosae* (Hymenoptera: Rhodotini) for *Wolbachia* infection, but found no direct evidence of horizontal transmission. Four parasitoid species were infected with distinct *Wolbachia* variants but none of them carried the variant from the host *D. rosae.*

Sexual generation *Biorhiza pallida* (Hymenoptera: Cynipidae: Cynipini) wasps induce multilocular 'apple' galls on *Quercus* oaks (Fig. 1.3), in which over 100 gall wasps may develop. In the UK, 23 species of parasitoid / inquiline wasp are associated with these galls (Fig. 2.1). *B. pallida* has previously been found to be infected with a single A-clade *Wolbachia* variant (Rokas *et al.*, 2002a). Another two *Wolbachia* variants were identified separately infecting the inquiline wasp species *Synergus gallaepomiformis* (Hymenoptera: Synergini) and *S. umbraculus*, both of which attack *B. pallida* galls in Europe (Rokas *et al.*, 2002a; Askew *et al.*, 2004).

In this study, the parasitoid wasp community associated with sexual generation *B. pallida* galls were tested for the incidence and diversity of *Wolbachia* using PCR amplification and sequence analysis of the *wsp* marker gene. The study aimed to determine the strength of evidence for horizontal transmission via parasitoid vectors within this community. Parasitoid wasps that emerged from *B. pallida* galls examined by Rokas *et al* (2001) were screened, facilitating direct comparison with results obtained in that study, in which only the gall causer and associated inquilines were tested.

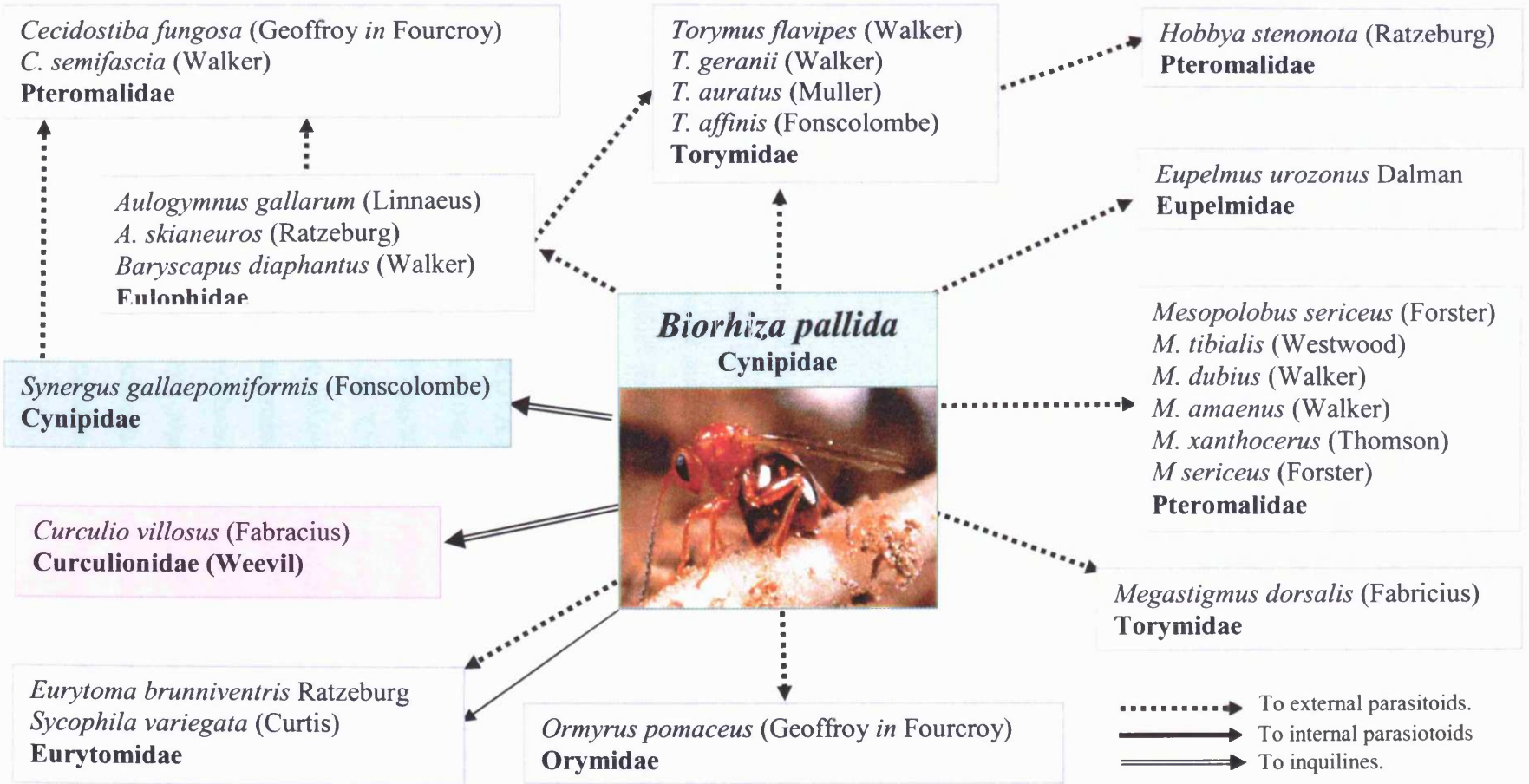

FIG. 2.1 The *Biorhiza pallida* (sexual generation) food web (Redfern & Askew, 1992; Williams, 2004). Parasitoid and inquiline wasp species associated with *B. pallida* galls in the UK are shown and the direction of feeding is indicated. Cynipid wasp species are coloured green, Chalcid wasp species lilac. Arrows point in direction of the consumer. Photo courtesy of G. N. Stone.

2.2 Materials & Methods

2.2.1 Insect samples

Wasps from sexual generation galls of *Biorhiza pallida*, which had been collected from eight locations in France, UK, Spain and Hungary (Table. 2.1), and provided by Graham Stone (Edinburgh University) in 100% ethanol, were used in this study. One to eight parasitoid wasps from each gall were tested. Wasps were identified with the help of Alex Heywood (Edinburgh University), using morphological keys by Askew & Thuroczy; G. N. Stone and colleagues (unpublished observations), and Graham & Gijswijt (1998). Molecular characterisation using the 28S rRNA gene (D2 expansion region) was also carried out (Section 2.2.3).

Drosophila simulans (Sturtevant) (Riverside strain) and *D. melanogaster* (Meigen) are naturally infected with *Wolbachia* and therefore laboratory stocks were used as positive controls. Flies treated with tetracycline to 'cure' them of the infection were used as negative controls. *Drosophila* stocks were kindly supplied by Henk Braig (Bangor University).

2.2.2 DNA extraction

Wasps were transferred to individual tubes of ethanol (100%) immediately following emergence and stored at –80°C until DNA was extracted up to a year later. Each sample was washed in 5% [vol / vol] Clorox solution (5.25% sodium hypochlorite) and serially rinsed in sterile distilled water, before the abdomen was dissected using a sterile scalpel blade and used for DNA extraction.

Tissue was homogenised in 100 µl of sterile salt homogenizing buffer (50 mM Tris-HCl pH 8.0, 0.4 M NaCl, 20 mM EDTA pH 8.0) using a sterile pestle, then a further 400 µl homogenisation buffer, 25 µl of 10% [wt / vol] SDS (0.5% final concentration) and 10 µl of 20 mg/ml proteinase K (380 µg/ml final concentration) were added. Samples were vortexed briefly and incubated at 55 °C for 3 h or overnight at 37 °C. Each sample was then treated with two cycles of the following: centrifugation at 15, 800g for 5 min, transfer of the supernatant to a sterile microfuge tube, addition of 170 µl of NaCl (5 M), 20 sec of vigorous shaking and 5 min incubation on ice. To the supernatant approximately 2 x volume of ice cold ethanol (100%) was added and mixed gently. After 10 min centrifugation (15, 800g), the supernatant was discarded and the pellet washed in 200 µl 70% ethanol, dried and finally resuspended in 50 µl sterile dH_20 (Stone & Cook, 1998). DNA was stored at -20 °C.

TABLE 2.1 Sampling locations: the locations from which each *Biorhiza pallida* induced-gall was collected by Rokas *et al.* (2001 & 2002).

Gall	N° wasps examined	Site	Country	Code	Longitude, Latitude
6	1	Oxford	UK	U1	0° 6' W 51° 6' N
11	5	Oxford	UK	U1	0° 6' W 51° 6' N
16	6	Oxford	UK	U1	0° 6' W 51° 6' N
18	3	Cambridge	UK	U2	0° 1' E 52° 2' N
30	5	Birnwood Forest	UK	U3	1° 27' W 51.75 N
33	3	Szentendre	Hungary	H1	19°03' E 47° 67' N
36	4	Szentendre	Hungary	H1	19°03' E 47° 67' N
37	7	Karceg	Hungary	H2	20° 88' E 47° 32' N
38	6	Visegrad	Hungary	H3	18° 97' E 47° 78' N
42	3	Cercedillo	Spain	S1	4° 07' W 40° 73' N
44	1	Cercedillo	Spain	S1	4° 07' W 40° 73' N
45	8	Cercedillo	Spain	S1	4° 07' W 40° 73' N
47	5	Cercedillo	Spain	S1	4° 07' W 40° 73' N
48	3	Cercedillo	Spain	S1	4° 07' W 40° 73' N
49	10	Rennes	France	F1	1° 67' W 48° 1' N
50	3	Rennes	France	F1	1° 67' W 48° 1' N
55	6	Rennes	France	F1	1° 67' W 48° 1' N
56	7	Rennes	France	F1	1° 67' W 48° 1' N

2.2.3 DNA amplification by polymerase chain reaction (PCR)

DNA samples extracted from each insect were used at templates in PCRs to amplify the following gene sequences: *Wolbachia* specific *wsp* gene; *Wolbachia* 16S rRNA gene; 28S rRNA gene of the host. In this way PCR was used to detect and confirm the presence or absence of the endosymbionts, and to check the quality of the DNA template. The primers and the PCR conditions for each primer pair are summarised in Table 2.2.

PCR reactions were performed under optimum conditions which were determined empirically by extensive experimental testing. Reactions included 1 x reaction buffer, 1.5 mM $MgCl_2$, 50 mM of each dNTP (dATP, dTTP, dGTP, dCTP), 1.25 U Taq DNA Polymerase, and 0.2 pmol of each primer. 1 µl template DNA (0.1 – 10 ng) was used for amplification of 16S and 28S rRNA gene sequences and 1.5 µl for the *wsp* gene. PCR amplifications were performed with a DNA Engine Dyad Thermal Cycler (MJ Research, Boston, MA, USA). A negative control in which no DNA was included, was performed with every set of reactions. DNA samples extracted from *D. simulans* (Riverside strain) or *D. melanogaster* were used as positive controls, and DNA from tetracycline-treated flies were used as a further negative control.

2.2.4 Agarose gel electrophoresis of PCR amplified gene fragments

Amplimers were separated and visualised by electrophoresis for 35 min at 85 V in 1.25% agarose gels [wt / vol] in TBE (40 mM Tris-base, 20 mM Boric acid, 1 mM EDTA pH8.0) and containing 0.8 µg/ml ethidium bromide, according to standard protocols (Sambrook *et al.*, 2001). Gel images were captured using the GeneSnap imaging software (Synegene, UK) and the size and concentration of DNA products was determined by comparison with Bioline Hyperladder DNA marker (Bioline) included in the gel.

2.2.5 DNA sequencing and sequence analysis

PCR amplified DNA was purified using the Qiaquick Gel Extraction kit™ (Qiagen), according to manufacturer's instructions, checked by agarose gel electrophoresis (2.2.3) and used for direct sequencing.

Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing Kit (PE Biosystems, Warrington, UK) with the appropriate primers (Table 2.2) and the products were analysed with an ABI PRISM 3100-Genetic Analyser

TABLE 2.2 Oligonucleotide primers used for PCR in this study

Gene	Primer	Nucleotide sequence (5'-3')	Approximate product size (bp)	Cycling conditions
wsp[a]	81f	TGGTCCAATAAGTGATGAAGAAAC	590-632	94.0°C for 4 min, 35 cycles of 92°C for 30 s, 56.7°C for 30 s, 75°C for 1 min, extension for 5 min at 72°C.
	691r	AAAAATTAAACGCTACTCCA		
16S rRNA[b]	16Sfor	TTGTAGCCTGCTATGGTATAACT	936	94°C for 4 min, 35 cycles of 94°C for 30 s, 50°C for 30 s, 75°C for 1 min, extension for 5 min at 72°C.
	16Srev	GAATAGGTATGATTTTCATGT		
28S rRNA[c]	28Sfor	CCCTGTTGAGCTTGACTCTAGTCTGGC	500-600	95°C for 5 min, 30 cycles at 95°C for 30 s, 58 °C for 30 s, 72 °C for 30 s, extension for 5 min at 72 °C.
	28Srev	AAGAGCCGACATCGAAGGATC		
28S rRNA D2 expansion region[d]	CP12	GTGGATCCAGTCGTGTTGCTTGATAGTGCAG	615-640	94°C for 5 min, 30 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, extension for 5 min at 72°C.
	CP15	GTGAATTCTTGGTCCGTGTTTCAAGACGGG		

[a] Braig *et al.*, 1998; [b] O'Neill *et al.*, 1992, 16Sfor and 16Srev correspond to *Escherichia coli* positions 76-99 forward and 1012-994 reverse, respectively (Brosius *et al.*, 1981); [c] Werren *et al.*, 1995; [d] Porter & Collins, 1996

(Applied Biosystems, Foster City, CA, USA). Sequence chromatographs were analysed using the BioEdit Sequence Alignment Editor software package (version 5.0.9; Hall, 1999) and a consensus sequence was generated for each sample from forward and reverse passes.

Homology searches for *wsp* sequences in GenBank DNA databases using BLASTN (Basic Local Alignment Search Tool) on the National Centre for Biotechnological Information site were used to confirm that the PCR products had been amplified from the *Wolbachia* specific *wsp* gene in each sample. Representatives of each of the recognised *Wolbachia* subgroups (Zhou *et al.*, 1998; van Meer *et al.*, 1999) and *wsp* sequences from members of other gall wasp communities were selected from GenBank for inclusion in a sequence alignment. A C-clade *wsp* sequence from the filarial nematode *Brugia malayi* (GenBank accession number AJ252061; Bazzocchi *et al.*, 2000) was also included as a root sequence for phylogenetic analysis. Sequences were aligned using ClustalX (Thompson *et al.*, 1997) with default settings and alignments were corrected manually. The third hypervariable region (519-559 bp) was excluded from analysis as in previous studies (Braig *et al.*, 1998; Kittayapong *et al.*, 2003; Rokas *et al.*, 2002a; Thiapaksorn *et al.*, 2003; van Meer *et al.*, 1999; Zhou *et al.*, 1998) as this region could not be aligned with confidence. Phylogenies were generated using distance methods using the TreeconW software package (Van de Peer & De Wachter, 1997). Jukes-Cantor (1969) was used to compare the aligned sequences, followed by Neighbour Joining (Saitou & Nei, 1987), and bootstrap analysis was carried out at 1000 repetitions. The derived amino acid sequence of the *wsp* gene is not usually used for phylogenetic analysis due to the uncertainties introduced by the hypervariable regions. Phylogenetic analysis using aligned derived amino acid sequences produced the same subgroup clusters as the nucleotide alignment, and the differences in tree topology that were seen did not affect the conclusions made in Sections 2.3 and 2.4.

28S rRNA gene and 28S D2 expansion region sequence alignments and phylogenetic trees were constructed as described above. These trees were rooted to the 28S rRNA gene and 28S D2 expansion region sequences *Myrmecia croslandi* (Hymenoptera: Apocrita: Aculeata).

2.2.6 Cloning of *wsp* gene sequences

Where direct sequencing produced multiple peaks indicative of the presence of more than one *Wolbachia* strain in an individual wasp, *wsp* PCR products were cloned and up to 10

clones were sequenced. PCR products (30 ng) were ligated into a T-tailed vector (pGEM-T Easy Vector system, Promega Ltd.) following manufacturer's instructions. Ligations were used to transform *E. coli* XL1-Blue and plated onto Luria-Bertani (LB) agar containing ampicillin (100 μg/ml), X-Gal (80 μg/ml), and IPTG (0.5 mM). Cultures were incubated at 37°C and transformants were identified by growth of the colony in the presence of amplicillin. The presence of an insert was confirmed by colony PCR with *wsp* specific primers. Plasmids were extracted from bacteria using the Wizard miniprep DNA purification systems kit (Promega Ltd.), according to manufacturer's protocol, and stored at -20°C.

2.3 Results

2.3.1 Host species identification

DNA was extracted from 82 wasp specimens collected from sites across Europe (Rokas *et al.*, 2001). Morphological examination identified the following seven species of wasp: *Torymus auratus* (*T. geranii*; see below) (Chalcidoidea: Torymidae) (46 specimens), *T. flavipes* (28 specimens), *B. pallida* (Cynipoidea: Cynipini) (1 specimen), *Synergus gallaepomiformis* (Cynipoidea: Synergini) (1 specimen), *Megastigmus almusiensis* (Chalcidoidea: Torymidae) (2 specimens), a braconid species (Ichneumonidae: Braconidae) (3 specimens) and a eupelmid species (Chalcidoidea: Eupelmidae) (2 specimens).

Each wasp specimen was labelled according to the species of the wasp itself, the specimen number, the species of the causer of the gall from which the specimen emerged, the number of that gall, and the sampling location. For example specimen Ta.1.Bp.45.S1 was the first *T. auratus* specimen that emerged from gall *B. pallida*-induced gall number 45, collected from sampling site S1. The species names abbreviations are given in the 'Abbreviations' table and the sampling locations are described in Table 2.1.

Although the primary aim of this study involved investigation of the parasitoid species, the cynipid specimens were included. It was not possible to distinguish between *T. auratus* and *T. geranii* specimens with absolute confidence using morphological analysis. No formal identification of the eupelmid species from Gall 18 (collected in Cambridge, UK) was made but only one species, *Eupelmus urozonus* is known to be associated with *B. pallida* galls in Britain (Askew *et al.*, 2004; Williams, 2004). Therefore, this species designation was employed.

Two specimens from galls collected in France and Spain were identified as *Megastigmus almusiensis*, by morphological analysis. This parasitoid species has previously only been found attacking *N. macropterus* galls in Turkey (Askew *et al.*, 2004). This may indicate that *M. almusiensis* distribution includes other European regions, and that this parasitoid is not specific to a single gall-causer host but also attacks galls induced by *B. pallida*.

Two molecular marker gene regions were used to confirm and extend the results of the morphological identifications. The 28S rRNA gene sequences from this study varied in length between 504 - 541 bp and the aligned dataset was 506 bp. The variable D2 expansion region (28S rRNA gene) produced sequences between 615 - 643 bp, and an aligned dataset of 620 bp was produced. Phylogenetic tree construction using aligned 28S

rRNA gene sequences confirmed that at least six different species of wasps were present but sequence divergence was not sufficient to discriminate between *Torymus* parasitoid species (Fig. 2.2). Analysis of the variable D2 expansion region revealed greater sequence divergence. The phylogenetic tree in Fig. 2.3 supported the morphology based species designation of the *T. auratus* and *T. flavipes* specimens and improved on this, indicating that samples Tg.1.Bp.55.F1, Tg.1.Bp.49.F1, Tg.2.Bp.49.F1, Tg.1.Bp.18.U2 and Tg.1.Bp.11.U1 belonged to a third species which was designated *T. geranii* (Table 2.3). The species assigned to each wasp sample examined in this study are summarised in Table 2.3.

2.3.2 Detection of *Wolbachia* in parasitoids from the *B. pallida*-induced gall community

Five of the eight species tested were found to be infected with *Wolbachia* (63%). Table 2.3 lists all wasp specimens, their species designation and *Wolbachia* infection status. Samples in which both *wsp* (and 16S rDNA, data not shown) and 28S rDNA were amplified successfully were recorded as infected (e.g. Fig. 2.4, lanes 11 & 14; Section 2.2.3). Absence of a *wsp* product was assumed to indicate the absence of *Wolbachia* infection only if amplification of the 28S rRNA gene was successful (e.g. lanes 3 - 10, Fig 2.4). Where amplification of a 28S rRNA gene sequence failed, template DNA was assumed to be of poor quality and samples were excluded from further study. A 93% infection rate was detected in *T. flavipes* (26 / 28 specimens), 67% in *T. geranii* (4 / 6 specimens), and 100% in the braconid sp. (3/3 specimens). The infected status of *B. pallida* (1 specimen) and *S. gallaepomiformis* (1 specimen) detected by Rokas *et al.* (2002a) was confirmed.

2.3.3 Analysis of *wsp* sequence information

Nucleotide sequences of the *wsp* gene fragments amplified from the wasp specimens varied in length between 575 - 605 bp. Study sequences were aligned with sequences from the GenBank database and the resulting aligned dataset was 463 bp in length. The *Wolbachia* subgroups proposed by Zhou *et al.* (1998) and extended by van Meer *et al.* (1999) have been highlighted in Fig. 2.5, which shows that six distinct *wsp* sequences, representing six different *Wolbachia* variants, infect members of the wasp community tested in this study (Table 2.3).

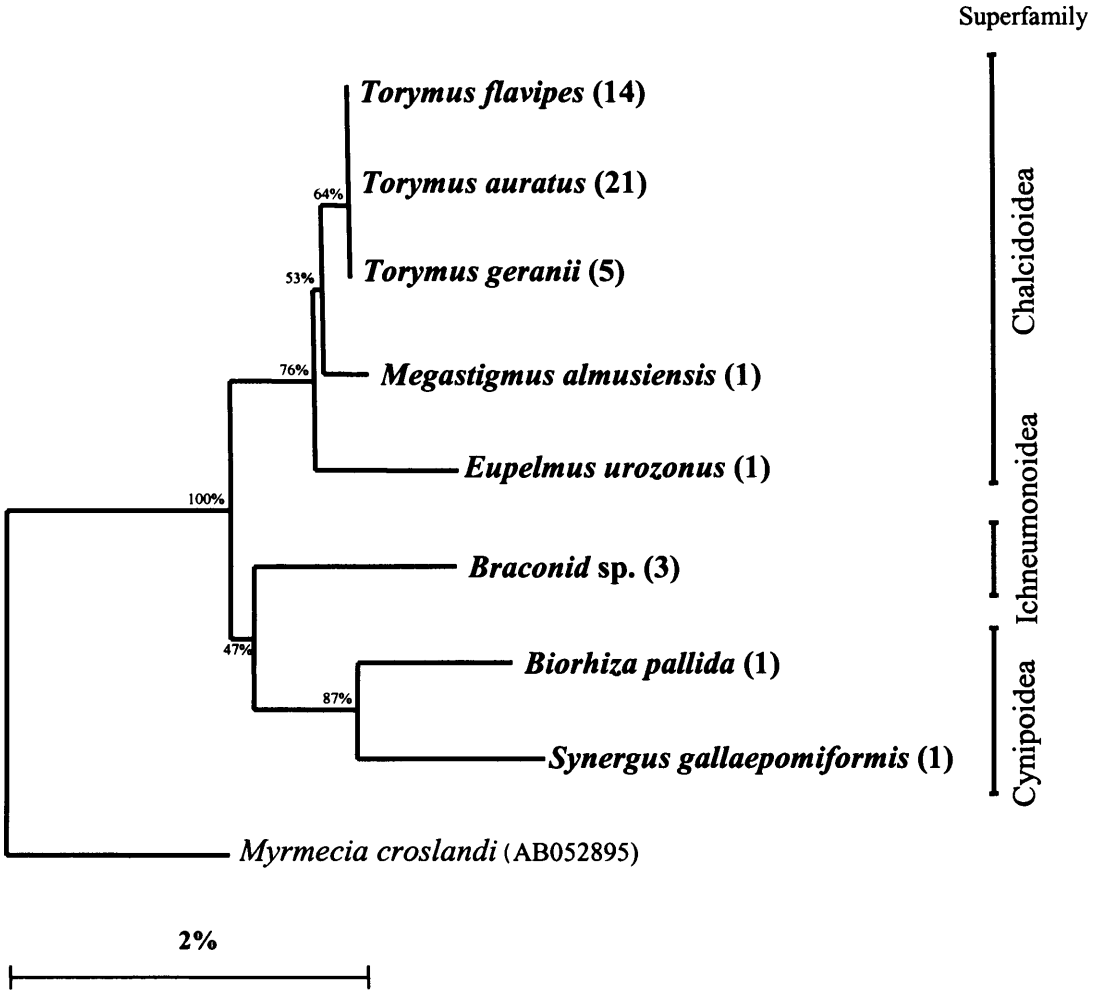

FIG. 2.2 Phylogenetic tree based on analysis of 28S rRNA gene sequences from members of the oak gall wasp community, constructed using Jukes-Cantor to compare ClustalX alignments (506 bp) followed by Neighbour Joining (Section 2.2.5). Sequences obtained in the present study are presented in bold and the number of samples from which that sequence was obtained is given in parenthesis. For all other sequences the accession number is given. The 28S rRNA gene sequence from *Myrmecia croslandi* (Hymenoptera: Apocrita: Aculeata) was used as a root and bootstrapping was carried out at 1000 replicates.

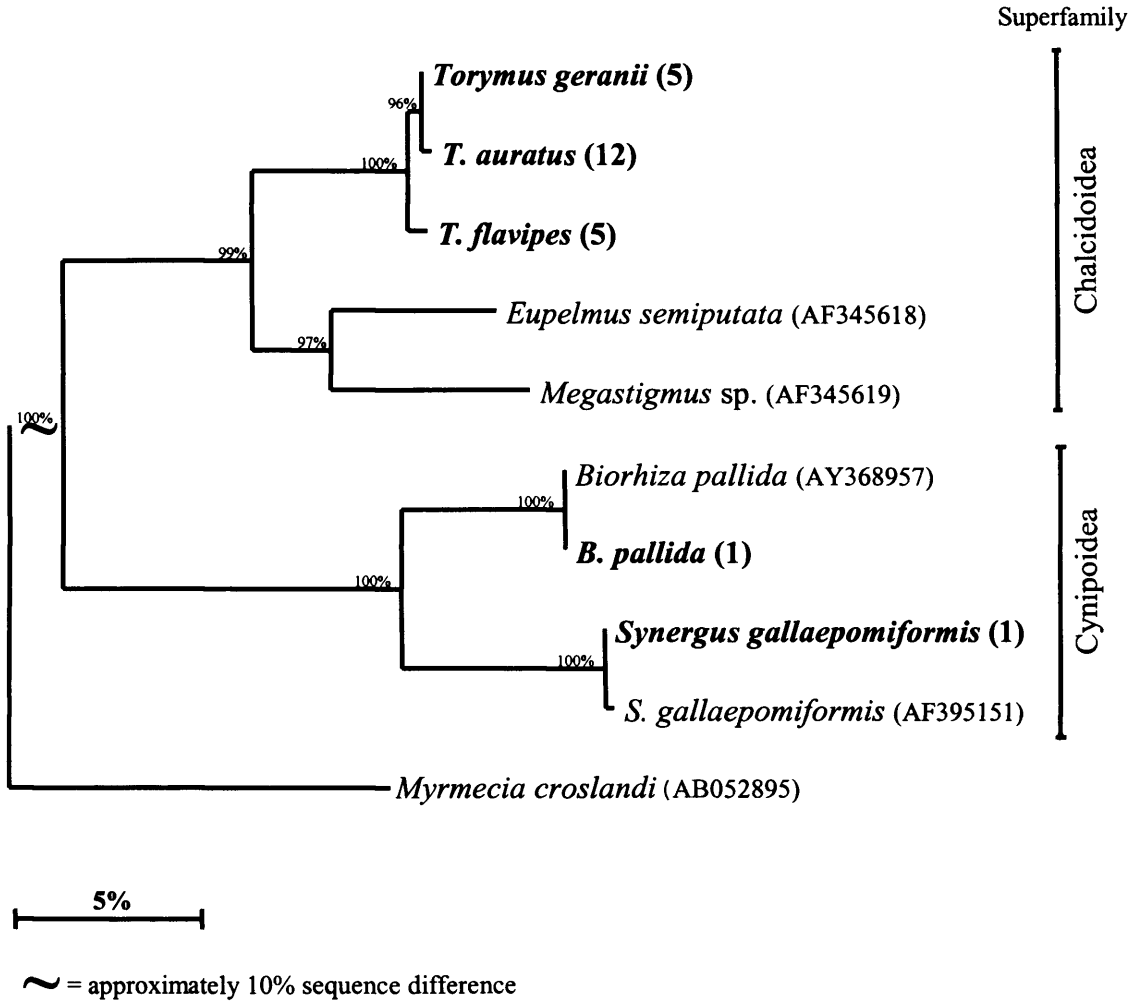

FIG. 2.3 Phylogenetic tree based on analysis of 28S D2 expansion region sequences from members of the oak gall wasp community, constructed using Jukes-Cantor to compare ClustalX alignments (620 bp), followed by Neighbour Joining (Section 2.2.5). Sequences obtained in the present study are presented in bold and the number of samples from which that sequence was obtained is given in parenthesis. For all other sequences the accession number is given. The 28S rRNA gene sequence from *Myrmecia croslandi* (Hymenoptera: Apocrita: Aculeata) was used as a root and bootstrapping was carried out at 1000 replicates.

TABLE 2.3 The infection status of each wasp tested during this study is presented with the number of infected (+) and uninfected (-) individuals of each species found in each gall. The subgroup to which each *Wolbachia* variant grouped, is also given [a]

Sample labels[b]	Species name	Infection status	Ratio + : -	*Wolbachia* subgroup [a]
Sg.1.Bp.6.U1	*Synergus gallaepomiformis* (Fonscolombe)	+	1:0	wMel
Tg.1.Bp.11.U1	*Torymus geranii* (Walker)	-	0:1	-
Ta.1-4.Bp.11.U1	*Torymus auratus* (Geoffroy in Fourcroy)	-	0:4	-
Ta.1-6.Bp.16.U1	*Torymus auratus*	-	0:6	-
Tg.1.Bp.18.U2	*Torymus geranii*	+	1:0	wUni & wCon
Ta.1-2.Bp.18.U2	*Eupelmus urozonus* Dalman, 1820	-	0:2	-
Tf.1-4.Bp.30.U3	*Torymus flavipes* (Geoffroy in Fourcroy)	+	4:0	wKue
Bp.1.Bp.30.U3	*Biorhiza pallida* (Olivier)	+	1:0	wMors
Ta.1-3.Bp.33.H1	*Torymus auratus*	-	0:3	-
Ta.1-4.Bp.36.H1	*Torymus auratus*	-	0:4	-
Ta.1-2.Bp.37.H2	*Torymus auratus*	-	0:2	wKue
Tf.1-5.Bp.38.H2	*Torymus flavipes*	+	5:0	wKue
Tf.1-6.Bp.38.H3	*Torymus flavipes*	+	6:0	wKue
Ta.1-3.Bp.42.S1	*Torymus auratus*	-	0:3	-
Ta.1.Bp.44.S1	*Torymus auratus*	-	0:1	-

TABLE 2.3 continued

Sample labels [b]	Species name	Infection status	Ratio + : -	*Wolbachia* subgroup [a]
Tf.1-8.Bp.45.S1	*Torymus flavipes*	+	8:0	wKue
Tf.1.Bp.47.S1	*Torymus flavipes*	+	2:2	wKue
Tf.4.Bp.47.S1	*Torymus flavipes*	+		wKue
Tf.2-3.Bp.47.S1	*Torymus flavipes*	-		-
Ta.5.Bp.47.S1	*Torymus auratus*	-	0:1	-
Ta.1-3.Bp.48.S1	*Torymus auratus*	-	0:3	-
Tg.1-2.Bp.49.F1	*Torymus geranii*	+	2:0	wUni & wCon
Ta.1-6.Bp.49.F1	*Torymus auratus*	-	0:6	-
Bsp.1-3.Bp.50.F1	Braconid sp.	+	3:0	XwBra [c]
Tg.1.Bp.55.F1	*Torymus geranii*	-	1:2	-
Tg.2.Bp.55.F1	*Torymus geranii*	+		wUni & wCon
Ta.4-5.Bp.55.F1	*Torymus auratus*	-	0:1	-
Mga.1.Bp.55.F1	*Megastigmus almusiensis* Doganlar	-	0:1	-
Ta.1-6.Bp.56.F1	*Torymus auratus*	-	0:6	-
Tf.1.Bp.56.F1	*Torymus flavipes*	+	0:1	wKue

[a] Zhou *et al.* (1998); van Meer *et al.*, 1999; Section 2.2.5; Fig. 2.5. [b] For an explanation of the sample labels see Section 2.3.1. The sample label has been abbreviated where multiple specimens of a given species were found in the same gall, e.g. Ta.1-3.Bp.48.S1 refers to samples Ta.1.Bp.48.S1, Ta.2.Bp.48.S1 and Ta.3.Bp.48.S1. [c] The *Wolbachia* variant identified in samples Bsp.1-3.Bp.50.F1 did not group within an established subgroup so the label XwBra was assigned (see Section 2.3.3).

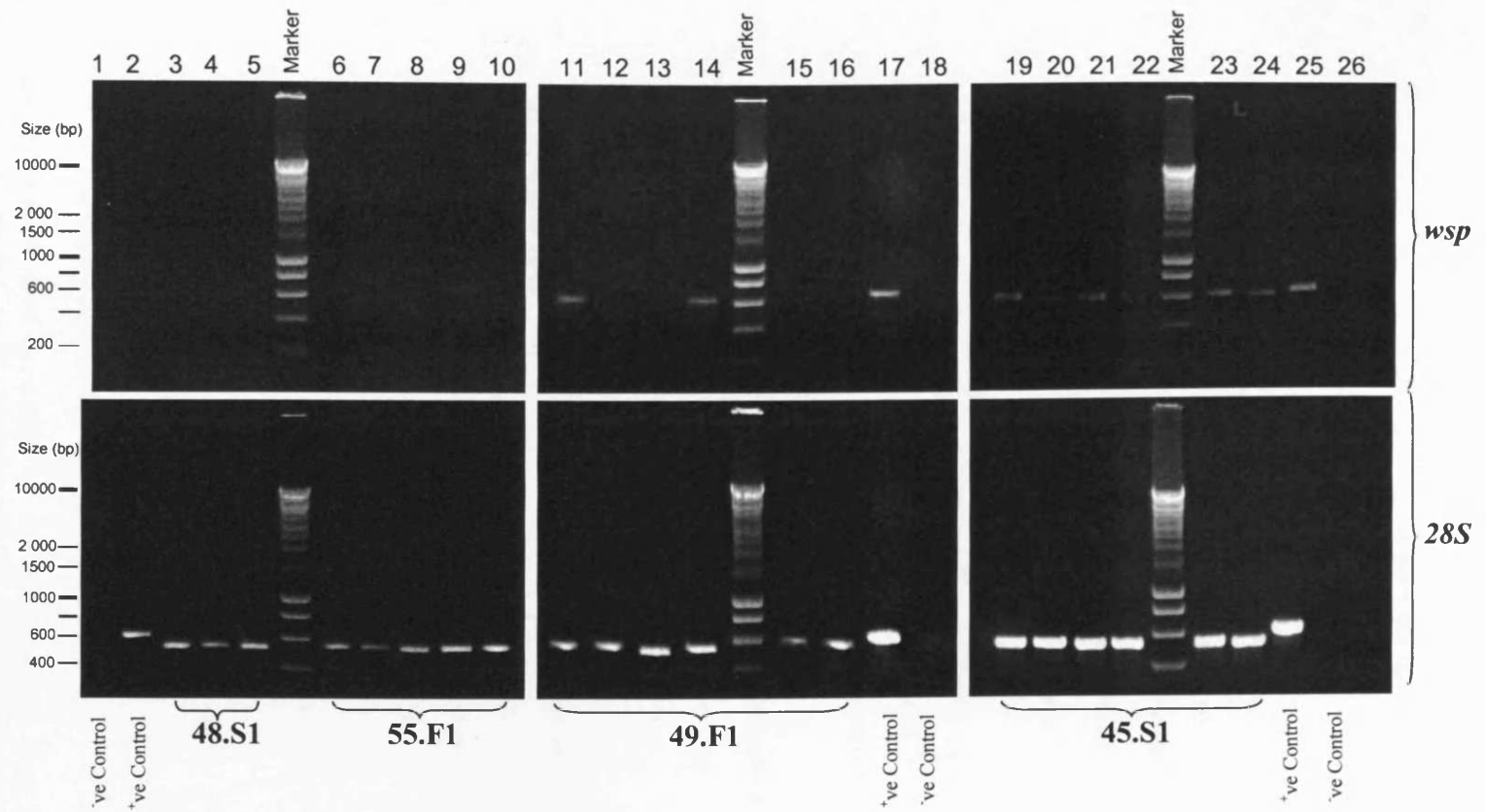

FIG. 2.4 PCR-amplified *wsp* (top) and 28S (bottom) fragments for each wasp specimen were run on agarose electrophoresis gels. Products from samples from galls 48.S1 (lanes 3-5), 55.F1 (lanes 6-10), 49.F1 (lanes 11-16), 45.S1 (lanes 19-24) are shown (Table 2.3). Negative controls in which no DNA was included in the PCR reaction were run in lanes 1, 18 & 26. Positive controls in which DNA from *D. simulans* (Riverside strain) was used as a template were run in lanes 2, 17 & 25.

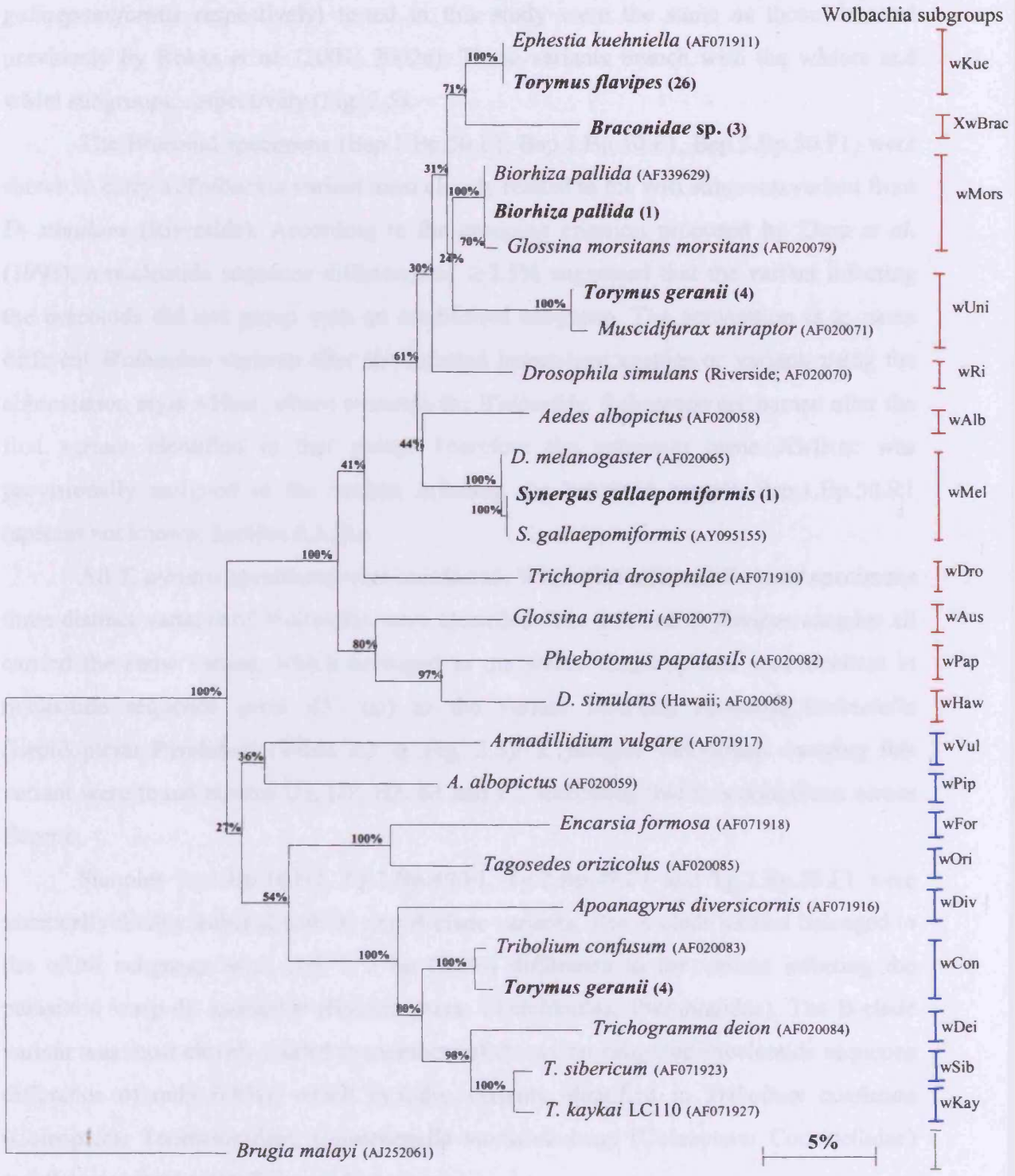

FIG. 2.5 Phylogenetic tree constructed based on analysis of *wsp* gene sequences from the oak gall wasp community, using Jukes-Cantor to compare ClustalX alignments (414 bp), followed by Neighbour Joining (Section 2.2.5). Bootstrapping was carried out at 1000 repetitions. Sequences in bold were obtained during the present study. The number of specimens from which each sequence variant was obtained is given in parenthesis. For all other sequences the accession number is given. The *Wolbachia* subgroups proposed by Zhou *et al.* (1998) and van Meer *et al.* (1999) are labelled. Red lines group together clade-A sequences, blue lines group sequences from clade-B and the green line highlights the sequence from the nematode clade-C, which was used to root the tree.

The *wsp* sequence variants from Bp.1.Bp.30.U3 and Sg.1.Bp.6.U1 (*B. pallida*, *S. gallaepomiformis* respectively) tested in this study were the same as those detected previously by Rokas *et al.* (2001; 2002a). These variants branch with the wMors and wMel subgroups, respectively (Fig. 2.5).

The Braconid specimens (Bsp.1.Bp.50.F1, Bsp.2.Bp.50.F1, Bsp.3.Bp.50.F1) were shown to carry a *Wolbachia* variant most closely related to the wRi subgroup variant from *D. simulans* (Riverside). According to the grouping criterion proposed by Zhou *et al.* (1998), a nucleotide sequence difference of ≥2.5% suggested that the variant infecting the braconids did not group with an established subgroup. The convention is to name different *Wolbachia* variants after the infected insect host species or variant, using the abbreviation style wHost, where w stands for *Wolbachia*. Subgroups are named after the first variant identified in that group. Therefore the subgroup name XwBrac was provisionally assigned to the variant infecting the braconid sample Bsp.1.Bp.50.R1 (species not known; Section 2.3.1).

All *T. auratus* specimens were uninfected. Within the infected *Torymid* specimens three distinct variants of *Wolbachia* were identified. The infected *T. flavipes* samples all carried the same variant, which belonged to the wKue subgroup and was identical in nucleotide sequence (over 463 bp) to the variant infecting *Ephestia kuehneiella* (Lepidoptera: Pyralidae) (Table 2.3 & Fig. 2.5). *T. flavipes* individuals carrying this variant were found at sites U3, H2, H3, S1 and F1, indicating that it is ubiquitous across Europe.

Samples Tg.1.Bp.18.U2, Tg.1.Bp.49.F1, Tg.2.Bp.49.F1 and Tg.2.Bp.55.F1 were identically doubly infected with A- and B-clade variants. The A-clade variant belonged to the wUni subgroup, with only a 2 bp (0.4%) difference to the variant infecting the parasitoid wasp *M. uniraptor* (Hymenoptera: Chalcidoidea: Pteromalidae). The B-clade variant was most closely related to members of the wCon subgroup (nucleotide sequence difference of only 0.8%), which includes variants identified in *Tribolium confusum* (Coleoptera: Tenebrionidea), *Coleomegella maculate lengi* (Coleoptera: Coccinellidae) and the parasitoid wasp *Torymus bedeguarius*.

2.4 Discussion

Wolbachia prevalence was investigated in members of the wasp community associated with sexual generation *B. pallida* galls, from eight sites across Europe (Fig. 2.1, Table. 2.1, Rokas *et al.*, 2001). Eight wasp species were screened for *Wolbachia* infection: two cynipid, one braconid (Hymenoptera: Ichneumonoidea: Ichneumonidae) and five chalcid species (Table 2.3). The presence of the braconid species was unexpected as most parasitoids of the Cynipini belong to the superfamily Chalcidoidea but occasional Ichneumonidae species have been reared from cynipid galls (Askew, 1984).

2.4.1 *Wolbachia* diversity in parasitoids of sexual generation *B. pallida* galls

Four diverse *Wolbachia* variants were identified in the parasitoid species tested, including one double infection (Table. 2.3). Three of the variants belonged to clade A and were spread throughout the clade (Fig. 2.5). Two of these grouped with sequence variants from the wKue and wUni subgroups (Zhou *et al.*, 1998; van Meer *et al.*, 1999). The third A-clade variant was identified in the braconid specimens (XwBrac). The high incidence of clade A *Wolbachia* in this wasp community was in agreement with previous surveys in the hymenoptera (Rokas *et al.*, 2002a; West *et al.*, 1998; Werren & Windsor, 2000).

One B-clade variant from the wCon subgroup was identified, occurring as a double AB infection with the wUni subgroup variant. Neither of these variants was detected as a single infection in any of the samples tested. Multiple (up to 5 variants in a single host insect) infections have been detected in a range of host species (Baurdry *et al.*, 2003; Breeuwer *et al.*, 1992; Ijichi *et al.*, 2002; Jamnongluk *et al.*, 2002; Kikuchi & Fukatsu, 2003; Kondo *et al.*, 2002; Mercot *et al.*, 1995; Mitsuhashi *et al.*, 2002; Nirgianaki *et al.*, 2003; Riegler & Stauffer, 2002; Van Borm *et al.*, 2003; Vavre *et al.*, 1999a; Wenseleers *et al.*, 1998; Werren & Windsor, 2000), including other parasitic hymenoptera, such as species from the families Pteromalidae, Braconidae, Figitidae and Eulophidae (Breeuwer *et al.*, 1992; Vavre *et al.*, 1999a; West *et al.*, 1998). This is the first example of a multiple infection in the family Torymidae.

2.4.2 *Wolbachia*-induced reproductive manipulation

It is not known whether the *Wolbachia* variants identified in this survey induce a phenotype in their hosts. *Wolbachia*-induced CI (Section 1.3) has been detected in haplodiploid wasp species such as *Nasonia vitripennis*, *Asobara tabida* and *Leptopilina heterotoma* (Perrot-Minnot *et al.*, 1996; Vavre *et al.*, 1999a), in which it results in the

production of an excess of male offspring (Breeuwer & Werren, 1990). In other species belonging to the Chalcidoidea and Cynipoidea, *Wolbachia* has been shown to be associated with PI (Stouthamer, 1997; Section 1.3). Plantard *et al.* (1998) determined that thelytokous parthenogenesis was induced by *Wolbachia* in several species of the Diplolepidini (previously known as the Rhoditini; Ronquist, 1999b) tribe and 'Aylacini' tribe (Hymenoptera: Cynipidae). However, *Wolbachia* is not involved in parthenogenic reproduction of species of the Synergini and Cynipini tribes (Rokas *et al.*, 2002a). *Wolbachia* induced thelytoky has also been identified in parasitic wasps including *Aphytis* species (Chalcidoidea: Aphelinidae) (Zchori-Fein *et al.*, 1995), *Trichogramma* species (Stouthamer *et al.*, 1990), *Muscidifurax* species (Chalcidoidea: Pteromalidae) (Stouthamer *et al.*, 1993), *Leptopilina clavipes* and *L. australis* (Eucoilidae: Leptopilina) (Werren *et al.*, 1995a) and *Encarsia formosa* (Chalcidoidea: Aphelinidae) (Werren *et al.*, 1995a).

Due to the obligate nature of the association between the parasitoids and the gall-causer or inquiline hosts, and between the gall-causer and the oak tissues (Askew, 1984; Plantard *et al.*, 1998; Rokas *et al.*, 2001; Stone *et al.*, 2002), the influence of *Wolbachia* on the reproduction of the parasitoid species from this study could not be tested directly using established curing and mating experiments (Stouthamer *et al.*, 1999; Jiggins *et al.*, 2000; 2001b). However, the *Wolbachia* variants identified here were similar, or identical to variants isolated from other hosts species in which the reproductive phenotype induced has been determined.

The A-clade variant from the doubly infected *T. geranii* was nearly identical (0.4% nucleotide difference, 2bp) to the variant that induces parthenogenesis in *M. uniraptor* (wUni) (van Meer *et al.*, 1999). The B-clade variant was most similar to the variant infecting *T. bedeguaris* (phenotype untested; van Meer *et al.*, 1999) and *Tribolium confusum* (MK & CI; Chang & Wade, 1996; Fialho & Stevens, 2000). The *T. flavipes* infecting variant was identical to the *Ephestia kuehneilla* (Lepidoptera) variant, which induces CI (Ikeda *et al*, 2003a) and the variant identified in the braconid specimens was most closely related to the wRi strain from *D. simulans* (Riverside), which also induces CI (Hoffmann & Turelli, 1997).

This assortment of phenotypes complicates inferences about the potential effects of *Wolbachia* on the study samples. Also, it has been proven that the phenotype induced is significantly influenced by the host species itself. For example, identical *Wolbachia* variants that occur naturally in *Tribolium madens* and *Tribolium confusum*, were shown to

induce MK and CI in their respective hosts (Fialho & Stevens, 2000). Experimental transfection of the CI-inducing variant from *Cadra cautella* (Lepidoptera), or the F-inducing variant from *Ostrinia scapulalis* (Lepidoptera) into *E. kuehniella*, resulted in expression of the MK phenotype (Fujii *et al.*, 2001; Sasaki *et al.*, 2002). Therefore similarity in nucleotide sequence is not sufficient to infer reproductive phenotype.

The expression of F, PI or MK phenotypes has in the past been inferred from a disturbance in the natural 1:1 ratio of male to female offspring in *Wolbachia* infected populations (Hurst *et al.*, 1997). Both male and female specimens were screened in this survey but larger sample numbers would be required to judge whether a sex ratio bias exists.

It is interesting to note that CI is the only *Wolbachia*-induced phenotype that is known to occur in multiply-infected hosts (Kondo *et al.*, 2002; Perrot-Minnot *et al.*, 1996; Riegler & Stuaffer, 2002; Vavre *et al.*, 1999a), though in several other species the phenotype of multiply infecting variants remains untested and expression of other phenotypes can not be ruled out (Jamnongluk *et al.*, 2002; Kikuchi & Fukatsu, 2003; Mitsuhashi *et al.*, 2002; Reuter & Keller, 2003; Van Borm *et al.*, 2003; Werren & Windsor, 2000). The *T. geranii* double infection might, therefore, include CI-*Wolbachia.* Co-infections of CI-strains are successful due to the selective advantage incurred by infected individuals, which allows the multiple infection to spread through, and be maintained in a population (Mouton *et al.*, 2003; Hoffmann & Turelli, 1997). There is currently no information about how PI-, MK- or F-inducing strains of *Wolbachia* might interact with each other if they occurred as multiple infections. However, 'neutral' variants that exert no affect on their hosts, may 'hitchhike' alongside PI-, MK- or F-inducing strains (Charlat *et al.*, 2004; Giordano *et al.*, 1995; Hoffmann *et al.*, 1996; Vavre *et al.*, 2002) and could occur as part of a multiple infection. *Wolbachia* infection dynamics were discussed in more detail in Section 1.3.1. MK- and PI-strains have yet to be identified in hymenopterans (Dyson *et al.*, 2002; Hurst *et al.*, 2003), therefore *Wolbachia* variants identified in this study are more likely to express CI, PI or be neutral, no firmer conclusions can be drawn based on the evidence available.

2.4.3 Incidence and prevalence of *Wolbachia* infection in the community associated with sexual generation *B. pallida* galls

Of a total of 28 *T. flavipes* specimens, 93% were infected. The uninfected individuals (x2) were from Gall 47, from Spain. However, infected *T. flavipes* specimens from the same

gall were identified; therefore this result may have been due either to genuine infection polymorphism (see below) or failure of the PCR to amplify low density infections. Infection frequency at all other sites was 100% therefore this infection appears to be ubiquitous.

In populations where *Wolbachia* infection occurs at a low frequency, screening of only a few individuals may give a misleading indication of the prevalence and diversity of infection. In this study every effort was made to screen maximum numbers of individuals but rarer species were represented by only a few specimens, and infections in these species may therefore have evaded detection. This also limits the ability to make inferences about the geographical variation in *Wolbachia* infection in these samples.

Single specimens of the gall causer and the only inquiline species known from *B. pallida* galls were tested, confirming infection with single *Wolbachia* variants identified previously (Rokas *et al.*, 2002a). Rokas *et al.* (2002a) detected *Wolbachia* in 85% of 206 *B. pallida* samples and the infection was either fixed or absent in populations. *Wolbachia* was detected in 85% of 34 *S. gallaepomiformis* samples and the populations sampled were polymorphic for the infection. The incidence of *Wolbachia* infection thus appears to be highly variable within the oak gall wasp community.

Infection frequency is rarely 100% in natural populations. For example in European populations of tephritid cherry fruit flies a *Wolbachia* prevalence of 99.8% was recorded (Riegler & Stauffer, 2002), and Abe & Muira (2002) found that infection frequency differed between two populations of *Andricus mukaigawae* (80% and 92%). Failure of *Wolbachia* infections to reach fixation in a host population (infection frequency of 100%) may be the result of fitness costs associated with infection. CI-inducing strains typically occur at a high prevalence, often near fixation. MK-strains normally show lower prevalence and PI- and F-inducing strains occur at a range of frequencies from fairly low to fixation (Jiggins *et al.*, 2001a). Inefficient vertical transmission is also important for maintaining polymorphism in infection in the host population (Merçot *et al.*, 1995). Several factors influence the efficiency of vertical transfer of *Wolbachia* and therefore prevalence, including the age of the host, presence of natural antibiotics, host diapause and exposure to elevated temperature (Hoffmann *et al.*, 1998; Hurst *et al.*, 2000; Perrot-Minnot *et al.*, 1996). In addition, there is evidence that 'repressor genes' that act against the effects of *Wolbachia* infection, have evolved in some species (Hoffmann & Turelli, 1997; Rigaud & Juchault, 1993). For example in *Armadillidium vulgare*, autosomal masculinising genes (M) have evolved in response to feminising factors, including an F-

inducing variant of *Wolbachia* (Rigaud & Juchault, 1993). This could be a possible explanation of why some of the parasitoid species were not infected, even though they share a similar ecological niche and physiology to infected species (West *et al.*, 1998). Differences in infection frequency could also reflect differences in the time elapsed since *Wolbachia* invaded the population (Shoemaker *et al.*, 2003).

2.4.4 Has horizontal transmission of *Wolbachia* occurred in the wasp community associated with sexual generation *B. pallida* galls?

The results of this study do not provide direct evidence that horizontal transmission of *Wolbachia* has occurred widely within the wasp community associated with *B. pallida.* The gall causer, inquiline and parasitoid wasp species tested in this study were found to be infected with highly divergent variants. Three *Torymus* species were examined in this study and displayed very different infections: *T. flavipes* was infected with a single *Wolbachia* variant from the wKue subgroup, *T. geranii* was doubly infected with variants from the wUni and wCon subgroups and *T. auratus* was uninfected. As a result of horizontal transmission, these species might be expected to harbour more similar infections because they provide physiologically similar host environments for the endosymbiont (Russel *et al.*, 2003). Conversely, the fact that such diverse infections have been detected, including a double infection, suggests that they have not arisen by divergence from an ancestral infection and that horizontal transmission has occurred at some time in the past.

Indirect evidence of horizontal transmission was been detected in oak gall wasps previously (Rokas *et al.*, 2002a). Several of the cynipid species tested, were found to carry the same, or very similar *Wolbachia* variants. For example, the variants infecting *B. pallida*, *Andricus solitarius* (variant 1), *Synergus crassicornis* and *Neuroterus macropterus* all belong to the wMors subgroup, and the variants infecting *A. solitarius* (variant 2 and variant 3), *S. umbraculus*, *S. diaphanus* and *S. reinhardi*, all belong to the wHaw subgroup (Fig. 2.5; Rokas *et al.*, 2002a). In addition, *A. solitarius* individuals were infected with more than one variant. These results suggested possible horizontal transfer from inquiline to causer species (or vice versa). However, as stated earlier (Section 2.1), none of these inquiline species have been reared from the galls induced by *B. pallida*, *A. solitarius* or *N. macropterus*, therefore transfer could not have occurred directly between these gall causers and these inquiline species.

Parasitoids may provide an explanation, as several of the parasitoid species which attack the gall causers listed above, also attack galls which are used by the inquilines infected with similar *Wolbachia*. For example, galls induced by *A. quercuscalicis* are used by *S. umbraculus*, *S. reinhardi* and *S. crassicornis* inquilines and are attacked by *Eurytoma brunniventris* parasitoids. *E. brunniventris* parasitoids also attack *B. pallida*, *A. solitarius* and *N. macropterus* galls (Askew, 1984) and therefore provide a potential route of transfer.

In fact, few parasitoid species are restricted to one oak gall or host species (Stone *et al.*, 2002). Each of the parasitoid species tested in this study (*T. auratus*, *T. flavipes*, *T. geranii*, *E. urozonus*) are known to attack several different cynipid species (Askew *et al.*, 2004; Askew, 1961; Williams, 2004; Stone *et al.*, 2002).

The absence of direct evidence in support of horizontal transfer of *Wolbachia* in the species tested in the present work, is not sufficient to rule out the possibility that horizontal transfer occurs within the oak gall wasp community as a whole. If the *Wolbachia* in this community are neutral or induce no fitness benefit, their ability to invade or be maintained in a new host species may be severely reduced (Hoffmann & Turelli, 1997). As a result, horizontal transmission may occur only rarely within this community and if it does occur, it may fail to result in stably inherited infections. Such transient infections are likely to be missed during PCR based studies due to insufficient sample numbers.

To gain more information about *Wolbachia* transmission in the oak gall wasp community, all possible routes of transfer should be examined by screening members of parasitoid assemblages associated with several other oak gall wasp species. High throughput screening techniques such as SSCP and DGGE may be useful alternatives to the labour intensive PCR and sequencing approach, facilitating larger scale screens that should reduce the likelihood of overlooking low level infections.

2.4.5 Wasp species identification using molecular markers

In this study two wasp gene sequences were employed as molecular markers: the 3' end of the 28S rRNA gene and the 28S rRNA D2 expansion region. Both were suitable for higher level phylogeny construction, successfully discriminating between the gall causer and inquiline species and separating the cynipid gall wasps from the chalcid parasitoids (Fig. 2.2 & Fig. 2.3). However, the rate of evolution of the 28S rRNA gene is relatively slow, and therefore it was found to be less suitable for phylogeny construction at the

species level (Rokas *et al.*, 2002c). The 28S D2 expansion region showed minimal sequence differences between the torymids, allowing tentative species identifications to be made but in future studies a more divergent marker gene should be used. Recently Rokas *et al.* (2002c) conducted an analysis of eight phylogenetic markers in gall wasps and determined that mitochondrial genes such as cytochrome *b* and cytochrome oxidase I subunit may be more suitable for construction of lower level phylogenies.

Chapter 3

Denaturing gradient gel electrophoresis: a rapid, reproducible technique for the detection of *Wolbachia* sequence variants in multiple insect specimens

3.1 Introduction

3.1.1 Studying *Wolbachia*

Symbiotic microorganisms are widespread in nature and the majority of insect species are associated with bacterial, viral, fungal or protist symbionts. The complexity of the association depends on the host-symbiont species combination and is influenced by several factors, including environmental pressures and other microorganisms. As such, the study of these interactions is of great ecological and evolutionary importance (Reeson *et al.*, 2003; Dillon & Dillon, 2004; Jeyaprakash *et al.*, 2003; Bourtzis & Miller, 2003, Moran & Wernegreen, 2000; Werren & O'Neill, 1997).

Wolbachia are obligate endosymbionts of insect (Jeyaprakash & Hoy, 2000; Kikuchi & Fukatsu, 2003; Kittayapong *et al.*, 2000; Reuter & Keller, 2003; Ricci *et al.*, 2002; Rokas *et al.*, 2001; Werren *et al.*, 1995a; West *et al.*, 1998), and as is true of most obligate intracellular bacteria, the study of *Wolbachia* has been confounded by the intimate nature of the association with the host. In the past, research has been restricted to laboratories with appropriate insect rearing facilities and to host species that are easily maintained (e.g. *Drosophila*). More recently, *in vitro* infections in insect cell lines have been established (Dobson *et al.*, 2002c; Fenollar *et al.*, 2003; Noda *et al.*, 2002; O'Neill *et al.*, 1997b).

The frequency and diversity of *Wolbachia* infections has been most commonly investigated using recombinant DNA and molecular phylogenetic techniques, which provide a means for studying microorganisms without the need for cultivation (Hugenholtz *et al.*, 1996; 1998; Section 1.6.1). Most studies involve PCR amplification of *Wolbachia*-specific marker genes, followed by cloning and DNA sequencing (O'Neill *et al.*, 1992; Werren *et al.*, 1995a; Zhou *et al.*, 1998). However this strategy is labour intensive, and as a result a given host species or population is often represented by only 1-5 individuals, which may not provide an accurate representation of the species / population (For example: Kikuchi & Fukatsu *et al.*, 2003; O'Neill *et al.*, 1992; Rokas *et al.*, 2002a, Wenseleers *et al.*, 1998; Werren *et al.*, 1995a). *Wolbachia* prevalence is highly variable and infection rarely occurs at a frequency of 100% (Abe & Muira, 2002; Riegler & Stauffer, 2002; Rokas *et al.*, 2002a; Stouthamer, 1997), as illustrated by the results

discussed in Chapter 2. The lower the frequency of infection, the greater the likelihood that the presence of *Wolbachia* will be missed by small sample sizes. Infection polymorphism was discussed in more detail in Sections 1.3.1 and 2.4. To gain a more accurate estimation of *Wolbachia* prevalence, high-throughput methods are needed to screen much larger population samples.

Several groups have used restriction fragment length polymorphism to distinguish between 16S rRNA, *ftsZ* or *wsp* sequence variants, reducing the need for DNA sequencing (Huigens *et al.*, 2004; Jamnongluk *et al.*, 2002; Kikuchi & Fukatsu, 2003; Kondo *et al.*, 2002; Mandel *et al.*, 2001; Merçot *et al.*, 1995; Mitsuhashi *et al.*, 2002; O'Neill *et al.*, 1997b; Reuter & Keller, 2003; Riegler & Stauffer, 2002; Van Borm *et al.*, 2003). This has allowed greater numbers of individuals to be screened for the presence of specific sequence variants more rapidly. In the studies listed above, different combinations of 2-3 restriction enzymes were used to detect the relative frequency of 1 to 3 *Wolbachia* sequence variants. However, to be truly useful, a screening technique that distinguishes between all *wsp* sequence variants is required.

Molecular typing methods enable the rapid screening of multiple samples with a greatly reduced need for cloning and sequencing. Typing methods vary in complexity and the method of choice depends on the type of community under investigation and the molecular markers in use. These methods (Table 1.4) are generally more cost-effective than sequencing and are very useful as prescreening techniques.

3.1.2 Profiling microbial populations using denaturing gradient gel electrophoresis

The DGGE technique has great potential as a rapid, simple discriminatory method for screening *Wolbachia* infected host specimens. DGGE facilitates the separation of PCR-amplified gene fragments of equal size, based on altered electrophoretic mobility resulting from nucleotide sequence differences. Fragments are subjected to electrophoresis in a polyacrylamide gel containing a linear gradient of chemical denaturants (formamide and urea). These, coupled with a high running temperature, cause the two strands to dissociate or 'melt', so increasing frictional drag between the DNA and the gel matrix. Fragments differing in nucleotide base composition and order demonstrate differences in electrophoretic mobility under denaturing conditions and migrate to different positions in the gel (Muyzer, 1999; Muyzer & Smalla 1998; Section 1.7.1). A major advantage of the DGGE technique over other profiling methods is the ability to

excise sequence variants from the gel and use the resuspended DNA as a template for PCR and DNA sequencing.

In the study of bacterial communities, DGGE has most frequently been applied to the 16S rRNA gene and several studies have used this technique to investigate the diversity of bacterial symbiont populations (Ashton *et al.*, 2003; Gillan *et al.*, 1998; Haynes *et al.*, 2003; Reeson *et al.*, 2003; Schabereiter-Gurtner *et al.*, 2003; Table 1.5). Recent literature has shown an increase in the number of studies exploiting functional genes, such as enzyme encoding genes, which generally have more sequence variation and are more useful as molecular markers of bacterial strains / variants rather than bacterial species (Hein *et al.*, 2003; Henckel *et al.*, 1999; Wawer & Muyzer, 1995; Wawer *et al.*, 1997; Webster *et al.*, 2002). This is true of the *wsp* gene employed in this study.

This study aimed to develop a novel approach based on the DGGE technique, for the rapid screening of insect populations for the presence or absence of *Wolbachia* endosymbionts, and for comparison of variant diversity in infected individuals. Each parameter (denaturant gradient, acrylamide concentration, porosity gradient, gel size, running time and temperature) was optimised using a series of experiments designed to maximise the separation of *wsp* (~600 bp) fragments, amplified from reference host species. The objective was to develop DGGE to facilitate the identification of novel *Wolbachia* strains through the discrimination of *wsp* amplimers differing in nucleotide sequence, without the need for extensive cloning and DNA sequencing. Host species infected with characterised variants, for which sequence information was available from GenBank, were chosen as references for this study. Singly, doubly and triply infected species were used to determine the effectiveness of the technique for detection of multiple infections. To test the discriminatory power of DGGE, highly divergent variants from the A and B *Wolbachia* clades were employed, as well as variants with a high percentage sequence identity.

3.2 Materials and Methods

3.2.1 Insect samples analysed during this study

Insects with characterised *Wolbachia* infections were used to assess the discriminatory power of the DGGE technique (Table 3.1). *Biorhiza pallida*, *Synergus gallaepomiformis*, a braconid sp. and *Torymid* parasitoid wasps were kindly supplied by Graham Stone, Edinburgh University (Section 2.2). Henk Braig, University of Wales, Bangor provided *Drosophila melanogaster* and *D. simulans* (Riverside strain) samples in 95% ethanol (Section 2.2). *Asobara tabida* (Hymenoptera, Braconidae), *Leptopilina heterotoma* (Hymenoptera, Figitidae), *D. ambigua* and *D. tristis* specimens were provided by James Cook, Imperial College London, Silwood Park.

3.2.2 Insect DNA extraction

Each sample was washed (30 s) in 5% [vol / vol] Clorox solution (5.25% sodium hypochlorite) and serially rinsed in sterile distilled water before DNA extraction. Modification of the method described by West *et al.* (1998) provided a rapid extraction procedure for the several hundred samples collected. Abdomens were homogenized using a sterile pestle in 50 µl extraction solution (5 % [wt / vol] Chelex (Bio-Rad), 10% proteinase K [wt / vol]), vortexed for 10 s, incubated at 56 °C for 35 min, vortexed for 15 s, incubated at 96 °C for 30 s, vortexed for 15 s and centrifuged at 15, 800g for 3 min. The supernatant was transferred to a sterile microfuge tube and stored at -20 °C.

3.2.3 PCR amplification of *Wolbachia* and host genes

For each DNA sample, amplification reactions were carried out for the *Wolbachia*-specific *wsp* gene, the *Wolbachia* 16S rRNA gene and the host mitochondrial cytochrome *b* gene, to detect and confirm the presence or absence of the endosymbiont and check the quality of the DNA template. PCRs were performed under optimum conditions which were determined empirically by extensive experimental testing. The sequences and the PCR conditions for each primer pair are summarised in Table 3.2. A 40 bp GC clamp was added to the 5' end of the reverse *wsp* primer to stabilise the PCR product and to prevent strand dissociation during DGGE analysis.

PCRs were prepared as described in Section 2.3 and included 1 x reaction buffer, 1.5 mM $MgCl_2$, 50 µM of each dNTP (dATP, dTTP, dGTP, dCTP), 1.25 U Taq DNA Polymerase, and 0.2 pmol of each primer (0.4 pmol clamped 691r primer). 1 µl template DNA (0.1 – 10 ng) was used for amplification of 16S rRNA and cytochrome *b* genes, and

TABLE 3.1 Reference insect samples used for optimisation of DGGE and the number of *wsp* sequence variants expected from each species

Species	*Wolachia* strains per individual	Reference
Torymus flavipes (Walker) sample Tf.1.Bp.45.S1[a]	1	This study
Leptopilina heterotoma Thompson[b]	3	Varve *et al.*, 1999
T. geranii (Walker) sample Tg.1.Bp.49.F1	2	This study
Drosophila simulans (Riverside strain) Sturtevant[c]	1	Braig *et al.*, 1998
Biorhiza pallida (Olivier) sample Bp.1.Bp.30.U3	1	Rokas *et al.*, 2001
D. ambigua Pomini	1	This study
D. tristis Fallén	1	This study
Asobara tabida Nees[d]	3	Varve *et al.*, 1999
D. melanogaster Meigen[e]	1	Braig *et al.*, 1998
Braconid species sample Bsp.1.Bp.50.F1	1	This study

[a] For sample label codes see Section 2.3.1 and Table of abbreviations. [b] *L. heterotoma* sequences; AF124860, AF124854, AF124858. [c] *D. simulans* sequence; AF020070. [d] *A. tabida* sequences; AF124857, AF124856, AF124859. [e] *D. melanogaster* sequence; AF020065.

TABLE 3.2 Oligonucleotide primers used for PCR in this study

Gene	Primer designation	Nucleotide sequence (5'-3')	Approx product size (bp)	Cycling conditions
wsp[a]	81f	TGGTCCAATAAGTGATGAAGAAAC	630-672	94°C for 10 min, 35 cycles of 92°C for 30 s, 56.7°C for 30 s, 75°C for 1 min, extension for 5 min at 72°C
	Clamped 691r	GCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCC CGCCCCAAAAATTAAACGCTACTCCA[b]		
16S rRNA[c]	16Sfor[d]	TTGTAGCCTGCTATGGTATAACT	936	94°C for 4 min, 35 cycles of 94°C for 30 s, 50°C for 30 s, 75°C 1 min, extension for 5 min at 72°C
	16Srev[d]	GAATAGGTATGATTTTCATGT		
Cytochrome *b*[e]	CB1	TATGTACTACCATGCGGACAAATATC	420	94°C for 4 min, 35 cycles of 92°C for 30 s, 50°C for 1 min, 72°C for 1 min, extension for 5 min at 72°C
	CB2	ATTACACCTCCTAATTTATTAGGAAT		

[a] Braig *et al.*, 1998. [b]GC-Clamp sequence as used by Muyzer (1993). [C]O'Neill *et al.*, 1992. [d]16Sfor and 16Srev correspond to *Escherichia coli* positions 76-99 forward and 1012-994 reverse respectively (Brosius *et al.*, 1981). [e] Jermiin & Crozier, 1994.

1.5 µl for the *wsp* gene. Gene amplimers were analysed by agarose gel electrophoresis as described in Section 2.2.

3.2.4 DGGE analysis of reference *wsp* sequence variants

DGGE analysis was carried out under the optimised conditions as determined by a series of experiments summarised in Table 3.3. Each gel included reference samples to facilitate comparison between gels (Table 3.1). The *wsp* sequences identified in the *T. geranii* wasp sample Tg.1.Bp.49.F1 (Section 2.2), were used as references throughout the study because they included one A-clade and one B-clade *Wolbachia* strain, which represented the extremes of the sequence difference observed in the DGGE profile. Following these experiments it was determined that the highest degree of separation attainable was ~5 cm between the A-clade and B-clade bands, and that this could be achieved using either a denaturant gradient of 20.0 - 27.5% in a 6% polyacrylamide gel, run at 60°C, or a 22.5 - 30.0% denaturant gradient and an acrylamide gradient of 5 - 6%, run at 58°C. The first set of experimental parameters was employed routinely.

PCR products (~100 ng of each product) were separated using a D-code Universal Mutation Detection System (Bio-Rad Laboratories) with 1 mm-thick (12 x 16 cm) polyacrylamide gels (6% [wt / vol] Acrygel 2.6 solution; acrylamide-N,N'-methylenebisacrylamide [37:1]; BDH laboratory supplies, Poole, UK) with a linear denaturant gradient between 20.0% and 27.5% (100% denaturant conditions were 7 M urea and 40% [vol / vol] formamide). Gels were poured with the aid of a 50 ml gradient mixer (Fischer Scientific, Loughborough, UK) and allowed to polymerise for a maximum of 1 h to minimise diffusion of the narrow gradient.

Gels were prepared with and electrophoresed in 1 x TAE buffer (pH 8, 40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and electrophoresis was carried out at 200 V for 5 h at a constant temperature of 60°C. Polyacrylamide gels were visualised under UV (302 nm) following staining with 1 x SYBR gold (Molecular Probes, Oregon, USA) according to manufacturer's instructions, and the image was captured using the GeneSnap imaging software (Synegene, UK).

3.2.5. Cloning and sequencing of *wsp* amplimers

Bands of interest were excised using sterile scalpel blades and stored at -20°C before homogenisation in 10 µl sterile distilled water and storage at 4°C overnight.

TABLE 3.3 Optimisation of parameters for DGGE protocol

Gradient of denaturants (%)	Duration of electrophoresis (h)	Length of gel[a] (cm)	Temperature (°C)	Percentage acrylamide	Approx distance separation[b] (cm)	Position of denaturing region[c]
0 - 35	4	12	60	6	1.0	M
10 - 30	4	12	60	6	2.0	M
10 - 30	1	12	60	6	0.0	U
10 - 30	2	12	60	6	0.2	M
10 - 30	3	12	60	6	1.4	M – L
10 - 30	4	12	60	6	1.7	M – L
10 - 30	5	12	60	6	1.8	L
10 - 30	6	12	60	6	1.9	L
15 - 30	5	12	60	6	3.0	M
20 - 30	5	12	60	6	3.3	M
20 - 27.5	5	12	60	6	3.5	M
20 - 27.5	5	16	60	5 - 6[d]	4.8	M
20 - 27.5	5	16	60	6	5.0	M
20 - 27.5	5	16	58	5 - 6	5.0	L
22.5 - 30	5	16	58	6	5.0	M

[a]All gels were 1 mm thick. [b]Distance between A-clade (lower) and B-clade (upper) bands from doubly infected reference sample Tg.1.Bp.49.F1. Measurements were taken from gel images and are approximate. [c]Relative position in gel to which amplimers migrate. U: Upper third of the gel, M: Middle third of the gel, L: Lower third of the gel. Optimum position for reference PCR products was M. [d]Gradient of acrylamide from top (5%) to bottom (6%) of gel.

Extracted DNA (1 μl) was then reamplified by PCR, and the purified products were used as templates for direct sequencing (Section 2.2.4).

Amplimers of the *wsp* gene from the reference samples were cloned as described in Section 2.2, and the amplified clones were re-analysed by DGGE to confirm their banding position.

Similarity matrices were constructed using the BioEdit sequence alignment editor software package (version 5.0.9; Hall, 1999) to establish the discriminatory power of the technique under the chosen conditions.

DNA sequences of all reference samples were aligned for phylogenetic analysis as described in Section 2.2.5. To assess the impact of excluding the third hypervariable region on the topology of the trees, analysis with this region included was also performed. The differences between the resulting trees did not affect the conclusions discussed in Sections 3.3 and 3.4, and so a 473 bp sequence alignment which included the third hypervariable region was used for construction of the phylogenetic trees.

3.3 Results

Partial *Wolbachia wsp* gene sequences were amplified from insect specimens in which the associated *Wolbachia* strains had previously been characterised (Braig *et al.*, 1998; Vavre *et al.*, 1999a; Chapter 2). The resulting amplimers were used as reference / marker sequence variants for the optimisation of a method for screening for the presence or absence of *Wolbachia* in insects, based on DGGE.

3.3.1 Sequence analysis of *wsp* amplimers from reference insect species

Sequences of the *wsp* gene (~600 bp) were obtained for each reference sample and aligned with sequences from GenBank database. Each variant grouped with one of the *Wolbachia* subgroups proposed by Zhou *et al.* (1998) and van Meer *et al.* (1999), according to the established grouping criterion of 97.5% nucleotide sequence similarity in the *wsp* gene.

The number of nucleotide differences between 468 bp fragments from the alignment (Fig. 3.1) ranged from 1 bp (0.3%) difference between *L. heterotoma* (variant 3) and sample Tf.1.Bp.45.S1, to 126 bp (26.9%) between samples Tg.1.Bp.49.F1A and Tg.1.Bp.49.F1B, which had previously been established to represent A- and B-clade sequences, respectively (Section 2.2). The *L. heterotoma* (variant 1) and *D. simulans* variants, and the *D. tristis* and *D. ambigua* variants shared identical *wsp* sequences. The phylogenetic tree constructed using the 468 bp nucleotide sequence alignment (Fig. 3.2), is colour-coded to facilitate comparison with the DGGE profile in Fig. 3.3 (see Section 3.3.2) and the summary diagram in Fig. 3.4 (see Section 3.4 for more detail).

3.3.2 DGGE analysis of PCR-amplified *wsp* fragments

Optimal DGGE conditions were defined as those that facilitated maximum PCR amplimer separation and band resolution, and caused denaturation of double stranded DNA molecules within a central region of the gel. This would accommodate *wsp* amplimers with higher or lower melting temperatures from future studies, above and below the reference bands. Optimal conditions were established empirically using over 30 experiments with varying gel and electrophoresis parameters. Each unique band position or DGGE variant was assigned an individual Roman numeral label. Table 3.4 shows the DGGE variants identified in each of the reference insect species.

			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
			A. tabida 3	Tf.1.Bp.45.S1	*L. heterotoma* 3	*L. hetertoma* 2	*D. simulans* (Riverside)	*L. heterotoma* 1	Bsp.1.Bp.50.F1	Tg.1.Bp.49.F1 A	*B. pallida*	*D. ambigua*	*D. tristis*	*A. tabida* 2	Sg.1.Bp.6.U1	*D. melanogaster*	*A. tabida* 1	Tg.1-2.Bp.49.F1 B
1	*Asobara tabida* 3	X	100	45	46	41	62	62	71	85	66	94	94	92	92	91	103	123
2	Tf.1.Bp.45.S1	III	90.4	100	1	8	45	45	39	50	29	48	62	60	61	59	98	119
3	*Leptopilina heterotoma* 3	IV	90.1	99.7	100	9	46	46	41	51	29	63	63	61	62	60	99	119
4	*L. hetertoma* 2	VI	91.2	98.2	98.0	100	41	41	37	50	29	59	59	57	58	56	95	115
5	*Drosophila simulans* (Riverside)	VI	86.8	90.3	90.1	91.3	100	0	44	62	29	71	71	70	71	70	94	115
6	*L. heterotoma* 1	VI	86.8	90.3	90.1	91.3	100	100	44	62	29	71	71	70	71	70	94	115
7	Bsp.1.Bp.50.F1	XIII	84.9	91.6	91.3	92.0	90.5	90.5	100	66	45	80	80	79	80	80	104	118
8	Tg.1.Bp.49.F1 A	II	81.8	89.3	89.1	89.3	86.8	86.8	85.8	100	38	72	72	72	73	72	107	126
9	*Biorhiza pallida*	XII	85.9	93.9	93.7	93.9	93.9	93.9	90.3	91.9	100	61	61	59	61	59	95	120
10	*D. ambigua*	XI	79.9	89.8	86.6	87.5	84.8	84.8	82.9	84.6	87.0	100	0	4	5	8	63	124
11	*D. tristis*	XI	79.9	86.8	86.6	87.5	84.8	84.8	82.9	84.6	87.0	100	100	4	5	8	63	124
12	*A. tabida* 2	VIII	80.3	87.2	87.0	87.9	85.0	85.0	83.1	84.6	87.3	99.1	99.1	100	1	4	61	123
13	Sg.1.Bp.6.U1		80.3	87.0	86.8	87.7	84.8	84.8	82.9	84.4	87.0	98.9	98.9	99.7	100	5	62	124
14	*D. melanogaster*	VII	80.6	87.5	87.2	88.1	85.0	85.0	82.9	84.6	87.3	98.2	98.2	99.1	98.9	100	65	125
15	*A. tabida* 1	IX	78.0	79.1	78.9	79.8	80.0	80.0	77.8	77.1	79.6	86.5	86.5	86.9	86.7	86.1	100	105
16	Tg.1.Bp.49.F1 B	I	73.8	74.6	74.6	75.5	75.4	75.4	74.7	73.1	74.4	73.5	73.5	73.7	73.5	73.3	77.5	100

FIG. 3.1 Sequence identity matrix constructed using BioEdit sequence alignment editor software package (version 5.0.9, Hall 1999) to compare ClustalX aligned *wsp* sequences, 468 bp in length. Results are expressed as bp differences (grey) and from these values, % similarities (white) were calculated. DNA sequences were named according to the insect specimen from with they were amplified and colour-coded to facilitate comparison with Fig. 3.2 and 3.3. Each DGGE banding position was given a unique Roman numeral label (total of 13 different positions).

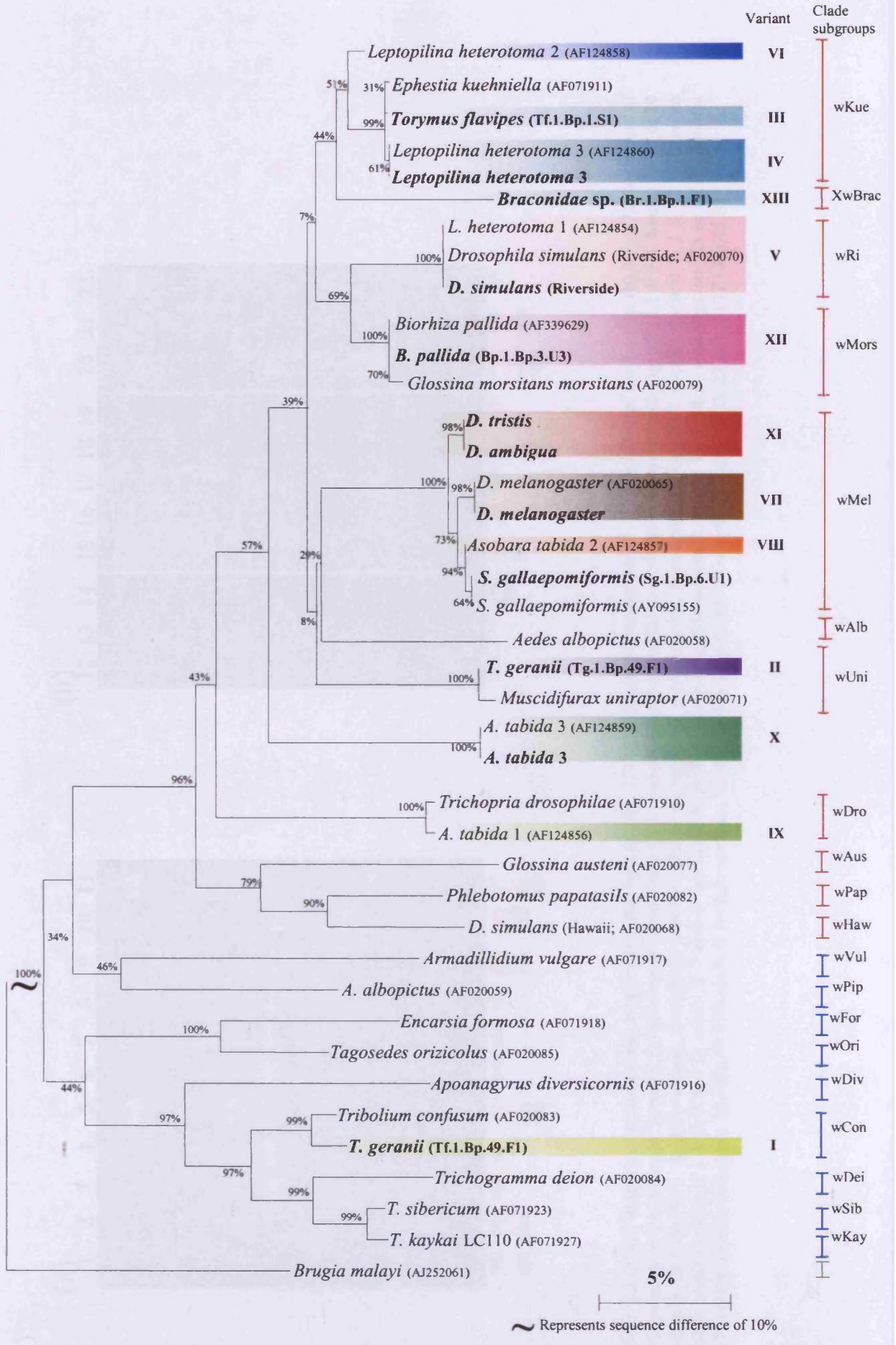

FIG. 3.2 Phylogenetic tree constructed based on analysis of *wsp* gene sequences using Jukes-Cantor to compare ClustalX alignments (473 bp), followed by Neighbour Joining (Section 4.2.5). Bootstrapping was carried out at 1000 repetitions. *wsp* variants identified in the present study are labelled and colour coordinated to facilitate comparison with Figs. 3.1, 3.3 & 3.4. Sequences in bold were obtained during the present study. For all other sequences the accession number is given. The *Wolbachia* subgroups proposed by Zhou *et al.* (1998) and van Meer *et al.* (1999) are labelled. Red lines group together clade-A sequences, blue lines group sequences from clade-B and the green line highlights the sequence from the nematode clade-C. which was used to root the tree.

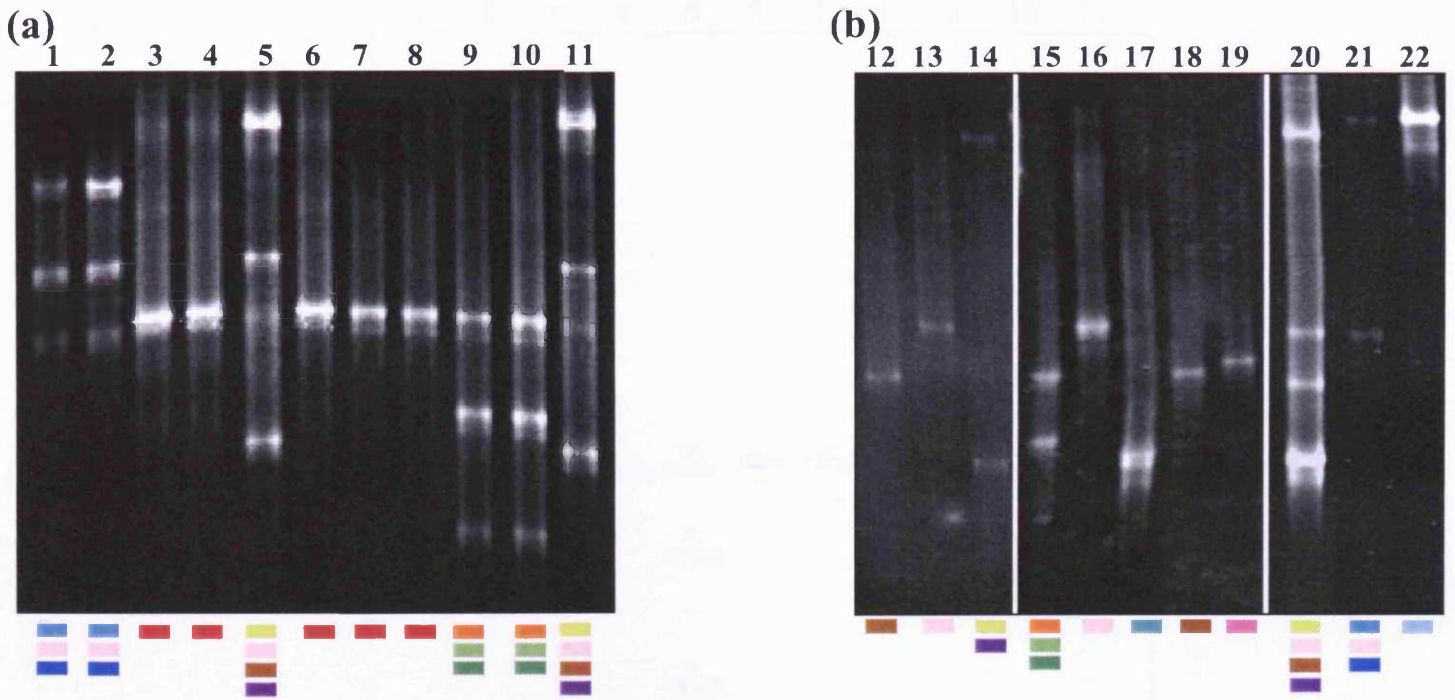

FIG. 3.3 DGGE analysis of reference *wsp* amplimers (~600 bp). **(a)** 20 - 27.5 % gradient of denaturants, 5 - 6 % gradient of acrylamide. **(b)** 20 - 27.5% gradient of denaturants, 6% acrylamide. Samples in each lane are listed from top to bottom and colour-coded to facilitate comparison with Fig. 3.1, 3.2 & 3.4. **Lanes**: **5**, **11** & **20** sample Tg.1.Bp.49.F1 B-clade variant, *Drosophila simulans* (Riverside strain) *D. melanogaster*, & sample Tg.1.Bp.49.F1 A-clade variant; **1**, **2** & **22** *Leptopilina heterotoma* variants 3, 1 & 2; **3** & **4** *D. tristis*; **6**, **7** & **8** *D. ambigua*; **9**, **10** & **15** *Asobara tabida* variants 2, 1 & 3; **12** & **18** *D. melanogaster*; **13** & **16** *D. simulans* (Riverside strain); **14** sample Tg.1.Bp.49.F1B-clade & A-clade variants; **17** sample Bsp.1.Bp.50.F1; **19** sample Bp.1.Bp.30.U3; **22** sample Tf.1.Bp.45.S1.

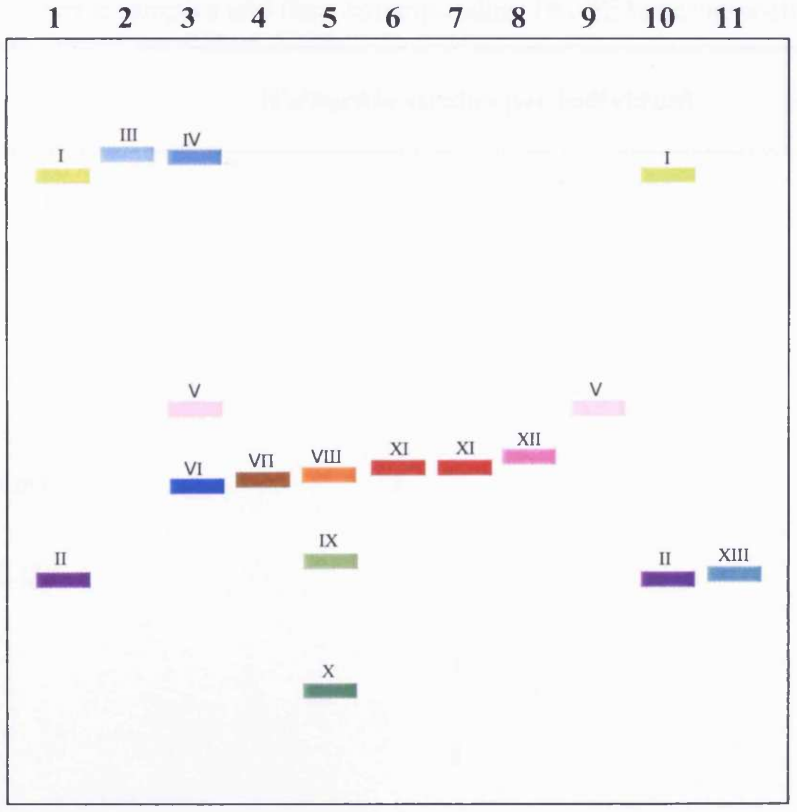

FIG. 3.4 Diagrammatic representation of DGGE profiles produced by analysis of *wsp* fragments amplified from reference insect samples under optimised DGGE conditions (Fig. 3.3b; section 3.2). Samples in each lane are listed in order from top to bottom and colour-coded to facilitate comparison with Figs. 3.1 – 3.3. A total of 13 banding positions are labelled in Roman numerals.

Lane: **1** sample Tg.1.Bp.49.F1 B-clade variant ( ) and A-clade variant ( ); **2** sample Tf.1.Bp.45.S1 ( ); **3** *Leptopilina heterotoma* variants 3, 1 & 2 ( ); **4** *Drosophila melanogaster* ( ); **5** *Asobara tabida* variants 2, 1 & 3 ( ); **6** *D. ambigua* ( ); **7** *D. tristis* ( ); **8** sample Bp.1.Bp.30.U3 ( ); **9** *D. simulans* (Riverside strain) ( ); **10** sample Tg.1.Bp.49.F1 B-clade variant ( ) and A-clade variant ( ); **11** sample Bsp.1.Bp.50.F1 ( )

TABLE 3.4 Reference samples and their corresponding DGGE banding positions (Fig. 3.4).

Species	***Wolbachia* strains per individual**	***wsp* DGGE variant (Fig. 3.4)**
Torymus flavipes sample Tf.1.Bp.45.S1[a]	1	III
Leptopilina heterotoma[b]	3	IV, V, VI
T. geranii sample Tg.1.Bp.49.F1	2	I, II
Drosophila simulans (Riverside strain)[c]	1	V
Biorhiza pallida sample Bp.1.Bp.30.U3	1	XII
D. ambigua	1	XI
D. tristis	1	XI
Asobara tabida[d]	3	VIII, IX, X
D. melanogaster[e]	1	VII
Braconid species sample Bsp.1.Bp.50.F1	1	XIII

[a] For sample label codes see Section 2.3.1 and Table of abbreviations. [b] *L. heterotoma* amplimers IV, V, VI = 3 (AF124860), 1 (AF124854) & 2 (AF124858), respectively. [c] *D. simulans* = AF020070. [d] *A. tabida* amplimers VII, IX, X = 2 (AF124857), 1 (AF124856) & 3 (AF124859) respectively. [e] *D. melanogaster*= AF020065.

Initially a 0 - 35% gradient in a 6% acrylamide gel (1 mm x 12 x 12 cm) was run at 60°C (200 V) for 4 h, yielding a 1 cm distance separation of the marker sequences from sample Tg.1.Bp.49.F1: variants I and II. Narrowing the gradient to 10 - 30% doubled the degree of separation. A time trial gel in which samples were applied to the gel at hourly intervals, revealed that increasing the running time to 5 - 6 h further increased the band separation to 2.65 - 2.80 cm (Fig. 3.5). Additional experiments in which the gradient was narrowed progressively from 15 - 30%, to 20 - 30%, to 20.0 - 27.5%, showed that a denaturant gradient of 20.0 - 27.5% produced a maximum distance of 3.5 cm between the reference bands, and this was increased to 5 cm when the gel was expanded to 12 x 16 cm. Experiments were conducted to investigate whether application of a 5 - 6% polyacrylamide gradient would aid DNA fragment separation and increase the resolution of the DGGE bands. However, no significant improvements were observed. Lowering the electrophoresis running temperature to 58°C in an attempt to reduce distortion of the low percentage acrylamide gel, altered the concentration of denaturants required to melt the DNA molecules. Adjustment of the denaturant gradient to 22.5 - 30% produced the same results as obtained previously using the 20.0 - 27.5% gradient at 60°C and the latter set of conditions were employed routinely.

In summary, the following running conditions were found to produce the greatest degree of band separation: 20 - 27.5% gradient of denaturants in a 6% acrylamide gel (12 x 16 cm) run at 60°C for 5 h. These parameters resulted in a separation of 5 cm between sequence DGGE variants I & II from Tg.1.Bp.49.F1.

The results of the DGGE analysis of reference *wsp* fragments are presented in Fig. 3.3 & 3.4, which show DGGE gels run under optimal conditions and a diagrammatic representation of all the DGGE variants. Each reference sequence variant produced the expected number of DGGE bands based on earlier sequence analysis (Section 3.2 & 3.3.1) and all variants could be distinguished from each other.

The double infection that occurs naturally in sample Tg.1.Bp.49.F1 and the triple infections from *A. tabida* and *L. heterotoma*, were clearly identifiable. As shown by the similarity matrix in Fig. 3.1, the two *wsp* sequence variants from sample Tg.1.Bp.49.F1 (lanes 1, 4, 8, 11 & 17 Fig. 3.3; Fig. 3.4), differed from each other by 126 bp (26.9%). The three variants from *A. tabida* (lanes 6, 7 & 12 Fig. 3.3; Fig 3.4) differed from each other by 61 bp (13.1%, between *A. tabida* variants 1 & 2), 92 bp (19.7%, between *A. tabida* variants 2 & 3), and 103 bp (22%, between *A. tabida* variants 1 & 3). The three variants from *L. heterotoma* (lanes 2 & 18 Fig. 3.3; Fig 3.4) showed nucleotide

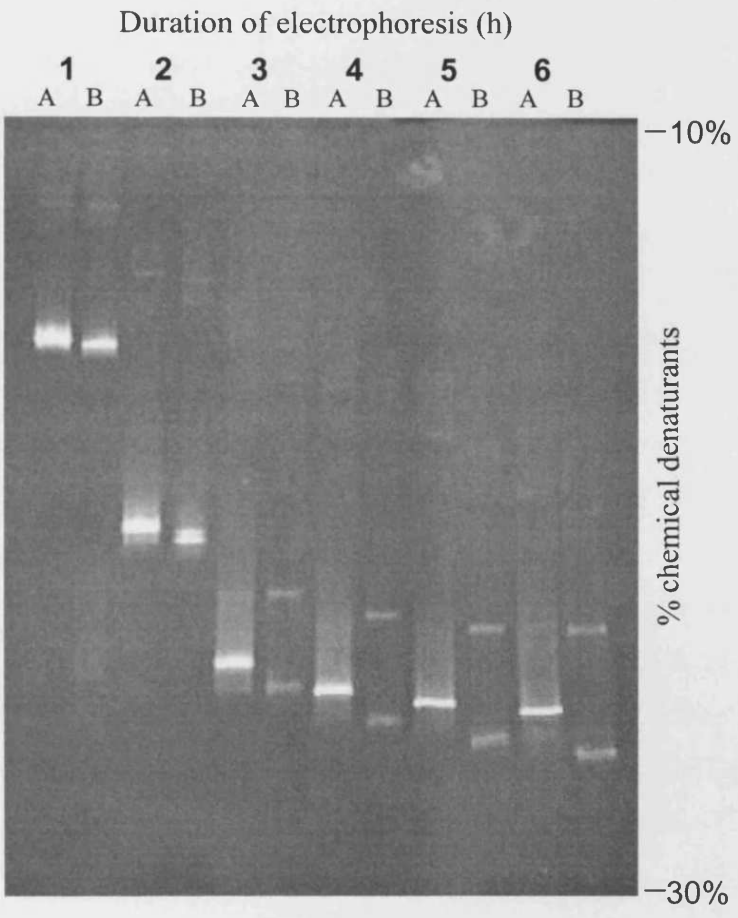

Fig3.5. The *wsp* sequence variants amplified from **A** - *Drosophila melanoagaster* and **B** - *Torymus geranii* sample 49(3) (DGGE variants I & II), were applied to a 6% polyacrylamide gel (12cm in length) containing a denaturant gradient of 10 – 30%, at hourly intervals and electrophoresed at 200V (60°C).

differences of 9 bp (2%, between *L. heterotoma* variants 2 & 3), 41 bp (8.7%, between *L. heterotoma* variants 1 & 2), and 46 bp (9.8%, between *L. heterotoma* variants 1 & 3). These comparisons were made over 468 bp of the *wsp* amplimer sequence and DGGE analysis of the complete ~600 bp PCR amplimer would have detected any further nucleotide differences.

3.4 Discussion

In this study DGGE was optimised and used successfully for the discrimination of closely related *Wolbachia* variants to provide a rapid, effective and practical approach to analysing *Wolbachia* in insects. Optimum gradient and running conditions were established through extensive experimentation, during which ~600 bp *wsp* amplimers from insects in which the *Wolbachia* strains had been previously characterised were employed as markers (Section 3.2.1).

Several parameters have been employed in the literature and were considered during this study: acrylamide concentration, use of porosity gradient, denaturant gradient (ranging from a 5% gradient to a 70% gradient), running time (ranging from 2 - 23 h), temperature (58 - 60°C), type of buffer and concentration of buffer, thickness of gel, inclusion of glycerol and voltage (40 - 200 V) (Hayes *et al.*, 1999).

A denaturant gradient of only 7.5% (20 - 27.5%) was found to denature all reference *wsp* amplimers tested and caused the *wsp* fragments from sample Tg.1.Bp.49.F1 to form DGGE bands 5 cm apart (under optimal conditions, see below). These two sequence variants had been found to show homology to *wsp* sequences in GenBank from A- and B-clade *Wolbachia*, and share a 73.1% nucleotide sequence identity (over 468 bp) (Fig. 3.1 & Chapter 2). The requirement for such a low denaturant concentration was surprising as a much higher *Tm* would be expected for a 600 bp product. The low *Tm* is the result of the low G / C content of the *wsp* gene (~38%) and of the *Wolbachia* genome as a whole (35.2%) (Wu *et al.*, 2004). This is not uncommon in obligate endosymbionts which undergo reductive evolution and often display a tendency towards A / T richness (Akman *et al.*, 2002; Tamas & Andersson, 2003; Moran & Wernegreen, 2000; Shigenobu *et al.*, 2000; Table 1.2).

A 6% acrylamide gel was used in this study to separate the ~600 bp *wsp* fragments. DNA fragments of approximately 200 bp in length are separated on an 8 or 9% acrylamide gel (Haynes *et al.*, 2003; Webster *et al.*, 2002) but for larger amplimers, a lower percentage is necessary to allow the DNA to pass through the gel matrix, whilst providing enough drag to separate different conformers. In the majority of studies in which DGGE has been employed, gene fragments of only 200 bp, to an upper limit of 500 bp have been used, which severely limits the potential for phylogenetic inference. It has been proposed that it should be possible to separate amplimers up to 1000 bp in length using DGGE (Myers *et al.*, 1985; Nollau *et al.*, 1997) but up to now, the largest gene fragment analysed using a single set of DGGE running conditions, is a 600 bp fragment

of the 16S rRNA gene, analysed by Kisand & Wikner (2003b) during investigation of the diversity of bacterial taxa in riverine sediments. A variant of DGGE, Two Dimensional Gene Scanning (TDGS), which combines DGGE in the first dimension with size separation in the second, could allow larger fragments of a chosen gene to be analysed on a single gel but the output is complex and specialised equipment is required (McGrath *et al.*, 2001; van Orsouw *et al.*, 1998).

A porosity gradient can be applied to increase band resolution where larger DNA molecules are being examined. This is commonly employed for the resolution of hetero- and homoduplexes which tend to produce smears during long separation times (Cremonesi *et al.*, 1999; Hayes *et al.*, 1999). Following several experiments during which a 5 - 6% gradient was applied, use of a porosity gradient was not found to be useful for the separation of *wsp* fragments.

It was established that for a fragment the size of the *wsp* amplimer (~600 bp + 40 bp GC-clamp) a high running temperature and low percentage acrylamide was required, to allow the DNA molecule to be denatured and pulled through the gel. However, these parameters would be expected to increase the rate of diffusion of the denaturant gradient employed, and as a consequence it was necessary to prepare DGGE gels and carry out electrophoresis on the same day. Electrophoresis was carried out at a high voltage (200 V) to increase the speed of amplimer migration, leading to a running time of only 5 h (and no longer than 6 h) to achieve maximum amplimer separation. The buffer used was 1 x TAE, as a lower buffer concentration would decrease the ion concentration and result in increased resistance and conversion of electrical energy to heat energy. Even under these fully optimised conditions some bowing was observed in the DGGE profile due to the fragility of the acrylamide gel.

The discriminatory power of DGGE was tested using the reference *wsp* amplimers and the parameters employed were successful in the detection and discrimination of all sequence variants. This technique confirmed the presence of *Wolbachia* strains in the reference samples, which had been characterised previously by other research groups: the triple infections identified in *L. heterotoma* and *A. tabida* (Vavre *et al.*, 1999a) and the double infection from sample Tg.1.Bp.49.F1 were clearly identifiable (Fig. 3.3 & 3.4). DGGE has previously been shown to discriminate between 200-500 bp 16S rRNA amplimers, differing by only 1 bp (Muyzer, 1999; Hayes *et al.*, 1999). In this study, the ~600 bp *wsp* fragments amplified from *L. heterotoma* (variant IV) and sample Tf.1.Bp.45.S1 (variant III), which differed by 1 bp were distinguished (Fig. 3.1 & 3.3).

This nucleotide sequence difference was confirmed by comparison of the full length sequences for these samples (560 bp and 579 bp, respectively). Currently, any difference in the DNA sequence of the *wsp* gene is used to classify *Wolbachia* as different variants. These results indicated that DGGE can be used to differentiate between *Wolbachia* variants from a variety of insect host species and between variants from different *Wolbachia* clades. However, further testing of B-clade variants should be carried out.

For the majority of the sequence variants, a correlation between the number of base differences and the separation achieved by DGGE was observed. Comparison of the DGGE profile and schematic in Fig. 3.3 & 3.4, with the phylogenetic tree in Fig. 3.2 and the similarity matrix in Fig. 3.1, highlighted this trend. For example, *wsp* fragments amplified from *D. melanogaster* (VII), *A. tabida* sequence variant 2 (VII), *D. tristis* (XI) and *D. ambigua* (XI) shared between 98.2 and 100% sequence identity (over the aligned 468 bp) and migrated together to the mid region of the gel (Figs. 3.2 - 3.4). This pattern was also demonstrated by the two variants (I & II) infecting sample Tg.1.Bp.49.F1, which differed by 126 bp (26.9%) and migrated to positions 5 cm apart.

However, the relationship between DNA melting point and nucleotide difference is imprecise and hard to predict (Kisand & Wikner, 2003a). PCR fragments that differed by ~10 bp (1 - 2%) migrated closely together but sequences that differed by ≥10% (~45 bp) migrated less predictably. For example, the DGGE variant II from sample Tg.1.Bp.49.F1, differed from variant XIII from the sample Bsp.1.Bp.50.F1 by ~66 bp (14%), yet the gene fragments produced bands in similar positions on a DGGE gel (Fig. 3.4). Conversely, *A. tabida* variant X, which also differs from variant XIII by ~15% (71 bp), migrated to the bottom of the gel and produced a band over 1 cm from variant XIII. Also, sample Tg.1.Bp.49.F1 variant I migrated very closely to the sample Tf.1.Bp.45.S1 variant III, yet these amplimers shared only 74.6% sequence identity. This lack of correlation between nucleotide sequence difference and banding position is because the nucleotide differences between the DGGE variants XIII and II are not the same as those between XIII and X. They differ in the position and type of substitution (transition / transversion) and both of these factors have a significant impact on the behaviour of the gene fragment as it migrates through the denaturant gradient, and therefore impacts upon the final position of the DGGE band.

In complex DGGE profiles, amplimers differing in nucleotide sequence may co-migrate and it may be necessary to excise and clone the DNA to identify the sequence variants (Schabereiter-Gurtner *et al.*, 2003; Vallaeys *et al.*, 1997). However, a maximum

of 5 *Wolbachia* variants have been detected in an individual host specimen (Jamnongluk *et al.*, 2002; Reuter and Keller, 2003), therefore relatively simple DGGE profiles are expected from screening *Wolbachia* infected insects populations. During this study the identity of each DGGE band was confirmed by PCR amplification and sequencing of gel extracted DNA, and in all cases a single sequence was recovered and no incidence of amplimer co-migration was detected.

A potential difficulty associated with use of the *wsp* gene, is the occurrence of length polymorphism of *wsp* amplimers from different variants, which range from 590–632 bp (Braig *et al.*, 1998). This could affect DGGE banding pattern irrespective of nucleotide differences. During preparation of the reference sequences in this study agarose gel electrophoresis did not detect any difference between product sizes, though differences of ≤10 bp might not be evident using this technique (Sambrook & Russell, 2001). To ensure the accuracy of DGGE, it may be necessary to check the nucleotide sequence of bands, selected at random to confirm that each band represents only one sequence variant and that the relationships inferred by the DGGE gel are correct.

DGGE has been shown to significantly increase the sample throughput relative to traditional screening methods based solely on PCR, cloning and sequencing. Some groups have used restriction fragment length polymorphism (RFLP) to discriminate between 16S rRNA, *ftsZ* or *wsp* sequence variants, in an effort to reduce the number of sequencing reactions required to screen a maximum number of samples (Huigens *et al.*, 2004; Jamnongluk, *et al.*, 2002; Kikuchi & Fukatsu, 2003; Kondo *et al.*, 2002; Mandel *et al.*, 2001; Merçot *et al.*, 1995; Mitsuhashi *et al.*, 2002; O'Neill *et al.*, 1997; Reuter & Keller, 2003; Riegler & Stauffer, 2002; Van Borm *et al.*, 2003). However, in most cases a maximum of 3 variants are present in the study population and the nucleotide sequence of each has already been determined. Even when combined with extensive cloning and sequencing, RFLP analysis failed to detect all *wsp* sequence variants present (Kikuchi & Fukatsu, 2003b).

Using DGGE, up to 20 samples can be applied to each gel and two gels can be run simultaneously in each gel tank. The ability to compare at least 40 samples at the nucleotide sequence level, within an eight hour period (3 h preparation, 5 h running duration) makes DGGE a very useful technique for the characterisation of *Wolbachia* infections in insect populations. The samples analysed in this investigation will be used as marker sequences in future studies. Amplimers banding to the same positions as the reference samples should represent identical sequences and therefore the same *Wolbachia*

variant. Only novel bands need then be characterised by DNA sequencing either directly from the host tissue, or following band excision. Newly identified sequence variants will then be added to the reference collection. By comparison with *wsp* gene fragments from known *Wolbachia* infections, strains infecting test samples can be identified without the need for extensive cloning and sequencing.

Chapter 4

Incidence and prevalence of *Wolbachia* in the oak gall wasp community: application of denaturing gradient gel electrophoresis

4.1 Introduction

Wolbachia are maternally transmitted endosymbionts that ensure their maintenance and spread within a host population by practising several forms of reproductive manipulation, and are considered to be one the most prevalent species of bacterial endosymbiont found in insects (Kikuchi & Fukatsu, 2003; Kittayapong *et al.*, 2000; Reuter & Keller, 2003; Ricci *et al.*, 2002; Rokas *et al.*, 2001; West *et al.*, 1998; Werren *et al.*, 1995a).

Wolbachia prevalence shows variation between different insect assemblages and several studies have detected intraspecific geographic variation in infection frequency and diversity (Abe & Muira, 2002; Dyson *et al.*, 2002; Keller *et al.*, 2004; Malloch *et al.*, 2000; Plantard *et al.*, 1998; Riegler and Stuaffer, 2002; Rokas *et al.*, 2001; Shoemaker *et al.*, 2003; Tsutsui *et al.*, 2003; Section 4.4.3). In many studies host species have been represented by only one or a few specimens and so estimates of infection frequency are likely to have been greatly underestimated, and may in fact be as high as 76% of all insect species (Jeyaprakash & Hoy, 2000).

It is widely accepted that larger sample numbers are required to allow accurate frequency estimations to be made. However this is both expensive and labour intensive. High throughput molecular typing methods would be highly advantageous to this research field (Muyzer, 1999; Section 1.7). Techniques such as single stranded conformation polymorphism (Hayashi *et al.*, 1999; Sheffield *et al.*, 1993) and denaturing gradient gel electrophoresis (Muyzer *et al.*, 1993; Muyzer & Smalla, 1998) could potentially be used to discriminate between *Wolbachia* variants, based on the separation of *wsp* (*Wolbachia* surface protein; Braig *et al.*, 1998) gene sequence variants (further information about molecular typing methods was given in Section 1.7).

Wolbachia are thought to undergo occasional horizontal transmission but direct evidence in support of this is limited (Heath *et al.*, 1999; Huigens *et al.*, 2000, van Meer & Stouthamer, 1999). Feeding communities in which several insect species interact on different trophic levels are ideal for investigating the hypothesis that horizontal transmission occurs between host species in close ecological associations. The incidence and diversity of *Wolbachia* has been investigated in feeding communities such as those associated with gall-inducing wasp species (Plantard *et al.*, 1999; Schilthuizen &

Stouthamer, 1998), including the oak gall wasp communities (Hymenoptera: Cynipidae: Cynipini) (Abe & Muira, 2002; Rokas *et al.*, 2001; 2002a; Section 2.1). However, the parasitoid assemblages associated with specific gall-causer species have not been studied extensively. Parasitoid wasps attack the larvae of gall-causer, inquiline and other parasitoid species, and therefore are potential vectors of *Wolbachia* infection between members of the oak gall wasp community (Askew, 1961; Noda *et al.*, 2001; Stone *et al.*, 2002; van Meer *et al.*, 1999; Vavre *et al* 1999a; Werren *et al.*, 1995b; Section 1.8.4).

In Chapter 2, parasitoid wasps collected from galls induced by the sexual generation of *Biorhiza pallida* were tested for *Wolbachia*. *B. pallida* and its inquiline species, *S. gallaepomiformis*, had been found to be infected with *Wolbachia* previously (Rokas *et al.*, 2002a) and three parasitoid species were also found to be infected (Sections 2.3 & 2.4). The *Wolbachia* variants identified in the chalcid parasitoids were not closely related to those infecting the cynipid (gall-causer and inquiline) members of this wasp community; however, indirect evidence of horizontal transmission was obtained by the discovery of a double infection in the chalcid species *Torymus geranii*, that consisted of diverse *Wolbachia* variants from different clades. The possibility that occasional parasitoid species could have been missed due to small sample numbers, or that generalist parasitoids could play an important role in the infection diversity in this community was discussed.

In this Chapter, the investigation in Chapter 2 was extended by the inclusion of four more gall-inducing wasp species and their associated wasp assemblages. All wasps that emerged from galls induced by the cynipid species *Andricus curvator*, *A. quadrilineatus*, *B. pallida*, *Neuroterus numismalis* and *N. quercusbaccarum* were screened for the presence or absence of *Wolbachia* infection. At least 30 parasitoid and inquiline wasp species are known to be associated with the sexual generation galls (asexual generation of *A. quadralineatus*) induced by these cynipid species in the spring-summer in the UK (Table 4.1). Few of these species are restricted to one gall; several of them attack four or five of the gall-causers listed above. This provides several potential routes through which *Wolbachia* could be transferred and should allow detection of horizontal transmission if it occurs in this community.

In this study denaturing gradient gel electrophoresis, optimised as described in Chapter 3, was used to compare ~600 bp fragments of the *Wolbachia* specific *wsp*

TABLE 4.1 Inquiline and parasitoid species associated with the summer galls induced by five gall wasp species in the UK (Askew, 2001; Williams, 2004)

Gall-causer species[a]		Parasitoid species																									Inquiline species							
		Eurytoma brunniventris	*Sycophila variegata*	*Megastigmus dorsalis*	*Torymus affinis*	*T. auratus*	*T. flavipes*	*T. geranii*	*T.notatus*	*Orymus pomaceus*	*Cecidostiba fungosa*	*C. semifascia*	*Hobbya stenonota*	*Mesopolobus albitarsis*	*M. amaenus*	*M. dubius*	*M. fasciiventris*	*M. fuscipes*	*M. sericeus*	*M. tibialis*	*M. xanthocerus*	*Eupelmus urozonus*	*Aulogymnus arsames*	*A. eudoreschus*	*A. gallarum*	*A. skianeuros*	*Baryscapus diaphantus*	*Synergus albipes*	*S. apicalis*	*S. crassicornis*	*S. gallaepomiformis*	*S. nervosus*	*S. thaumacerus*	*Hemiteles* sp.	*Aprostocetus aethiops*
A. curvator	(Bisex)	*		*			*	*	*					*	*		*	*	*	*		*	*		*			*		*	*	*		
A.quadralineatus	(Unisex)						*														*		*	*				*			*	*		*	
B. pallida	(Bisex)	*	*	*	*	*	*	*		*	*	*	*		*	*			*	*	*	*			*	*	*				*				
N. numismalis	(Bisex)	*					*										*	*		*			*					*							*
N. quercusbaccarum	(Bisex)	*				*	*												*	*		*	*					*	*		*	*	*		
Number of gall-causer species not listed that each wasp species attacks		19	2	10	0	9	9	6	0	1	4	2	1	0	6	6	8	11	14	8	7	13	3	0	1	0	0	9	3	3	14	12	3	0	6

[a]The generation of the gall induced in the summer is given alongside the species name.

gene from individual insects. This allowed the diversity of infection and frequency of multiple infections to be investigated in larger sample numbers, more rapidly and with a reduced need for sequencing and cloning. Samples were collected from several sites across South Wales to investigate the possibility of small scale geographic variation in infection frequency and diversity.

4.2 Materials and Methods

4.2.1 Insect sample collection and handling

Five species of oak gall wasp, together with their associated parasitoids and inquiline species were surveyed. Wasps were reared from galls collected from *Quercus robur* between May 15th and June 10th 2002, and between May 15th and May 20th 2003. Sampling locations formed a transect, running from South to North initially (15 miles) and then South West to South East (35 miles) over approximately 50 miles (Fig. 4.1). The collection locations and grid references are listed in Table 4.2, and the site code and the numbers of trees sampled are given. Conditions were generally damp to wet, throughout the sampling period, therefore the galls were transferred to individual containers at the earliest opportunity. The galls were identified according to Redfern & Askew (1992).

Following collection, individual galls were kept in plastic containers covered with a fine cotton mesh, and a thin layer of moist calcium sulphate was maintained at the base of each container to prevent galls from becoming too dry. A piece of Nesco-film™ was placed between the gall and the calcium sulphate to keep the galls from becoming too damp and prevent fungal growth. During 2002 a solution of methyl paraben (p-hydroxybenzoic acid methyl ester) was used as an anti-fungal agent but it was determined that better regulation of the moisture level within the rearing container was more effective. Samples were kept in the laboratory at approximately 20°C and emerging adults were removed daily.

Some of the *B. pallida* galls produced over 100 gall-causer adults. During the study, most galls were represented by one to three samples because infected *B. pallida* populations had previously been shown to be infected at a frequency of 100% (Rokas *et al.*, 2001). To check that this was the case in this sampling region, 10 *B. pallida* samples from the same gall were tested for infection and all 10 were positive for *Wolbachia*. For all other species, every specimen that emerged was tested for infection.

Galls of the asexual generations were collected for *Andricus curvator, Neuroterus quercusbaccarum* and *N. numismalis* during August and October of 2002, and kept at 8°C to induce emergence but this was not successful. Therefore only sexual generation wasps were tested during this study.

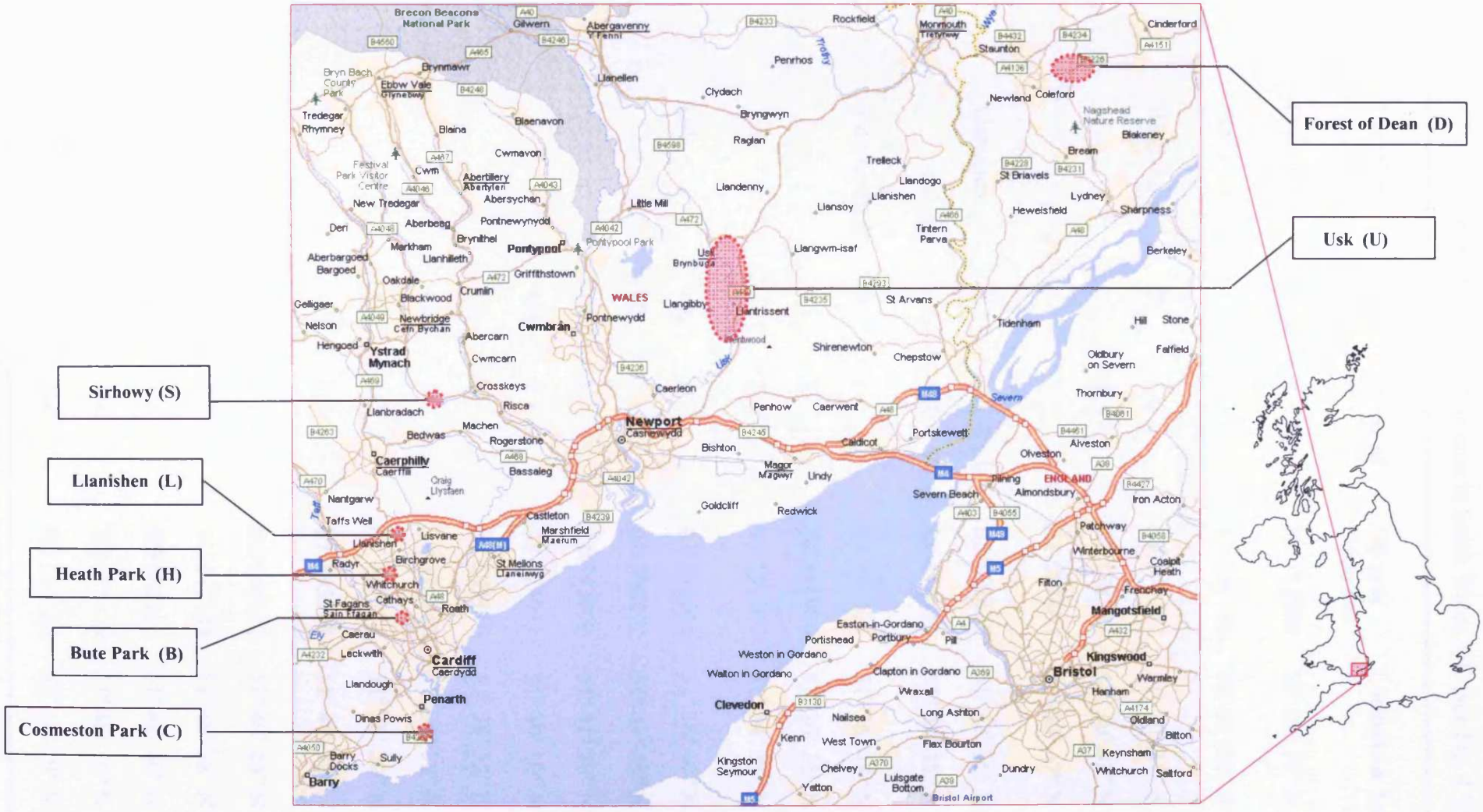

FIG. 4.1 Map of sampling locations in South Wales (see Table 4.2). Site codes C, B, H, L, S, U, and D were used throughout the report.

TABLE 4.2 Gall collection sites in South Wales, UK (see Fig. 4.1 for map)

Location	Site[a]	Number of each tree	OS grid reference	Latitude & Longitude
Cosmeston	C	1 + 2	ST 172696	51° 25' 10" N 3° 11' 30" W
Bute Park	B	1 + 2	ST 168779	51° 29' 39" N 3° 11' 57" W
Heath Park	H	1-6	ST 179797	51° 30' 37" N 3° 11' 04" W
		7, 8	ST 178798	51° 30' 42" N 3° 11' 05" W
		9	ST 177800	51° 30' 47" N 3° 11' 13" W
Llanishen	L	1-4, 10-14	ST 178833	51° 32' 36" N 3° 11' 11" W
		5-9	ST 177833	51° 32' 35" N 3° 11' 17" W
Sirhowy Park	S	1	ST 190911	51° 36' 48" N 3° 10' 12" W
		2	ST 195907	51° 36' 35" N 3° 09' 46" W
		3 - 6	ST 193907	51° 36' 35" N 3° 09' 58" W
Usk	Ua	10	ST 374945	51° 38' 47" N 2° 54' 22" W
	Ub	1 + 2	SO 396002	51° 41' 49" N 2° 52' 32" W
	Uc	1	ST 393992	51° 41' 20" N 2° 52' 45" W
	Ud	1 + 2	ST 375952	51° 39' 10" N 2° 54' 13" W
	Ue	2	ST 389946	51° 38' 52" N 2° 53' 00" W
	Uf	1	ST 390942	51° 38' 38" N 2° 52'59" W
	Ug	1-3	ST 387937	51° 38' 20" N 2° 53' 11" W
	Uh	1-5	ST 395974	51° 40' 20" N 2° 52' 31" W
Forest of Dean	Da	1 + 2	SO 556122	51° 48' 25" N 2° 38' 42" W
	Db	1-7	SO 612144	51° 49' 38" N 2° 33' 47" W
	Dc	1-6	SO 642126	51° 48' 40" N 2° 31' 12" W
	Dd	1-4	SO 617119	51° 48' 16" N 2° 33' 24" W
	De	1-4	SO 559120	51° 48' 19" N 2° 38' 23" W

[a]Site codes C, B, H, L, S, U & D were used throughout the report.

4.2.2 Species identification by morphological analysis

Gall wasps, inquiline and parasitoid species were identified using keys and descriptions by Williams (2004), which are based on earlier works of R.R Askew and others. A high degree of confidence can be attached to the identifications of the gall-causer species as they have distinctive morphologies and are gall-specific. The species assignments made for the inquiline and parasitoid specimens should be considered more tentative. These wasps are not gall specific and share extremely similar morphological characteristics. The samples were not compared with type specimens, and the sequence data (see below) available in the National Centre for Biotechnology Information (NCBI) GenBank databases for comparison with these species was limited. Voucher specimens of all species are to be deposited in the National Museum & Galleries of Wales, Cardiff.

Each wasp specimen was labelled according to the species of the wasp itself, the number of that specimen, the species of the causer of the gall from which the specimen emerged, the number of that gall, and the sampling location and number of the tree from which the gall came. For example, specimen Sap.3.Nq.2.H.3 was the third *Synergus apicalis* specimen that emerged from the second *N. quercusbaccarum*-induced gall collected from tree number 3 at sampling site H. The species abbreviations are given in the 'Table of abbreviations' and the sampling locations are described in Table. 4.2.

4.2.3 Insect sample DNA extraction

Wasps were transferred to individual tubes of ethanol (100%) immediately following emergence and stored at –80°C until DNA was extracted up to a year later. Each sample was washed in 5% [vol / vol] Clorox solution (5.25% sodium hypochlorite) and serially rinsed in sterile distilled water, before the abdomen was dissected using a sterile scalpel blade and used for DNA extraction. The extraction method is given in Section 3.2.2.

4.2.4 PCR amplification of *Wolbachia* and host marker genes

For each insect sample, amplification reactions were carried out for the *Wolbachia*-specific *wsp* gene, the *Wolbachia* 16S rRNA gene and the cytochrome *b* gene of the host, to detect and confirm the presence or absence of the endosymbiont and check the quality of the DNA template.

PCR reactions were performed under optimum conditions which were determined experimentally. The primers and the PCR conditions for each primer pair are summarised in Table 4.3. 1 µl template DNA (0.1 – 10 ng) was used for amplification of cytochrome *b*

TABLE 4.3 Oligonucleotide primers used for PCR in this study

Gene	Primer	Nucleotide sequence (5'-3')	Approx product size (bp)	Cycling conditions
Wsp[b]	81f	TGGTCCAATAAGTGATGAAGAAAC	630-672	94°C for 10 min, 35 cycles of 92°C for 30 s, 56.7°C for 30 s, 75°C for 1 min, extension for 5 min at 72°C.
	Clamped 691r	GCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCC CCGCCCCAAAAATTAAACGCTACTCCA		
16S[c]	16WolSpfor[a]	GATGAGCCTATATTAGATTA	470	94°C for 4 min, 35 cycles of 92°C for 30 s, 54°C for 30 s, 75°C 1 min, extension for 5 min at 72°C.
	16SWolSprev[a]	CTGGTGTTCCTCCTAATATT		
Cytochrome *b*[d]	CB1	TATGTACTACCATGCGGACAAATATC	420	94°C for 4 min, 35 cycles of 92°C for 30 s, 50°C for 1 min, 72°C for 1 min, extension for 5 min at 72°C.
	CB2	5'ATTACACCTCCTAATTTATTAGGAAT		

[a] 16Sfor and 16Srev correspond approximately to *Escherichia coli* positions 227-246 and 703-722, respectively (Brosius *et al.*, 1981). [b] Braig *et al.*, 1998.
[c]This study. [d]Jermiin & Crozier, 1994

gene and 16S rRNA gene fragments, and 1.5 μl for *wsp* gene fragments. All other conditions were as used in Section 2.2.3. Primers specific to the *Wolbachia* 16S rRNA gene were designed using the PRIMROSE software package (Ashelford *et al.*, 2002), for increased confidence in the specificity of this reaction and the ability to amplify all possible *Wolbachia* sequences. Positive and negative controls were included with each set of reactions (Section 2.2.3)

4.2.5 Denaturing gradient gel electrophoresis (DGGE) analysis of *wsp* PCR amplimers

DGGE analysis was carried out under the conditions given in Section 3.2.4. Each gel included at least three marker lanes, consisting of *wsp* amplimers from insects infected with known *Wolbachia* variants to facilitate comparison between gels. These included amplimers from *Torymus geranii* sample Tg.1.Bp.49.F1, *Asobara tabida*, *Leptopilina heterotoma*, *Drosophila melanogaster*, *D. simulans* (Riverside strain) and *B. pallida* (Chapter 3). Therefore up to 22 samples were analysed on each gel.

Gels were run for 5 h routinely; though a 6 h running time was used on one occasion to increase confidence in the observed banding pattern. Samples that produced bands that migrated to novel positions in the gel were examined by direct sequencing of the purified PCR product or the gel-extracted, re-amplified product (Sections 3.2.4 & 2.2.4).

4.2.6 DNA sequencing and sequence analysis

DNA sequences were obtained from the wasp-extracted DNA or resuspended gel-extracted DNA as described in Sections 2.2.5 & 3.2.5. All *wsp* sequences were compared with each other and with sequences from GenBank. Representative sequences were used for the construction of phylogenetic trees using Jukes-Cantor (1969) to compare ClustalX alignments (Thompson *et al.*, 1997), followed by Neighbour Joining (Saitou & Nei, 1987) using TreeconW (Van de Peer & De Wachter, 1997). It is common practice to remove the third hypervariable region (519-559 bp) from the sequence alignment due to the difficulty in aligning the region accurately (Braig *et al.*, 1998; Kittayapong *et al.*, 2003; Rokas *et al.*, 2002; Thiapaksorn *et al.*, 2003; van Meer *et al.*, 1999; Zhou *et al.*, 1998). Due to the inclusion of relatively short DNA sequences, a dataset of 376 bp was produced, which did not include the third hypervariable region (Fig. A1). Bootstrap analysis was carried out at 1000 repetitions. The *wsp* tree was rooted to a C-clade *wsp* sequence from the nematode

Brugia malaya (Bazzocchi *et al.*, 2000). Phylogeny reconstruction using aligned derived amino acid *wsp* sequences produced the same subgroup clusters as the nucleotide alignment, and the differences in tree topology that were seen did not affect the conclusions discussed in Sections 4.3 and 4.4.

Cytochrome *b* sequences were compared with each other and with GenBank sequences in the same way, in order to support the results of the morphological identifications. The dataset was 356 bp in length and is presented in Fig. A2. A cytochrome *b* sequence from the bee species *Trigona hockingsi* was used as a root (Franck *et al.*, 2004), and bootstrapping was carried out at 1000 replicates.

4.2.7 Statistical analysis

The frequency of infected individuals at each sampling site was compared for each species using the Fisher's Exact test, to determine if there was any heterogeneity across the sites with respect to the level of infection. The same test was applied to compare the rate of parasitoid / inquiline attack of galls of each gall-causer species, to determine if there was any heterogeneity across the sites. Fisher's exact test was calculated using StatXact version 4.0.1, Cytel software corporation, Cambridge, Massachusetts.

4.3 Results

4.3.1 The oak gall wasp community samples

Several hundred sexual generation galls induced by *A. curvator*, *B. pallida*, *N. numismalis* and *N. quercusbaccarum* and the asexual generation of *A. quadrilineatus* were collected from locations across South Wales in May-June of 2002 and 2003 (Table 4.2). The five gall wasp species were often found occurring together on the same tree. Galls of *Andricus* and *Neuroterus* species are monolocular (Redfern & Askew, 1998; Stone & Cook, 1998; Williams, 2004; Section 1.8.3), therefore one adult gall wasp was collected from each gall. *Biorhiza pallida* galls are multilocular and produced anything from 1 to over 100 gall wasps.

Wasps emerged from 253 galls, totalling 595 wasps from 19 species. In addition to the gall-causer species, morphological examination identified 5 inquiline species and 9 parasitoid species (Table 4.4). The identifications were confirmed using PCR amplification and DNA sequencing of part of the cytochrome *b* gene. The alignment was 356 bp in length and is given in the Appendix (Fig. A2). This marker gene was found to be most useful for distinguishing between the parasitoid species, clearly separating the *T. auratus*, *T. geranii* and *T. flavipes* specimens (Fig. 4.2). The results for the *Synergus* species were less clear, and cytochrome *b* failed to separate clearly the species *S. albipes* and *S. nervosus* (Fig 4.2).

Sample Tf.1.Ac.5.S.4 was identified as *T. flavipes* following morphological examination, and this was confirmed by R. R. Askew, an independent expert in the field. However, cytochrome *b* analysis, however, showed that this sample differed from the other *T. flavipes* specimens. Little is currently known about torymid haplotypes and ecotypes, and therefore if this specimen is genuinely *T. flavipes*, it may represent natural cytochrome *b* sequence diversity in *T. flavipes*, or it could be a novel haplotype that has arisen through hybridisation with a closely related species as a result of rare interbreeding (Dr G.N. Stone, personal communication).

The rate of parasitoid and inquiline attack varied between gall-causer species and sampling sites (Tables 4.5 & A1-5), and the number of inquilines or parasitoids collected varied between 1 and 20 individuals in each gall. At site C, there were relatively few *B. pallida* galls and a high rate of parasitism was detected (52 parasitoid wasps from 10 galls). Conversely, at site B, the trees were heavily laden, so the chance that the collected galls had been attacked may have been reduced, and of the nine galls that yielded gall-causer wasps, no parasitoids and only a single inquiline specimen emerged. The point

TABLE 4.4 Incidence of *Wolbachia* infection in each species tested in this study [a]

Wasp species	Sampling location [b] C		B		H		L		S		U		D		Total	
Gall causer wasp species	2002	2003	2002	2003	2002	2003	2002	2003	2002	2003	2002	2003	2002	2003	2002	2003
Andricus curvator Hartig	-	-	-	-	0/3	0/3	0/1	0/2	-	-	0/2	0/1	0/17	0/2	**0/23 (0%)**	**0/8 (0%)**
A. quadralineatus Hartig	-	-	-	-	-	-	1	-	-	-	-	-	-	-	**0/1 (0%)**	-
Biorhiza pallida [c] (Olivier)	15/15 (3)	3/3 (7)	5/5 (7)	10/10 (3)	6/6 (5)	4/4 (5)	6/6 (4)	1/1 (3)	5/7 (6)	6/6 (11)	23/27 (28)	30/31 (25)	2/2 (1)	1/1 (2)	**62/68 (91%)**	**55/56 (98%)**
Neuroterus numismalis (Geoffroy in Fourcoy)	-	-	-	-	-	-	-	0% 0/1	-	-	0/1	-	0/1	0/4	**0/2 (0%)**	**0/5 (0%)**
N. quercusbaccarum (Linnaeus)	-	-	-	-	-	0/3	0/16	0/31	-	0/1	0/4	0/9	0/4	0/23	**0/24 (0%)**	**0/67 (0%)**
Inquiline wasp species	2002	2003	2002	2003	2002	2003	2002	2003	2002	2003	2002	2003	2002	2003	2002	2003
Synergus albipes Hartig [d]	-	-	-	-	-	-	-	0/6	1/1	-	-	0/2	-	1/4	**1/1 (100%)**	**1/12 (8%)**
S apicalis Hartig	-	-	-	-	4/4	-	-	-	5/6	-	-	-	-	-	**4/4 (100%**	-
S. gallaepomiformis Fonscolombe	-	-	-	2/2	-	7/11	11/19	2/6	4/5	-	16/25	5/5	-	-	**32/50 (63%)**	**16/24 (67%)**
S. nervosus Hartig [d]	-	-	-	-	-	-	1/1	3/3	2/2	-	3/4	3/3	-	-	**6/7 (86%)**	**6/6 (100%)**

TABLE 4.4 continued

	Sampling location [b]															
	C		B		H		L		S		U		D		Total	
Parasitoid wasp species	2002	2003	2002	2003	2002	2003	2002	2003	2002	2003	2002	2003	2002	2003	2002	2003
Torymus auratus (Walker)	0/34	-	-	-	0/2	-	-	-	0/66	-	-	-	-	-	**0/102 (0%)**	-
T. flavipes (Walker)	8/9	-	-	-	1/1	5/5	1/1	-	5/7	1/1	20/24	6/6	6/6	-	**41/48 (85%)**	**12/12 (100%)**
T. geranii (Walker)	-	-	-	-	-	-	-	-	1/1	-	-	-	-	-	**1/1 (100%)**	-
Megastigmus dorsalis (Fabricius)	0/9	-	-	-	-	-	-	-	-	-	-	-	-	-	**0/9 (0%)**	-
M. fasciiventris (Westwood)	-	-	-	-	0/1	1/1		1/1	-	-	0/1		-	-	**0/2 (0%)**	**2/2 (100%)**
M. tibialis (Westwood)	-	-	-	-	-	1/1	1/1	1/3	-	-	-	-	-	-	**1/1 (100%)**	**2/4 (50%)**
M. sericeus (Forster)	-	-	-	-	-	1/1	-	-	-	-	-	-		1/1	-	**2/2 (100%)**
Cecidostiba sp.	-	-	-	-	0/1	-	-	-	-	-	-	-	-	-	**0/1 (0%)**	-
Aulogymnus sp.	-	-	-	-	-	-	-	-	-	-	0/1	-	-	-	**0/1 (0%)**	-

[a] Percentage infected specimens are followed by total number of specimens in brackets. [b] For sampling locations see Table 4.2. [c] The number of *B. pallida* galls sampled is given in parenthesis. 100% of *B. pallida* galls carried infected individuals. [d] A total of 49 *S. albipes* or *S. nervosus* specimens could not be separated with confidence and therefore were not included in the table. If all *S. albipes* and *S. nervosus* samples are treated as one group, the infection frequency of the group is 35% (26/75).

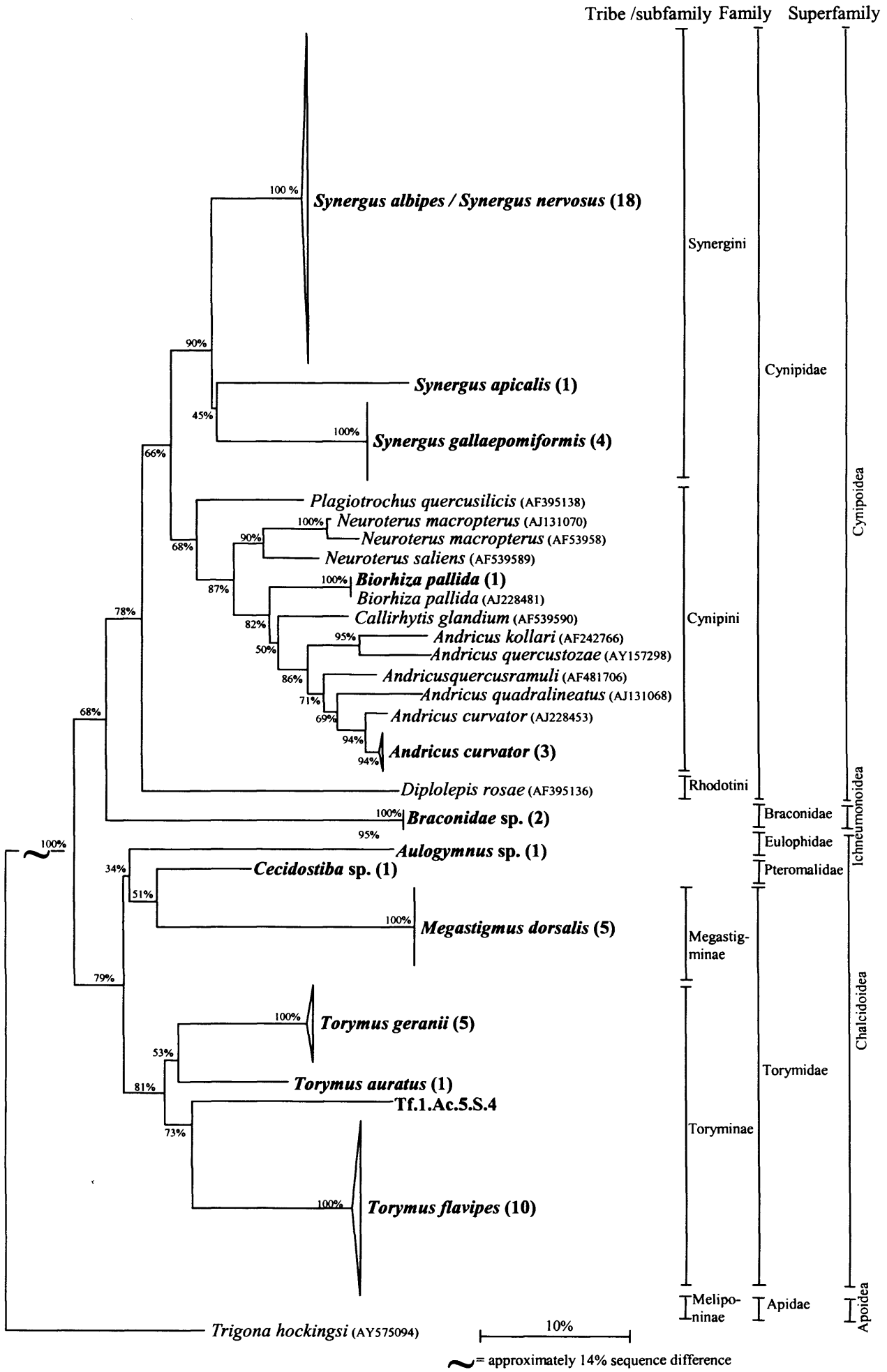

FIG. 4.2 Phylogenetic tree based on analysis of cytochrome *b* gene sequences from members of the oak gall wasp community, constructed using Jukes-Cantor to compare ClustalX aligned sequences (356 bp) followed by Neighbour Joining (Section 4.2.5). Sequences obtained in the present study are presented in bold and the number of samples from which that sequence was obtained is given in parenthesis. For all other sequences the accession number is given. The cytochrome *b* sequence from *Trigona hockingsi* (Hymenoptera: Apocrita: Aculeata) was used as a root and bootstrapping was carried out at 1000 replicates. The tribes (family Cynipidae only; Section 1.8.1), subfamilies, families and superfamilies are labelled.

TABLE 4.5 The percentage of galls of each gall-causer species, from which parasitoid (unshaded) or inquiline (shaded) wasps emerged at each location[a]

	Sampling location													
Gall causer species	**C**	**B**	**H**	**L**	**S**	**U**	**D**	**C**	**B**	**H**	**L**	**S**	**U**	**D**
Andricus curvator	-	-	0% (0/6)	0% (0/4)	12.5% (1/8)	25% (1/4)	7% (1/14)	-	-	0% (0/6)	25% (1/4)	75% (6/8)	0% (0/4)	14% (2/14)
A. quadralineatus	-	-	-	0% (0/1)	0% (0/4)	-	-	-	-	-	100% (1/1)	100% (4/4)	-	-
B. pallida	70% (7/10)	0% (0/9)	36% (4/11)	0% (0/7)	62.5% (10/16)	33% (16/48)	0% (0/3)	0% (0/10)	11% (1/9)	100% (11/11)	71% (5/7)	19% (3/16)	17% (8/48)	66% (2/3)
Neuroterus numismalis	-	-	100% (2/2)	100% (4/4)	-	50% (1/2)	0% (0/5)	-	-	0% (0/2)	0% (0/4)	-	0% (0/2)	0% (0/5)
N. quercusbaccarum	-	-	54% (7/13)	18% (3/17)	12.5% (1/8)	17% (3/18)	18% (7/38)	-	-	38% (5/13)	18% (3/17)	75% (6/8)	22% (4/18)	13% (5/38)

[a]The numbers of attack/ not attacked galls are given in parenthesis.

in the season at which the galls were collected (gall maturity), the weather at the time and how the galls were handled (condition of the gall) could also have affected the likelihood of the wasps emerging. However, all galls were collected within a short period as galls reached maturity, and were all handled in the same way.

There was a significant difference between the number of galls that were successfully parasitised at different sampling sites for *B. pallida* and *N. numismalis*-induced galls (Fisher's Exact Test, $P < 0.05$), and between the number of galls successfully attacked by inquilines for *B. pallida* and *N. quercusbaccarum*-induced galls (Fisher's Exact Test, $P < 0.05$) (Table 4.5). The rate of successful attack at each site (i.e. number of galls from which parasitoid / inquiline wasps emerged) was compared statistically because it could have a significant influence on the potential for horizontal transfer and therefore on the prevalence and diversity of infection at different locations, however the pattern of parasitoid / inquiline attack differed for each gall-causer species and the results were open to no specific interpretation.

As shown by Table 4.6, all *A. curvator*, *A. quadralineatus*, *N. numismalis* and *N. quercusbaccarum*-induced galls produced either parasitoids or inquilines, never both, and in most galls either the gall-causer, or a parasitoid species, or an inquiline species emerged (survived to adulthood). Very rarely (one instance for *A. curvator* and *A. quadralineatus* galls, four for *N. quercusbaccarum* galls) did a gall-causer wasp and a parasitoid species emerge from the same gall, and only in one instance (*N. quercusbaccarum* gall) did a gall-causer and inquiline wasp emerge from the same gall. Parasitoids were always singular but more than one inquiline emerged from some galls, and where the species could be identified all individuals from one gall were found to be the same species. Every wasp combination was seen to emerge from *B. pallida* galls due to the multilocular nature of these galls (Table 4.6).

4.3.2 Incidence and prevalence of *Wolbachia* infection in the oak gall wasp communities

In all, 593 wasps were tested for infection by PCR amplification of *wsp* and 16S rRNA genes. Samples which gave a negative result for *Wolbachia* infection were checked by amplification of cytochrome *b*. Where this reaction failed template DNA was assumed to be of poor quality and such samples were excluded from further study.

In this study, samples from seven sites in South Wales were collected for testing.

TABLE 4.6 Frequency at which gall causers, inquilines or parasitoids, or combinations thereof, emerged from each gall type

	Gall causer only	**Parasitoid only**	**Inquiline only**	**Gall causer & Parasitoid**	**Gall causer & Inquiline**	**Parasitoid & Inquiline**	**Gall causer, Parasitoid & Inquiline**
Andricus curvator	24	2	9	1	0	0	0
A. quadralineatus	0	0	4	1	0	0	0
B. pallida	58	23	10	12	3	2	2
Neuroterus numismalis	7	6	0	0	0	0	0
N. quercusbaccarum	83	16	22	4	1	0	0

(Table 4.2; Fig. 4.1). Nineteen species from five oak gall wasp communities were tested for *Wolbachia* infection and 10 species were found to be infected (Table 4.4); a frequency of 53%. A total of 256 wasps (43%) were found to be infected with *Wolbachia*. Positive samples for which species identification could be made with confidence were analysed by DGGE (see Section 4.3.3).

Wolbachia prevalence varied between 8% and 100% in infected species (Table 4.4). Table 4.4 shows the total frequency of infection detected for each species tested, regardless of which gall the wasps emerged from, as all parasitoid / inquiline specimens of a given species at a given location can potentially interbreed, and can therefore be regarded as a single population. However, the infection frequency of each species isolated from each gall community is also given separately in Tables A1-A5 of the Appendix and showing the same relative infection frequencies.

Of the three (6%) uninfected *B. pallida* specimens, two were from location S and one from location U (Table 4.4). The specimen from location U came from a gall from which no other wasps emerged, so it could be that the gall was of poor condition, and that environmental conditions lead to the loss of the *Wolbachia* infection. However, the other two uninfected specimens came from the same gall, from which one other *B. pallida* specimen was tested and found to be positive for *Wolbachia*. Also, an infected *T. flavipes* and several uninfected *T. auratus* specimens were also obtained from this gall. Therefore, environmental conditions during gall formation cannot explain the appearance of these uninfected individuals.

Forty-nine *Synergus* specimens proved difficult to identify but were narrowed down to *S. nervosus* or *S. albipes* (Section 4.3.1). These specimens were not included in the infection frequency calculations. If all the *S. nervosus* and *S. albipes* samples from the study were grouped together with these 49 specimens, the infection frequency was estimated at 35% (75 samples).

4.3.3 Determination of the diversity of *Wolbachia* present using DGGE and DNA sequencing

A total of eight different variants that migrated to distinct positions in the DGGE gel were identified: I, II, III, XII, XIV, XV, XVI and XVII (Figs. 4.3 and 4.4). Each unique variant was assigned a Roman numeral label, to extend the list of reference DGGE variants developed in the work in Chapter 3. Fig. 4.4 is a diagrammatic summary of 29 DGGE profiles. Sequencing reactions were carried out for up to nine replicates of each of

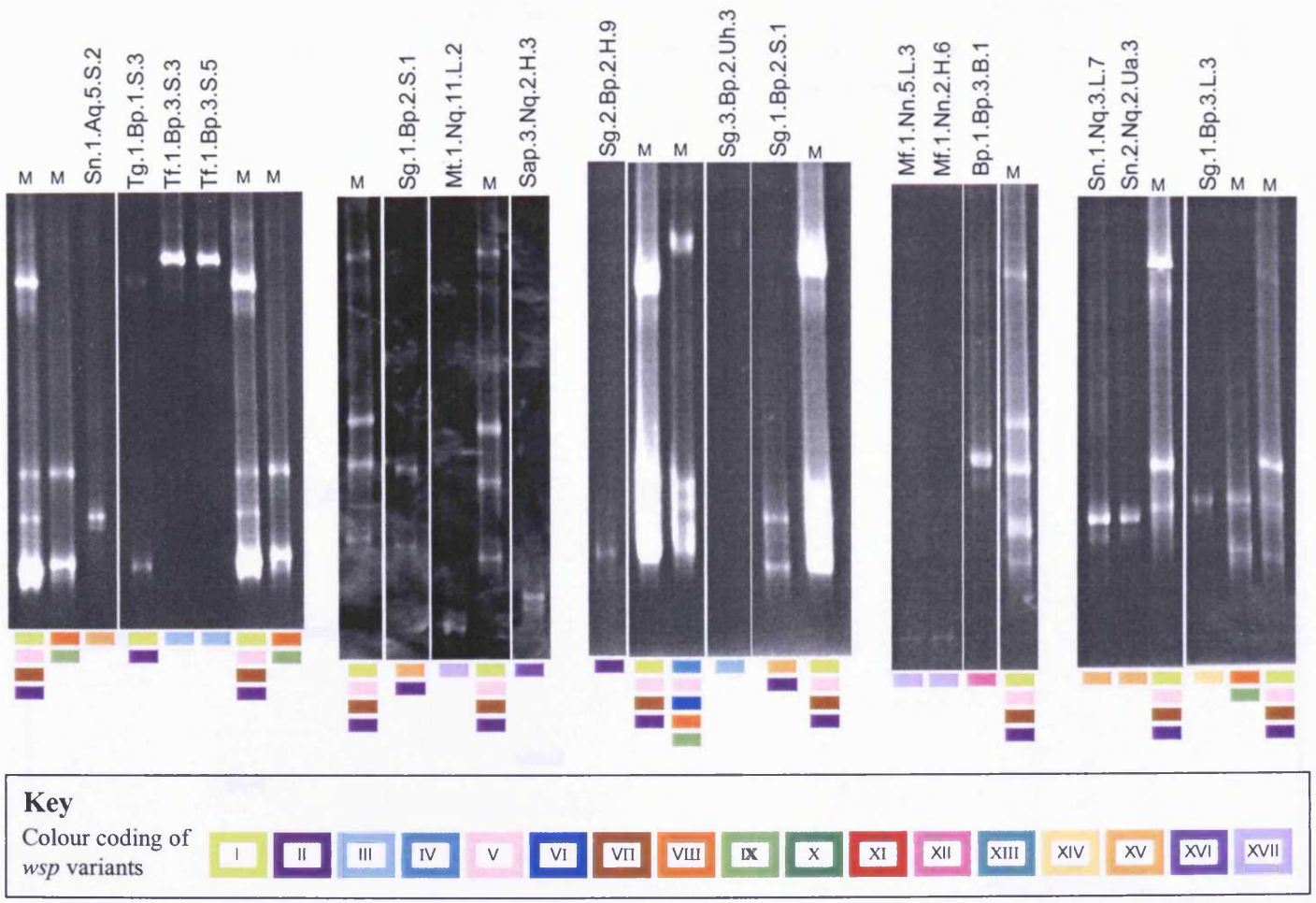

FIG. 4.3 Banding profiles produced by DGGE analysis of *wsp* fragments amplified from members of the oak gall wasp community. Representatives of each *wsp* variant identified in the present study are shown, labelled according to the wasp sample from which they were amplified. Two double infections identified (samples Sg.1.Bp.2.S.1 and Tg.1.Bp.1.S.3) have also been included (see section 4.2.2 for an explanation of the sample names). Marker variants (M) characterised in Chapter 3 were run alongside. Variants are colour-coded according to the key, to allow comparison with Figs. 4.4, 4.5 and 4.6.

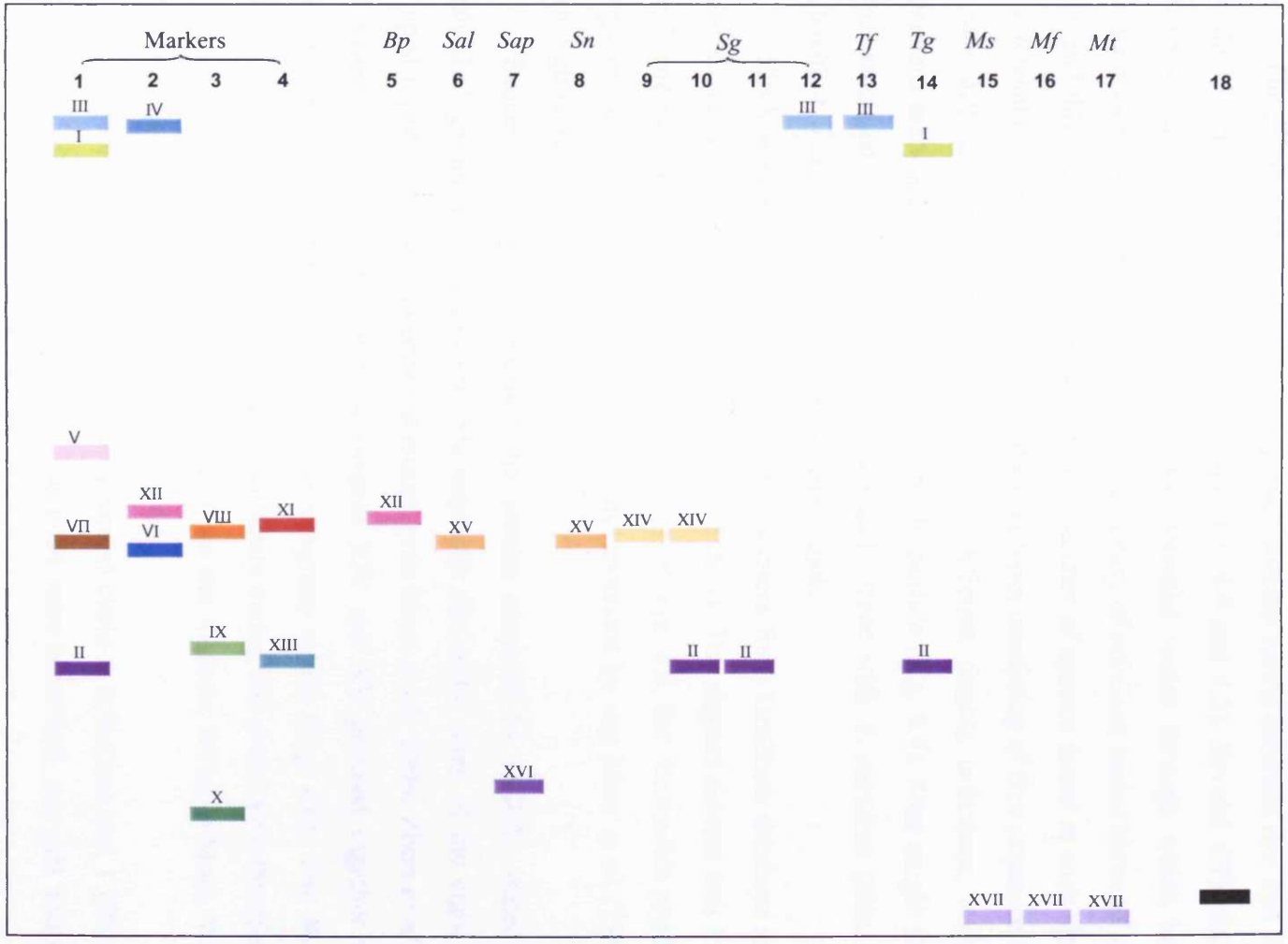

FIG. 4.4 Diagrammatic summary of the DGGE analysis of *wsp* fragments amplified from the oak gall wasp community. Each DGGE variant has been assigned a colour and Roman numeral label to facilitate comparison with Fig. 4.5 and Fig.4.6. Lanes 1-4 contain reference *wsp* DGGE variants (section 3.3). Lanes 5-17 show the single and double infections detected in each *Wolbachia* infected species. The band in lane 18 is discussed in section 4.3.5 & 4.4.6 *Bp = Biorhiza pallida; Sal = Synergus albipes; Sap = S. apicalis; Sn = S. nervosus; Sg = S. gallaepomiformis; Tf = Torymus flavipes; Tg = T. geranii; Ms = Mesopolobus sericeus; Mf = M. fasciiventris; Mt = M. tibialis.*

the DGGE banding positions from both gel-extracted DNA and PCR fragments amplified from the original DNA extraction. This was done in order to confirm that each band represented a unique sequence variant and that the band identified at a given position corresponded to the *wsp* sequence obtained from the extracted DNA.

The number of variants infecting each species varied between one and four, and two double infections were detected (Figs. 4.3, 4.4 and 4.5). Several different species carried similar variants, revealing several potential routes through which horizontal transfer may have occurred (Fig. 4.5). The diversity of infection varied between sampling sites, and this appeared to correspond to the number of species found at each site (Table 4.7). A total of 6 different *Wolbachia* infection types consisting of five sequence variants, in four different single infections and two different double infections, occurred in specimens associated with galls induced by *B. pallida* (Fig. 4.4). Four single infections were associated with *N. quercusbaccarum* galls, three with *A. curvator* galls, and one each with *A. quadralineatus* and *N. numismalis* galls.

DNA sequences were aligned with sequences from GenBank database and those from the reference *wsp* amplimers (Chapters 2 & 3). The aligned dataset was 376 bp in length and is given in the Appendix (Fig. A1). In Fig. 4.6, the *Wolbachia* phylogenetic subgroups proposed by Zhou *et al.* (1998) and extended by van Meer *et al.* (1999) have been highlighted.

Sequence analysis confirmed the results obtained by DGGE. Based on the established grouping criterion of 97.5% sequence similarity, some of the eight variants grouped together in *wsp* sequence subgroups (van Meer *et al.*, 1999; Zhou *et al.*, 1998). For example, DNA sequences from variants XIV and XV grouped together with the marker variants VII, VIII and XI in *wsp* subgroup wMel (Fig. 4.6). The *Wolbachia* variants identified in the oak gall wasp community during this study were therefore found to belong to five different subgroups, four from the A-clade; wKue, wMors, Wmel and wUni and one from the B-clade; wCon (Fig. 4.6).

Of the 19 species tested, 10 (53%) carried clade-A infections and 1 (5%) carried the clade-B infection and two double infections were identified, one AB and one AA infection.

The first double infection occurred in one *T. geranii* specimen isolated from a *B. pallida*-induced gall (Fig. 4.4, lane 14), and was identical in sequences and banding positions to the infection identified in this species in Chapter 2. The B-clade sequence variant from this infection (I) was found only as part of this double infection. The A-clade

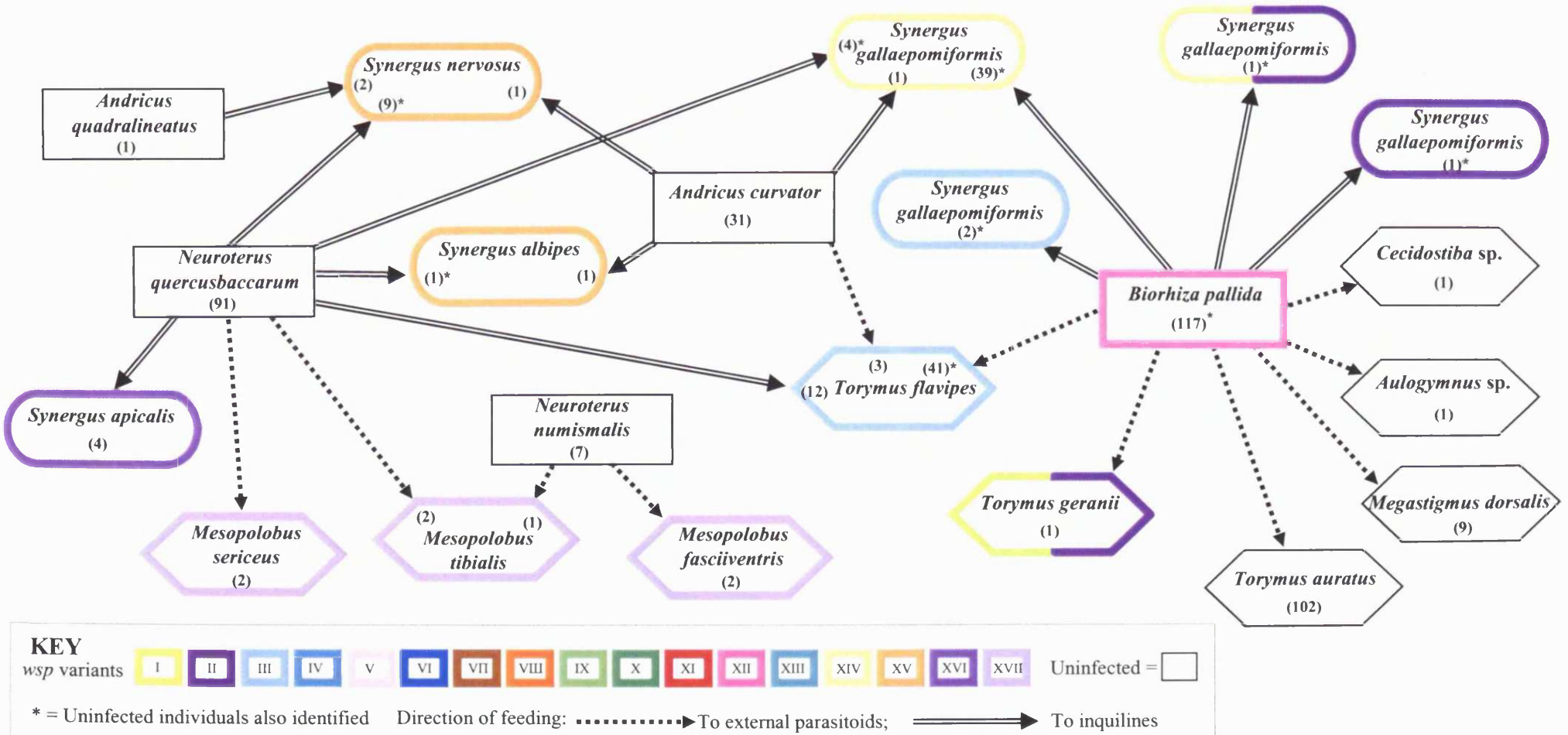

FIG. 4.5 Foodweb diagram showing the associations between the wasp species examined in the present study, and their *Wolbachia* infection status. Wasp species are shown linked to the causer species of the gall from which they emerged. Gall-causer species are shown in rectangles, inquiline species in oblongs and parasitoid species in hexagons. The *wsp* variants detected in *Wolbachia* infected species are colour-coded according to the key, to facilitate comparison with Figs. 4.4, 4.6 and Chapter 3. The number of specimens in which each infection type (single, double or uninfected) was detected, is given in parenthesis for each species. For parasitoid and inquiline species, these numbers may be divided between the galls from which they emerged.

TABLE 4.7 Diversity of *Wolbachia* variants detected at each sampling site

Sampling site[a]	Infected species present[b]	Variants detected	Total number of variants
C	Bp Tf	**III XII**	2
B	Bp Sg	**XII XIV**	2
H	Bp Sap Sg Tf Mf Ms Mt	**II III XII XIV XV XVII**	6
L	Bp Sg Sn Tf Mf Mt	**III XII XIV XV XVII**	5
S	Bp Sal Sg Tg Tf	**I II III XII XIV XV**	6
U	Bp Sg Tf	**III XII XIV**	3
D	Bp Sal Tf Ms	**III XII XV XVII**	4

[a] For location of sample sites see Table 4.2. [b]Bp = *Biorhiza pallida*; Tf = *Torymus flavipes*; Sg = *Synergus gallaepomiformis*; Sap = *S. apicalis*; Mf = *Mesopolobus fasciiventris*; Ms = *M. sericeus*; Mt = *M. tibialis*; Sn = *S. nervosus*; Sal = *S. albipes*.

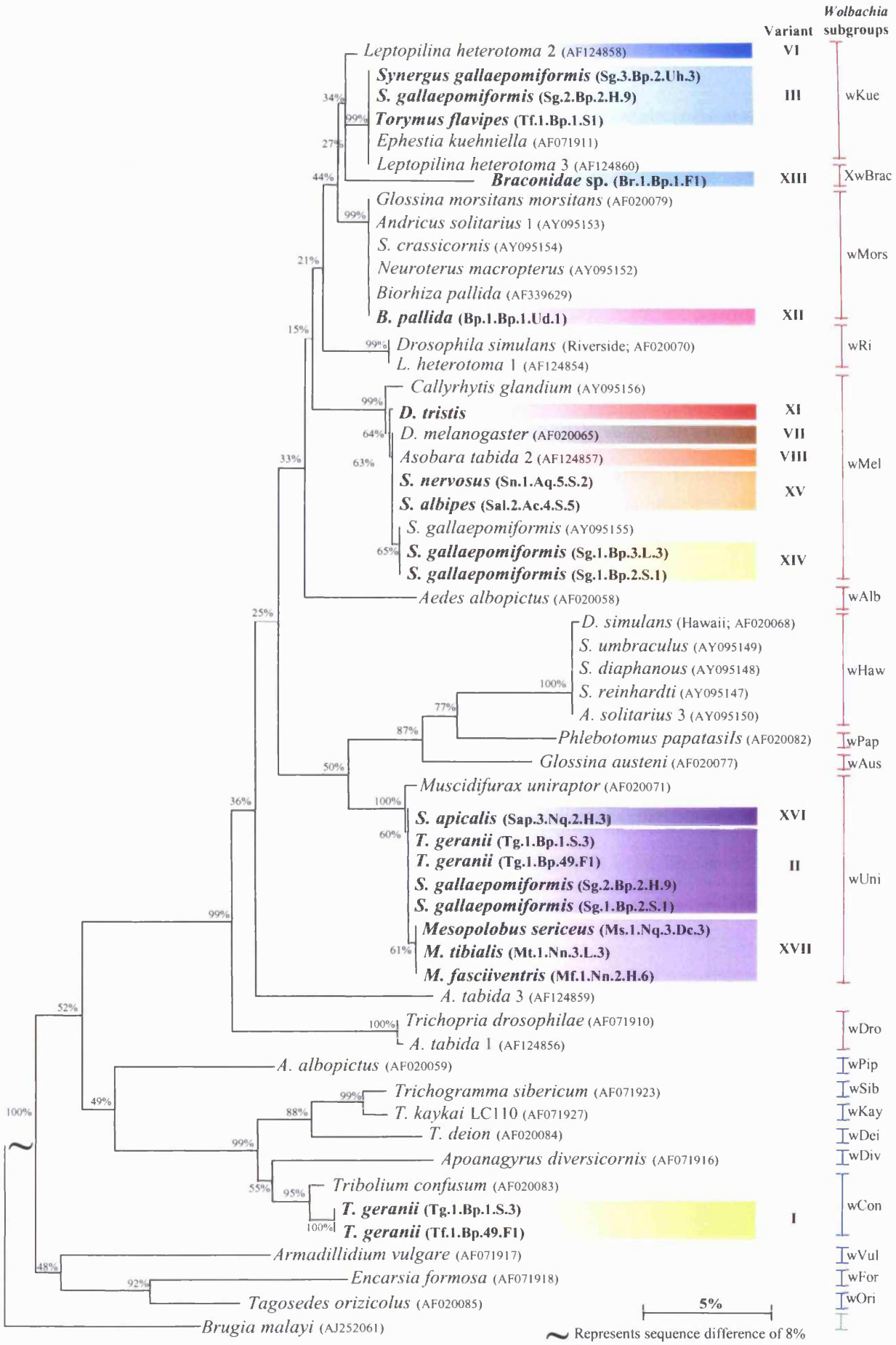

FIG. 4.6 Phylogenetic tree based on analysis of *wsp* gene sequences from the oak gall wasp community, constructed using Jukes-Cantor to compare ClustalX alignments (376 bp) followed by Neighbour-Joining (Section 4.2.5). Bootstrapping was carried out at 1000 repetitions. *wsp* variants identified in the present study are labelled and colour coordinated to facilitate comparison with Figs. 4.3 – 4.5. Sequences in bold were obtained during the present study, Chapter 2 and Chapter 3. For all other sequences the accession number is given. The *Wolbachia* subgroups proposed by Zhou *et al.* (1998) and van Meer *et al.* (1999) are labelled. Red lines group together clade-A sequences, blue lines group sequences from clade-B and the green line highlights the sequence from the nematode clade-C, which was used to root the tree.

sequence variant (II) however, was also found to occur as a single infection in one *S. gallaepomiformis* specimen (Fig.4.4, lane 11) and also as a second double infection in *S. gallaepomiformis* (Fig. 4.4, lane 10), along with variant XIV. This species also carried single infections of the XIV variant (Fig. 4.4, lane 9) and variant III (Fig. 4.4, lane 12). *S. albipes* and *S. nervosus* were found to be singularly infected with variant XV (Fig. 4.4, lanes 6 & 8 respectively), which was very similar to variant XIV. *B. pallida* carried a single sequence variant (variant XII; Fig. 4.4, lane 5). *T. flavipes* was singly infected with a wKue subgroup sequence variant (variant III; Fig. 4.4, lane 13), which had been identified in this species previously (Chapter 2). *M. sericeus, M. tibialis* and *M. fasciiventris* were all singly infected with a wUni sequence variant (variant XVII; Fig. 4.4, lanes 15-17). *S. apicalis* was found to carry a very similar sequence variant (variant XVI; Fig. 4.4, lane 7).

Variant XVI differed from variant II by a single base at the 5' end of the *wsp* fragment but due to the inclusion of shorter *wsp* sequences in the alignment used to construct the phylogenetic tree in Fig. 4.6, this sequence difference was not demonstrated by the tree. However, it was shown clearly by the DGGE profile in Fig 4.3. To demonstrate this, and other differences in DNA sequence that were not fully demonstrated by Fig. 4.6, a full length sequence alignment has been included in the Appendix (Fig. A3). The alignment shows that variants IV and III differed by 1 bp (over 560 bp; Fig. A3; Fig. 3.1), and variants VII and XV differed from variant VIII by 4 bp (over 560 bp bp; A3; Fig. 3.1).

4.3.4 Sensitivity of DGGE *wsp* screen

Sequence variants of approximately 610 bp in length, that differed by as little as 1 bp, were distinguished by the DGGE screen (Chapter 3; Fig. 3.1). For example variant XIV differed from variant XV by a single base and they banded consistently at slightly different positions (Fig 4.3; Fig 4.4, lanes 8 and 9). Variants II and XVI also differed from each other by a single base and produced bands at quite distinct positions in the gel (Fig. 4.3; Fig 4.4, lanes 11 and 7 respectively).

In one case, sequence information did not agree with the DGGE result. Variant XV (Fig. 4.4, lane 8: Fig. 4.6) appeared to be identical in nucleotide sequence to the marker variant VIII (Fig. 4.4, lane 3) yet migrated to a position just below it. Variant XIV (Fig. 4.4, lane 9) showed one base difference to variants VIII and XV, but appeared to migrate to the same position as variant VIII and above XV. The nucleotide sequence

obtained for VIII is approximately 19 bases shorter than the XV and XIV sequences so it is possible that a sequence polymorphism indicated by DGGE but not detected by DNA sequencing, occurs in this region. It is due to this uncertainty that variants VIII and XV have been categorised as different variants and assigned their different designations.

4.3.5 Amplification of a retrotransposase using general *wsp* primers

Contrary to initial evidence, the gall-causer *A. curvator* was found to be uninfected. PCR amplification using general *wsp* primers (Table 4.3) produced a band of expected size (610 bp) following agarose electrophoresis, and the product gave a consistent band in repeated DGGE profiles (Fig 4.3; lane 18). However, sequence analysis showed that this product had no nucleotide sequence similarity to the *wsp* gene. A BLASTN search across NCBI databases failed to identify any significant matches in the database, but searches using BlastX revealed low percentage matches over the full length *A. curvator* sequence (175 aa), to several reverse-transcriptase and maturase gene products that may be indicative of a retrotransposon or retrovirus (Mohr *et al.*, 1993; Xiong & Eickbush, 1988). The best protein sequence match was to a reverse transcriptase synthetic construct from a eukaryotic non-long terminal repeat retrotransposon (AF025672.2: E = 5e-09).

When the putative translated product was compared with the genome sequence of the wMel *Wolbachia* variant using BlastP, the highest similarity was to putative reverse transcriptase genes: WD0693, WD0995 and WD1138 (Fig. 4.7; Fig. 1.2). The match showed only 26% sequence identity (43% sequence similarity) over 94 amino acids (E = 0.032), the significance of which is borderline, but several residues that are conserved among reverse-transcriptases were present (Xiong & Eickbush, 1988).

Interestingly, part of the complement to the forward *wsp* primer sequence occurs in the nucleotide sequence of the wMel transposase gene WD0693: 8 bases from the 3' end of the primer sequence match exactly with the complement of the region from base 670140 to 670166 of the wMel genome sequence (Fig. 4.7; Fig. 1.2). Another five bases match 10 bases upstream. The reverse primer sequence was not found but sequencing reactions carried out with both primers were successful.

This evidence strongly suggested that the fragment amplified from *A. curvator* belongs to a gene encoding a retrotransposase. It may be possible that a transposon from the *Wolbachia* genome was transferred to *A. curvator* from an ancient infection that has since been lost. However, the match to eukaryotic retrotransposase genes also suggested

A BLASTP results

	Score (bits)	E value
WD0693 reverse transcriptase, putative {Wolbachia pipient...	73	0.032
WD0995 reverse transcriptase {Wolbachia pipientis wMel}	73	0.032
WD1138 reverse transcriptase, putative {Wolbachia pipient...	73	0.032

B Alignment with WD0693

```
>WD0693 reverse transcriptase, putative {Wolbachia pipientis wMel} Length = 515

Score = 73 (30.8 bits), Expect = 0.033, Sum P(2) = 0.032
Identities = 25/94 (26%), Positives = 41/94 (43%)


Query:     4 GLRQGCPLSPTLFAILIADMEGKLEAXXXXXXXXXXXXXXWS----LAYADDIVLLAKSEE 59
             G  QG  +SP L  + +  +E  LE+              S    + YADD ++   + E
Sbjct:   230 GTPQGSIISPILANLALNGLEKSLESQFGKLGSKRRSKIRSGVNVIRYADDFIISGITRE 289


Query:    60 ALKEMMKRL-RRYLDKNRLELNAEKSKVMVFRKG 92
              L+  +K L   +L +  L L+ EK+K+      G
Sbjct:   290 VLENEVKPLVSSFLQERGLILSEEKTKITSITTG 323


 Score = 36 (17.7 bits), Expect = 0.033, Sum P(2) = 0.032
 Identities = 9/26 (34%), Positives = 12/26 (46%)

Query:   102 KWKGKAVQAVKEFVYLGFLFRRNGGV 127
             KWK    + VK+   L   F R G +
Sbjct:   476 KWKKYFDERVKQTKMLASSFSREGSL 501
```

C Potential binding site for *wsp* primer in the reverse transcriptase gene

```
wsp 81f primer  3' CAAAGAAGTAGT-------GAATAACCTGGT 5'
WD0693     5'670140 CTTTCTTCAGGAGAGAGGTCTTAT----CC- 670166 3'
```

FIG. 4.7 The product amplified from *A. curvator* using the general *wsp* primers (Section 4.2.4), was compared with the *Wolbachia* (wMel) genome sequence (Wu *et al.*, 2004). The translated amino acid sequence produced a match with three putative reverse transcriptase genes (A) using BlastP which searches for similar protein sequences. The alignment is shown in box B. A potential binding site for the 81f *wsp* primer was identified (C) in the coding strand of the WD0693 gene (genome coordinates 669520 – 671067).

that the transposon may occur naturally in *A. curvator* and amplification with *wsp* primers was coincidental. Amplification of this product occurred in only 50% (15) of *A. curvator* samples and showed no connection to the date of the PCR reaction, gall collection area or host tree.

4.4 Discussion

4.4.1 Incidence of *Wolbachia* infection in the oak gall wasp communities

It is clear that levels of infection vary between different host assemblages and intraspecifically, and that some taxonomic groups may be more likely to acquire and maintain *Wolbachia* infections than others (Werren & Windsor, 2000). In the present study, 53% (10 / 19) of the species tested were infected with *Wolbachia*. Previous estimates of infection frequency range from 17% to 76% of insect species tested (Jeyaprakash & Hoy, 2000; Kikuchi & Fukatsu, 2003; Kittayapong *et al.*, 2000; Reuter & Keller, 2003; Ricci *et al.*, 2002; Rokas *et al.*, 2001; Werren *et al.*, 1995a; Werren & Windsor, 2000; West *et al.*, 1998). For example, in the survey carried out by Werren *et al.* (1995a), 157 neotropical insect species were tested for the presence of *Wolbachia*, including species from eight different arthropod orders. In that study 20% of the 19 wasp species were found to be infected, but this may be an underestimate because each species was represented by only a single specimen. Jeyaprakash & Hoy (2000) also tested single specimens from 63 arthropod species and found that eight of 11 wasp species were infected (72%).

Infection frequency estimates will be strongly influenced by variation in DNA extraction methods, PCR amplification procedures, including choice of target gene and primer sequence, and by the host species tested. Jeyaprakash & Hoy (2000) suggested that arthropod DNA interfered with *Taq* DNA polymerase activity and used a combination of two enzymes (*Taq*I and *Pwo*I) to increase the sensitivity of the PCR assay. Thipaksorn *et al.* (2003) also found that Long-PCR detected *Wolbachia* in samples where standard PCR produced negative results, but other studies have found no advantage to using this procedure (Jiggins *et al.*, 2001a; Tsusui *et al.*, 2003). Here we used the *Taq*-only protocol to allow comparison with the majority of published studies.

Wolbachia has previously been detected in members of the oak gall wasp community (Abe & Muira, 2002; Rokas *et al.*, 2002a). Rokas *et al.* (2002a) detected *Wolbachia* in 9% of 53 gall-causer species. In the present study (and Chapter 2), the infected status of *B. pallida* and uninfected status of *A. curvator*, established in Rokas *et al.* (2002a) was confirmed, and three other gall-causer species were tested. Only one of the five gall-causer species was infected (20%) but this value is likely to be less statistically accurate due to the small number of species tested. It is clear however, that the gall-causers show a lower incidence of infection relative to the inquiline species. In this study, all five inquiline species were infected and 9/10 species tested by Rokas *et al.*

(2002a) were also positive; an average incidence of 93%. A high incidence of infection was also found in the parasitoid members of the community. Ten species were tested and six were found to carry *Wolbachia* (60%). Again, this estimate may be biased due to the relatively low numbers of species sampled, but the higher frequency of infection in the parasitoid and inquiline wasps, which live in close proximity to each other and develop at the expense of the gall-causers, suggests that these species are more prone to acquiring *Wolbachia* infections, and this is likely to occur via horizontal transfer (see Section 4.4.5).

4.4.2 Diversity of infection

The number of *Wolbachia* variants identified at each sampling site showed a clear association with the presence of greater numbers of infected species. Specimens collected from locations H, L and S showed the greatest diversity in infection (Table 4.7). Considering each gall community separately, the greatest diversity of infection was seen in *B. pallida* galls (Section 4.3.3; Fig. 4.5). This is most likely due to the multilocular nature of *B. pallida* galls, which provide multiple host larvae for attack by multiple inquiline and parasitoid wasps, and as shown in Tables 4.1 and 4.8, *B. pallida* galls host a greater number of inquiline and parasitoid species compared with *A. curvator*, *A. quadralineatus*, *N. numismalis* and *N. quercusbaccarum* galls.

A greater diversity of infection was detected in the four inquiline species (five variants) and nine parasitoid species (four variants) examined in the present study, compared with the five gall wasp species (one variant). Most of the *Wolbachia* positive species were infected by only one variant across all sampling sites. However, three *Wolbachia* variants were found in the inquiline *S. gallaepomiformis*, occurring as three single and a double infection (Fig. 4.5). A double infection was also found in the parasitoid *T. geranii*.

Multiple infections occur in numerous species (Baudry *et al.*, 2003; Breeuwer *et al.*, 1992; Dobson *et al.*, 2004; Ijichi *et al.*, 2002; Jamnongluk *et al.*, 2002; Kikuchi & Fukatsu, 2003; Kondo *et al.*, 2002; Malloch *et al.*, 2000; Merçot *et al.*, 1995; Mitsuhashi *et al.*, 2002; Nirgianaki *et al.*, 2003; Reuter & Keller, 2003; Riegler & Stauffer, 2002; Rokas *et al.*, 2002a; Rousset & Solignac, 1995; Sinkins *et al.*, 1995; Vavre *et al.*, 1999a; van Borm *et al.*, 2003; Wenseleers *et al.*, 1998; Werren & Windsor, 2000), at varying frequencies, which suggests that multiple infections are more easily established or maintained in some species compared with others. Of the eight *Wolbachia* variants

TABLE 4.8 Parasitoid and inquiline wasp species associated with the the bisexual and sexual generation galls of the gall-causers screened in this study and four others, across Europe (Askew, 2002; Williams, 2004)

	Parasitoid species																																						
Gall-causer species	*Ceroptres cerri*	*C. clavicornis*	*Eurytoma brunniventris*	*E. pistacina*	*E. querceticola*	*E. spinipes*	*Sycophila biguttata*	*S. flavicollis*	*Sycophila variegata*	*Megastigmus almusiensis*	*M. dorsalis*	*Torymus affinis*	*T. auratus*	*T. flavipes*	*T. geranii*	*T. nobilis*	*T.notatus*	*T. roboris*	*Orymus nitidulus*	*O. pomaceus*	*Caenacis lauta*	*Cecidostiba fungosa*	*C. ilicina*	*C. semifascia*	*Hobbya stenonota*	*Mesopolobus albitarsis*	*M. amaenus*	*M. dubius*	*M. fasciiventris*	*M. fuscipes*	*M. mediterraneus*	*M. sericeus*	*M. tarsatus*	*M. tibialis*	*M. xanthocerus*	*Eupelmus annulatus*	*E. rostratus*	*E. urozonus*
Andricus curvator		*	*	*			*		*		*			*	*		*			*		*				*	*	*	*	*		*	*	*	*			*
A.quadralineatus		*	*		*		*							*						*	*						*		*			*		*	*			*
Biorhiza pallida			*	*		*	*		*		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		*	*	*		*	*		*	*	*	*	*
Neuroterus numismalis		*	*	*			*							*						*								*	*	*	*			*				*
N. quercusbaccarum	*		*	*			*		*			*	*	*	*		*			*							*	*	*	*	*	*		*	*	*		*
N. macropterus	*		*	*			*	*	*	*	*								*	*	*	*			*			*								*		*
A. solitaris		*	*	*			*		*		*									*					*	*			*	*	*		*	*	*	*		*
Plagiotrochus quercusilicis			*				*	*	*					*		*				*			*				*			*		*		*	*	*		*
Callirhytis glandium							*				*																												

TABLE 4.8 continued

Gall-causer species	Parasitoid species																																
	Aulogymnus arsames	*A. euedoreschus*	*A. gallarum*	*A. eudoreschus*	*A. skianeuros*	*Baryscapus anasillus*	*B. diaphantus*	*B. pallidae*	*Synergus albipes*	*S. apicalis*	*S. crassicornis*	*S. flavipes*	*S. incrassatus*	*S. gallaepomiformis*	*S. nervosus*	*S. pallicornis*	*S. rotundiventris*	*S. thaumacerus*	*S. ruficornis*	*S. umbraculus*	*S. variabilis*	*Hemiteles spp.*	*Aprostocetus aethiops*	*A. biorrhizae*	*Arthrolytus nanus*	*Macroneura vesicularis*	*Pediobias rotundatus*	*P. lysis*	*Saphanecrus connatus*	*Cirrospilus diallus*	*C. viticola*	*Closterocerus tridaciatus*	*Minotetrastichus ecus*
Andricus curvator	*		*		*				*		*		*	*	*	*	*		*	*			*			*			*				
A.quadralineatus	*	*	*	*					*					*	*							*											
Biorhiza pallida	*		*		*	*	*	*					*	*	*			*		*	*			*		*							
Neuroterus numismalis	*		*						*					*	*	*							*					*		*	*	*	*
N. quercusbaccarum	*		*						*	*	*			*	*	*		*					*		*	*	*	*	*				
N. macropterus												*					*				*												
A. solitaris			*						*					*	*																		
Plagiotrochus quercusilicis	*										*												*			*	*						
Callirhytis glandium																												*					

identified in the present study, seven belonged to clade-A and only one to clade-B; one AA double and one AB double infection were detected. Vavre *et al.* (1999a) also found a higher proportion of A-clade variants in parasitoid species associated with *D. simulans*, and a higher frequency of A-clade infections were detected in other Hymenoptera by West *et al.* (1998) and Werren & Windsor (2000). This may reflect a difference in the ability of A- and B-*Wolbachia* to infect and be maintained in different taxa (Werren & Windsor, 2000). A higher frequency of AB double infections than expected by chance has been detected in other surveys (Werren *et al.*, 1995a, Werren & Windsor, 2000), though AA / BB double infections and triple infections may have been overlooked in the past due to the use of clade-specific primers.

The *S. gallaepomiformis* double infection (variants II and XIV) was detected in only 1% of the samples tested. This could be explained if the infection had been acquired relatively recently and if the spread of infection was purely by stochastic processes. The possible phenotypic effects of *Wolbachia* in members of the oak gall wasp assemblage were discussed in more detail in Section 2.4, but if no phenotype was induced, and there was no fitness benefit associated with the double infection, the infection would spread only slowly due to chance (Mouton *et al.*, 2003).

The most common *S. gallaepomiformis* variant (XIV) was found at all sampling sites where this species was collected, and has also been detected in specimens from elsewhere in the UK and Europe (Sections 2.3 & 2.4; Rokas *et al.*, 2002a). At site H, all three single infection types were detected. Interestingly, the double infection was found at a different site to the single variant II infection, but this may be due to limited sample sizes at these sites. In species that carry more than one infection type, host populations in which several of these infection types occur together have been identified (Baudry *et al.*, 2003), though different infection types may be restricted to different host populations (Riegler & Stauffer, 2002). This has lead to the hypothesis that CI type *Wolbachia* infections could contribute to the reproductive isolation of host species (see Section 1.3).

4.4.3 Prevalence and geographic variation in *Wolbachia* infection in species from the oak gall wasp community

Every effort was made to obtain large numbers of specimens of each species but some galls, for example those induced by *A. quadralineatus* and *N. numismalis*, occurred at low frequencies and the rate of wasp emergence from their galls was also low. Therefore, some species were represented by relatively small sample numbers and as a

result the estimated frequency of *Wolbachia* infection is subject to some statistical error. For example, only single *A. quadralineatus* and *T. geranii* specimens were tested, leading to infection estimates of 0% and 100%, respectively, but it is highly likely that greater sample numbers would reveal infection frequencies that deviate from these estimates.

In species where larger sample numbers were surveyed, both infected and uninfected individuals were detected, suggesting that a *Wolbachia* infection is generally not fixed in any of the species from this community. Most natural populations exhibit some polymorphism in their infections, due to loss of infection resulting from 'curing' by natural antibiotics, high temperatures, host diapause or populations bottlenecks (Hoffmann *et al.*, 1998; Hurst *et al.*, 2000; Perrot-Minnot *et al.*, 1996; Section 1.3.1).

Geographic variation in *Wolbachia* infection has been described in the European cherry fruit-fly *Rhagoletis cerasi*, the European raspberry beetle *Byturus tomentosus*, the argentine ant species *Linepithema humile*, the butterfly *Hypolimnas bolina*, the Neotropical beetle *Chelymorpha alternans*, the rose gall wasp *Diplolepis spinosissimae* and the oak gall wasps *B. pallida* and *A. mukaigawae* (Abe & Muira, 2002; Dyson *et al.*, 2002; Keller *et al.*, 2004; Malloch *et al.*, 2002; Plantard *et al.*, 1998; Riegler & Stauffer, 2002; Rokas *et al.*, 2001; Shoemaker *et al.*, 2003; Tsusui *et al.*, 2003). Analysis of *ftsZ* and 16S rRNA gene marker sequences have shown variation in the presence or absence of *Wolbachia*, and in the proportion of infected individuals in different host populations. More recently, application of the *wsp* marker gene has allowed variation in the type of infection to be detected. For example, Keller *et al.* (2004) found that populations of the Neotropical beetle *Chelymorhpa alternans* varied in the presence of single or double *Wolbachia* infections, and determined that this was a result of loss of one strain in some populations due to differences in environmental conditions.

The sampling strategy adopted in this study aimed to allow detection of more localised variation in infection frequency and diversity. However, the number of gall and wasp specimens found at each site varied considerably between species and only *B. pallida* was present at all sampling sites. Little is known about the distance of dispersal or the feeding habits of the adult gall-causer, inquiline and parasitoid wasps of the species examined in this study. Both generations of *B. pallida* oviposit on *Q. robur* oaks (Section 1.8), and migrate from the roots of the tree (asexual generation), to the twigs where the sexual generation galls are induced (Askew, 1984). Consequently, *B. pallida* wasps may not disperse over large distances to locate a potential mate or food source, and it could be hypothesised that these relatively isolated wasp populations would be more likely to show

variation in *Wolbachia* prevalence. The inquiline and parasitoid species however, may disperse over greater distances in search of their hosts, allowing *Wolbachia* to spread between sites. The frequency of infection was not found to vary significantly between sites in any of the species examined in this study ($P > 0.05$, Fisher's Exact test). It is possible that the sampling sites in this study were too close together to be considered treated as independent populations.

Fourteen of the 19 species surveyed in this study showed either 100% or 0% infection at all sites where they were found. *Biorhiza pallida* showed 100% infection at most sites, uninfected individuals were found at sampling sites S and U (Section 4.3.2). These individuals may be an indication of a genuine infection polymorphism in *B. pallida* in this population, and could have resulted from inefficient maternal transmission during oogenesis (Section 1.3.1). Unlike galls induced by *A. curvator*, *A. quadralineatus*, *N. numismalis* and *N. quercusbaccarum*, galls induced by *B. pallida* contain many larvae, some of which may have come from different mothers (Atkinson *et al.*, 2003). Therefore infection polymorphism in a *B. pallida*-induced gall could be the result of infected and uninfected offspring produced from one mother or due to multiple founding by both infected and uninfected mothers.

A total of 7 uninfected *T. flavipes* individuals were identified out of 60 specimens collected at locations C, S and U. At all other locations, the infection prevalence was 100% but the sample size was much smaller (Table 4.4). A *T. flavipes* population polymorphic for *Wolbachia* infection (91%) has been identified in a previous study (Sections 2.3 & 2.4). *S. gallaepomiformis* populations were polymorphic for *Wolbachia* infection at most sampling sites and this species has also been found to be polymorphic at sites in Europe (Rokas *et al.*, 2002a).

Both infected and uninfected *T. flavipes* individuals were found occurring together in three *B. pallida*-induced galls. The same was true for the inquiline *S. gallaepomiformis*. In *B. pallida* galls, due to the large numbers of gall-causer larvae available, galls are likely to contain parasitoids / inquilines from several mothers that may or may not be infected (Askew, 1984: Stone *et al.*, 2002). In the other gall types, because the galls are small, fewer parasitoid / inquiline larvae can develop in each gall. The larvae compete for space and resources and it could be assumed that eggs laid earlier will develop ahead of, and out-compete larvae from eggs laid later. Therefore, it seems likely that in most instances all inquiline wasps that emerge from an *A. curvator*, *A. quadralineatus*, *N.*

numismalis or *N. quercusbaccarum*-induced gall were siblings. Only single parasitoid specimens emerged from these gall types (Section 4.3.1).

Where a parasitoid and gall-causer emerged together (Table 4.6) it is possible that an inquiline or another parasitoid wasp had been present originally, but was attacked by the second parasitoid. Emergence of an inquiline with the gall-causer could occur if the gall was large enough to accommodate both wasps and there was no competition for space and food, hence this was seen more commonly in *B. pallida* galls (Askew, 1984; Table 4.6).

These patterns of wasp emergence are significant when considering the possible routes of transfer of *Wolbachia* and the chances of recognising such transfers. *B. pallida* galls show a much higher rate of gall-causer plus parasitoid / inquiline emergence (Table 4.6). There were also two instances where a parasitoid and an inquiline emerged from the same gall and one instance where the gall-causer, a parasitoid species and an inquiline species all emerged from the same gall. This may indicate that *B. pallida* galls provide a more suitable environment for the interspecific transfer of *Wolbachia*.

4.4.4 Horizontal transfer

Although *Wolbachia* are maternally (vertically) transmitted, the lack of concordance between the *Wolbachia* strains and the insect hosts strongly suggests that horizontal transfers between unrelated hosts has taken place at high frequency in the past (Rokas *et al.*, 2002a; Werren *et al.*, 1995b; Werren & Windsor, 2000).

There are at least four possible mechanisms by which horizontal transfer could occur between members of the oak gall wasp community. (a) Tissue damage resulting from conflict between an inquiline and its host during competition for space (Stone *et al.*, 2002), or damage caused as the cuticle is pierced by the ovipositor of the egg laying parasitoid could allow *Wolbachia* to be transferred between the insect haemolymph (Rigaud & Jachault, 1995). (b) An infection could be transmitted on the ovipositor of a parasitoid as it probes infected and uninfected hosts. (c) Transfer of an infection could occur between two parasitoids during super-parasitism of a single host that may not itself become infected (Schilthuizen & Stouthamer, 1997). (d) Horizontal transfer could occur through ingestion of infected food (Huigens *et al.*, 2000; Mitsuhashi *et al.*, 2002; Niebylski *et al.*, 1999), therefore parasitoids could become infected through ingestion of infected gall-causer, inquiline or parasitoid larvae, and gall-causer or inquiline species

could acquire an infection by consuming infected gall tissue (Mitsuhashi *et al.*, 2002), which could become infected during wasp conflict or oviposition as described above.

It is questionable as to whether *Wolbachia* could survive passage on the ovipositor of a parasitoid or through the hosts digestive system, but it is already known that *Wolbachia* have adapted to life in a diverse range of hosts and so may represent a stage in bacterial evolution that is intermediate between a mutualistic nutritional endosymbiont and an extracellular pathogen. *Wolbachia* may therefore have maintained the ability to survive inside the insect gut possessed by many of its free-living relatives. *Wolbachia* have been found in the intestines of some host species (Mitsuhashi *et al.*, 2002; Oh *et al.*, 2000) and has the ability to mediate its own entry into host cells (Noda *et al.*, 2002). *Wolbachia* have not been cultivated in cell-free media but comparative analysis of the wMel genome sequence with that of the tsetse fly symbiont *Sodalis glossinidius*, which has been cultivated in cell-free media, may provide information about the potential for extracellular survival (Akman *et al.*, 2001; Dale & Maudlin, 1999; Wu *et al.*, 2004; Section 5.0).

Evidence of potential *Wolbachia*-host co-evolution and of horizontal transmission was found in this study: co-evolution was indicated by the occurrence of similar *Wolbachia* variants in closely related species. For example, three *Mesopolobus* species were infected with variant XVII, and two variants from the wMel subgroup (variants XIV & XV; Figs. 4.4 & 4.5) were found to infect three *Synergus* species. However, as these species are associated with the same gall communities, the possibility that horizontal transfer is the cause of the similarity in infection could not be ruled out.

The variant from *B. pallida* was very similar to the variants detected in two other gall-causing species, *N. macropterus* and *A. solitarius*, and the inquiline species *S. crassicornis* by Rokas *et al.* (2002a). This could therefore represent an ancestral infection that has been maintained in these species since before the divergence of the Cynipini and Synergini and / or have been acquired by *S. crassicornis* by horizontal transfer.

Horizontal transmission was indicated by the variety of *Wolbachia* variants identified in the parasitoid and inquiline species, including four *Wolbachia* variants from different subgroups in the inquiline *S. gallaepomiformis*, and also by the identification of two double infections in the community as a whole. The double infection detected in the parasitoid *T. geranii* consisted of both A- and B-clade *Wolbachia* (variants I and II from the wCon and wUni subgroups; Figs. 4.4 & 4.5). The AA double infection from *S. gallaepomiformis* also included variant II coupled with variant XIV (wMel subgroup;

Figs. 4.4 & 4.5). The fact that variants II and XIV were also found as single infections, suggests that the double infection in *S. gallaepomiformis* could have arisen from horizontal transfer of one variant into an individual singly infected with the other variant. As variant II is also part of the double infection in *T. geranii*, this variant may be able to become established in different host species following horizontal transfer more easily than other *Wolbachia* variants.

Evidence of horizontal transmission in the oak gall wasp community was also found by Rokas *et al.* (2002a). Inquiline species were found to be infected with variants from three different subgroups (wHaw, wMors and wMel) and a triple infection was detected in the gall-causer *A. solitarius*. Two possible horizontal transmission events were indicated: the variant from *A. solitarius* (variant 3) was identical to that found in *S. umbraculus*, *S. diaphanus* and *S. reinhardi*; and the variant found in the inquiline *S. crassicornis* was identical to those found in *A. solitarius* (variant 1) and *N. macropterus*. Direct horizontal transfer from the gall-causer to the inquiline or vice versa was ruled out however, because none of the inquilines were known to be associated with the gall-causer communities in question. Common parasitoids were suggested as a possible vector of infection (Rokas *et al.*, 2002a).

In the present study, a high rate and diversity of infection was found in the parasitoid species tested, therefore they are a likely route through which *Wolbachia* could be horizontally transmitted. Torymid parasitoids can be phytophagous, entomophagous, or both, and may attack other *Torymus*, *Mesopolobus*, *Eurytoma* or *Aulogymnus* parasitoid species, as well as inquiline and cynipid species (Askew, 1984). Several inquiline and parasitoids were found to carry the same *Wolbachia* variant and to be associated with more than one gall-causer, presenting several potential horizontal transfer events. For example, *S. gallaepomiformis* and *T. flavipes* were associated with *A. curvator, B. pallida* and *N. quercusbaccarum* galls (Fig. 4.5). *T. auratus* can develop as an inquiline, consuming gall tissue (Askew, 1961; 1965), which may explain its uninfected status even though two other torymid species were positive for *Wolbachia*.

Horizontal transmission of symbionts is thought to be more likely to occur between more closely related host species that provide similar physiological backgrounds (Huigens *et al.*, 2004; Russell *et al.*, 2003). Thus transfer could occur more frequently between the hymenopteran species in the oak gall wasps communities than has been seen in other host-parasitoid systems, in which the host and parasitoid belong to different insect orders. However, similar *Wolbachia* variants have been identified in fly species

such as *Protocalliphora* and *Drosophila*, and their respective parasitoids *Nasonia giraulti* and *Asobara tabida* (Vavre *et al.*, 1999a; Werren *et al.*, 1995b), and between the moth *Ephestia kuehneilla* and its *Trichogramma* spp. parasitoids (van Meer *et al.*, 1999).

In contrast, in the present study there was no similarity between the variant in the only infected gall-causer, *B. pallida* and any of the parasitoid or inquiline species tested. It may be that horizontal transmission is more likely to occur from a host to a parasitoid, rather than from the parasitoid to the host, because the attacked wasp does not normally survive the association (Csóka *et al.*, 2004; Jervis *et al.*, 2004; Stone *et al.*, 2002). Gall-causers may occasionally survive an attack by a parasitoid, by suppressing the parasitoids development (Jervis *et al.*, 2004), and this could lead to transfer of *Wolbachia* from the parasitoid to the gall-causer. As shown by Table 4.6, there were instances where parasitoid and gall-causers both emerged from *B. pallida* galls but this is most likely to be due to the large numbers of *B. pallida* larvae in the gall, some of which will be parasitised, some of which will not.

Based on the sequence evidence, horizontal transfer may have occurred between the inquiline and parasitoid species and may not have involved the gall-causer. This could take place if the parasitoid developed alongside an inquiline, following attack of the gall-causer, or if the parasitoid attacked the inquiline larva. Therefore transfer of *Wolbachia* could have occurred during attack of the inquiline *S. gallaepomiformis*, by the parasitoids *T. geranii* (variant II) and *T. flavipes* (variant III).

However, even though transfer may seem more likely to occur as a result of attack of an inquiline by a parasitoid (i.e. from inquiline to parasitoid), in this case the evidence suggested that transfer occurred in the opposite direction, from parasitoid to inquiline. The variant II and variant III infections in *S. gallaepomiformis* were detected in very few samples, whereas the variant III occurred in 53 *T. flavipes* specimens. Variant II was found in only one *T. geranii* specimen in this survey but had been identified in another four specimens from the UK and Europe in a previous study, at an infection frequency of 67% (Section 2.3). These infections therefore seem to be more common in the parasitoids and the rare occurrences in the inquiline are probably the result of relatively recent acquisition from the parasitoids via horizontal transfer. Interestingly, the Japanese oak gall wasp *A. mukaigawae* is known to also carry variant II (Abe & Muira, 2002).

The greatest variety of infections was found in the community associated with *B. pallida* galls, and this is a strong indication that horizontal transmission occurred more

frequently in these galls due to the potential for large number of species to inhabit simultaneously the same gall environment (Fig. 4.5; Table 4.7). This could explain why *B. pallida* was the only gall-causer species that was infected; the other gall-causers may remain uninfected because there are fewer opportunities for horizontal transfer in these galls. Alternatively the wasps themselves could be less susceptible to horizontal transfer for some reason and / or they may simply be less suitable hosts for *Wolbachia*. Other host species such as *Anopheles* mosquitoes have been shown to be uninfected even though closely related species carry *Wolbachia* (Sinkins, 2004).

Fourteen species of parasitoid and inquiline species associated with the five gall wasp species were surveyed during the present study, but it is important to consider other species which were not found at the sampling sites in South Wales and are known to be associated with sexual and asexual generation *A. curvator*, *A. quadralineatus*, *B. pallida*, *N. numismalis* and *N. quercusbaccarum* galls elsewhere in the UK and Europe. As shown in Table 4.1, the species tested in this survey represent only a small proportion of the species that can associate with these gall-causers in the UK, and when the whole of Europe is considered (Table 4.8), the number of associated species increases further. This could explain why the *B. pallida* infection was shared with the other species tested in the present study, even though the evidence suggested that horizontal transmission occurred in these galls.

In addition, several of the parasitoid or inquiline species tested are known to attack four or five of the gall wasp species tested, even though they may not have been found in all of these galls in the present study e.g. *T. flavipes*. Therefore there is huge potential overlap between the foodwebs of each gall wasp species (Tables 4.1 and 4.8) In Table 4.8, 4 other gall-causing species which were found to be infected with *Wolbachia* by Rokas *et al.* (2002a) were included to show other gall-causer members of the community that could contribute to the infection patterns detected. Of these, only *A. solitarius* is known to occur in the UK but all are worth considering because species distributions change over time, and species that were once restricted to mainland Europe may now be found in the UK (Askew, 1984; Stone *et al.*, 2002).

4.4.5 Denaturing gradient gel electrophoresis (DGGE): a useful alternative to cloning and sequencing for detecting *Wolbachia* diversity in a field study

In Chapter 2, 85 samples from the *B. pallida* community were surveyed. A minimum of 68 sequence reactions and several cloning reactions were required to

determine the incidence and prevalence of each infection type from 34 *Wolbachia* infected samples. In this Chapter, 593 samples from 19 species of wasp, from five different oak gall wasp communities were screened for the presence of *Wolbachia* and the infection types compared using DGGE. Only 16 sequencing reactions (forward and reverse reads) were required to establish the similarity in nucleotide sequence of the *wsp* variants from the entire community due to the use of DGGE. As this was the first time that DGGE had been applied to this system, several more reactions were carried out in order to be completely sure the results were accurate.

In several studies in the literature, frequency of infection was determined using PCR screening of large numbers of insect specimens but the diversity of the infection was established by analysing the nucleotide sequence of very few specimens (Jeyaprakash & Hoy, 2000; Kikuchi & Fukatsu *et al.*, 2003; O'Neill *et al.*, 1992; Rokas *et al.*, 2002a; Wenseleers *et al.*, 1998; Werren *et al.*, 1995a; Werren & Windsor, 2000). As some of the sequence variants may occur at very low frequencies (Section 4.4.3), insufficient sample numbers would result in *Wolbachia* variants remaining undetected. The double infection in *S. gallaepomiformis* for example, occurred in only a single specimen, a frequency of 1.4%. Seventy-four specimens had to be examined in order to detect one infected with the double infection, yet this provided strong evidence that horizontal transmission has occurred, which would be essential information if use of *Wolbachia* as a biocontrol system for this host species was in consideration (Section 1.4).

Variability in conditions during gel preparation often resulted in variation between the gel profiles from day to day. Marker DGGE variants aided comparison between gels, but as the number of variants increased it became more difficult to judge the identity of closely migrating variants. It is likely that the magnitude of this problem will increase as greater numbers of variants are examined in future studies, therefore computational software should be employed to ensure accuracy in judging relative migration distances through the denaturant gradient.

Although DGGE allowed the nucleotide sequence similarity of 40 samples to be compared within an eight hour period, this is still limited to the lower end of the scale of molecular typing methods and future studies will require even greater throughput to achieve truly meaningful results. Automation of techniques such as SSCP (single stranded conformation polymorphism) could provide the answer (Table 1.4). PLACE-SSCP (Post Labeling Automated Capillary Electrophoresis-SSCP) is an advancement on traditional SSCP in which fluorescently labeled single stranded conformers are separated on

automated sequencing gels or capillaries. This results in greater resolution and automation but currently is limited to gene fragments of ~200 bp (Hayashi *et al.*, 1999). T-RFLP is another sensitive automated technique and is based on restriction digestion of marker gene sequences. This technique is only likely to be useful for discriminating between broad groups of *Wolbachia* variants, because large numbers of restriction enzymes would be required to recognize all sequence polymorphisms, resulting in extremely complex outputs. Heteroduplex analysis has already been applied to discriminate between three *wsp* fragments (300 bp) by Rousset *et al.* (1999), but the mutation detection rate is reported to fall to 80% over larger fragments (Nollau *et al.*, 1997). As with DGGE however, careful optimisation may allow this technique to be applied more widely in this research field.

The specificity and range of the general *wsp* primers has previously been called into question (Dr J.M. Cook, personal communication; Mitsuhashi *et al.*, 2002; Tsusui *et al.*, 2003) and during this study a putative retrotransposase gene was amplified and sequenced using these primers (Section 4.3.5). This raises questions about other studies in which sequence information was not used to confirm the identity of the PCR product, and highlights the need to confirm the identity of each novel DGGE variant using DNA sequencing, and to carry out random checks to ensure all results are genuine.

4.4.6 Wasp samples from the oak gall wasp community

In Chapter 2, the D2 variable region of the 28S rDNA gene was used as a marker for wasp species identification, clearly distinguishing the cynipids (gall-causers and inquilines) and chalcids (parasitoids) from each other, but the sequence diversity was not sufficient for confident species-level identification (Section 2.4.5). Cytochrome *b* and cytochrome oxidase subunits are faster-evolving loci that have been found to be useful for recovering inter- and intrageneric, and intra-specific phylogenies (Rokas *et al.*, 2002c; Stone & Cook, 1998).

Comparison of cytochrome *b* nucleotide sequences allowed the parasitoid species to be separated with confidence and also confirmed the identity of the *S. gallaepomiformis* specimens (Fig. 4.2). However, this gene was not able to distinguish between two other inquiline species, *S. nervosus* and *S. albipes*. These species are also very similar morphologically and therefore species designations for these wasps were given tentatively.

The use of mitochondrial genes for the reconstruction of phylogenies of *Wolbachia* infected hosts has been called into question due to the possibility that *Wolbachia* can induce cytoplasmic sweeps that result in reduced diversity in the mitochondrial genome of host species populations (Jiggins, 2003; Rokas *et al.*, 2001; Hoffmann & Turelli, 1997; Section 1.3). This could cause different species to be grouped as one, based on similarity of their mitochondrial marker sequences. For this reason, nuclear gene markers should be used in conjunction with mitochondrial markers. It is not known whether *Wolbachia* induce a reproductive phenotype, and as a result cause a cytoplasmic sweep in the species used in this investigation. The possibility of a *Wolbachia*-induced mitochondrial sweep has been tested by Rokas *et al.* (2001) in the gall-causing species *B. pallida* and it was concluded that *Wolbachia* was not responsible for the reduced mitochondrial diversity detected in populations of this species.

This study attempted only to confirm that specimens identified as different from each other using morphological keys are truly different species, and did not attempt to infer the degree of phylogenetic relatedness between the wasp species using this gene.

4.4.7 Concluding remarks

Through the use of DGGE, close to 600 samples from the oak gall wasp community were screened for the presence or absence of *Wolbachia* infection and the strength of evidence of horizontal transfer was determined, with greatly reduced need for sequencing and cloning. Wasp species from five different oak gall wasp communities were screened to allow all potential routes of transfer to be examined. Unlike some previous studies, the maximum available number of samples was screened for each species, and the nucleotide sequence of every infected individual was analysed using DGGE, ensuring that all sequence variants were detected. This revealed rarer infection types that would otherwise have been missed, and provided evidence of horizontal transmission in the wasp community.

5.0 Discussion

Wolbachia are obligate endosymbionts of arthropods and filarial nematodes, and display a range of effects on their hosts including mutualistic association, reproductive parasitism, lethality and fecundity enhancement. Through sampling of relatively small numbers of individuals from many species, researchers have established that *Wolbachia* are extremely common in arthropods throughout the world. In several of the infected species the reproductive phenotype induced has been characterised, and population and cytological studies have gradually unravelled the mechanism of manipulation.

The dynamics of infection in arthropods has been modelled extensively for cytoplasmic incompatibility-inducing strains (CI), and the potential for application of CI-*Wolbachia* for the control of agricultural pest species and disruption of vector borne disease is currently being explored (Section 1.4). Measurements in the field have shown that infection dynamics can be significantly affected by factors such as paternal transmission, antibiotic curing, host diapause and elevated temperatures (Hoffmann *et al.*, 1998; Hurst *et al.*, 2000; Perrot-Minnot *et al.*, 1996; Section 1.3.1). Horizontal transmission between closely related and distantly related host species is believed to have occurred frequently in the past (Rokas *et al.*, 2002a; Werren *et al.*, 1995b; Werren & Windsor, 2000) and can lead to multiple infections and complex incompatibility patterns (Hoffmann & Turelli, 1997).

In this study, a novel approach based on DGGE was developed for visually comparing ~600 bp PCR-amplified *wsp* fragments to investigate the diversity of *Wolbachia* variants in infected individuals. The DGGE technique was found to be sensitive, reproducible, and relatively quick and inexpensive compared with the standard cloning and sequencing screening approach.

By using the standard approach, six different *wsp* sequence variants were identified in 34 infected wasp specimens, using 68 sequencing and several cloning reactions (Chapter 2). The use of DGGE allowed *wsp* sequence variants from 256 infected wasps from five oak gall wasp communities to be compared, without the need for extensive sequencing and cloning reactions. Only 16 sequencing reactions were required to characterise the 8 sequence variants found, including both A and B-clade sequence-variants (Chapter 4). Furthermore, the technique successfully identified double and triple infections that could be characterised directly from the polyacrylamide gel, reducing the need for cloning. Thus, cloning reactions were only required to produce stocks of variants

for use as markers in future experiments. DGGE therefore resulted in a significant reduction in the labour required for host screening, and it is envisioned that this technique will facilitate the screening of larger numbers of infected host specimens than have been tested in many studies in the past.

Recently, the importance of including large sample numbers when characterising *Wolbachia* infection of an insect population has been recognised (Kittayapong *et al.*, 2002; Kondo *et al.*, 2002; Rokas *et al.*, 2002a; Shoemaker *et al.*, 2003). In the past, estimates of infection incidence were based on screens in which each species was represented by very few (1-3) individuals (Kikuchi & Fukatsu *et al.*, 2003; O'Neill *et al.*, 1992; Rokas *et al.*, 2001; 2002a; Wenseleers *et al.*, 1998; Werren *et al.*, 1995a). While these small sample sizes may resulted in the identification of several *Wolbachia* variants in several host species, evidence of geographic variation in the presence of infection, the distribution of multiple infections, and rare infection types are likely to have been missed.

If *Wolbachia* are to be successfully employed as a method of biocontrol for agricultural pest and disease spreading insect species, greater understanding of their infection dynamics, the occurrence of natural infections and their mode of transmission must be attained through thorough population screening. Cytoplasmic incompatibility-inducing *Wolbachia* variants may be useful as a means of population suppression in pest species (Section 1.4). However, any undetected *Wolbachia* variants in the native host population could interact with and alter the dynamics of the introduced population. A further significant potential issue is the possibility that *Wolbachia* variants could be horizontally transmitted to other insect species associated with the native host population, introducing strains that could interfere with the dynamics of the invading population, and providing the potential for transgenic *Wolbachia*-variants or other cytoplasmic elements to be transmitted to other insect populations or species.

The present study showed that when larger numbers of samples were tested, rare infection types were revealed (Chapter 4), infections that could have a significant impact on the success or failure of a biocontrol programme if they were overlooked due to insufficient sampling. Furthermore, the diversity of infections revealed suggested that horizontal transfer of *Wolbachia* may have occurred between the inquiline and parasitoid wasp species (Section 4.4.4). This is in agreement with the hypothesis that there is greater potential for horizontal transmission of *Wolbachia* between intimately associated host species, and highlights the value of studying whole feeding communities.

The results of this study may reflect a difference in the suitability for infection by certain *Wolbachia* variants of the different host species tested: the difference in the life cycles of the gall-causer, inquiline and parasitoid wasp species may prevent variants adapting to the new host environment subsequent to horizontal transfer, and this could be the reason why no evidence of transfer of an infection to or from the gall-causer species was detected. These results may, however also reflect the fact that *A. curvator*, *A. quadrilineatus*, *B. pallida*, *N. numismalis*, *N. quercusbaccarum*-induced galls are associated with many more wasp species than those that emerged from galls from this study (Table 4.9), suggesting that direct routes of transfer may only be elucidated through testing of complete foodwebs.

As more information about *Wolbachia* genetics is gained through genomic sequencing and phylogenetic studies, it is becoming evident that the current typing system of *Wolbachia* variants is inadequate (see Chapter 1). How many different species / strains there are in the *Wolbachia* genus remains a primary unknown.

These is no clear definition of a bacterial species, but similar strains are grouped if they share a high degree of similarity in several independent features including genomic, phenotypic and phylogenetic properties (Oren, 2004; Rossello-Mora & Amann, 2001; Stackebrandt, 2003; Wertz *et al.*, 2003). Genomic properties include DNA base ratio (G + C%) and DNA-DNA reassociation values, and genomic sequences such as 16S rRNA gene sequences have been used widely to estimate phylogeny (Coenye & Vandamme, 2004; Dykhuizen & Green, 1991; Oren, 2004; Stackebrandt, 2003; van Belkum *et al.*, 2001).

Species / strains have been typed based on microbial genotype or DNA sequence comparisons (genomospecies) using techniques such as those discussed in Section 1.7 and others such as whole chromosome PFGE (pulsed field gel electrophoresis) and amplification of repetitive elements such as REP (repetitive extragenic palindromic), BOX, ERIC (enterobacterial repetitive intergenic consensus) and IS (insertion sequence) elements (Beuzon *et al.*, 2004; Olive & Bean, 1999; Stackebrandt, 2003; van Belkum *et al.*, 1998; Versalovic *et al.*, 1991).

Phylogenies based on single gene sequences can be confused by the transfer of genes laterally between species, resulting in organisms from different species sharing identical gene sequences, and closely related organisms carrying different gene sequences. A relatively new approach to bacterial taxonomy involves sequence analysis of several core, or housekeeping genes, that encode universal metabolic functions.

Housekeeping genes are under stabilising selection and evolve more slowly than auxiliary genes that are under positive selection. They are less likely to be transferred laterally than positively selected genes and are generally present as single copies. They therefore they provide a more reliable indication of genetic relationships (Santos & Ochman, 2004; Urwin & Maiden, 2003; Wertz *et al.*, 2003).

These genes however, will still be of limited use individually because of the slow rate of sequence evolution, and because the possibility of differences in selective pressures and lateral gene transfer events cannot be ruled out. The value of these genes is in combining the sequence data from several different housekeeping genes. The data produced can be analysed in terms of allelic differences, each sequence variant being assigned a unique allele number regardless of the amount of sequence difference; or the degree of sequence similarity at each locus can be taken into account to allow phylogenetic inferences to be made.

It has been proposed that a similar approach should be employed for strain typing in future studies of *Wolbachia* (Boutzis, 2003). Comparison of the recently published genome sequence of the wMel *Wolbachia* strain (Wu *et al.*, 2004; Fig. 1.2) with the data from other current genome sequencing projects (Foster *et al.*, 2004), should generate several suitable marker genes. A collaborative project is currently underway in which sequence information will be obtained for the following genes: *gltA*, *dnaA*, *groEL*, *aspAT*, *gyrB*, *wsp*, *ftsZ* and 16S rRNA (Fig. 1.2) from several representative *Wolbachia* strains from each subgroup (Bourtzis, 2003). In addition, the information generated by MLST can be supplemented with data from faster evolving genes to increase phylogenetic resolution (Urwin & Maiden, 2003). The vast amount of information for the *wsp* gene already available could therefore be included.

The wMel genome sequence has already revealed at least two potential strain markers that could enhance strain typing by MLST. The wMel genome contains a uniquely high proportion of repetitive DNA and mobile genetic elements, much higher than other intracellular species such as *Buchnera*, *Rickettsia*, *Chlamydia* and *Wigglesworthia*, all of which have undergone substantial genome reduction (Wu *et al.*, 2004; Section 1.2.1; Table 1.2). The copy number of some of the identified repeat elements varies between *Wolbachia* strains, and therefore might be useful for strain typing. In addition, two divergent paralogs of the *wsp* gene that cannot be amplified using the *wsp* general primers (Braig *et al.*, 1998) have been identified in the wMel genome.

The *wspB* gene appears to be evolving at a faster rate than *wsp* and therefore might also be useful as a strain marker.

With proper optimisation DGGE could be used to separate the gene fragments from each of the genes employed during MLST, in a similar way to the original technique of multi-locus enzyme electrophoresis, in which the enzymes encoded by several genes were separated on the basis of their electrophoretic mobility. Using DGGE to pre-screen the MLST amplimers would reduce the number of sequencing reactions required. The disadvantage of using DGGE is that each gene locus would almost certainly require its own set of DGGE conditions and amalgamation of the gene DGGE profiles into a single MLST profile could be complex. However, although automated DNA sequencing is becoming less expensive, DGGE might still be useful as a relatively inexpensive pre-screening system.

Population screening and strain typing using MLST may eventually help to establish exactly how many strains of *Wolbachia* exist. This will allow a more reliable phylogeny to be reconstructed and could provide information about the evolution of the reproductive manipulations induced by different strains. In combination with genome sequence analysis it may eventually be possible to identify the genes involved. The genome sequence has already revealed a large number of genes that encode proteins that contain ankyrin repeats. These may be involved in regulating host cell-cycle or cell division, or interacting with the host cytoskeleton, processes that are altered during expression of cytoplasmic incompatibility or parthenogenesis induction (Bourtzis *et al.*, 2003; Huigens & Stouthamer, 2003; Veneti *et al.*, 2004; Wu *et al.*, 2004; Section 1.3).

Cultivation of *Wolbachia* in cell-free media has not been possible due to the obligate nature of the relationship with its host. However, the secondary symbiont of *Glossina morsitans*, *Sodalis glossinidius*, has been cultured on agar media (Dale & Maudlin, 1999), and sequencing of its genome is near completion (Aksoy *et al.*, 2005). Therefore comparative genome analysis with this endosymbiont, and other cultivated / uncultivated intracellular bacteria, could help to explain why *Wolbachia* remains uncultivated, and may allow a cell-free culture system to be designed. For example, the genome sequence of wMel shows that arthropod-associated *Wolbachia* have lost several classes of genes as a result of reductive evolution, including genes involved in cell envelope biogenesis (Wu *et al.*, 2004). These genes have also been lost by *Buchnera* (Moran & Mira, 2001; Shigenobu *et al.*, 2000), and *Anaplasma* and *Ehrlichia* species (Lin & Rikihisa, 2003) that are also uncultivated in cell-free media. In addition, like many

obligate endosymbionts *Wolbachia* has a limited set of regulatory systems (Wilcox *et al.*, 2003; Wu *et al.*, 2004) and it may therefore never be possible to cultivate these bacteria outside of the stable intracellular environment.

The inability to cultivate *Wolbachia in vitro* could impact negatively on the organism's potential for use in biotechnology (Section 1.4). On the other hand, with the growing range of cell culture lines in which *Wolbachia* can be maintained, it may not be necessary to culture the endosymbiont *in vitro* to study the interactions with the host. The ability to culture *Wolbachia* in insect and mammalian cell lines has provided information about the organism's host cell range and this could ensure a supply of inocula for microinjection into other host species, for example pest species (Dobson *et al.*, 2002; Fenollar *et al.*, 2003; O'Neill *et al.*, 1997; Noda *et al.*, 2002). DNA micro-array technology may facilitate examination of changes in host and symbiont gene expression associated with host cell invasion, or differences between strains inducing different phenotypes (Hinton *et al.*, 2004). Cell lines also provide the potential to create novel multiple infections to monitor the occurrence of recombination events.

As molecular methods provide more information about uncultivated prokaryotic groups, it is becoming evident that innumerable symbiotic interactions exist between prokaryotes and eukaryotes. The complete genome sequence of the *Wolbachia* (wMel) genome has provided new insights into the possible mechanisms of the endosymbionts interaction with its hosts, and in the future, genomic and proteomic technologies will shed even more light on the mechanisms and evolution of symbiotic interactions.

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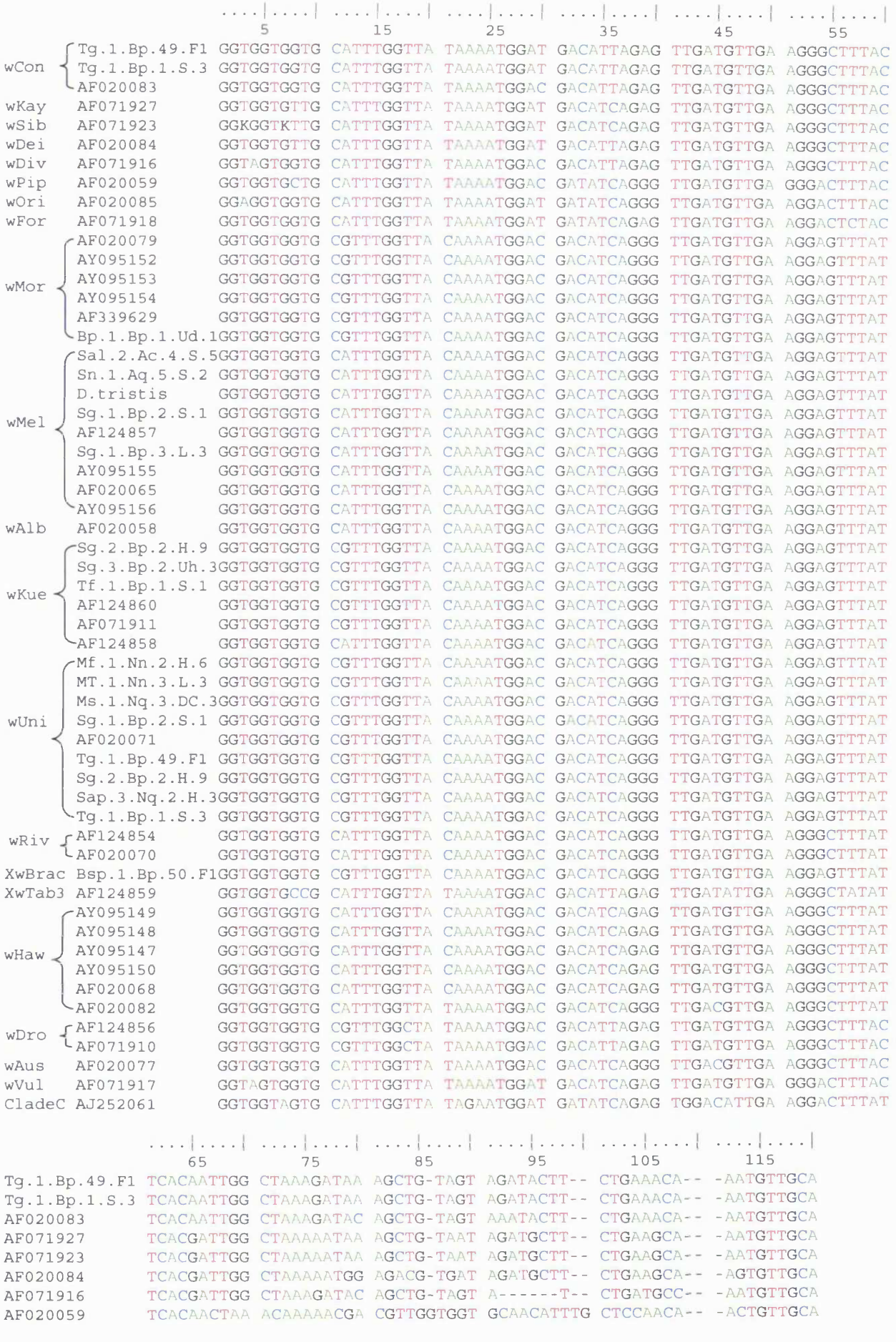
Appendix

Fig A1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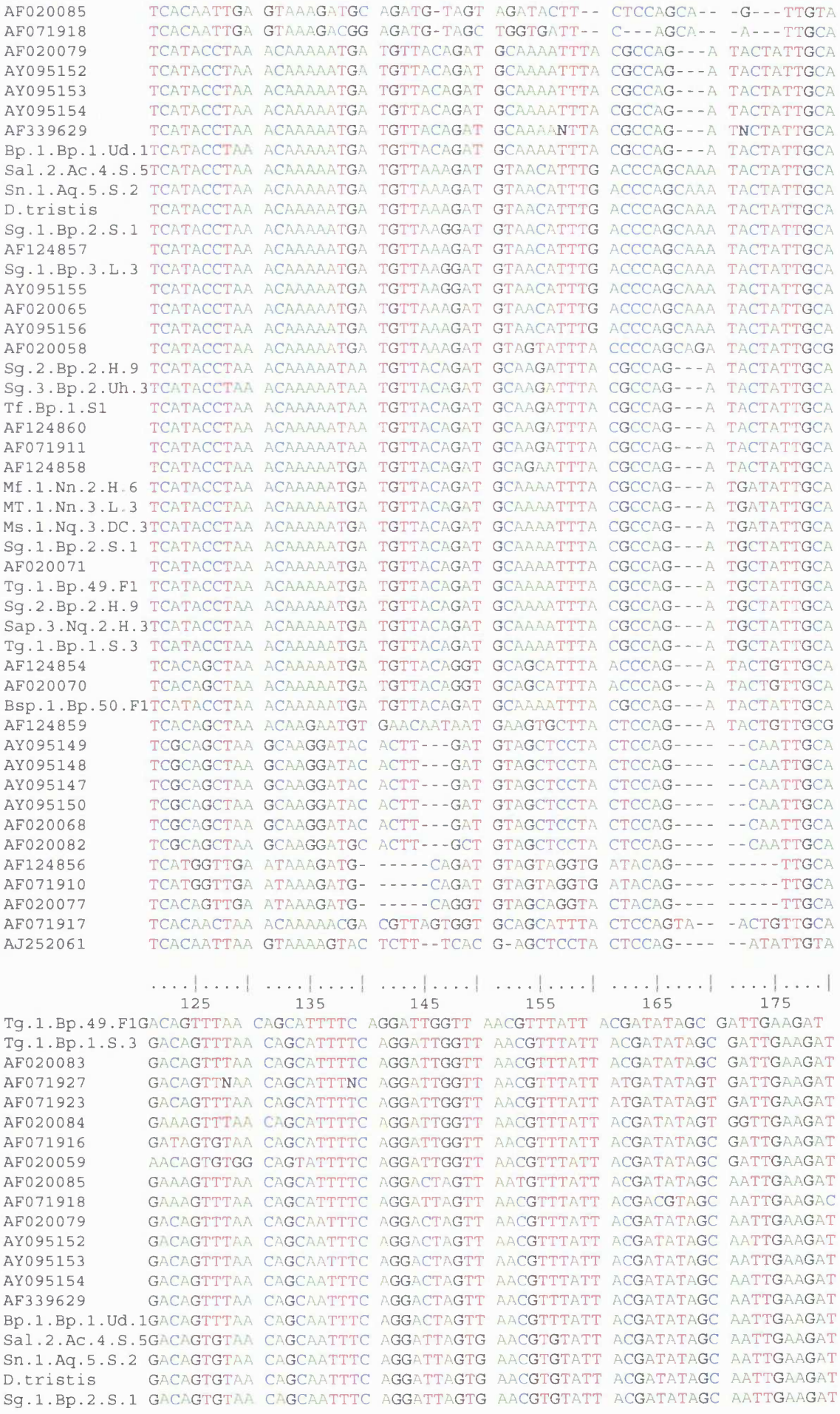
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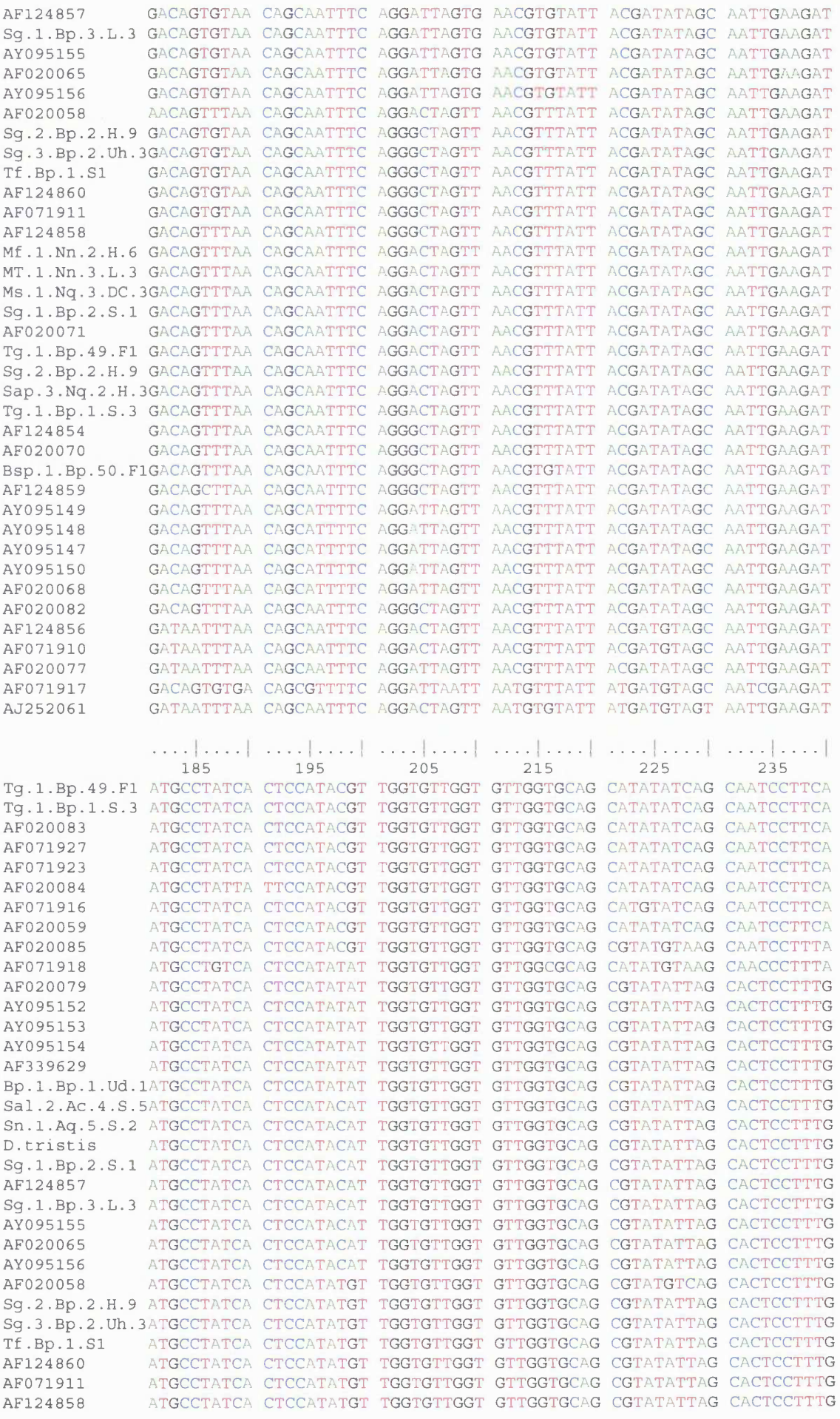
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Fig A1

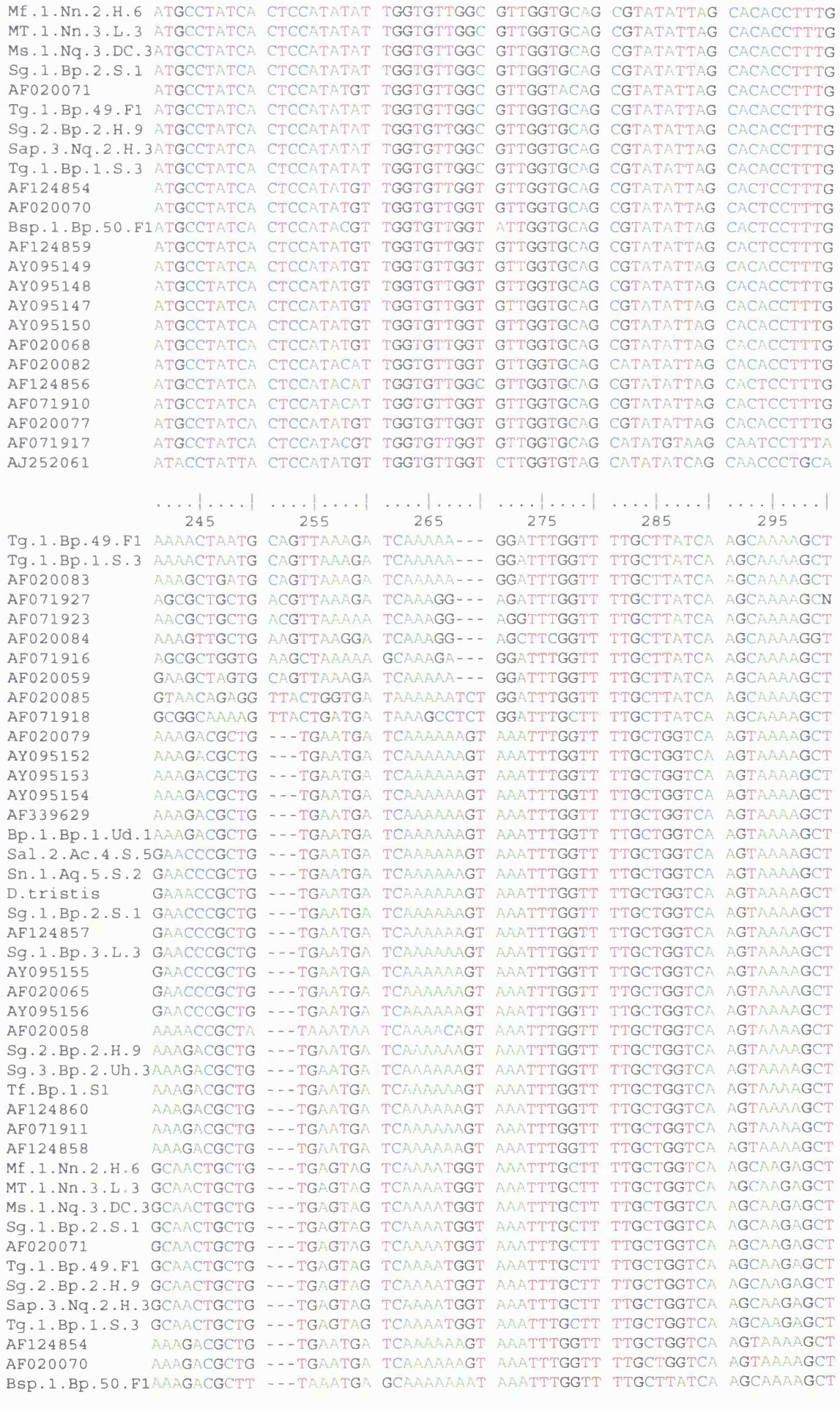
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Fig A1

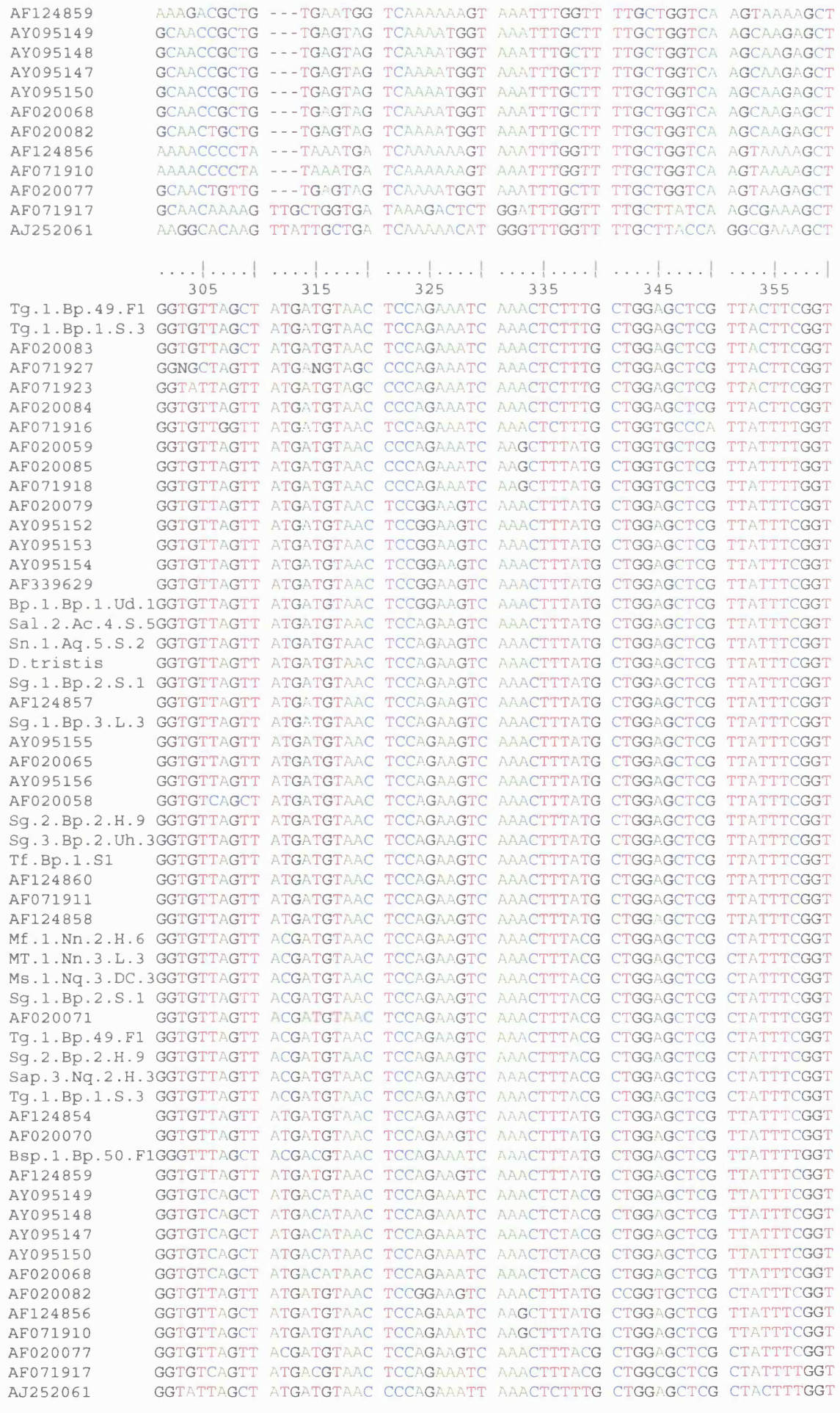
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Fig A1

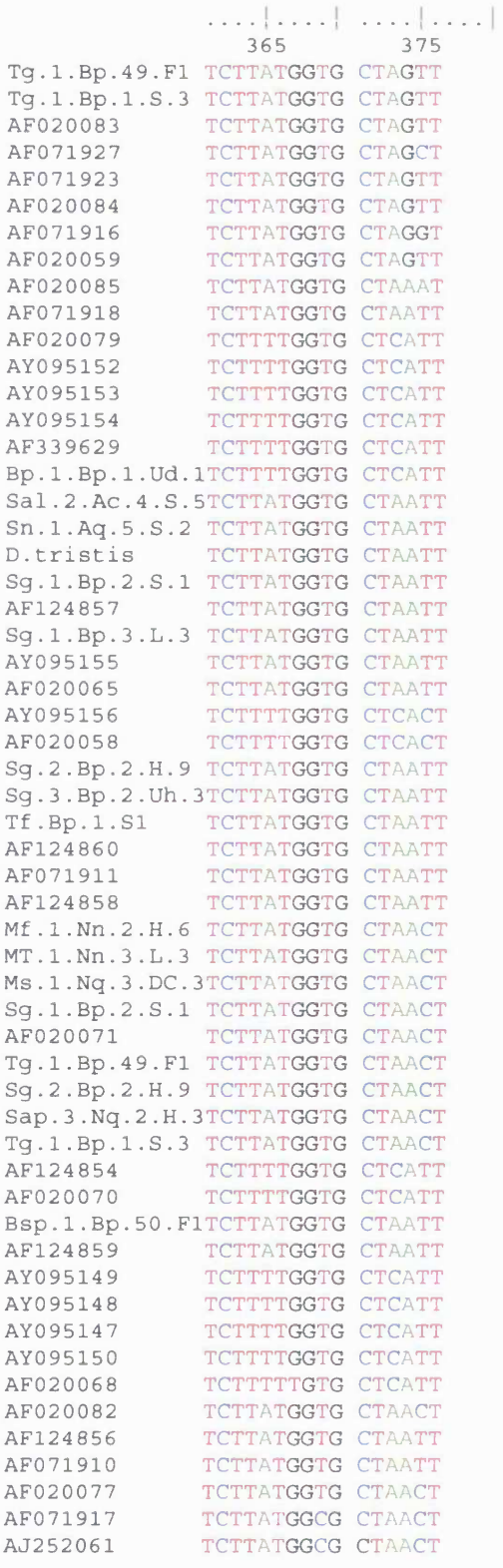

FIG. A1 ClustalX alignment of *wsp* sequences used to construct the phylogenetic tree in Fig. 4.6. The *Wolbachia* subgroups identified in Fig. 4.6 are shown.

Fig A2

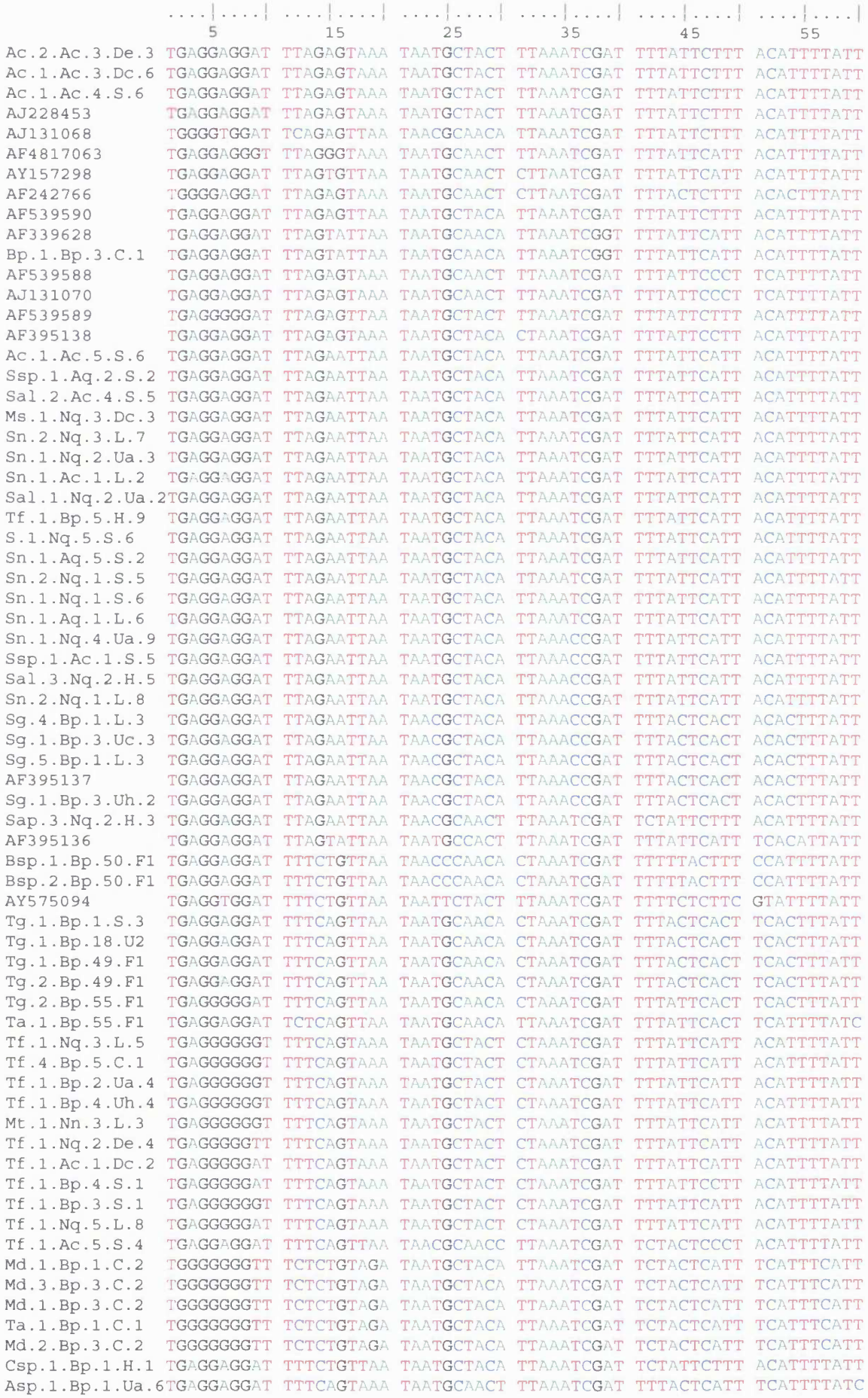
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Fig A2

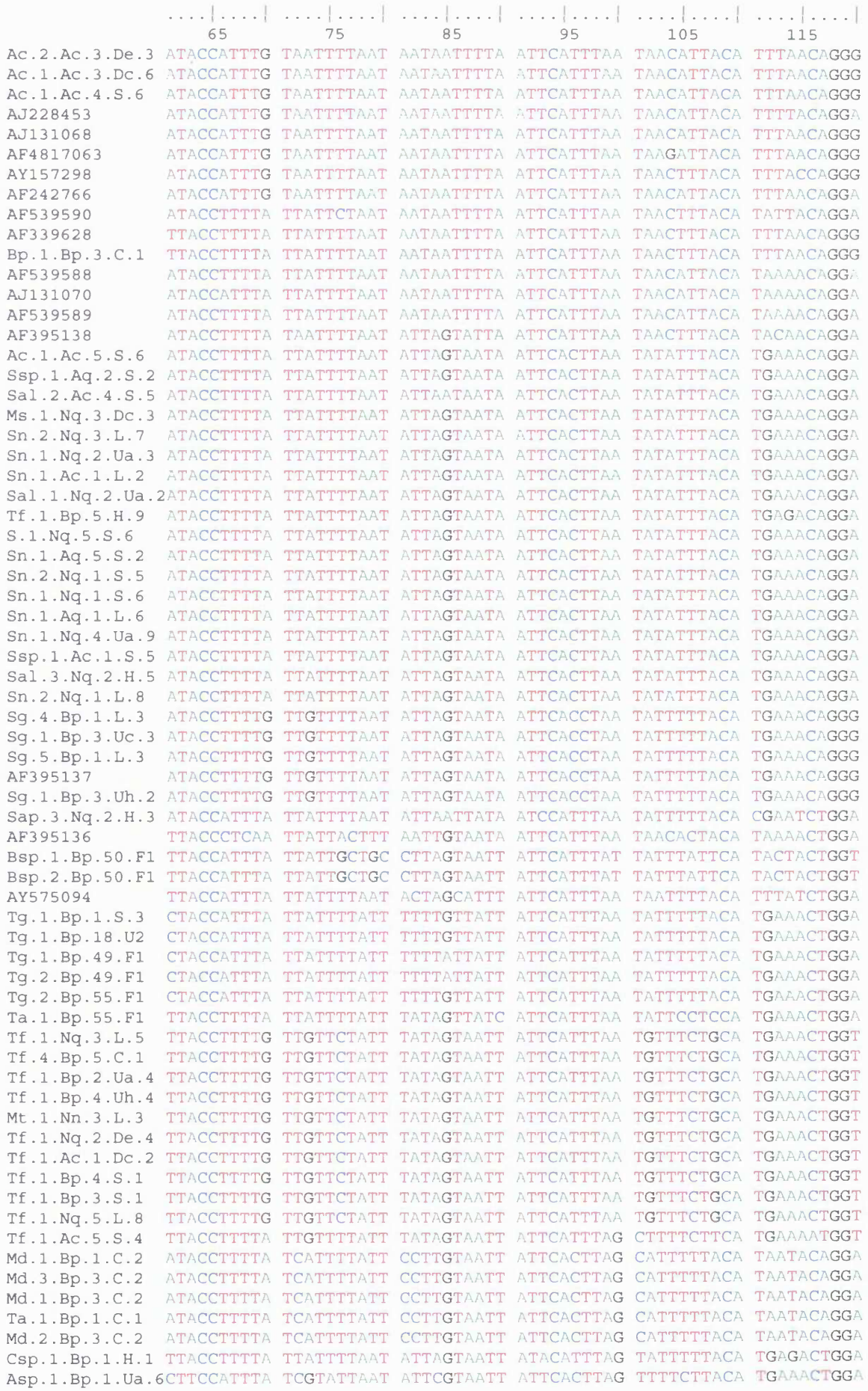
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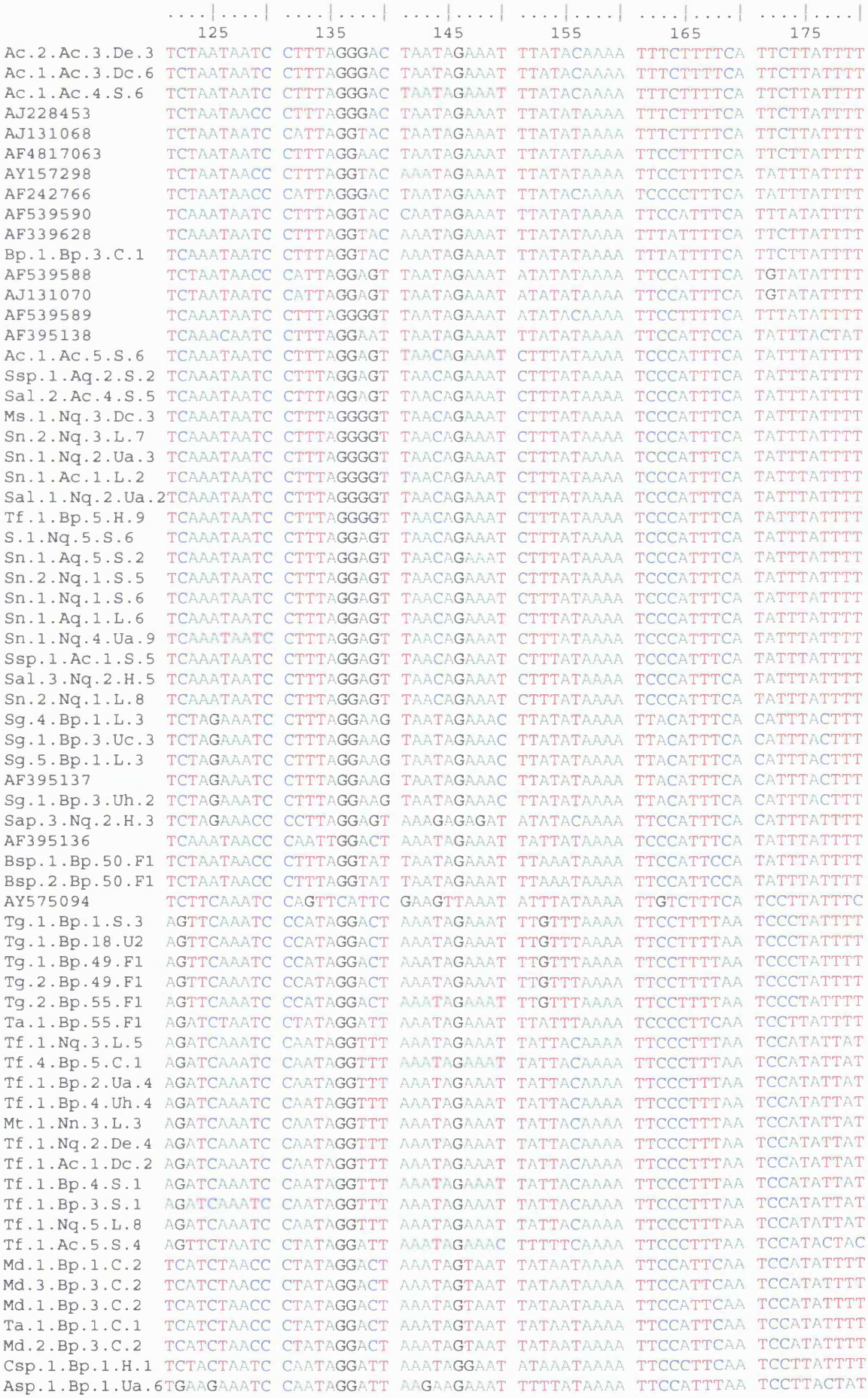
Fig A2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Md.1.Bp.3.C.2 TCATCTAACC CTATAGGACT AAATAGTAAT TATAATAAAA TTCCATTCAA TCCATATTTT
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Fig A2

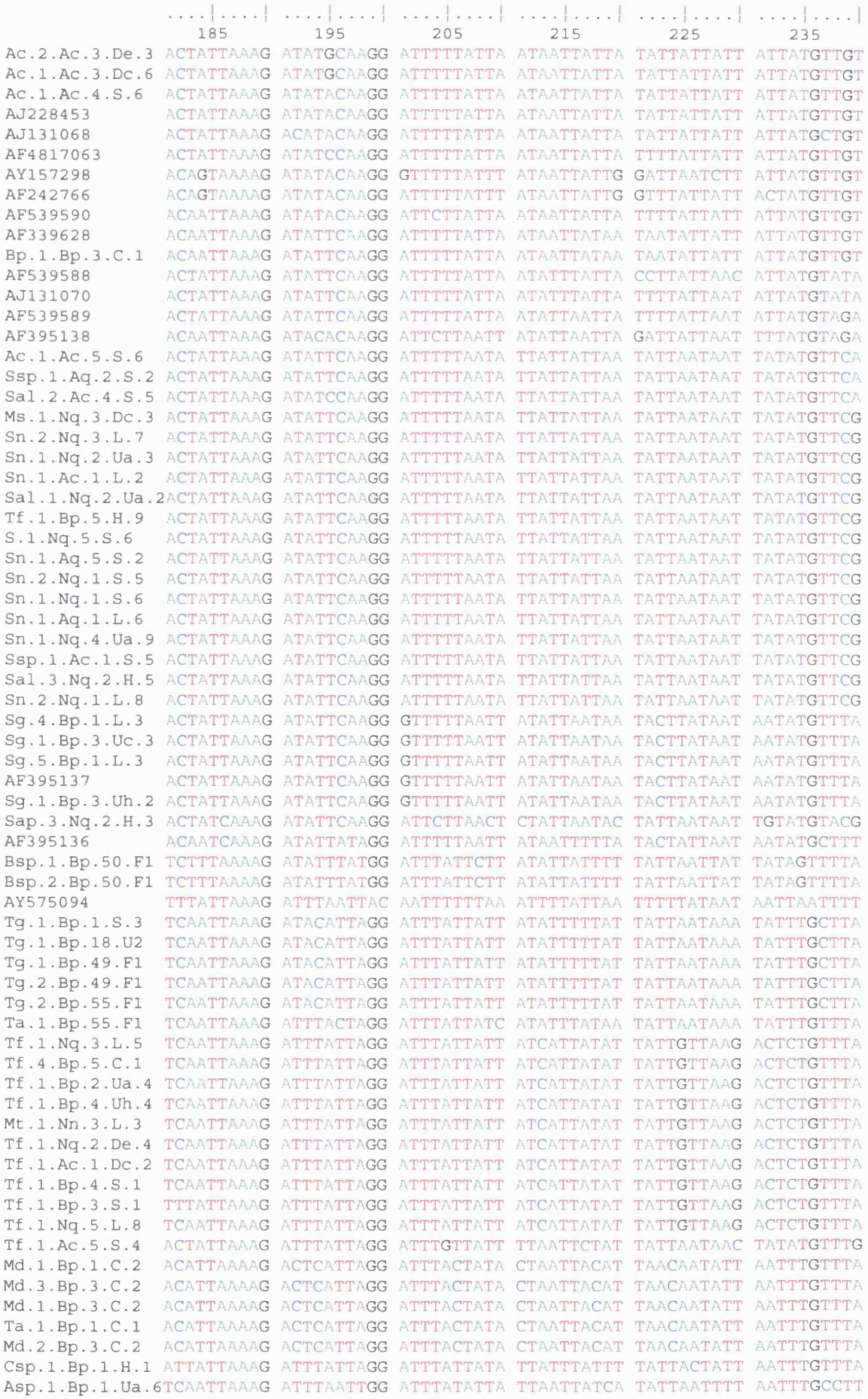
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Fig A2

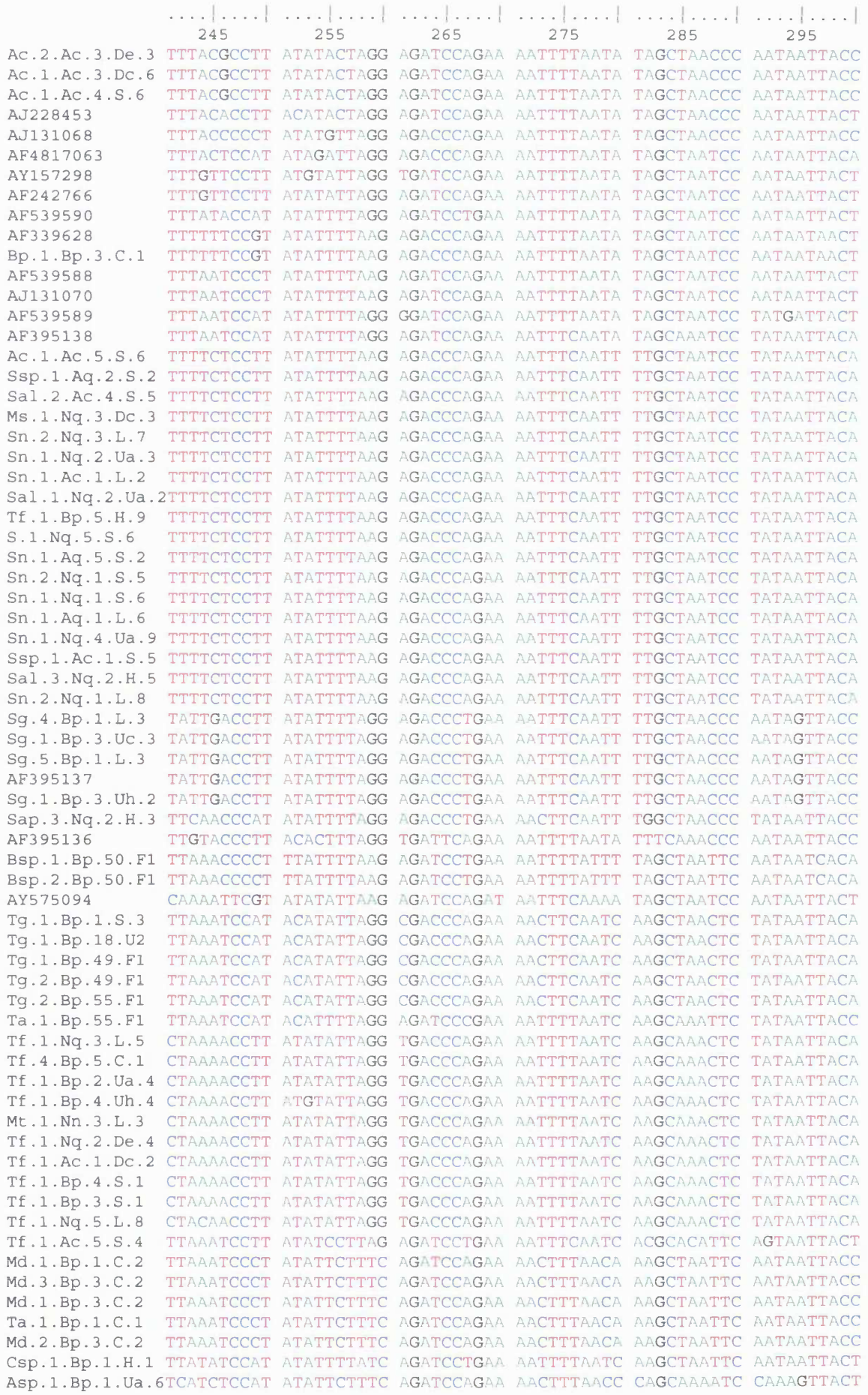
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Fig A2

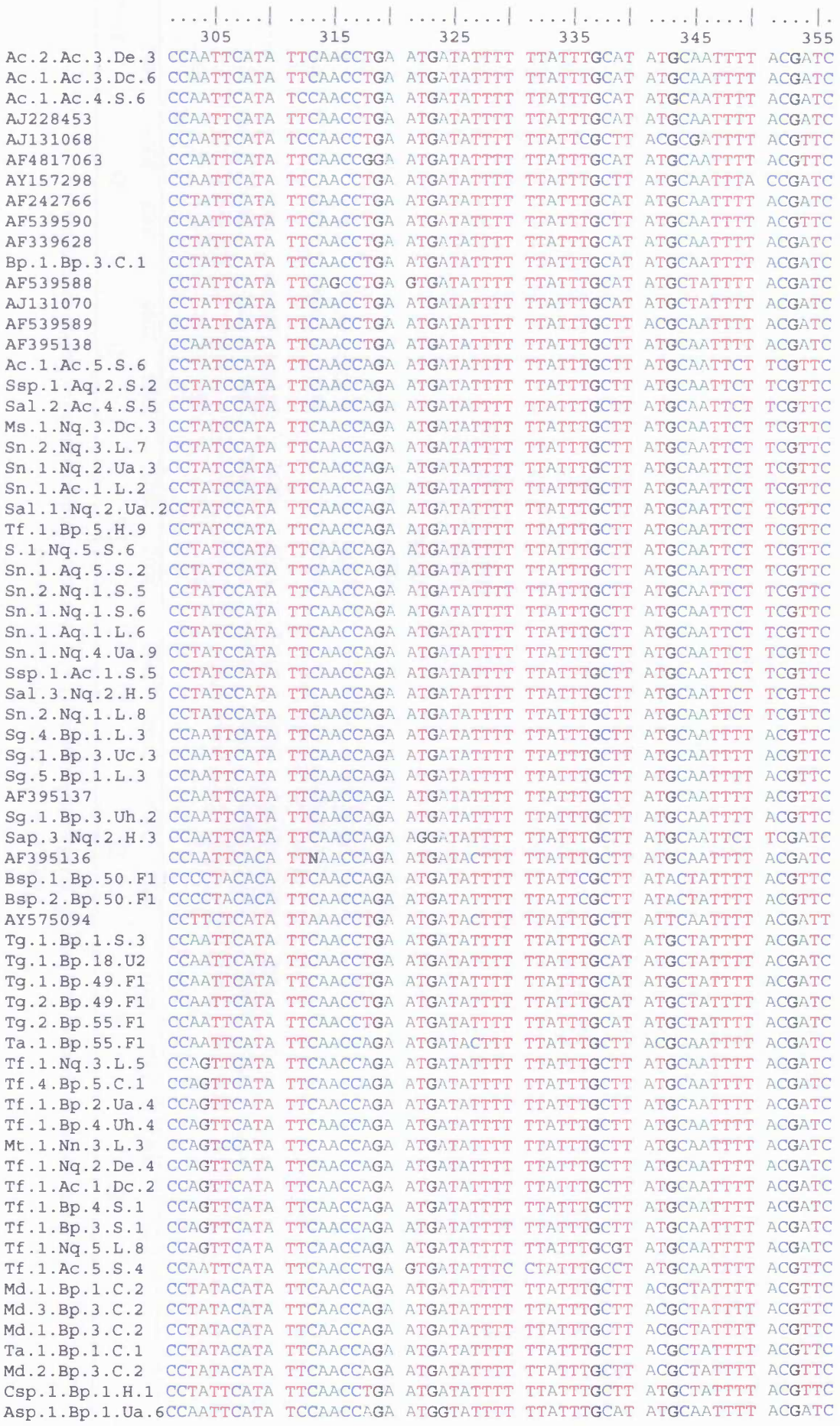


FIG. A2 ClustalX aligned cytochrome *b* sequences from members of the oak gall wasp community from the present study and from GenBank that were used for the construction of the phylogenetic tree in Fig. 4.2.

TABLE A1 Galls of *Biorhiza pallida*:
Wasp species that emerged from *B. pallida*-induced galls and the ratio of infected to uninfected specimens

Site[a]	**C**		**B**		**H**		**L**		**S**		**U**		**D**		**Total**	
Year	2002	2003	2002	2003	2002	2003	2002	2003	2002	2003	2002	2003	2002	2003	**2002**	**2003**
N° of galls	7	3	2	7	5	6	3	4	10	6	26	22	2	1	**55**	**50**
Biorhiza pallida (Olivier)	100% (15/15)	100% (3/3)	100% (5/5)	100% (10/10)	100% (6/6)	100% (4/4)	100% (6/6)	100% (1/1)	71% (5/7)	100% (6/6)	85% (23/27)	97% (30/31)	100% (2/2)	100% (1/1)	**91% (62/68)**	**98% (55/56)**
Torymus flavipes (Walker)	89% (8/9)	-	-	-	-	100% (1/1)	-	-	66% (4/6)	-	81% (17/21)	100% (5/5)	100% (3/3)	-	**82% (32/39)**	**100% (6/6)**
T. auratus(Walker)	0% (0/34)	-	-	-	0% (0/2)	-	-	-	0% (0/66)	-	-	-	-	-	**0% (0/102)**	-
T. geranii (Walker)	-	-	-	-	-	-	-	-	100% (1/1)	-	-	-	-	-	**100% (1/1)**	-
Megastigmus dorsalis (Fabricius)	0% (0/9)	-	-	-	-	-	-	-	-	-	-	-	-	-	**0% (0/9)**	-
Synergus gallaepomiformis Fonscolombe	-	-	-	100% (2/2)	-	70% (7/10)	58% (11/19)	33% (2/6)	-	-	64% (16/25)	100% (5/5)	-	-	**61% (27/44)**	**70% (16/23)**
Cecidostiba sp.	-	-	-	-	0% (0/1)		-	-	-	-	-	-	-	-	-	**0% (0/1)**
Aulogymnus sp.	-	-	-	-	-	-	-	-	-	-	0% (0/1)	-	-	-	-	**0% (0/1)**

[a]See Table 4.2 for sampling site information

TABLE A2 *Andricus curvator* galls:
Wasp species that emerged from *A. curvator*-induced galls and the ratio of infected to uninfected specimens

Site[a]	**H**		**L**		**S**		**U**		**D**		**Total**	
Year	2002	2003	2002	2003	2002	2003	2002	2003	2002	2003	**2002**	**2003**
N° of galls	3	3	1	3	8	-	3	1	12	2	**27**	**9**
Andricus curvator Hartig	0% (0/3)	0% (0/3)	0% (0/1)	0% (0/2)	-	-	0% (0/2)	0% (0/1)	0% (0/17)	0% (0/2)	**0% (0/23)**	**0% (0/8)**
Torymus flavipes (Walker)	-	-	-	-	100% (1/1)	-	100% (1/1)	-	100% (1/1)	-	**100% (3/3)**	-
Synergus gallaepomiformis Fonscolombe	-	-	-	-	100% (1/1)	-	-	-	-	-	**100% (1/1)**	-
S. albipes Hartig	-	-	-	-	100% (1/1)	-	-	-	-	-	**100% (1/1)**	-
S. nervosus Hartig	-	-	-	100% (1/1)			-	-	-	-	-	**100% (1/1)**
Synergus sp.	-	-	-	-	18% (2/11)	-	-	-	0% (0/8)	-	**11% (2/19)**	

[a]See Table 4.2 for sampling site information

TABLE A3 *Neuroterus quercusbaccarum* galls:
Wasp species that emerged from *N. quercusbaccarum*-induced galls and the ratio of infected to uninfected spe

Site[a]	**H**		**L**		**S**		**U**	
Year	2002	2003	2002	2003	2002	2003	2002	2003
N° of galls	4	9	14	3	6	2	7	11
Neuroterus quercusbaccarum (Linnaeus)	-	0% (0/3)	0% (0/16)	0% (0/31)	-	0% (0/1)	0% (0/4)	0% (0/9)
Torymus flavipes (Walker)	100% (1/1)	100% (4/4)	100% (1/1)	-	-	100% (1/1)	100% (2/2)	100% (1/1)
Mesopolobus tibialis (Westwood)	-		100% (1/1)	-	-	-	-	-
M. sericeus (Forster)	-	100% (1/1)	-	-	-	-	-	-
Synergus gallaepomiformis Fonscolombe	-	0% (0/1)	-	-	80% (4/5)	-	-	-
S. nervosus Hartig	-	-	-	100% (2/2)	100% (1/1)	-	75% (3/4)	100% (3/3)
S apicalis Hartig	100% (4/4)	-	-	-	-	-	-	-
S. albipes Hartig	-	-	-	0% (0/6)	-	-	-	0% (0/2)
Synergus sp.	25%	-	-	0%	50%	-	0%	-

TABLE A4 *Neuroterus numismalis* galls:
Wasp species that emerged from *N. numismalis*-induced galls and the ratio of infected to uninfected specimens are given

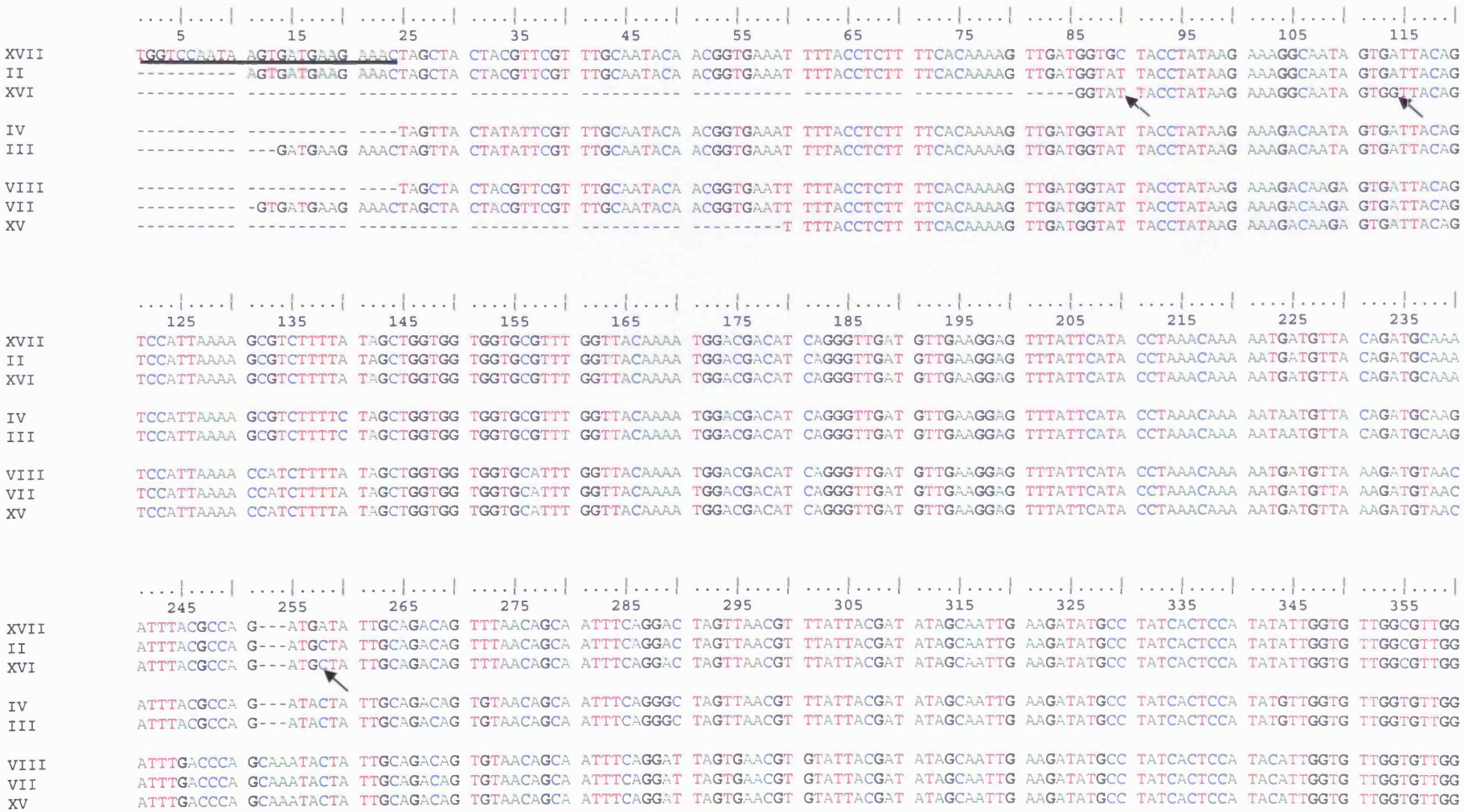
Site[a]	**H**		**L**		**U**		**D**		**Total**	
Year	2002	2003	2002	2003	2002	2003	2002	2003	**2002**	**2003**
N° of galls	1	1	-	4	2	-	1	4	**4**	**9**
Neuroterus numismalis (Geoffroy in Fourcoy)	-	-	-	0% (0/1)	0% (0/1)	-	0% (0/1)	0% (0/4)	**0% (0/2)**	**0% (0/5)**
Mesopolobus fasciiventris (Westwood)	0% (0/1)	100% (1/1)	-	100% (1/1)	0% (0/1)	-	-	-	**0% (0/2)**	**100% (2/2)**
Mesopolobus tibialis (Westwood)	-	-	-	66% (2/3)	-	-	-	-	-	**66% (2/3)**

[a]See Table 4.2 for sampling site information

TABLE A4.5 *Andricus quadralineatus* galls:
Wasp species that emerged from *A. quadralineatus*-induced galls and the ratio of infected to uninfected specimens are given

Site[a]	**L**		**S**		**Total**	
Year	2002	2003	2002	2003	**2002**	**2003**
N° of galls	1	-	4	-	**5**	-
Andicus quadralineatus Hartig	0% (0/1)	-	-	-	**0% (0/1)**	-
S. nervsus Hartig	100% (1/1)	-	100% (1/1)	-	**100% (2/2)**	-
Synergus sp.	-	-	100% (2/2)	-	**100% (2/2)**	-

[a]See Table 4.2 for sampling site information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II ---------- AGTGATGAAG AAACTAGCTA CTACGTTCGT TTGCAATACA ACGGTGAAAT TTTACCTCTT TTCACAAAAG TTGATGGTAT TACCTATAAG AAAGGCAATA GTGATTACAG
XVI ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----GGTAT TACCTATAAG AAAGGCAATA GTGGTTACAG
IV ---------- ---------- ----TAGTTA CTATATTCGT TTGCAATACA ACGGTGAAAT TTTACCTCTT TTCACAAAAG TTGATGGTAT TACCTATAAG AAAGACAATA GTGATTACAG
III ---------- ---GATGAAG AAACTAGTTA CTATATTCGT TTGCAATACA ACGGTGAAAT TTTACCTCTT TTCACAAAAG TTGATGGTAT TACCTATAAG AAAGACAATA GTGATTACAG
VIII ---------- ---------- ----TAGCTA CTACGTTCGT TTGCAATACA ACGGTGAATT TTTACCTCTT TTCACAAAAG TTGATGGTAT TACCTATAAG AAAGACAAGA GTGATTACAG
VII ---------- -GTGATGAAG AAACTAGCTA CTACGTTCGT TTGCAATACA ACGGTGAATT TTTACCTCTT TTCACAAAAG TTGATGGTAT TACCTATAAG AAAGACAAGA GTGATTACAG
XV ---------- ---------- ---------- ---------- ---------- ---------T TTTACCTCTT TTCACAAAAG TTGATGGTAT TACCTATAAG AAAGACAAGA GTGATTACAG
125 135 145 155 165 175 185 195 205 215 225 235
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245 255 265 275 285 295 305 315 325 335 345 355
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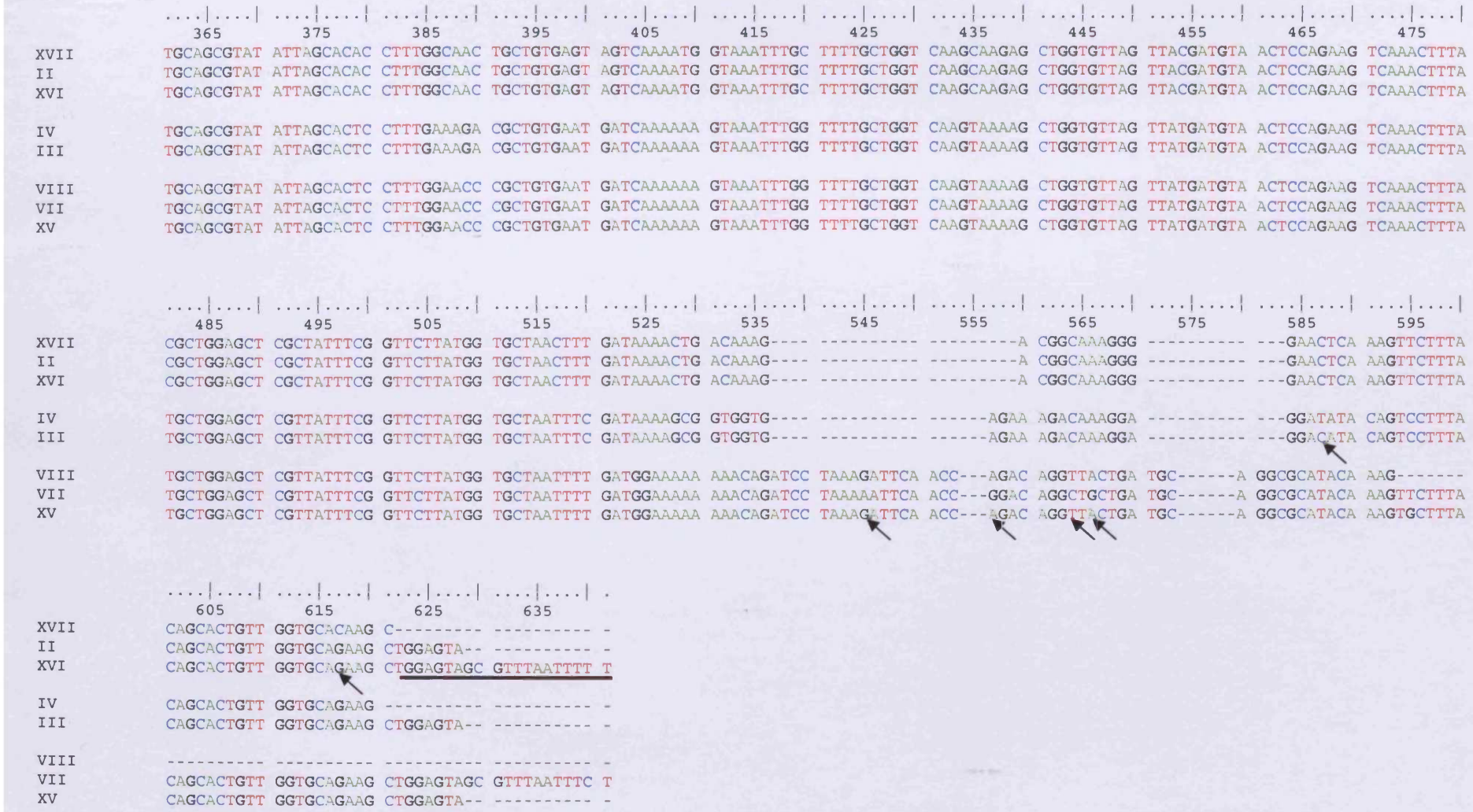

FIG. A3 Full length ClustalX alignment of *wsp* DNA sequences from groups of variants that could not be distinguished in the phylogenetic tree in Fig. 4.6 but were separated using DGGE. The forward and reverse primer binding sites are underline at the 5' and 3' ends of the alignment. Arrows indicate the position of the sequence polymorphisms.