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Interaction of European Chalcidoid Parasitoids with the Invasive Chestnut Gall Wasp, *Dryocosmus kuriphilus*

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Declaration

The work contained within this thesis has been composed by myself and is my own work unless otherwise stated. Aspects of this work were made possible by collaboration and data sharing with individuals and institutions presented here.

Italy

Data for *D. kuriphilus* and its parasitoid associates were made available by:

The department of exploitation and protection of agricultural and forestry resources (DIVAPRA) in Turin (Italy). Prof. Alberto Alma, Dr. Ambra Quacchia and Dr. Chiara Ferrancini provided data from the North and Centre of Italy and suggested field sites for gall collections. They also provided space and staff hours to rear *D. kuriphilus* parasitoids collected by Marloes de Boer and myself in Sicily.

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Slovenia

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Croatia

Dr. Dinka Matosevic at the Croatian Forest Research Institute (Croatia) provided *D. kuriphilus* data and samples, field sites for gall collections and information on the spread of *D. kuriphilus* in Croatia.

I have received samples of *D. kuriphilus* parasitoids, for molecular analyses, with permission, from the above stated institutions and it was agreed to return samples to their rightful owners after completion of this project.

All data involving oak gall parasitoids have been collected and reared by myself and my field assistants under my supervision. Field assistants included Laura Riggi for spring collections 2011, Gethin Evans for autumn collections 2011 and Marloes de Boer for spring collections 2012.

A subset of oak gall parasitoid identifications carried out by myself were verified by Dr. George Melika from the Plant Health and Molecular Biology Laboratory, National Food Chain Safety Office in Budapest, Hungary, an expert in cynipid taxonomy and their associates.

Signed: ______________________________ Date:____________________

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Abstract

Insect herbivores and their parasitoids are estimated to comprise between one and two thirds of all multicellular life on earth. Insect herbivores are key primary consumers, and occupy economically important roles as agricultural pollinators and pests. Natural insect parasitoid enemies of insect herbivores can inflict very high mortality, and provide economically important biological control of many insect pests. While the processes involved in some specific host-parasitoid interactions have been studied in detail, the recruitment of parasitoids to herbivore hosts in nature remains poorly understood.

In this thesis I consider the recruitment of native European parasitoids to an invading herbivore – the Asian chestnut gall wasp, *Dryocosmus kuriphilus.* Originating from China, *D. kuriphilus* has rapidly become an economically important pest of sweet chestnut, *Castanea sativa*, in Europe, where it is now found from Portugal to Turkey and from southern Italy to the U.K. Since its arrival in *ca*. 1996, it has become locally super-abundant and has recruited over 30 native chalcid parasitoids as opportunistic enemies. Most are known to attack native oak gall wasp hosts. This thesis seeks to understand the processes underlying recruitment of native enemies to the novel host. Specifically, I seek to understand whether recruitment of native parasitoids is a rare and localised process, or a frequent and widespread one. I address this question using widespread geographic sampling of oak and chestnut gall wasps, rearing their parasitoids and using multi-species analyses of community structure and composition. Because some currently recognised parasitoid morpho-species have been shown to include cryptic molecular taxa, I explore the consequences of a DNA barcoding approach for analyses of the parasitoid communities attacking *D. kuriphilus* and native gall wasp hosts.

At my study sites I found *D. kuriphilus* to be attacked by 29 parasitoid morpho-species, extended by DNA barcoding to a total of 39 molecular and morphological taxa. The majority of native cynipid galls in Europe are associated with oak and most of the parasitoid species found to attack *D. kuriphilus* are also known to attack gall wasp hosts on oak. My data provide new records of parasitoid species recruited to *D. kuriphilus* on chestnut. This includes parasitoids known to attack non-oak cynipid galls suggesting that other sources for the recruitment of parasitoids need to be considered. Multi-species community analyses suggest that parasitoid host shifts to chestnut have happened repeatedly in multiple locations. My study thus suggests that, while gall wasps are highly specific to particular tree taxa, their chalcidoid natural enemies are not so constrained.

Chapter 1 – Introduction

1.1 Community Interactions and Biological Invasions

Mankind is causing increasingly rapid environmental changes through land-use intensification, environmental pollution, and the global conveyance of goods. These practices progressively contribute to four key processes in anthropogenic extinctions (the 'evil quartet' of Diamond 1989): habitat destruction, over-exploitation, extinction cascades and biological invasions. Since Elton (1958) recognised biological invasions as a significant driver of ecosystem change, invasion biology has been integrated as an important discipline within community ecology (Diamond 1989). Invasions can cause restructuring of biological communities, displacing native species through direct or indirect interactions and can, over time, cause extinctions (Atkinson 1989, Diamond 1989, Pimentel 2011, Sax et al 2005, Settle & Wilson 1990, Yoshida et al 2007). Given the importance of the provision of ecosystem services such as pollination or biological control, it is crucial that we understand how invasions affect ecological communities (Cavender-Bares et al 2009, May 1990, Montoya et al 2006, Ricklefs 1987 & 2008, Suttle et al 2007) for the sake of effective ecosystem management. One key observation in relation to alien species that become invasive is the absence of natural enemies that can control their populations (Enemy Release Hypothesis, Keane & Crawley 2002, Roy et al 2011). Yet some gall wasps, alien to the UK, having arrived over the last 200 years are known to have recruited parasitoid natural enemies from the invaded range species pool quite quickly (Schönrogge et al 2012). Parasitoid recruitment to alien species is still not particularly well understood, in part because invasions may be common, but often proceed unobserved at least for periods of time so that early interactions with the native community remain unknown (Williamson 1996).

Biological invasions can be detrimental both economically and to native biodiversity, but many have only limited impact. In fact, the '10's rule' (Williamson 1996) posits that only 10% of colonising species establish populations in the invaded range followed by, possibly, only 10% (1% of the original colonisers) being able to spread and only 10% of those (0.1% of the original total) becoming harmful as invaders to the environment, economy or both. This pattern was observed with escaping British crop plants but has subsequently been found to be context specific (Williamson 1996, Jeschke & Strayer 2005). Whether or not alien species are perceived to be harmful, they provide research

opportunities to study community assembly and species interactions. They represent large-scale natural experiments (following Diamond & Case 1986) in which native community responses can be studied after perturbation by the arrival of an alien invader (Hails et al 1990, Yoshida et al 2007). A particular area of interest in community studies of biological invasions has been to assess how quickly and in what manner invading species recruit assemblages of natural enemies from a given native community (Cornell & Hawkins 1993, Schönrogge et al 1995, Godfray et al 1995, Grobler & Lewis 2008). Species could recruit to an invading alien host (IAH) repeatedly from local species pools throughout the invaded range of the IAH (the Local Recruitment Hypothesis), or they could recruit very rarely – perhaps in a single location – and then track the IAH as it expands its range (the Host Tracking Hypothesis). Studies of invasive plants and herbivores and their associated natural enemy assemblages, involving sampling along invasion routes, or comparisons between native and invaded ranges, have revealed general patterns in species interactions underlying biological invasions. For example, 1) rapid colonisation of new resources (such as invading plant - or herbivore hosts) by native natural enemies is common; 2) enemy species richness associated with an invading host is correlated with but generally lower than that in its native range; and 3) invader-centred assemblages are more dominated by generalists than assemblages in the invader's native community (e.g. Cornell & Hawkins 1993). Studies of biological invasions have also proven useful in demonstrating the relative importance of apparent competition (Settle & Wilson 1990, Holt & Lawton 1993), regional variation in community composition (Hernandez-Lopez et al 2012), and the relative importance of biotic and abiotic factors in novel community assembly (Tack et al 2010).

1.2 Value of the Gall Wasp System in Studies of Community Interactions

Cynipid gall wasps have been a convenient system for community studies over the last 65 years. Their galls are relatively easy to identify, census, and collect because they are sessile and often conspicuous on the tree. The vast majority of cynipid galls in the Western Palaearctic are associated with oak, *Quercus* spp. . They have complex, cyclically parthenogenetic life cycles, which means that they exhibit alternating sexual and asexual generations (Askew 1984, Stone et al 2002 & 2008). The sexual generation develops during spring, with haploid male and diploid female adults emerging from spring generation galls and the asexual generation develops during the autumn of each year with only diploid females emerging from autumn generation galls. These alternating generations develop often strikingly different gall morphologies both in physical structure and in gall location on a given host plant. Parasitoid assemblages associated with cynipid galls in Europe are closed

and well understood. Comprehensive morphological species keys of gall morphologies (associated with the herbivore host) and parasitoid adult morphology have been developed for use by non-specialists (Buhr 1965, Ambrus 1974, Redfern & Shirley 2002, Askew & Thúroczy unpublished).

Cynipid galls support relatively simple and well-studied communities, comprising 4 major guilds at 3 trophic levels: (i) gall inducing cynipids (Hymenoptera: Cynipidae: Cynipini), (ii) inquiline gall wasps that are close relatives to the inducer and exploit its gall tissue (Hymenoptera: Cynipidae: Synergini); (iii) parasitoids and (iv) hyper-parasitoids (Hymenoptera: Chalcidoidea) that attack one or both herbivore guilds as well as, in the case of hyper-parasitoids, other chalcidoid gall inhabitants (Csoka et al 2005, Figure 1.1). Species in each guild show varying levels of specificity to host plants or host insects.

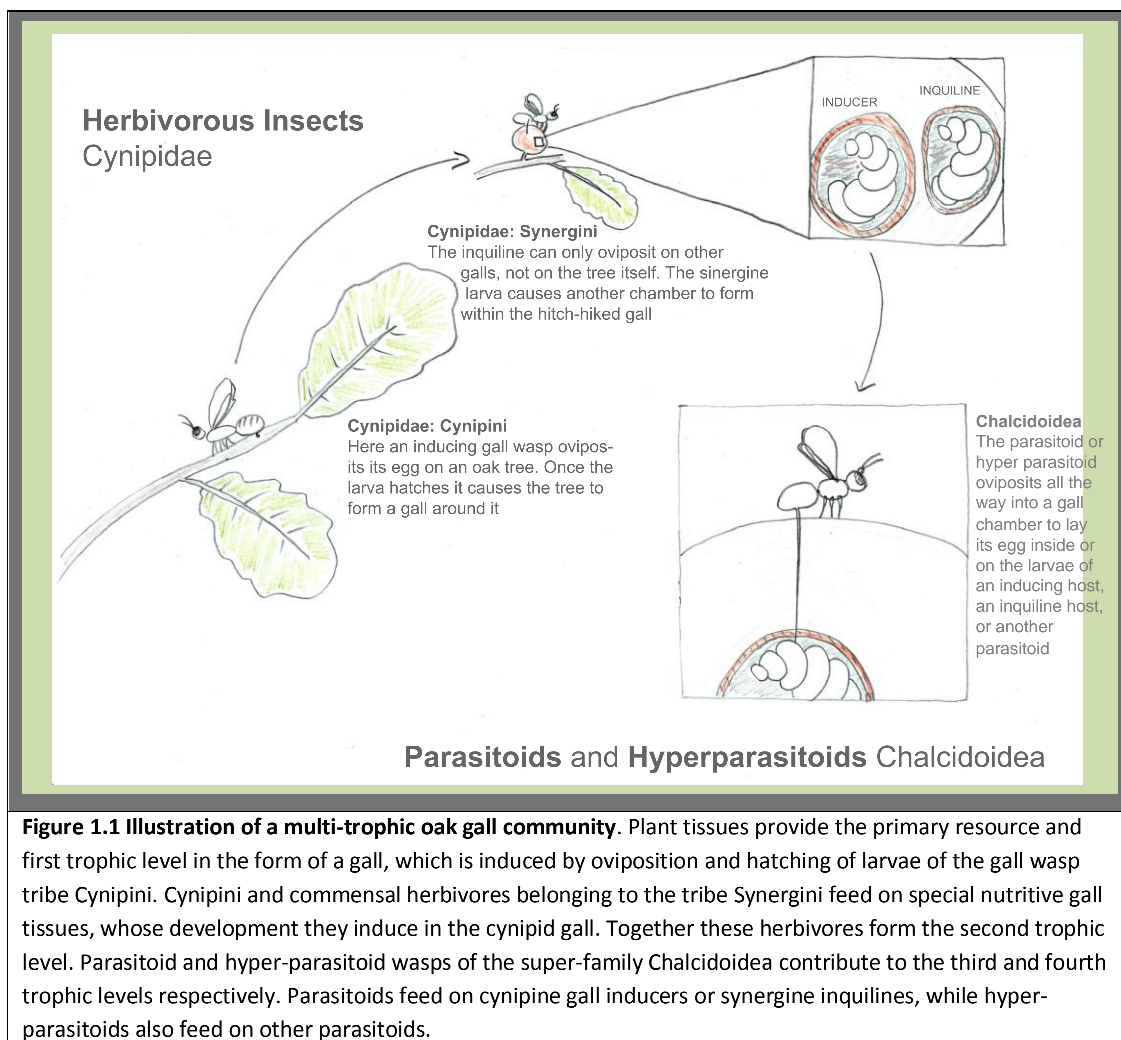

Figure 1.1 Illustration of a multi-trophic oak gall community. Plant tissues provide the primary resource and first trophic level in the form of a gall, which is induced by oviposition and hatching of larvae of the gall wasp tribe Cynipini. Cynipini and commensal herbivores belonging to the tribe Synergini feed on special nutritive gall tissues, whose development they induce in the cynipid gall. Together these herbivores form the second trophic level. Parasitoid and hyper-parasitoid wasps of the super-family Chalcidoidea contribute to the third and fourth trophic levels respectively. Parasitoids feed on cynipine gall inducers or synergine inquilines, while hyper-parasitoids also feed on other parasitoids.

Cynipid galls are physically closed ecosystems (Harper et al 2004) and the communities associated with them can also be considered 'ecologically closed'. This means that very few of the associated species are linked trophically to hosts outside oak cynipid galls. Cynipid gall inducers and their inquilines are both specialized to feed on nutritive tissue within the gall. They are, in turn, primarily attacked by parasitoids that, with very few exceptions, attack only the gall inhabiting herbivores (Askew 1961, Stone et al 2002). Exceptions include *Pediobius saulius* (Hymenoptera: Chalcidoidea), which attacks oak galls and lepidopteran leaf miners (Hernandez-Lopez 2012), *Cirrospilus diallus* which most commonly attacks leaf mining Lepidoptera but also sometimes attack hosts in *Neuroterus* cynipid galls, and *Eupelmus urozonus*, an extreme generalist chalcidoid which attacks a range of insect host lineages on plants in several families including Fagaceae, Rosaceae, and Sapindaceae (Askew et al 2006 & 2013). Because the host plant, the cynipid galler and its parasitoids present a relatively closed assemblage, it is reasonable to consider their dynamics in isolation from the wider community outside the gall (Askew 1961, Askew 1980,

Askew et al 2006 & 2013, Csoka et al 2005, Stone et al 2002). However, morphological identification of community members is challenging and requires substantial taxonomic expertise and access to extensive collections of reference specimens. Although the closed community aspect simplifies work on oak galls, misidentification of morphologically similar taxa is likely. Molecular studies of European cynipid gall parasitoids have revealed morphologically cryptic species (independently evolving lineages that are morphologically indistinguishable, Ács et al 2007, Kaartinen et al 2010, Nicholls et al 2010b). As species level taxa are usually the principle unit of ecological study, identification errors and the presence of cryptic species pose potentially serious problems (Armstrong & Bar 2005). Consequently it is becoming increasingly common within ecological studies to supplement morphological identifications with molecular methods such as DNA barcoding (see Box 1).

Box 1. A short explanation of DNA barcoding detailing its use and application

DNA barcodes are short sequences from a standardised region of DNA – usually the 'Folmer' region of the mitochondrial cytochrome c oxidase subunit I gene (Hebert et al 2003). Based on the assumption that species level taxa are monophyletic at the barcode locus and that variation within species is less than variation between species, the primary application of DNA barcoding is as a means of assigning query specimens to existing taxa if they differ from voucher sequences by less than a specified threshold (Hebert et al 2003, Ratnasingham and Hebert 2007).

DNA barcoding can also offer a tool for assessing the accuracy of morphological species classifications (Hebert & Gregory 2005). If barcodes from multiple species are grouped into molecular operational taxonomic units (MOTUs) based on sequence similarity (Blaxter et al 2005), then the assumption that variation within species is less than variation between species will be characterised by a barcoding gap (Acs et al 2010, Meyer and Paulnay 2005, Wiemers et al 2007). Where a gap is observed, MOTUs within it are likely to represent species under the phylogenetic species concept (Baum & Shaw 1995), and can be compared with existing classifications to identify discordance. If specimens from a single morphological species occur within multiple MOTUs then it suggests the presence of cryptic lineages.

Additionally, DNA barcodes can be used as a molecular marker for phylogenetic and/or phylogeographic analyses to make inferences about the evolutionary history of species and populations (Hajibabaei et al 2007) and the relationships between them. Such analyses however will usually incorporate additional independently evolving (unlinked) molecular markers for improved resolution and error control (Lohse et al 2010).

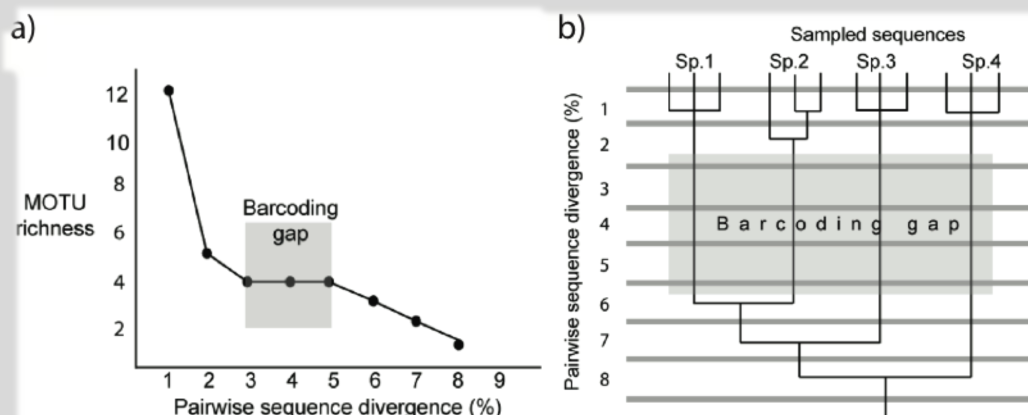

Illustration of barcoding gap (Ács et al 2010). a) Barcoding gaps are an apparent plateau (grey box) in a graph that plots number of MOTUs observed (y-axis) against the % pairwise sequence divergence (x-axis) between sequences. Over the plateau, the number of MOTUs does not change despite an increase in % difference between fragments. b) These plateaux correspond to deep splits in a phylogenetic tree where branches remain the same despite moving backwards in evolutionary time. Barcoding gaps therefore suggest the presence of divergent clades amongst a given number of samples.

Detailed morphological (e.g. Askew 1961, Askew et al 2006 & 2013, Csoka et al 2005, Pujade-Villar et al 2003), ecological and phylogenetic research (e.g. Kaartinen et al 2010, Nicholls et al 2010a&b, Rokas et al 2003a&b, Ronquist 1999, Stone et al 2002, 2009 & 2012) on gall communities in Europe has provided a comprehensive record of the parasitoid and inquiline species associated with the approximately 140 European cynipid gall wasp species throughout their life cycles. Outside Europe, oak gall wasps are found throughout the temperate Holarctic, with approximately 1400 gall wasp species known worldwide (Ronquist 1999, Csoka et al 2005). Studies in East Asia suggest that many more species remain to be discovered (Melika et al 2011, Tang et al 2012a, 2012b & 2013). Yet wherever they are found, oak cynipid gall communities contain members of a consistent set of insect taxa. The ecological closure and defined taxonomic membership of oak gall communities make them analogous to other widely studied microcosmic insect communities, such as fig wasps (Hawkins & Compton 1992, Segar et al 2014, Wang et al 2015), leaf miners (Askew & Shaw 1974, Rott & Godfray 2000, Grobler & Lewis 2008) and seed feeders (Aebi et al 2008).

Cynipid gall wasps have, in particular, proven to be useful study systems in invasion ecology and community assembly. Thirteen species have become established in the British Isles over the last 200 years and all but *Andricus kollari*, whose galls were imported as a source of tannin during the 'ink trade' in the 19th century (Walker et al 2002, Stone et al 2001), arrived there within the last 60 years (Schönrogge et al 2012). Most of these invasions resulted from natural range expansions by European gall wasps following the Europe-wide introduction of a host tree that is an obligatory part of their life cycle, the Turkey oak *Quercus cerris* (Schönrogge et al 1995, 1996a&b, 1998, Stone et al 1995, Walker et al 2002, Schönrogge et al 2012). These studies show that the species richness of invader-centred assemblages increases over time and declines with increasing distance from the site of origin. Schönrogge et al (1995) also showed that, with time, assemblages of alien-associated parasitoids tend to become more similar to assemblages from their native range, suggesting that the same species attacking them in the native areas were recruited in the new populations given enough time. One species, *Megastigmus stigmatizans* is thought to have been introduced to the UK together with their host cynipid galls, imported for the ink or tanning industry (Nicholls et al 2010a). To-date, however, there has been no strong supporting evidence for the Host-Tracking-Hypothesis from the alien cynipids study systems although it has been suggested that host tracking is important for *Pediobius saulius* attacking the IAH *Cameraria ohridella*. (Schönrogge et al 1995, Hernandes-Lopez et al 2012). More generally, the species richness of invader-centred assemblages also increases with the number and diversity of locally available plant and herbivore hosts (Cornell & Hawkins 1993).

One example of this suite of patterns is the recruitment of enemies to the galls of *Andricus quercuscalicis*, one of the gall wasp invaders to the UK (Hails et al 1990, Schönrogge et al 1995, 1996a&b, 1998, 2000, 2012, Schönrogge & Crawley 2000, Stone et al 1995). These galls have several structural attributes not exhibited by local UK gall species and interpreted as anti-parasitoid defences, including a coating of sticky resins and a larval chamber that is surrounded by an air-space (Bailey et al 2009). Furthermore, the galls are induced on acorns which is also a novel gall location relative to native UK oak gall cynipids. These galls were free of parasitism for about 40 years, suggesting that novel gall attributes can delay recruitment of native natural enemies. Over time, many of the parasitoid species that are native both to the original range of *Andricus quercuscalicis* and to the UK have recruited to the invading gall community in Britain. The similarity of parasitoid populations in these two regions has been taken to indicate that gall traits, including morphology and location, can act as a strong ecological filter in community assembly (Collins et al 1983, Hails et al 1990, Schönrogge et al 1995, 2012). *Dryocosmus kuriphilus*, being purely asexual and univoltine, has a less complex life-cycle than other known oak cynipids in Europe. Its gall structure appears to be packing less defenses compared to many European cynipids, including *A. quercuscalicis*, mentioned above. *Dryocsmus kuriphilus* galls are relatively soft and multi-chambered providing an easy-to-access food source. The fact that they occur on the European sweet chestnut, *Castanea sativa*, a tree species that prior to *D. kuriphilus* arrival in Europe was not host to any cynipid gallers, suggests that host tree may provide a significant barrier (filter) to the local species of parasitoids that are available for recruitment to the novel host.

1.3 The Chestnut Gall Wasp *Dryocosmus kuriphilus*

The Chinese chestnut gall wasp, *D. kuriphilus* (Hymenoptera: Cynipidae: Cynipini), is the only cynipid known to gall chestnuts (Fagaceae: *Castanea* spp). *Dryososmus kuriphilus* attacks *Castanea mollissima, C. seguinii* and *C. henryi* from its native range in China, as well as *C. crenata* native to Japan, *C. dentata* in the USA and *C. sativa* in Europe (Moriya et al 1990, Payne 1978, Payne et al 1976, Aebi et al 2006). Three biological characteristics set *D. kuriphilus* apart from oak cynipid gall wasps: an unusual host tree association, lack of a sexual generation in its life cycle and the capacity to seriously harm its host tree (reviewed in EFSA 2010). *Dryocosmus kuriphilus* reduces the development of chestnut fruit by galling the trees' shoots and leaves and stunting their growth (Figure 1.2).

Figure 1.2 *Dryocosmus kuriphilus* attack on *Castanea sativa*. Visible stunting of leaves (and in severe cases, fruit and shoots) is caused by the invader ovipositing in buds and manipulating their growth early in development.

Infestation can reach 100% of trees and affect 100% of branches on a given tree, resulting in loss of up to 80% of fruit (Zhang et al 2009, Breisch & Streito 2004). High infestation may eventually kill trees (Miyashita et al 1965, Dixon et al 1986), although death may occur indirectly, through exit wounds of the gall facilitating attack by pathogens (e.g. *Cryphonectria parasitica*, the chestnut blight, see Prospero & Forster 2011, EFSA 2010). *Dryocosmus kuriphilus* is a holarctic pest, damaging chestnuts in Japan (Yasumatsu 1951, Oho and Umeya 1975), Korea, Nepal, the USA and - since the beginning of the 21st century - Europe (Abe et al 2007, Aebi et al 2006 & 2007, Cooper & Rieske 2011, Forster et al 2009, Schönrogge et al 2006, Quacchia et al 2008). It is thought to have arrived in Europe in 1996 due to the import of an infested *C. mollissima* sapling from China to the Piedmont region in Italy (Brussino et al 2002, Aebi et al 2006, Graziosi & Santi 2008). The first official record of *D. kuriphilus* in Europe was reported in 2002 (EPPO 2015), but pin-pointing the exact time or source of introduction is difficult with so many potential source populations around the world. Since its arrival in Italy, *D. kuriphilus* has quickly spread to France, Spain, Portugal and Great Britain to the west, Switzerland, Austria, Germany and the Netherlands to the north and Slovenia, Czech Republic, Slovakia, Hungary, Croatia, Greece and Turkey to the

east. Table 1.1 shows the areas of infestation recorded in Europe as of August 2015 and figure 1.3 shows a map of spread by countries within Europe (and provinces within Italy). In Europe, spread is thought to occur through both, accidental introduction of infested trees (Aebi et al 2006, EFSA 2010, EPPO 2015) and natural range expansion by the winged adult gall wasps across native stands of European sweet chestnut, *C. sativa* (EFSA 2010). Unaided short-distance dispersal by adults is estimated at a rate of 3-12 km per year (EFSA 2010). The known environmental envelope of *D. kuriphilus* suggests that this pest will continue to spread throughout the range of chestnuts in Europe (Aebi et al 2006, EFSA 2010). In fact, *D. kuriphilus* has spread through much of Europe since the onset of this thesis and has finally arrived in the UK in 2015 (EPPO 2015), probably through human-aided long distance dispersal.

Table 1.1 Invasion status of *D. kuriphilus* in Europe. Summary of pest indices released by the European and Mediterranean Plant Protection Organisation (EPPO 2015) relating to *D. kuriphilus*, giving an indication of the spread of *D. kuriphilus* across Europe over time.

First recorded in:	Country Reported	Italian Province	Management Status	Mode of spread if known	Other	Source
2002	Italy	Cuneo	present	introduced	First record in Europe; biological control initiated in 2005	EPPO 2003
2005	France		eradicated	natural spread		EPPO 2008
2005	Slovenia		transient, under eradication	introduced	eradication assumed unsuccessful	EPPO 2006, Knapic et al 2009
2006	Italy	Abruzzo	present			EPPO 2006
2006	Italy	Lazio	present			EPPO 2006
2006	Italy	Lombardia	present			EPPO 2006
2007	Italy	Liguria	present			EPPO 2008
2007	Italy	Trentino-Alto Adige	present			EPPO 2009
2007	Italy	Veneto	present			EPPO 2008
2007	France		present	natural spread	biological control initiated in 2011 (Borowiec et al 2014)	EPPO 2012, Borowiec et al 2014
2008	Italy	Campania	present			EPPO 2009
2008	Italy	Emilia-Romagna	present			EPPO 2009
2008	Italy	Toscana	present			EPPO 2009
2008	Italy	Sardegna	present			EPPO 2008
2009	Italy	Calabria	present			EPPO 2009
2009	Italy	Marche	present			EPPO 2009
2009	Italy	Friuli-Venezia Giulia	present			EPPO 2009
2009	Italy	Umbria	present			EPPO 2009
2009	Hungary		eradicated	introduced		EPPO 2013
2009	Switzerland		transient, under eradication		eradication assumed unsuccessful	EPPO 2011
2010	Italy	Molise	present			EPPO 2010
2010	Italy	Sicily	present			EPPO 2010
2010	Hungary		eradicated	introduced		EPPO 2013
2010	Netherlands		absent, eradicated			EPPO 2013

2010	Croatia		present		suspected to be present since 2007-2008 based on gall load, biological control initiated in 2013	EPPO 2011, Matosevic et al 2010, Matosevic et al 2013
2010	Corsica		present			EPPO 2011
2012	Spain		present			EPPO 2014
2012	Germany		present			EPPO 2013
2012	Czech Republic		transient, under eradication			EPPO 2012
2013	Austria		present			EPPO 2013
2013	Hungary		present	natural spread		EPPO 2013
2014	Portugal		present	introduced	potential biological control from 2015	EPPO 2014
2014	Turkey		present	introduced		EPPO 2014
2015	United Kingdom		present, under eradication			EPPO 2015
2015	Netherlands		present			EPPO 2015

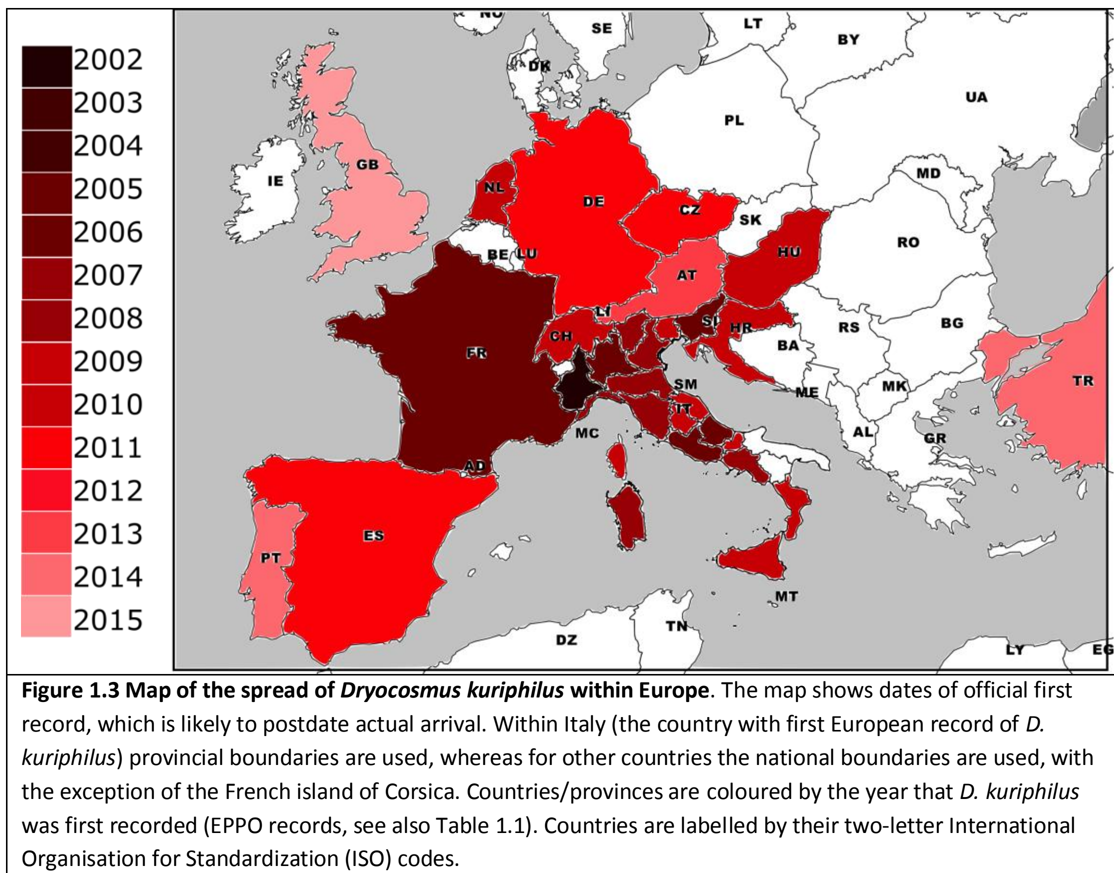

Figure 1.3 Map of the spread of *Dryocosmus kuriphilus* within Europe. The map shows dates of official first record, which is likely to postdate actual arrival. Within Italy (the country with first European record of *D. kuriphilus*) provincial boundaries are used, whereas for other countries the national boundaries are used, with the exception of the French island of Corsica. Countries/provinces are coloured by the year that *D. kuriphilus* was first recorded (EPPO records, see also Table 1.1). Countries are labelled by their two-letter International Organisation for Standardization (ISO) codes.

Dryocomus kuriphilus most likely arrived in Europe in its egg stage within an infested sapling, both invisible to the naked eye and free from natural enemies. Whether Europe constituted 'enemy free space' for *D. kuriphilus* (*sensu* Jeffries & Lawton 1984, Aebi et al 2006) upon arrival therefore depended on whether native parasitoids could locate and exploit it. If parasitoids preferentially associate with a particular host plant, such as oak sections in Europe (Bailey et al 2009), and do not readily exploit hosts on another plant, then we might expect colonisation of novel galls on a novel food plant to be a slow process. However, in contrast to this expectation, *D. kuriphilus* has recruited native parasitoid species rather quickly. It has now been recorded to host 42 native European parasitoid species representing 6 families (Aebi et al 2007, Quacchia et al 2013, Matosevic et al 2013, Panzavolta et al 2013, Palmeri et al 2014, Kos et al 2015) that are primarily associated with native oak cynipid galls (Aebi et al 2006, Askew et al 2006 & 2013). The presence of such a broad spectrum of native parasitoids on *D. kuriphilus* has implications for native oak gall communities through potential indirect competition between native and invasive gall makers, as they are attacked by shared parasitoids (Morris et al 2004, van Veen et al 2006). Alternatively, native parasitoids could act as local forms of pest control if the mortality they cause to the invader reaches significant levels (Aebi et al 2008). Work on native oak gall communities in Hungary has shown that the host oak section on which a gall develops is the best predictor of the

associated natural enemy community (Bailey et al 2009). This has also been observed anecdotally by Askew (1961) related to parasitoid assemblages on British cynipids and has been reported for UK parasitoids on invading oak gall wasp hosts (Schönrogge & Crawley 2000).

Here, I extend knowledge of parasitoid recruitment to invading hosts by focussing not on a natural range expansion, but on a long-range anthropogenic gall wasp introduction. I investigate the assembly of the parasitoid community associated with the Chinese chestnut gall wasp, *Dryocosmus kuriphilus,* in southern Europe.

1.4 Classical Biological Control of *D. kuriphilus*

To reduce the impact of the chestnut gall wasp, a Chinese natural enemy, *Torymus sinensis* Kamijo (Hymenoptera: Torymidae), has been released as a biological control agent in Japan (Moriya et al 1989), Korea, Nepal, the USA (Cooper & Rieske 2007) and Europe (Aebi et al 2006, EFSA 2010). *Torymus sinensis* was introduced in Italy in 2005 (Aebi et al 2006, EPPO 2015) and in Croatia in 2013 (Matosevic et al 2013). It effectively reduced gall load (% of buds galled on a given tree) from over 80% to below 30% in Japan (Gyoutoku & Uemura 1985, Murakami et al 2001) and is also starting to significantly reduce chestnut gall load in Piedmont, the original site of introduction of both the pest and the biological control agent in Europe (Quacchia 2014a).

Torymus sinensis is considered a specialist parasitoid of chestnut gall wasps in its native range (Murakami et al 1977, Zhang et al 2009). Field surveys in China (Murakami et al 1980, Zhang et al 2009) and laboratory experiments in Italy (Graziosi & Rieske 2013, Quacchia et al 2008) suggest that *T. sinensis* attacks only chestnut gall wasps. Screening methods carried out so far have been criticised as insufficient by Gibbs et al (2011) to assess the specificity of this biological control agent. They called for improved methods to assess *T. sinensis* to prevent any undesirable biological impact of *T. sinensis* on native communities. Suggestions involved: (i) more rigorous screening of gall communities in the native range of *T. sinensis*; (ii) expansion of laboratory gall host preference experiments to a wider and more phenologically relevant selection of oak gall types than those chosen by Quacchia et al (2008); (iii) testing for hybridisation of native *Torymus* species with the biological control agent (but see Quacchia et al 2014b). The latter is of particular concern because *T. sinensis* displaced a native relative, the oak gall parasitoid *T. beneficus*, in as little as 4 years at a field site in Obuse, Japan, by means of hybridisation (Yara 2006, Yara et al 2007). This underlines the need for careful monitoring of *T. sinensis* and its interaction with native *Torymus* species in Europe. At least so that, if hybridisation or host shifts should occur, the efficiency of *T.*

sinensis as a biological control agent remains effective and, in the wider sense, to observe unwanted effects on the native community.

1.5 Questions and Hypotheses

The arrival of *D. kuriphilus* in Europe - without observed natural enemies from its native range, lack of physical connection to its native range, and with the observed recruitment of 42 European parasitoid species to date - provides an excellent opportunity to test community assembly hypotheses as it expands its range throughout Europe. In this thesis I focus on the following questions:

1) What parasitoid species now attack *D. kuriphilus* in Europe and which native host-parasitoid assemblages do they come from? For reasons explained below, my ability to answer question (1) requires that the parasitoid assemblages associated with different potential source host plants differ in turn.

2) Do parasitoids attacking *D. kuriphilus* in Europe recruit locally, as previously observed for other invading cynipids in Europe? Discrimination between alternative recruitment processes in question (2) is linked to whether parasitoid communities differ between regions, as explained below.

To answer the questions above my results in Chapter 3 focus on whether parasitoid communities differ between hosts on different plants, or between geographic regions.

3) How have parasitoid assemblages changed over time since the arrival of *D. kuriphilus* in Europe? Is species richness increasing over time and how stable are the newly assembled parasitoid communities?

4) How does the use of DNA barcoding for a subset of morpho-species influence my answers to questions (1)-(3)?

I consider three hypotheses associated with questions 1 and 2.

The **Random Shift hypothesis** is a null hypothesis based on Hubbell's Neutral Theory of Biodiversity (2001). It postulates that enemies associated with an invading host represent a random subset of the available species pool. This pool would include all parasitoids, whatever hosts they feed on. This hypothesis would be rejected by the demonstration that parasitoids recorded from *D. kuriphilus* in Europe are a non-random subset of available species.

Alternatives to a neutral model are niche-based models, which predict that community membership is dependent on species niche requirements. In this case recruitment of enemies to a novel host is filtered according to host traits and the specific

requirements of each enemy species in the available species pool. For example, in *D. kuriphilus,* one form of filter might be that only parasitoids able to attack other cynipid galls have the traits necessary to exploit the invader. Niche based models can be considered along a continuum reflecting the strength of filtering of available species based on host traits. If only parasitoids with a very specific set of traits are able to exploit the new resource, then ecological filtering is strong and addition of parasitoids to the new host (recruitment) is rare. If, however, many parasitoids with a wide range of traits are able to exploit the new host, then ecological filtering is weak and recruitment occurs more readily. The opposing ends of this spectrum incorporate the two alternative niche-based hypotheses to the neutral model. Host tracking implies a strong ecological filter, while Local Recruitment implies a weaker filter.

The **Host Tracking hypothesis** predicts that host shifts occur very rarely (Community Phylogeography *sensu* Poulin 1999). In host-parasitoid terms, this is expected to result in a consistent set of associations, resulting from pursuit of the host through space and time (host tracking) by those parasitoid lineages able to switch to it. This hypothesis has been shown to apply over longer timescales to oak gall wasp associations with host plant lineages (Stone et al 2009) over the last 25 million years, as well as to associations between parasitoids and their cynipid hosts over the last 2 million years (Stone et al 2012), and to tracking of human-assisted range expansion of host-alternating oak gall wasps by the parasitoid *Megastigmus stigmatizans* (Hymenoptera: Chalcidoidea) (Nicholls et al 2010a).

Furthermore, Panzavolta et al (2013) released a record of parasitoid species reared from *D. kuriphilus* galls in Tuscany where the invader has arrived in 2008 (see table 1.1). Their records show reared parasitoid species to be a subset of approximately half of those *D. kuriphilus*-parasitoids reared in the site of origin in North Italy. This suggests that parasitoids may be tracking *D. kuriphilus* from the North of Italy to the South.

The **Local Recruitment hypothesis** (also termed the Local Host Shift hypothesis by Weiher & Keddy 1999) predicts that species shift to novel resources relatively frequently, with the resulting species assemblage on the new host reflecting the locally available species pool.

The extent to which these hypotheses can be differentiated depends on the extent to which local source communities for parasitoids available to attack *D. kuriphilus* vary in space, and between alternative source assemblages at a given site. If local parasitoid source assemblages show very low spatial variation, then the resulting parasitoid assemblages associated with chestnut galls could contain the same species in different locations

regardless of whether they result through Local Recruitment, Host Tracking or Random Shifts. However, if local parasitoids source assemblages vary in space, then the three hypotheses make differing predictions. The **Host Tracking hypothesis** predicts a very similar and specific set of parasitoids to be associated with *D. kuriphilus* in all locations. The **Local Recruitment hypothesis** predicts that membership of the *D. kuriphilus* community will reflect local variation in an ecologically specified (non-random) source pool. The **Random Shift hypothesis** predicts that membership of the *D. kuriphilus* community will constitute a random draw from the total locally available source pool.

If the parasitoid assemblages associated with alternative sets of hosts (in this thesis, galls associated with two different groups of oaks) are also different in a given location, then it may be possible to say which native community acted as the sources of natural enemies recruiting to *D. kuriphilus* in a given location, and to examine how consistent any pattern is across sites.

1.6 Thesis Outline

This thesis uses a combination of ecological surveys and DNA barcoding to investigate assembly of the parasitoid community attacking *D. kuriphilus* in Europe.

Chapter 2 describes the methods used, including field sites, gall sampling and rearing, my strategy for DNA-sequence based identification of taxa, and statistical analyses used.

Chapter 3 summarises my results relating to the community assembly hypotheses described above. Specifically I ask: (i) are native parasitoid communities structured by host oak section and/or geographical location? (ii) do parasitoid assemblages differ between native hosts and *D. kuriphilus*? and (iii) how have the parasitoids associated with *D. kuriphilus* changed over time in the original site of invasion (North of Italy). For each question, I compare the answers obtained using two alternative datasets: one using only adult morphological information (the morpho-dataset), and a second incorporating DNA barcode information for selected morpho-taxa (the barcode-informed dataset).

The discussion, Chapter 4, uses the results in Chapter 3 to assess the evidence for alternative models of community assembly in *D. kuriphilus.* I examine the impact of DNA barcoding for my conclusions, and set my findings in the context of previous research on oak gall and other ecological systems. I address the limitations of my approach, and suggest avenues for further research.

Chapter 2 – Methods

2.1 Fieldwork

2.1.1 Sampling Strategy and Gall Collection

I intended to capture the geographic distribution of *D. kuriphilus* as well as its range expansion in Europe over time (Figure 1.3). Previous studies have found that gall wasps and their associates naturally spread at a rate of 8-10km per annum, excluding human-facilitated movement or extreme winds (Stone & Sunnucks 1993, EFSA 2010, Gilioli et al 2013). Sites were therefore separated by at least 20km, situated in North Italy, Switzerland, Slovenia, Central Italy, Croatia and Sicily. *Dryocosmus kuriphilus* is thought to have been introduced to Europe in North Italy around 1996 (Brussino et al 2002). This is also where its biological control agent, *T. sinensis,* was introduced in 2005. In Switzerland, Slovenia and Central Italy, *D. kuriphilus* was first recorded in 2009. And discovery of the chestnut gall wasp in Croatia and Sicily dates to 2010 (EPPO 2015). Collections of oak and chestnut galls were carried out in a mixture of managed forests that contained both oaks and chestnuts as well as some abandoned chestnut groves within mixed forests containing oaks. Hawkins et al (1997) found that management degrees of a forest have no effect on the species richness of parasitoid assemblages and the different management types (mixed forests versus plantations) were treated as the same in this thesis.

Chestnut gall data available for this study cover collections from 2006 to 2013 depending on the geographic regions from which they were sampled (Table S2.1). Collections in the North of Italy were carried out twice every year since 2006 by collaborators at the Department of Exploitation and Protection of Agricultural and Forestry Resources (DIVAPRA) in Turin Italy. Their rearing data and sample specimens have been kindly made available for this thesis. *Dryocosmus kuriphilus* from Switzerland, Slovenia and Croatia were collected twice every year from 2010 until 2013 and reared by collaborators in the respective regions including Dr. Alexandre Aebi, Dr. Katarina Kos and Dr. Dinka Matosevic respectively to comply with phyto-sanitary recommendations for the pest (EPPO 2015). In Sicily, I collected *D. kuriphilus* galls in 2012 and 2013, which were then reared by collaborators at DIVAPRA.

Cynipid oak galls were collected in spring and autumn 2011 following the protocol in Box S2.1 and augmented in the spring of 2012 with further oak galls collected from Sicily (Table S2.2). Native oak species that were recorded at the sites included section Quercus *sensu stricto* (*Q. petraea, Q. pubescens, Q. robur*) and section Cerris (*Quercus cerris, Q. ilex, Q.*

suber). Oaks were identified to species whenever possible but this can be hard due to phenotypic plasticity and frequent hybridisation resulting in sometimes intermediate morphology of trees (Petit et al 2002, but see Kremer et al 2002). At each site I sampled all available oak species to ensure the collection of as broad a sample of oak cynipid galls as possible. Since previous studies have found that European parasitoid communities are structured by host oak section rather than species (Askew 1961, Bailey et al 2009), oak section was considered an appropriate alternative level of identification. Rose bushes (*Rosa canina*) were also surveyed for galls whenever present at the selected sites. The morphology of galls produced by the Cynipini represents an extended phenotype of the gall wasp larva (Stone & Cook 1998, Bailey et al 2009), and keys to Western Palaearctic species based on gall morphology are available (Buhr 1965, Ambrus 1974, Redfern & Shirley 2002). All collected galls comprised 1 rose gall wasp species and 63 oak gall wasp species. Nineteen of the encountered oak gall wasp species were collected in their sexual (spring) and asexual (autumn) generations meaning that out of 63 oak gall wasp species, 82 morphological gall types were collected. Collections were carried out between May and June for spring (sexual) generation galls and August-September for autumn (asexual) generation galls. At these stages of the season galls have matured but parasitoid emergences are still to commence. This collection method facilitates collection of all parasitoids, attacking at early and late stages of gall development, and will result in the broadest possible collection of community members (Askew 1980, Stone et al in prep). Previous studies have concluded that the minimum number of collected galls to obtain adequate data for robust analyses per species lies between 100 (Askew 1980) and 150 (Schönrogge 1994, unpublished PhD thesis). This would mean that species with a 5% attack rate would have a detection probability between 95 – 99%. Although individual gall species may not have been collected to that extent, due to patchiness of occurrence, pooling oak cynipid galls yielded well over 100 galls per site (~1900 galls in total per site, average number of galls per species and site ~70) this high number of pooled galls should reduce sampling bias caused by local or temporal disturbances despite increasing the variance of the pooled data in general. Galls were identified to species and generation, as spring and autumn generations of the same gall species have strikingly different morphology and different parasitoid communities associated with them. Autumn and spring galls were identified using a gall morphology picture guide used by the Stone group and corroborated between them and collaborators across Europe.

2.1.2 Rearing of Gall Inhabitants

After identification in the field, oak galls were reared individually as far as possible to facilitate quantification of each gall. Conversely, *D. kuriphilus* galls were reared in large mass rearings to maximise the probability of detecting parasitoids despite low reported attack rates (Quacchia et al 2013). In all cases galls were regularly checked for adult insect emergences as detailed in the fieldwork protocol (Box S2.1). Emerging insects were sorted by guild including inducers (Hymenoptera: Cynipini), inquilines (Hymenoptera: Synergini), chalcid parasitoids (Hymenoptera: Chalcidoidea) and, in the case of oak gall rearings, additional non-hymenopteran gall inhabitants (Diptera, Lepidoptera and Coleoptera). All insects were placed in 99% ethanol and are kept with the Stone Group at the University of Edinburgh. Chalcid parasitoids were further identified to species by myself using a morphological key for adult parasitoids of known European oak galls (Askew & Thuroczy unpublished). A representative subset of identified parasitoids was confirmed by Dr. George Melika (Hungarian Directorate of Plant Protection) - an established expert in the morphology of cynipids and their associates. Parasitoids reared from *D. kuriphilus* were identified by collaborators. Parasitoids reared from *D. kuriphilus* and sent to me for molecular identification had their morpho-identity confirmed by myself before molecular analyses.

2.2 Molecular Methods

2.2.1 Sample Selection for DNA Barcoding

To test the impact of morphological versus molecular identification on the analysis of community interactions I chose 4 morphologically identified parasitoid species that I considered most likely to contain cryptic lineages. These, out of all parasitoid species collected and reared during fieldwork, were chosen based on previous research. *Eupelmus urozonus* and *Eurytoma brunniventris* have been extensively investigated within my research group (Stone Group unpublished data) and *Megastigmus dorsalis* (Nicholls et al 2010b) and *Torymus flavipes* (Kaartinen et al 2010) have previously been published. All four of these species have been shown to contain morphologically conserved cryptic lineages. Furthermore, these species were recorded at all sites and possible re-allocation of individuals within them to discrete MOTUs was considered to have greatest potential to alter conclusions based on morpho-species distributions if molecular identification has any effect on the way we interpret community interactions. All four species were among the most abundant across my sampling area and of high relative importance for at least some community contrasts investigated in this thesis (see results chapter ahead).

I chose 20% of individuals of each target species in each region for sequencing: North (North Italy & Switzerland), Centre (Central Italy), South (Sicily) and East (synonymously the Balkans: Slovenia & Croatia). This strategy follows twice the 10% advocated for subsampling by Smith et al (2011) to reveal cryptic molecular lineages and misidentifications by means of molecular barcoding. Twenty per cent provides greater confidence when allocating the remaining 80% of specimens to MOTUs for statistical analyses based on molecular identification (see below). In practice, I achieved 16%, primarily due to DNA degradation and non-amplification in some samples (Table 2.1). This included a fairly even coverage of individuals across each candidate morpho-species (*E. urozonus* = 16.86%, *E. brunniventris* = 17.33%, *M. dorsalis* = 16.23% & *T. flavipes* = 18.79% sequenced) and each variable used in analyses: host plant (Quercus = 19.6%, Cerris = 18.46% & Castanea = 16.48%), region (Balkans = 17.25%, Sicily = 19.6%, Central Italy = 18.79% & North = 16% sequenced).

Table 2.1. Summary of sequencing success. The table includes samples assessed in the morphological species analysis, samples available for sequencing, samples chosen for sequencing and how many were and were not successful. Numbers have been divided into species and location (rows) as well as host plant (columns).
✓ = successful sequencing, ✗ = failed sequencing

	Tree Type	Quercus					Cerris					Castanea				
SPECIES	**Location**	No.	No. available	No. to sequence	✓	✗	No.	No. available	No. to sequence	✓	✗	No.	No. available	No. to sequence	✓	✗
E. urozonus	Balkans	12	12	12	12	0	2	2	2	2	2	261	197	40	35	5
	Sicily	13	13	5	5	0	5	5	4	4	0	1	1	1	1	0
	Central Italy	0	0	0	0	0	0	0	0	0	0	3	3	3	3	0
	North	3	3	3	3	0	0	0	0	0	0	561	49	49	37	12
E. brunniventris	Balkans	43	43	9	9	0	1	1	1	1	0	52	52	10	8	2
	Sicily	85	85	14	14	0	17	17	6	4	2	0	0	0	0	0
	Central Italy	28	28	10	8	2	0	0	0	0	0	0	9	0	0	0
	North	11	11	5	5	0	0	0	0	0	0	6	17	5	3	2
M. dorsalis	Balkans	19	19	19	18	1	0	0	0	0	0	296	166	45	31	14
	Sicily	70	70	10	10	0	6	6	6	6	0	0	0	0	0	0
	Central Italy	23	23	10	10	0	0	0	0	0	0	0	51	0	0	0
	North	4	4	4	4	0	0	0	0	0	0	1883	60	60	46	14
T. flavipes	Balkans	42	42	43	43	0	4	4	4	4	0	995	852	162	138	24
	Sicily	110	110	39	38	1	26	26	26	26	0	383	383	39	39	0
	Central Italy	12	12	9	9	0	1	1	1	1	0	0	59	0	0	0
	North	12	12	9	10	0	0	0	0	0	0	13	1	1	1	0

The individuals chosen to represent each morpho-species were selected in a hierarchical manner across regions, followed by host plants, given available samples. The 20% of individuals of each species from each region were subsampled across host plants (Quercus/Cerris/Castanea) based on proportions found on each. For example, if 100 *T. flavipes* individuals were found in a region, 60 from Quercus, 20 from Cerris and 20 from Castanea, then I would choose 20 individuals, comprising 12 from Quercus, 4 from Cerris, and 4 from Castanea for sequencing. Samples chosen for barcoding from Quercus and Cerris were then selected from as many available host galls as possible. Furthermore, samples were chosen from all available sites within each region, again, with an effort to represent collection sites proportionate to the incidence of samples.

Not all of the individuals represented in the data sets for the previous chapter were available for molecular work. If fewer individuals were available for barcoding than the target 20%, I sequenced as many individuals as possible from each tree type, gall and site following the proportional representation rules described above.

2.2.2 DNA Extraction and PCR Amplification

To extract DNA from each individual, a meta-thoracic leg (including coxa) was removed and placed in 50µL of chelex solution (5% chelex in double distilled water). This was then incubated over night with 5µL of 10mg/mL Proteinase K at 37°C and subsequently heated to 95°C for 15 minutes to deactivate the enzyme. This method has the advantage of leaving the specimen relatively intact to preserve a morphological reference while still producing ample DNA for molecular analyses. Different primers were used for each species to produce the barcode DNA sequence for each individual. For *E. urozonus* and *T. flavipes* I amplified a COI fragment (mtDNA) overlapping with the 3′ end of the Folmer fragment (the classic DNA barcode based on Hebert et al (2003)) by 458bp and can hence be used for wider analyses within the BOLD community (Ratnasingham & Hebert 2007). COI PCR was performed with either forward primer COI_pF2 (5′ ACC IGT DAT RAT RGG DGG ITT YGG DAA TT 3′) or forward primer COI_pF1 (5′ AGG RGY YCC WGA TAT AGC WTT YCC 3′) and reverse primer COI_2413d (5′ GCT ADY CAI CTA AAA ATY TTR ATW CCD GT 3′). COI_2413d has been developed within the Stone lab (Nicholls unpublished) from primer C1-J2441 (Simon et al 1994) creating a 656-698 base-pair fragment depending on whether it is paired with the pF1 or pF2 forward primer respectively (both forward primers were designed by Nicholls unpublished). These tailored fragments avoid a poly-T sequence present at the 5′ end of the classic Folmer regions in many chalcid parasitoids. Such a

repetitive sequence causes slippage of the *Taq* polymerase used for DNA amplification while copying the DNA strands and results in undecipherable sequence data.

For *E. brunniventris* and *M. dorsalis* the COI region mentioned above is more problematic to sequence and a cytB fragment (mtDNA) has proven more successful (Nicholls et al 2010a&b, Stone Group unpublished data). CytB PCR was carried out using primers CB1 (5′ TAT GTA CTA CCA TGA GGA CAA ATA TC 3′) as a forward primer and either CB2 (5′ ATT ACA CCT CCT AAT TTA TTA GGA TA 3′) or CP2 (5′ CTA ATG CAA TAA CTC CTC C 3′) as a reverse primer. The primer combination CB1/CB2 produces a 433 base-pair fragment whereas the primer pair CB1/CP2 produces a slightly longer fragment in the 3′ direction than CB1/CB2. Primers CB1 and CB2 were originally designed by Jermiin & Crozier (1994) and primer CP2 was designed by Auger-Rozenberg et al (2006) together with a CP1 forward primer not used in this thesis. The concentrations of reagents and PCR conditions used are listed in box S2.2.

PCR products were checked for quality by running them on a 2 % agarose gel with 1.5µL SYBRSafe per 40mL agarose solution for 30 min at 120V. Running the gel at this speed gave a good resolution to interpret bands confidently using a UV light imager. If bands were too faint (suggesting some but not enough DNA fragments within the PCR product for sequencing) the PCR products were rePCRed with the same procedure as stated in box S2.2 replacing DNA extracts with PCR products from the previously insufficient PCR run. These products contained the desired DNA fragment already and a second run ensured enough product for sequencing.

PCR products were cleaned by adding 2.5 µL SAP/Exo solution (1µL Shrimp Alkaline Phosphatase (SAP), 0.075µL Exonuclease 1 (Exo1) & 1.425 µL dilution buffer) to each sample and incubating at 37°C for 40 min followed by incubation at 94°C to deactivate the enzyme. Clean PCR products were PCRed in the forward direction using the corresponding PCR forward primer and ABI BigDye (Perkin Elmer Biosystems, Waltham, MA). BigDye fragments were submitted to the Edinburgh Genomics Facility for sequencing. Returned sequences were edited using Sequencher (version 5.3, Gene Codes Corporation, Ann Arbor, MI USA). Fragments were cut to variable lengths based on primers used and read quality of fragments. All fragments for each species were combined into a fasta file using Sequencher. The file produced was augmented with samples of the same species previously sequenced within the Stone group and as many species of the same genus as possible were added as well. Reference samples obtained from collections carried out within the Stone Group included a broad spectrum of parasitoids previously tested for correct species and lineage allocation. Reference samples were used to ensure that samples would be allocated to the right molecular taxonomic unit as far as can be known. Reference samples

included previously tested individuals from across Europe ranging from Ireland to Iran across longitudes (as well as some Eastern Asian and Northern American samples in the case of *Torymus*) and from Southern Spain to Finland across latitudes. Reference samples also included specimens from as wide a spectrum of section Cerris oak and section Quercus oak gall species as well as rose gall parasitoids where available (and chestnut gall parasitoids species in the case of *Torymus*). Sequences were aligned using MUSCLE (Edgar 2004) and converted to nexus format. Alignments were checked for quality by eye and trimmed to a standard length of 565 bp for *Eupelmus,* 607bp for *Torymus*, 394 bp for *Megastigmus* and 404bp for *Eurytoma.*

2.2.3 Allocation of Specimens to Molecular Operational Taxonomic Units

The aligned nexus file of each genus was introduced to jMOTU (Jones et al 2011) and analysed using recommended settings. jMOTU was set to allocate samples to molecular operational taxinimic units (MOTUs) according to 1-20% sequence divergence within clusters. BLAST identity filter was set to 95%. Sequence alignment overlap was set to the default 60% but since all sequences had previously been trimmed, overlap was 100% in all cases. jMOTU analyses were run and output (including visualisations and MOTU allocations based on all defined divergence scenarios) saved.

After MOTU analyses were carried out, the graphical representation of the output was assessed in search of a barcoding gap. Barcoding gaps are an apparent plateau in a graph that plots number of MOTUs observed against the per cent of base-pair differences between the assessed sequences. When a plateau is reached MOTUs do not change despite an increase in per cent differences between fragments. These correspond to deep splits in a phylogenetic tree and suggest the presence of divergent clades amongst a given number of samples (see Box 1, Chapter 1). If a barcoding gap was present this was used to re-allocate specimens to MOTUs as detailed in the results section below. If no barcoding gap was present a 2% cut-off was used, following Avise and Walker (1999).

The molecular data produced together with reference samples and outgroups were reduced to haplotypes using FaBox (Villensen 2007) a free online haplotype collapsing tool. The resulting haplotype alignment was converted to nexus format and introduced to PAUP* (Swofford 2002) to build distance-based neighbour joining (NJ) trees. These trees may not be rigorous enough to define species, as is the case for single-locus definition of MOTUs used here, but they are adequate to allocate individuals to the right clades to then compare them with the extensive list of reference samples used in this study. NJ trees were used to quality check the outcome as illustrated in box 1 of chapter 1 by checking MOTU correspondence to splits in the NJ tree.

Morphological data used in this thesis were altered by re-assigning morphologically identified specimens of the four chosen species to MOTUs identified during jMOTU analyses. The number of individuals of each morpho-species associated with a given host tree in a given region was then multiplied by the proportion of each MOTU representing it. MOTUs were named after the reference samples they grouped with or, when grouping on their own, kept their numerical reference. Morphological data of the four species was then replaced by their MOTU data for the molecular part of analyses below and statistical analyses were carried out in the same way for both, the purely morphological and the barcode-informed versions of my dataset.

2.3 Statistical Analyses

2.3.1 Summary Statistics Used

In my data, I investigate the extent to which parasitoid assemblages differ between host plant section (oak section Quercus *sensu stricto* vs. section Cerris), between host plant genera (Quercus vs. Castanea), and between regions. Oak section, initially incorporated as a factor in analysis of parasitoid community structure (below), was later excluded from further analyses of parasitoid communities associated with native (oak) and invading (chestnut) gall wasp hosts. I also investigate differences between parasitoid associations with *D. kuriphilus* over time. I explore these patterns by comparing the number of species present in each assemblage (species richness), how evenly individuals are distributed amongst species (effective species richness) and species compositions (multivariate log-abundances) associated with parasitoid assemblages between host plants, regions and years since invasion. The attributes used are described in more detail below.

Species richness is the simplest measure of community diversity. It is the sum of all species represented in a set of interacting species (Jost 2006, Ricklefs 1987). Here, species richness is the sum of parasitoid species recorded from samples of galls at the appropriate scale (oak section, tree genus, region, time since invasion).

The effective species richness is an estimate of species richness given the assumption that all species are equally abundant and therefore gives an estimation of the evenness of species distribution within a multi-species assembly when compared with species richness. It is calculated as the exponential of the Shannon-Wiener diversity index.

$$\exp(H') \quad \exp(\quad \sum_{i=1}^{S} p_i \ln(p_i)) \qquad \textbf{(eqn 1)}$$

In the above equation S is the total number of species in all samples and p_i is the proportion of individuals belonging to the ith species. While the Shannon-Wiener index estimates the richness and heterogeneity of a community in one number, the exponential of this index converts it back into an effective species number standardised by heterogeneity (Jost 2006). Effective species richness and species richness would be the same if all species in a given environment were equally abundant. Where abundances are uneven, effective species richness is lower than the direct count. The two measures represent a raw and a standardised version of species richness where the difference between the two reflects the unevenness in the species abundance distribution (Jost 2006).

Species richness and effective species richness were analysed as generalised linear models (GLM) in R. For species richness a model with quasi-Poisson errors, accounting for over-dispersion, and a log-link function was fitted. In contrast to Poisson errors, a quasi-Poisson error distribution assumes the variance of the data to be a linear function of its mean as was the case for my data. The model was checked for heteroscedasticity and normality of errors by inspecting mean-variance plots and normality (Q-Q) plots to confirm an appropriate fit (Crawley 2007). The analysis was then carried out using a standard F-test. The effective species richness was analysed in the same manner but with a Gaussian error distribution and an identity link function, which provided the best match for the model assumptions for heteroscedasticity and normality.

The analysis of differences in species composition between assemblages was carried out in the form of multivariate log-abundance general models (mvGLMs), which are an extension of standard univariate generalized linear models (Warton 2011, Wang et al 2012). It allows assessment of the impacts of explanatory environmental variables in a standard modelling framework where multivariate abundance data represent clusters and the number of species defines a cluster size. Clustered data like this are often analysed by Generalised Estimating Equations (GEE) for instance in microarray analyses or analyses of time series. The methods devised by Warton (2011) allow the application of the same principles to multivariate cluster analyses where n, the number of species in the community, can be large in relation to K - the number of communities in the analysis. The details of the methods are described by Warton (2011), and I follow their use described in Wang et al (2012) as implemented in the package *mvabund* in R (R Core Team 2013). Power analyses have shown that mvGLM is considerably more sensitive than other standard methods of multivariate analyses based on distance matrices (Wang et al 2012, Warton 2011, Oksanen et al 2013, Moorhouse et al 2013). Sampling sizes of individual gall types varied considerably ranging from one to hundreds and in the case of *D. kuriphilus* thousands due to the natural patchiness in both tree and gall distributions. The total number of parasitoids reared per

sample was used as an offset in all models to account for the sample size – species richness relationship (equivalent to species area relationships Preston 1962a&b). Using the number of parasitoids in a given sample as an offset weighs the number of species against the number of parasitoid individuals reared within a given factor before assessing the power this factor has as an explanatory variable.

Community differences were analysed as mvGLMs using the R package *mvabund* (Wang et al 2013). After assessing explanatory variables independently models were fitted with the same interaction term and the same offset as the analyses above. Models were fitted with negative-binomial error distribution and checked for an appropriate fit by inspecting Q-Q plots and residual plots for mean – variance independence. Significance was tested using likelihood-ratio tests, calculated using 999 bootstraps.

Multivariate GLMs are difficult to visualise and I follow examples in the literature (Nooten et al 2014) by plotting non-parametric multi-dimensional scaling (NMDS) results based on a Bray Curtis similarity matrix as implemented in the package *vegan* (Oksanen et al 2013). An NMDS visualises the degree of clustering (dispersion) or differentiation (location) of samples by reducing the stress (mis-match) between the multiple dimensions associated with the Bray-Curtis dissimilarity matrix and a two-dimensional graphical space. The data then represented in a two-dimensional space can be more easily interpreted visually such as illustrated in figure 2.1.

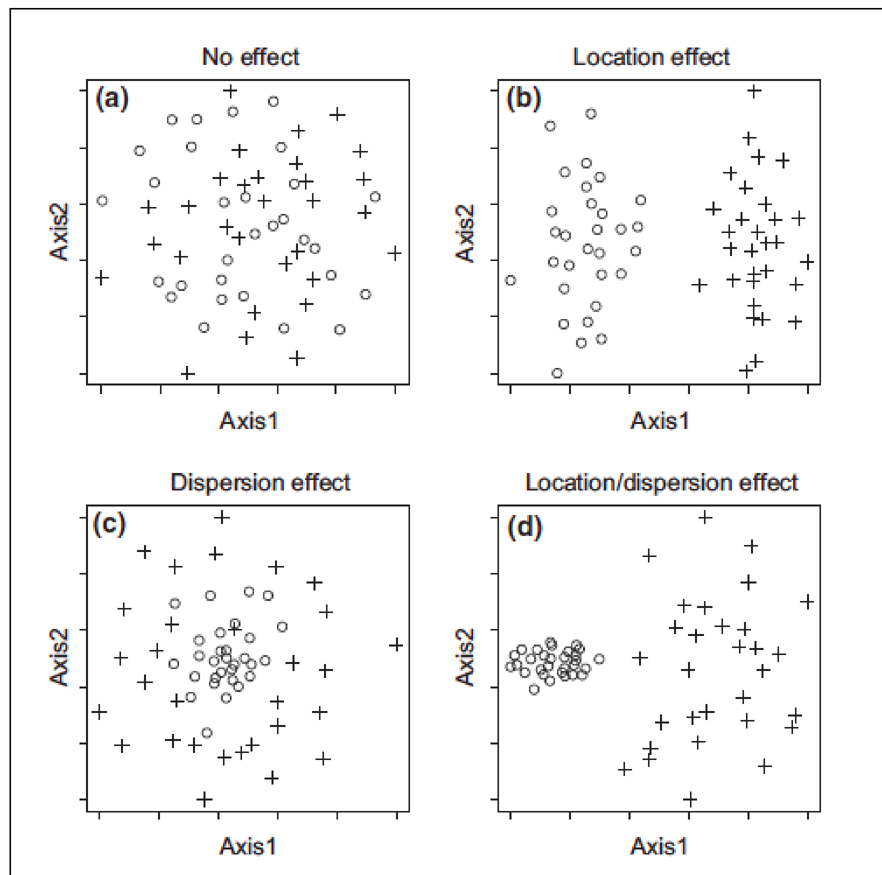

Figure 2.1 Non-metric Multi-Dimensional Scaling (NMDS) interpretation guide. Multivariate data plotted on an NMDS graph can reveal multivariate compositional community patterns by re-arranging the data in a 2-dimensional space. On the plot more similar data points group more closely together and hence the illustration gives information about dispersion (meaning variance of the data) and location (association with a particular factor). This illustration was taken from Warton *et al* (2012).

2.3.2 Subsets of Data Used to Define Parasitoid Assemblages

Qualitative observations of the parasitoid assemblages associated with galls on oak and chestnut

This section serves to make initial qualitative observations about the parasitoid community encountered during this study. I used parasitoid samples from all sites collected by myself and collaborators from 2011-2013 to make initial inferences about the available oak gall parasitoid pool and their potential association with chestnut galls.

Investigating traits that structure native parasitoids of oak cynipid galls on section Quercus and section Cerris trees

Any attribution of parasitoid recruitment from galls on oak to section Quercus or section Cerris requires differences in parasitoid association between the two. These differences are being explored here. In this analysis I used parasitoid samples from all sites collected in spring 2011 and autumn 2011, as well as samples from Sicily collected in spring 2012 to assess any structure in the available oak gall parasitoid community.

Comparison of parasitoid assemblages associated with *D. kuriphilus* and native oak associated gall wasps

This analysis comprised the oak gall wasp data specified above as well as parasitoids reared from *D. kuriphilus* galls from all regions collected in 2011 and 2012 and additional collections from Sicily from 2013.

Community assembly of parasitoids attacking *D. kuriphilus* over time

This analysis included parasitoid data from *D. kuriphilus* collections at sites that were revisited every year from 2006 – 2011 (Robilante, Peveragno and Boves in North Italy).

Chapter 3 – Results

Section 3.1 summarises information on the community composition of oak- and chestnut-associated cynipid gall communities, based on adult morphology. I summarise qualitative patterns in parasitoid community richness across plant taxa, and across the sampled geographic regions. In section 3.2, I summarise the results of DNA barcode analysis of four focal parasitoid morpho-species, detailing the way in which sequence data changes parasitoid species allocations. Section 3.3 extends the barcoding results to the creation of a new barcode-informed parasitoid dataset, and repeats the plant- and geography-based qualitative analyses of section 3.1, highlighting changes that arise through barcoding of specific morpho-taxa. Section 3.4 presents statistical analyses of patterns in parasitoid assemblages between plant taxa, geographic regions, and through time since the introduction of *D. kuriphilus* in North Italy.

3.1 Qualitative Analysis of the Parasitoid Community Using Morpho-taxa

Table 3.1 summarises all parasitoids reared from the years 2011-2013 for all 4 sampling regions and all 3 host plant taxa. Overall, 51 parasitoid morpho-species were associated with the three host tree taxa. Samples that could only be identified to genus (primarily due to low sample quality) are included in the table but only individuals identified to species were included in statistical analyses (section 3.4).

Table 3.1 List of reared parasitoids across regions and host trees. Data collected on all host tree types from 2011 until 2013. Parasitoids in the table are grouped by family. Parasitoids that could only be identified to genus are at the end of the table.

	Region	**EAST** Slovenia & Croatia			**NORTH** North Italy & Switzerland			**CENTRE** Central Italy			**SOUTH** Sicily		
	Host Plant	**Castanea**	**Cerris**	**Quercus**	**Castanea**	**Cerris**	**Quercus**	**Castanea**	**Cerris**	**Quercus**	**Castanea**	**Cerris**	**Quercus**
	Galls Reared	11761	249	1113	8000	25	336	3000	70	266	5607	383	1859
	Species Recorded	19	13	27	15	6	22	7	6	8	12	16	33
Parasitoid Species	**Family**												
Eurytoma brunniventris	Eurytomidae	40	1	43	6		11			28		17	81
E. pistacina	Eurytomidae			13	558	2	2						25
Sycophila biguttata	Eurytomidae	27	25	2	17		7	5	9		24	40	21
S. binotata	Eurytomidae		2				1					3	
S. variegata	Eurytomidae	5			40			5	3			53	17
Megastigmus dorsalis	Torymidae	88		19	1782		4			23		6	70
M. synophri	Torymidae				1								
Torymus auratus	Torymidae	5		6			18				3		261
T. cerri	Torymidae						2	1					
T. cyaneus	Torymidae				27								
T. flavipes	Torymidae	601	4	42			12		1	12	383	26	109
T. formosus	Torymidae	1											
T. geranii	Torymidae	12					1						6
T.nobilis	Torymidae	1											5
T.scutellaris	Torymidae	1											
Ormyrus nitidulus	Ormyridae		2	1									10
O. pomaceus	Ormyridae	165	13				7				1		6
Cecidostiba atra	Pteromalidae			3									3
C. fungosa	Pteromalidae		68	18		5	2					10	26
C. ilicina	Pteromalidae												2
C. saportai	Pteromalidae			2		1							
C. semifascia	Pteromalidae			34			2						7
Cyrtoptyx robustus	Pteromalidae										5		
Hobbya stenonota	Pteromalidae												66

Table continued on the next page

Mesopolobus albitarsus	Pteromalidae			6			1			1			3
M. amaenus	Pteromalidae		3	7					2	1		1	1
M. dubius	Pteromalidae	6			1		1		4		3		24
M. fasciiventris	Pteromalidae	4		4	13								26
M. fuscipes	Pteromalidae			2			1						
M. lichtensteinii	Pteromalidae			1							2		
M. mediterraneus	Pteromalidae			7							1		5
M. sericeus	Pteromalidae	25		21			1				36		24
M. tarsatus	Pteromalidae											1	5
M. tibialis	Pteromalidae	142	1	6	2					4	376	2	23
M. xanthocerus	Pteromalidae		4	1		1	3						17
Ormocerus latus	Pteromalidae			1				1					
Eupelmus annulatus	Eupelmidae	12		4	512	1	5	130		1	2	3	102
E. rostratus	Eupelmidae				141								
E. splendens	Eupelmidae				1			83					
E. urozonus	Eupelmidae	84	2	12	544		3	3			1	5	13
Macroneura vesicularis	Eupelmidae	1											
Aulogymnus arsames	Eulophidae						1						
A. eudereschus	Eulophidae											1	1
A. gallarum	Eulophidae		10			2	38						42
A. skianeuros	Eulophidae	1	7	5	180		1					1	1
A. testaceoviridis	Eulophidae											2	
A. trilineatus	Eulophidae												4
Aprostocetus aethiops	Eulophidae			3					1				
A. cerricola	Eulophidae			4									
Baryscapus pallidae	Eulophidae			1									2
Pediobius rotundatus	Eulophidae									2		7	27
Samples IDed to genus only													
Sycophila sp	Eurytomidae			11			11			3			32
Megastigmus sp	Tormyridae	1											
Torymus sp	Tormyridae	4		17			5				1		
Ormyrus sp	Ormyridae			22									
Cecidostiba sp	Pteromalidae			18						11			20

Table continued on next page

Mesopolobus sp	Pteromalidae						1	2		6			
Eupelmus sp	Eupelmidae						1	2			3		
Aulogymnus sp	Eulophidae									1			
Aprostocetus sp	Eulophidae		1										
Baryscapus sp	Eulophidae			8									
Pediobius sp	Eulophidae												1

3.1.1 Comparison of Parasitoid Assemblages Between Host Plants

Of all 51 morpho-species in table 3.1, 43 (84.3%) were sampled from oak galls while 29 (56.9%) were sampled from *D. kuriphilus* galls. Of the 43 parasitoid morpho-species reared from oak galls, almost all taxa (42, 97.7%) were reared from galls on section Quercus oaks, while only 25 (55.8%) attacked section Cerris galls. 19/43 morpho-species (44.2%) were found only on section Quercus but not section Cerris, while only 1/43 (2.3%), *Aulogymnus testaceoviridis*, was recorded from galls on section Cerris trees but not section Quercus. This is considered a sampling artefact as *A. testaceoviridis* is known, from previous research, to also attack galls on section Quercus oaks (Askew et al 2013).

Twenty-one parasitoid morpho-species (41.2%) were reared from both, oak galls and *D. kuriphilus*. Twenty-two (43.1%) parasitoid taxa were only reared from oak galls while 8 (15.7%) were reared only from *D. kuriphilus.* Of the 8 private parasitoid species I reared from *D. kuriphilus*, 7 are known from other studies to primarily attack oak galls. Two interesting species among these 7 are *Megastigmus synophri*, which has previously only been reared from cynipid galls on section Cerris oaks (Askew et al 2013), and *Eupelmus splendens*, which is primarily known from the galls of the sycamore gallwasp *Pediaspis aceris,* (Askew et al 2006). *E. splendens* has one record of being associated with section Cerris oaks (Noyes 2015), though this record is not confirmed by the largest survey of oak gall parasitoids to date (Askew et al 2013). Community summaries of parasitoid morpho-taxa associated with each host plant taxon are shown in table 3.3, section 3.3, with quantitative analysis in section 3.4.

3.1.2 Comparison of Parasitoid Assemblages Between Regions

Parasitoid morpho-species varied qualitatively among regions (Table 3.4 assessed quantitatively in section 3.4). Oak cynipid parasitoid richness was lower in the Central region (12 taxa) than in the other three regions (23 in the North, 30 in the East and 35 in the South). In comparison to the other three regions, for example, oak galls in Central Italy lacked many Torymidae, virtually all Pteromalidae and virtually all Eulophidae.

The richness of parasitoid species reared from *D. kuriphilus* varied among geographic regions, and of the 51 taxa in the morpho-dataset, a maximum of 19 taxa (37.3%) were recorded in a single region – the East. Lower proportions of the total richness were recorded in the North (15 taxa, 29.4%), Centre (7 taxa, 13.7%) and South (12, 23.5%).

The proportion of oak-reared parasitoid taxa that were also recorded from *D. kuriphilus* varied widely among regions. Near the invasion origin in the North, almost 35% of parasitoid taxa reared from oak galls in that region were also reared from *D. kuriphilus*, with corresponding values for the East, Centre and South of 40%, 33% and 29% respectively.

Overall 21 of 43 parasitoid morpho-species (48.8%) found on oak across the entire study area were also found attacking the new invader.

The sets of species attacking *D. kuriphilus* varied among regions. For example, of the 29 species attacking *D. kuriphilus* throughout my sampled range, 14 (48.3%) were not found around its introduction site in Northern Italy, i.e. parasitoid communities from central and southern Italy are not subsets of those recorded for Northern Italy the area where *D. kuriphilus* was first recorded. Species recorded at southern locations, but not in the North are likely to have been recruited from the local species pool.

Widely distributed parasitoid morpho-species are over-represented in the community attacking *D. kuriphilus*. The number of regions occupied by a parasitoid species is roughly uniformly distributed for my morpho-taxa sampled on oak: 10 morpho-species (19.6% of the total of 51) occur in all 4 regions, 13(25.5%) in 3, 15(29.4%) in 2 and 13(25.5%) in 1. However, widely distributed species are over-represented in the set that attack *D. kuriphilus*. 9/10 (90%) species found in all 4 regions attack *D. kuriphilus,* while the proportions of species found in 3, 2 and 1 region are lower at 7/13 (53.85%), 6/15 (40%) and 7/13 (53.85%) respectively.

3.2 Barcode-informed Identification of Four Focal Species

Use of DNA barcode information for the 4 candidate parasitoid morpho-species divided each into 3-8 MOTUs (*Eupelmus urozonus* 5 MOTUs, *Eurytoma brunniventris* 8 MOTUs, *Megastigmus dorsalis* 3 MOTUs, *Torymus flavipes* 8 MOTUs). DNA barcode identification resulted in three types of change, each of which applied to most or all of the 4 candidate species. Overall, DNA barcoding increased parasitoid species richness from 51 (morpho-species only) to 66 (morpho-species and MOTUs). DNA barcode identification resulted in three types of change:

(a) transfer of some morpho-specimens to other species already present in the morpho-community. This changed relative abundance between the species in question but not overall species richness (*Eupelmus*, *Eurytoma*, *Torymus)*

(b) sequence-based identification of taxa not identified in my morphological analysis, but which can be matched to Linnean species using reference sequences. This issue applied to all 4 morpho-species and added 10 species overall (*Eupelmus fulvipes*, *Eurytoma adleriae*, *E. hypochoeridis*, *Megastigmus dorsalis* species A/B, *M. stigmatizans*, *Torymus affinis*, *T. auratus*, *T. cyaneus* species 3, *T. rubi*, and *T. sinensis*).

(c) sequence based identification of morphologically cryptic taxa that do not group with any available reference sequence. These specimens may belong to known species for which no

reference sequence exists, or represent entirely new taxa. Specimens belonging to this group added 7 species to total species richness (*Eupelmus, Eurytoma*, *Torymus*).

I detail the impacts of DNA barcoding for each of the four candidate species in turn below. Table 3.2 gives the number of individuals for each MOTU of each species by host plant taxon and region. Combined morphological and MOTU-based identification of parasitoid taxa is summarised in table S3.1.

Table 3.2 Morpho-species assigned to MOTUs. The table shows which MOTU has been identified within each morpho-species and which known lineages they correspond to when available. The table also shows which MOTUs could not be allocated to known lineages. Numbers show how species associations to trees and regions have changed due to morpho/MOTU re-allocations.

			EAST Slovenia & Croatia			**NORTH** North Italy & Switzerland			**CENTRE** Central Italy			**SOUTH** Sicily		
Morpho-Species	**MOTU**	**Molecular Identification**	**Castanea**	**Cerris**	**Quercus**	**Castanea**	**Cerris**	**Quercus**	**Castanea**	**Cerris**	**Quercus**	**Castanea**	**Cerris**	**Quercus**
Eupelmus urozonus (5 MOTUs)	**TOTAL**	Individuals	36	2	14	38	0	3	3	0	0	1	4	9
		MOTUs	2	1	2	4	0	2	2	0	0	1	2	1
	MOTU0002	***E. urozonus***	9	2	13	12		2	2			1	3	9
	MOTU0003	***E. fulvipes***	27			22		1	1					
	MOTU0004	***E. annulatus***			1	3								
	MOTU0008	**Unknown**				1								
	MOTU0015	**Unknown**											1	
Eurytoma brunniventris (8 MOTUs)	**TOTAL**	Individuals	8	1	18	3	0	5	0	0	8	0	4	14
		MOTUs	2	1	2	1	0	2	0	0	2	0	1	4
	MOTU0001	***E. adleriae***			1						1			2
	MOTU0002	***E.brunniventris***	3		17			4			7		4	10
	MOTU0013	**Unknown**				3								
	MOTU0026	***E. hypochoeridis***	5											
	MOTU0035	**Unknown**												1
	MOTU0047	**Unknown**												1
	MOTU0053	***E. pistacina***						1						
	MOTU0068	**Unknown**		1										
Megastigmus dorsalis (3 MOTUs)	**TOTAL**	Individuals	31	0	18	46	0	4	0	0	10	0	6	10
		MOTUs	3	0	3	1	0	1	0	0	1	0	2	2
	MOTU0001	***M. dorsalis* spA**	16		8						10		3	8
	MOTU0002	***M. dorsalis* spB**	15		9	46		4					3	2
	MOTU0006	***M. stigmatizans***			1									
Torymus flavipes (8 MOTUs)	**TOTAL**	Individuals	137	4	40	1	0	10	0	1	9	40	26	38
		MOTUs	6	1	2	1	0	1	0	1	1	3	1	3
	MOTU0003	***T. auratus***	6											11
	MOTU0004	***T. flavipes* sp1**	111		39			10			9	37		25
	MOTU0005	***T. geranii***	7											
	MOTU0006	***T. rubi***	2									2		
	MOTU0007	***T. cyaneus* sp3**	9	4	1					1			26	2
	MOTU0010	***T. affinis***										1		
	MOTU0011	***T. sinensis***				1								
	MOTU0012	**Unknown**	2											

3.2.1 *Eupelmus urozonus*

A phylogenetic tree for the *Eupelmus* DNA barcode sequences is shown in Figure S3.2. *Eupelmus* showed a barcoding gap between 3% and 10% sequence divergence (Figure 3.1). Using a 3% cut-off for MOTU discrimination, individuals morphologically identified as *E. urozonus* in table 3.1 were divided into 5 MOTUs (Table 3.4). MOTU0002 represented the Linnean species *E. urozonus* (48.18% of morpho-specimens). Two MOTUs were matched to reference sequences for other *Eupelmus* species. MOTU0004 was matched to *E. annulatus* (3.64%), already recorded in the community, while MOTU0003 was matched to a previously unrecorded species, *E. fulvipes* (46.36%). Two MOTUs, MOTU0008 (0.91%, closest to *E. annulatus* within the genus) and MOTU0015 (0.91%, closest to *E. cerris*) did not match any available *Eupelmus* reference sequences and hence remain unknown here.

DNA barcoding of *Eupelmus urozonus* thus divided specimens among 5 taxa: *E. urozonus, E. annulatus,* and 3 new taxa (*E. fulvipes* and 2 unidentified taxa). Two taxa (*E. urozonus* and *E. fulvipes*) contributed over 90% of specimens.

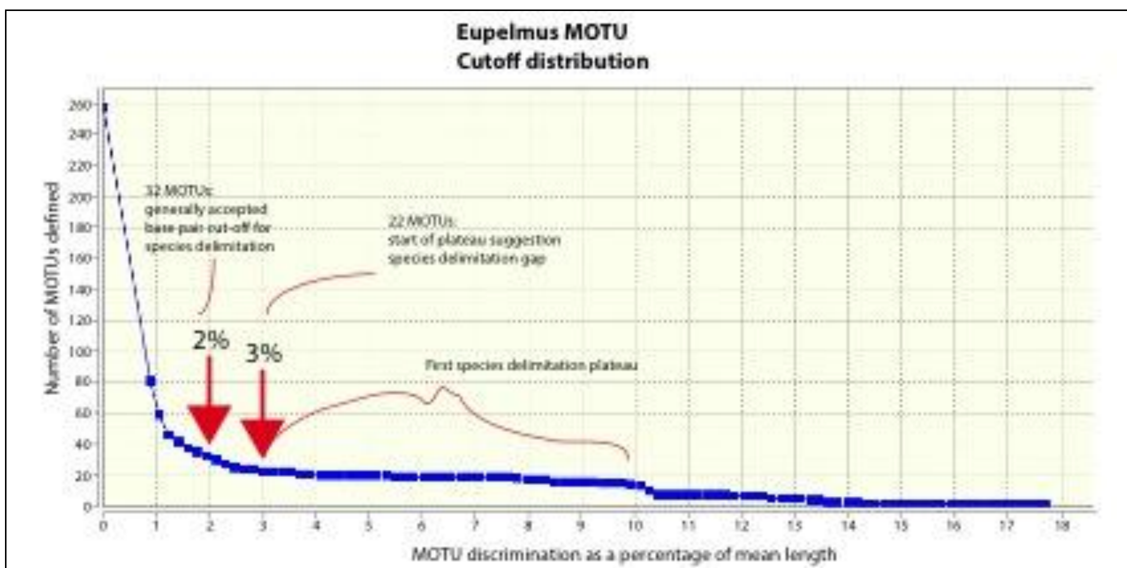

Figure 3.1 Per cent differences in base-pairs versus MOTUs for Eupelmus. MOTUs are defined by jMOTU. Annotations show where the generally accepted species cut-off lies, indicating how well this corresponds with the data specific cut-off. The cut-off for *Eupelmus* lies at 3% (17bp), which is at the beginning of the first plateau. This corresponds well with MOTU allocations to clades in the supplementary figure S3.2.

3.2.2 *Eurytoma brunniventris*

A phylogenetic tree for the *Eurytoma* DNA barcode sequences is shown in figure S3.3. The *Eurytoma* dataset showed no identifiable barcoding gap (Figure 3.2). I therefore split morphologically identified *E. brunniventris* into MOTUs using the widely accepted 2% sequence divergence threshold (see Chapter 2, Methods). This split *E. brunniventris* into 8

MOTUs (Table 3.2). MOTU0002 grouped with reference sequences for the Linnean species *E. brunniventris* (73.77% of morpho-specimens). Three MOTUs were matched to reference sequences for other *Eurytoma* species. MOTU0053 was matched with *E. pistacina* (1.64%), already recorded in the community, while MOTU0001 grouped with *E. adleriae* (6.56%) and MOTU0026 with *E. hypochoeridis* (8.20%), both previously unrecorded. MOTU0068 (1.64%) did not group with any reference samples but was placed within the Eurytoma clade. Another 3 MOTUs, MOTU0013 (5%), MOTU0035 (1.6%), MOTU0047 (1.6%) fall outside the Eurytoma clade and may not belong to this genus. DNA barcoding of *Eurytoma brunniventris* thus divided specimens among 8 taxa: *E. brunniventris, E. pistacina,* and six new taxa (*E. adleriae, E. hypochoeridis* and 4 unidentified taxa). True *E. brunniventris* contributed almost 75% of all specimens.

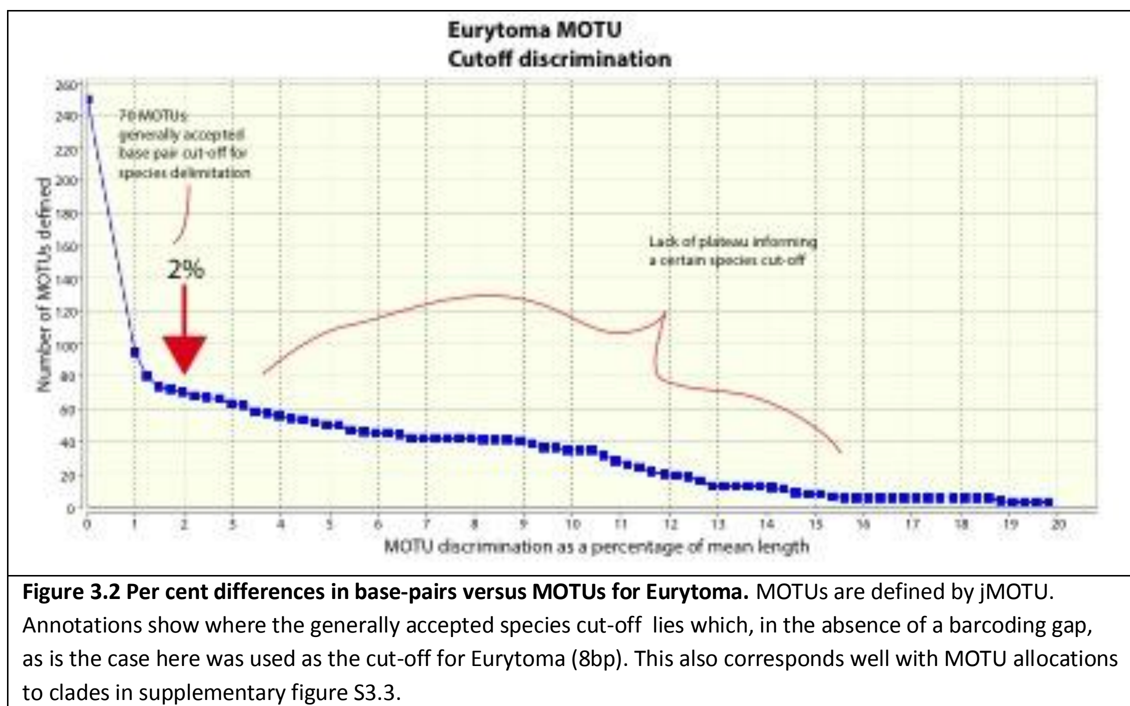

Figure 3.2 Per cent differences in base-pairs versus MOTUs for Eurytoma. MOTUs are defined by jMOTU. Annotations show where the generally accepted species cut-off lies which, in the absence of a barcoding gap, as is the case here was used as the cut-off for Eurytoma (8bp). This also corresponds well with MOTU allocations to clades in supplementary figure S3.3.

3.2.3 *Megastigmus dorsalis*

A phylogenetic tree for the *Megastigmus* DNA barcode sequences is shown in figure S3.4. *Megastigmus* showed a barcoding gap between 2% and 6% (Figure 3.3). Morphologically identified *M. dorsalis* were split into 3 MOTUs according to a 2% cut-off (Table 3.4). The 3 MOTUs comprised of 2 cryptic lineages known within the *M. dorsalis* morpho-species (Nicholls et al 2010b) with MOTU0001 representing *M. dorsalis* species A (36.00%) and MOTU0002 representing *M. dorsalis* species B (63.20%). MOTU0006 grouped with *M. stigmatizans* (0.80%). DNA barcoding of *M. dorsalis* thus divided specimens among 3

taxa: *M. dorsalis* species A and B, and 1 new taxon (*M. stigmatizans*). This increases overall species richness by 2. *Megastigmus dorsalis* species A represented over 60% of all sampled individuals while the remainder, species B, comprised all but one specimen (*M. stigmatizans*).

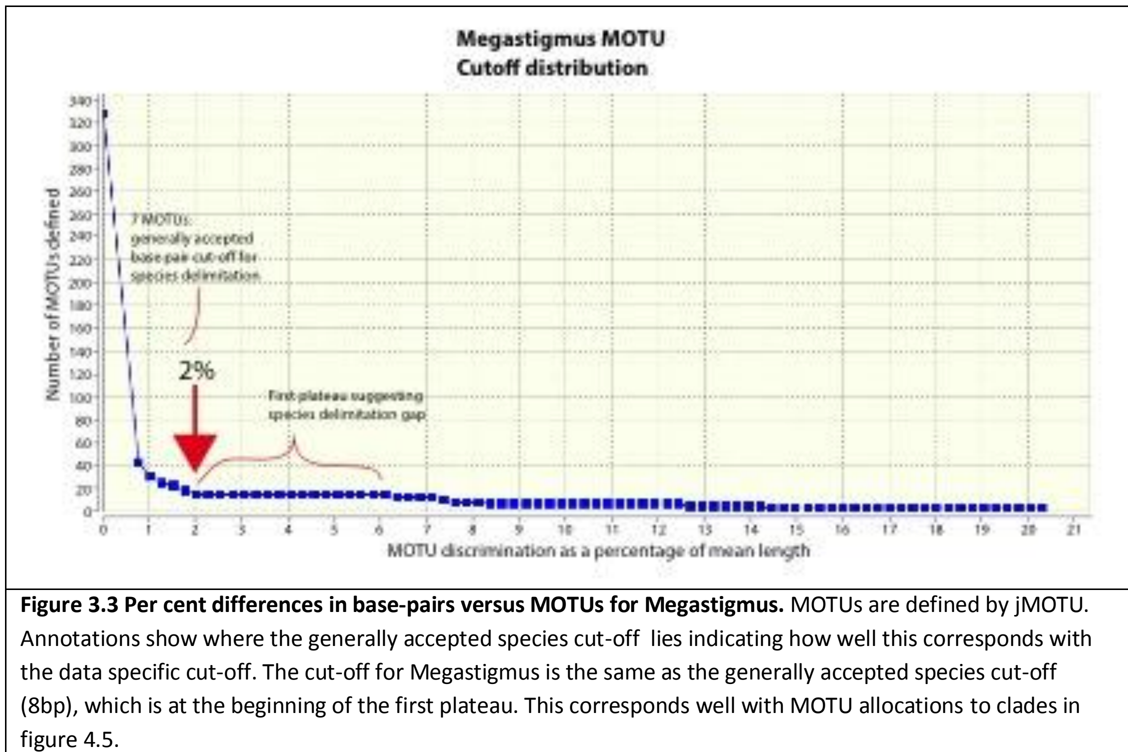

Figure 3.3 Per cent differences in base-pairs versus MOTUs for Megastigmus. MOTUs are defined by jMOTU. Annotations show where the generally accepted species cut-off lies indicating how well this corresponds with the data specific cut-off. The cut-off for Megastigmus is the same as the generally accepted species cut-off (8bp), which is at the beginning of the first plateau. This corresponds well with MOTU allocations to clades in figure 4.5.

3.2.4 *Torymus flavipes*

A phylogenetic tree for the *Torymus* DNA barcode sequences is shown in figure S3.5. *Torymus* showed an apparent barcoding gap at 4-7% sequence divergence (Figure 3.4) but assessing re-allocation of individuals according to a 4% cut-off gave discordant results to previously known molecular species allocations within the genus. After error checking, comparison of various cut-off points and comparing the MOTU allocation to a phylogeny of the same samples (Figure S3.5) the final cut-off point chosen was 8.89%. This resulted in *T. flavipes* being split into 8 MOTUs. MOTU0004 grouped with *T. flavipes* species 1 (75.49% of all morphological samples) a cryptic taxon known to share the Linnean species *T. flavipes* with another taxon *T. flavipes* species 2 (0% sampled). Six out of 8 MOTUs grouped with reference sequences of other known species of which MOTU0005 grouped with *T. geranii* (2.29%), already recorded in the community. Five MOTUs were not previously recorded in the community, MOTU0003 grouped with *T. auratus* (5.56%), MOTU0010 with *T. affinis* (0.33%), MOTU0011 grouped with *T. sinensis* (0.33%), MOTU0006 with *T. rubi* (1.31%) and

MOTU0007 grouped with *T. cyaneus* species 3 (14.05%), a cryptic taxon within the Linnean species *T. cyaneus*. The last, MOTU0012 (0.7%), grouped outside of the *Torymus* clade, could not be identified, and may not belong to this genus. DNA barcoding of *Torymus flavipes* thus divided specimens among 8 taxa: *T. flavipes* species 1, *T. geranii* and 6 new taxa (*T. auratus, T. affinis, T. sinensis, T. rubi, T. cyaneus* species 3, and 1 unidentified taxon). A cryptic taxon of the Linnean *Torymus flavipes*-complex (species 1) comprises more than 75% of sampled specimens.

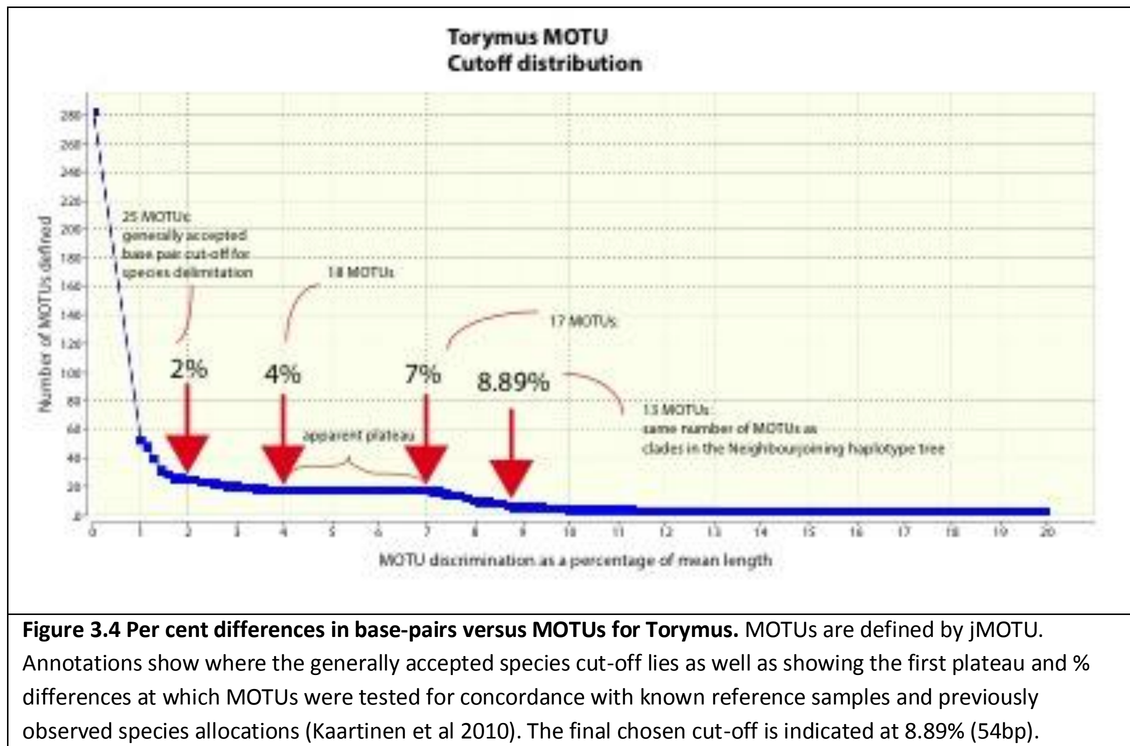

Figure 3.4 Per cent differences in base-pairs versus MOTUs for Torymus. MOTUs are defined by jMOTU. Annotations show where the generally accepted species cut-off lies as well as showing the first plateau and % differences at which MOTUs were tested for concordance with known reference samples and previously observed species allocations (Kaartinen et al 2010). The final chosen cut-off is indicated at 8.89% (54bp).

3.3 The Impact of DNA Barcode Information on Community Composition

I combined barcode-informed taxonomy for the four focal species with morphology-based information for all other specimens into a new dataset, hereafter termed the barcode-informed dataset. Reallocation of specimens to taxa for this new dataset is shown in supplementary table S3.1.

3.3.1 Impact of DNA Barcode Identification on Differences in Parasitoid Assemblages between plant taxa

DNA barcoding of 4 focal species increased the richness of parasitoid taxa from 51 to 66. General patterns closely paralleled those observed in the morpho-dataset. Refined parasitoid taxon identification does influence their distribution across plant taxa. For example, on the basis of morphological identifications, *Torymus flavipes* was reared from

galls on all 3 plant groups (section Cerris oaks, section Quercus oaks, chestnut) and in all 4 regions (3/4 for chestnut). Addition of barcode information resulted in allocation of *T. flavipes*-identified individuals to other MOTUs, such that barcode-confirmed *T. flavipes* (MOTU0004 *T. flavipes* species 1) was only reared from section Quercus oaks and chestnut, and from chestnut in only 2/4 regions.

As for the morpho-dataset, barcode-informed parasitoid species richness was higher in galls sampled from section Quercus (46 of 52 taxa on oaks) than from section Cerris (29 of 52 taxa). Twenty-one taxa (40.4% of oak-associated parasitoids) were reared from section Quercus alone, including two MOTUs (Eurytoma MOTU0035 and Eurytoma MOTU0047) originally identified as *Eurytoma brunniventris* but whose generic placement based on DNA sequence is uncertain. Four parasitoids (7.7%) were reared only from section Cerris. These include two taxa (Eurytoma MOTU0068 originally identified as *Eurytoma brunniventris* and Eupelmus MOTU0015 originally identified as *Eupelmus urozonus*) that cannot be identified to species using available reference sequences. The other two taxa, *Torymus cyaneus* and *Aulogymnus testaceoviridis,* are known to attack galls on both oak sections, despite having only been collected on section Cerris in this study (Askew et al 2013).

As for the morpho-dataset, barcode-informed parasitoid species richness was also higher in oak galls than in *D. kuriphilus.* Of the 66 taxa in table S3.1, 52 (78.8%, a lower proportion than was observed in the morpho-dataset) were collected on oak and 39 (59.1%, a higher proportion than was observed in the morpho-dataset) were collected on chestnut. Overlap in parasitoid species composition between the sampled oak and chestnut communities was extensive: 25 out of 66 species (37.9%) found on oak across the entire study area also attacked *D. kuriphilus*. 27 (40.9%) were collected exclusively on oak, while 14 (21.2%) were collected only on chestnut, including two MOTUs whose generic identification is uncertain (Eurytoma MOTU0013 and Torymus MOTU0012).

Interestingly, two of the newly identified chestnut-associated parasitoids are explicitly not associated with oak-feeding hosts: *Eurytoma hypochoeridis,* so far reared only from cynipid herb galls on Asteraceae; and *Torymus rubi*, so far predominantly associated with *Diastrophus* cynipid galls on *Rubus* (bramble, Rosaceae) and some rose and herb cynipid galls (Askew et al 2006).

The numbers of parasitoid taxa in the morpho and barcode-informed datasets associated with specific plant groups are compared in Table 3.3. In overview, comparison of values for the morpho and barcode-informed datasets shows the following trends:
In both datasets:

(i) parasitoid species richness is higher in oaks than in chestnut.

(ii) parasitoid species richness is higher in section Quercus than in section Cerris.

(iii) A consistently high proportion (>40%) of the parasitoids were reared only from oaks and not chestnut

(iv) A low proportion of parasitoids were reared only from chestnut galls.

The two datasets differ in that

(i) The barcode-informed dataset has 30% more parasitoid taxa.

(ii) Parasitoid richness increases for all tree categories, but more so for chestnut (29->39, 134% increase) than for oak (43->52, 120% increase).

(iii) The proportion of parasitoid taxa found only on oaks declines (43.1%-> 40.9%), but the proportion found only on chestnut increases (15.7% ->21.2%).

Table 3.3 The species richness of parasitoid taxa reared from galls on different plants, for both the morpho and barcode-informed datasets. % values refer to the total taxon richness for each dataset, given in the row 'TOTAL'. The row 'chest &oak' refers to parasitoid taxa shared by any oak and chestnut.

	Species richness associated with a plant taxon				Species richness associated EXCLUSIVELY with a plant taxon			
Host Plant Taxon	Morpho-Data		Barcode-informed Data		Morpho-Data		Barcode-informed Data	
	Count	%	Count	%	Count	%	Count	%
Chestnut	29	56.9	39	59.1	8	15.7	14	21.2
Oak	43	84.3	52	78.8	22	43.1	27	40.9
Cerris	25	55.8	29	55.8	1	2.0	4	6.1
Quercus	42	97.7	46	88.5	19	37.3	21	31.8
Chest&Oak	21	41.2	25	37.9	-	-	-	-
TOTAL	51		66		51		66	

3.3.2 Impact of DNA Barcode Identification on Differences in Parasitoid Assemblages Between Geographic Regions

Barcode-based identification of parasitoids did result in changes in taxon distributions between regions. Subdivision of the morpho-species *Megastigmus dorsalis* provides an example. Identification of species A and B within this taxon enhances differentiation between regions, because in the North only *M. dorsalis* spB was collected (on both section Quercus oaks and chestnut), while in Central Italy only *M. dorsalis* spA was collected (and only on section Quercus oaks), and in the East both A and B MOTUs were reared from both oak and chestnut. Finally, Sicily showed a 50/50 split between both MOTUs on section Cerris oaks while on section Quercus oaks *M. dorsalis* spA represented the larger proportion in an 80/20 split.

As for the morpho-species data, parasitoid composition varied qualitatively among regions (summaried in table 3.4, assessed quantitatively in section 3.4). Oak parasitoid richness (total across all regions 52 taxa) was again lower in the Central region (13 taxa, 25%) than in the other three regions (24/46.2% in the North, 36/69.2% in the East and 40/76.9% in the South). While total taxon richness increased, the percentage of all species found in a given region declined for all regions (Table 3.4), implying that significant numbers of the taxa added by barcoding are less shared among regions (i.e. only found in a subset).

The richness of parasitoid species reared from *D. kuriphilus* varied among geographic regions, and of the 66 taxa in the barcode-informed dataset, a maximum of 25 taxa (37.9%) were recorded in a single region – the East. Lower proportions of the total richness were recorded in the North (18 taxa, 27.3%), Centre (8 taxa, 12%) and South (14, 21.2%).

The proportion of oak-reared parasitoid taxa that were also recorded from *Dryocosmus kuriphilus* varied widely among regions. Near the invasion origin in the North, 8/24 of the parasitoid taxa reared from oak galls in that region (33.3%) were also reared from *D. kuriphilus*, with corresponding values for the East, Centre and South of 13/36 (36.1%), 3/13 (23.1%) and 11/40 (27.5%) respectively. Overall 25 of 52 parasitoid taxa (48.1%) found on oak across the entire study area were also found attacking the new invader.

The sets of species attacking *D. kuriphilus* varied among regions. For example, of 39 species attacking *D. kuriphilus,* 13 (33%) were recorded only outside the Northern region, and hence away from the introduction site. As before based on morpho-taxonomy there is clearly a significant proportion of parasitoid species that were recruited by *D. kuriphilus* outside the area where it was first recorded and again there is no indication the hierarchical subsets of species composition exists along the invasion route consistent with the Local Recruitment hypothesis.

The barcode-informed data repeat the over-representation of geographically widespread taxa in the *D. kuriphilus* community apparent in the morpho-data. The distribution of taxa in the barcode-informed dataset across numbers of regions is even less than in the morpho-data, with 13.6% of the 66 taxa found in all 4 regions, 25.7% in 3 regions, 24.2% in 2 regions and 36.4% in 1 region. This contrasts sharply with the representation of parasitoid taxa in these four categories in the *D. kuriphilus* community. 88.9% of species found in all 4 regions attack *D. kuriphilus*, compared to 64.7% of parasitoids found in 3 regions and 50% found in either 2 or 1 region.

Table 3.4 Parasitoid taxon richness by region for the morpho and barcode-informed datasets. (a) Oak-associated parasitoids, with percentages given relative to total oak-associated taxon richness. (b) Chestnut-associated parasitoids, with percentages given relative to total taxon richness. (c) Parasitoids shared between chestnut and any oak, with percentages given relative to the total parasitoid richness on oak in the same region.

(a) Parasitoids on Oak

Region	Morpho-Data		Barcode-informed Data	
	No	%	No	%
Centre	12	27.9	13	25.0
East	30	69.8	36	69.2
North	23	53.5	24	46.2
South	35	81.4	40	76.9
TOTAL	43		52	

(b) Parasitoids on Chestnut

Region	Morpho-Data		Barcode-informed Data	
	No	%	No	%
Centre	7	13.7	8	12.1
East	19	37.3	25	37.9
North	15	29.4	18	27.3
South	12	23.5	14	21.2
TOTAL	51		66	

(c) Oak Parasitoids also recorded on Chestnut

Region	Morpho-Data		Barcode-informed Data	
	No	%	No	%
Centre	4	33.3	3	23.1
East	12	40.0	13	36.1
North	8	34.8	8	33.3
South	10	28.6	11	27.5

3.4 Quantitative Analyses of Variation in Parasitoid Assemblages Between Plant Taxa and Between Geographical Regions.

3.4.1 Comparison of Parasitoid Assemblages Associated with Native Galls on Oak Sections Quercus and Cerris

Here I investigate the species richness, effective species richness and species composition of parasitoid communities associated with cynipid galls on oaks in section Cerris and section Quercus. The species richness of oak parasitoid morpho-species differed significantly between regions (df=3, dev=43.932, p=0.028, Figure 3.5 plot a), but not between oak sections or oak sections within regions. The equivalent barcode-informed dataset showed no significant effects of oak section or region (Table 3.5, Figure 3.5 plot b). Species richness and effective species richness for different oak sections within each geographic region are shown in Fig. 3.6, for both the morpho-taxon (a, c) and barcode-informed datasets (b, d). Across sites and plants, effective species richness values (which incorporate relative abundance of species in a sample) have both lower means and lower variance than observed species richness counts. This difference reflects the presence of rare species in all sampled communities, and is common to both the morpho-taxon and barcode-informed datasets.

Table 3.5 GLMs and mvGLMs for oak parasitoid assemblages. Analyses comparing species richness, effective species richness and species composition between regions on oak, between oak sections across regions and a region:oak section interaction. Interaction terms are not shown if their introduction caused no significant change in deviance. If introduction of interaction between terms did explain the data better, main terms are not shown.

Response Variable	Explanatory Variable	DF	Morphological Data					Barcode-Informed Data				
			Deviance	Res. DF	Res. Dev.	F	p-value	Deviance	Res. DF	Res. Dev.	F	p-value
Species Richness	Region	3	43.932	29	94.255	3.507	0.028	45.434	29	153.04	2.221	0.107
	Oak Section	1	>0.001	31	138.19	0	0.999	2.0463	31	196.43	0.254	0.618
	Interaction Deviance Change	3	21.617	25	72.255	1.812	0.171	29.535	25	118.14	1.566	0.222
Effective Species Richness	Region	3	8.432	29	97.115	0.839	0.483	12.871	29	154.77	0.804	0.502
	Oak Section	1	5.522	31	100.03	1.711	0.2	16.772	31	150.87	3.446	0.073
	Interaction Deviance Change	3	22.746	25	92.553	1.229	0.3201	24.637	25	114.66	1.174	0.339
Similarity - Species Composition	Interaction Deviance Change	3	77.71	25	-	-	0.013	74.26	25	-	-	0.003
	Region	3	172.4	29	-	-	0.134	203.7	29	-	-	0.107
	Region:Oak Section	4	150.4	25	-	-	0.009	183.1	25	-	-	0.005

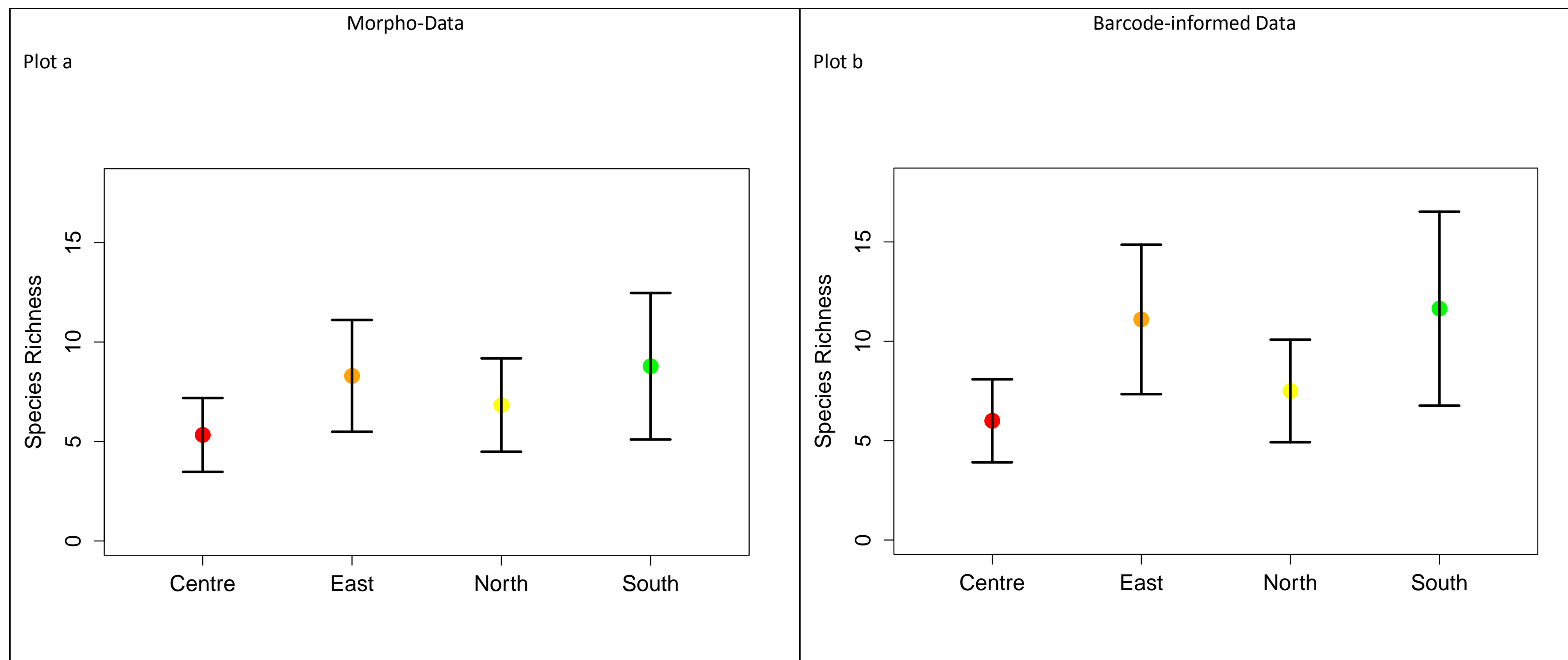

Figure 3.5 Variation in parasitoid species richness on oak (both sections combined) among geographic regions. Values shown are predicted means with their associated standard errors. Results are shown for the morpho-data (plot a), and the barcode-informed data (plot b). Regional colour coding is consistent in the following figures in this chapter.

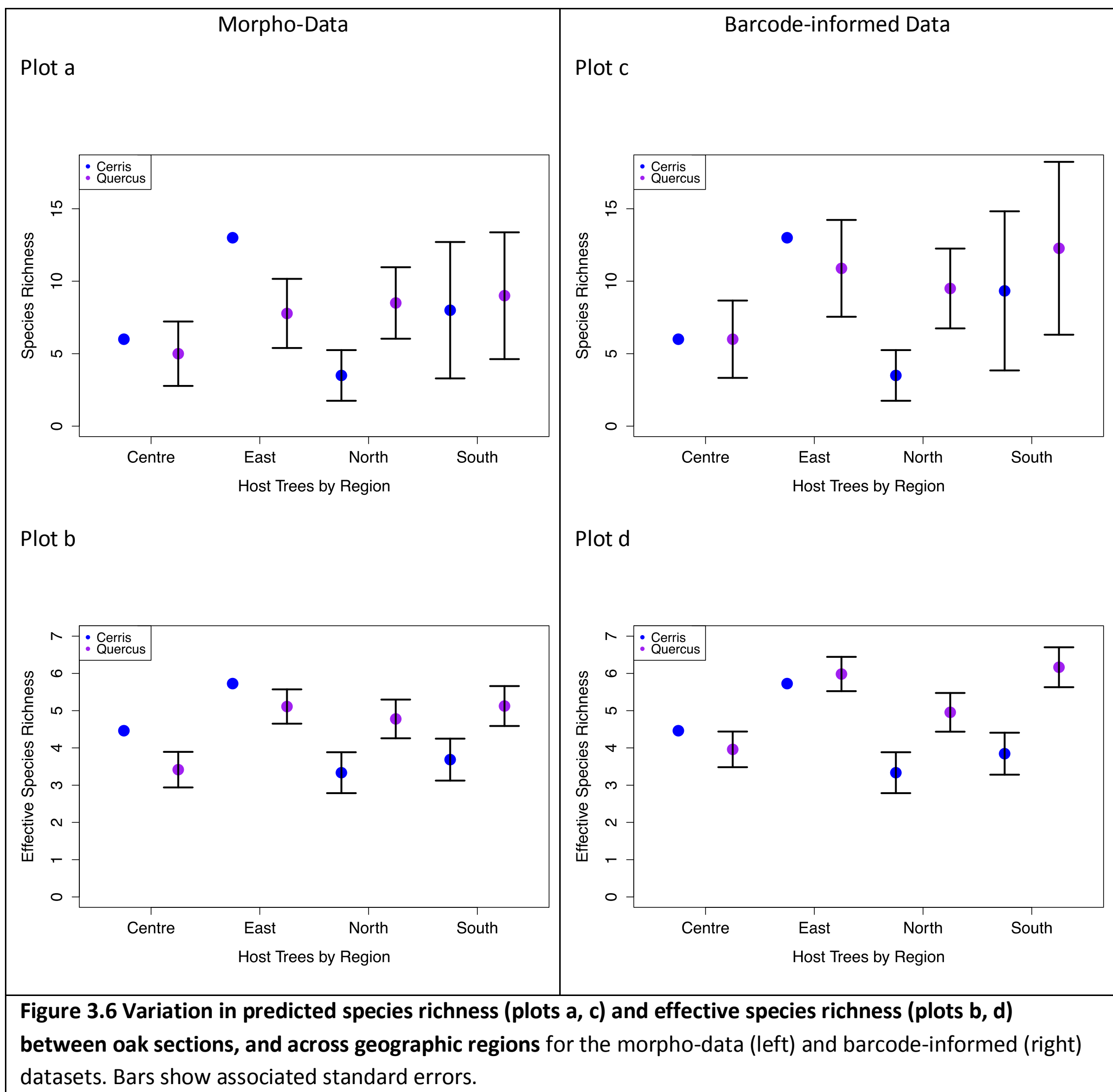

Figure 3.6 Variation in predicted species richness (plots a, c) and effective species richness (plots b, d) between oak sections, and across geographic regions for the morpho-data (left) and barcode-informed (right) datasets. Bars show associated standard errors.

Parasitoid assemblage composition showed a significant oak section by region interaction, such that differences between oak sections depended significantly on the region sampled. This was true for both the morpho-data and barcode-informed datasets (mvabund morpho: df=4, dev=150.4, p=0.009 and molecular: df=4, dev = 183.1, p = 0.005 respectively) (Table 3.5). These results are visualised using NMDS in Figure 3.7 plots a and b. The oak section by region interaction is exemplified by the following: for the Southern region, ellipsoids representing 95% confidence limits around the centroids for data corresponding to the two oak sections are largely overlapping, representing very similar parasitoid assemblages. In contrast, in the North, the ellipsoids for the two oak sections are entirely separate, indicating greater differences in assemblage composition. While the main effect of oak section was non-significant for the morpho-dataset, it was significant for the barcode-informed data (morpho: df=1, dev=68.3, p=0.086 and molecular: df=1, dev=97.2, p=0.024; Table 3.5). The most notable difference between morphology-based and barcode-informed comparison of regions in NMDS space is that the barcode-informed data better separate northern assemblages from other regions. This may be due to re-allocation of morpho-*M. dorsalis* to spatially separated, cryptic lineages (see table 3.2). The parasitoid species contributing most statistical signal to the observed patterns, in terms of deviance explained in the model, are shown in table 3.6. Inspection of table 3.6 shows that barcode information did not visibly alter interpretation of the parasitoid species composition and its key players in differentiating assemblages on a given oak section in a given region (but note morpho-*M. dorsalis* versus barcode-informed *M. dorsalis* spA).

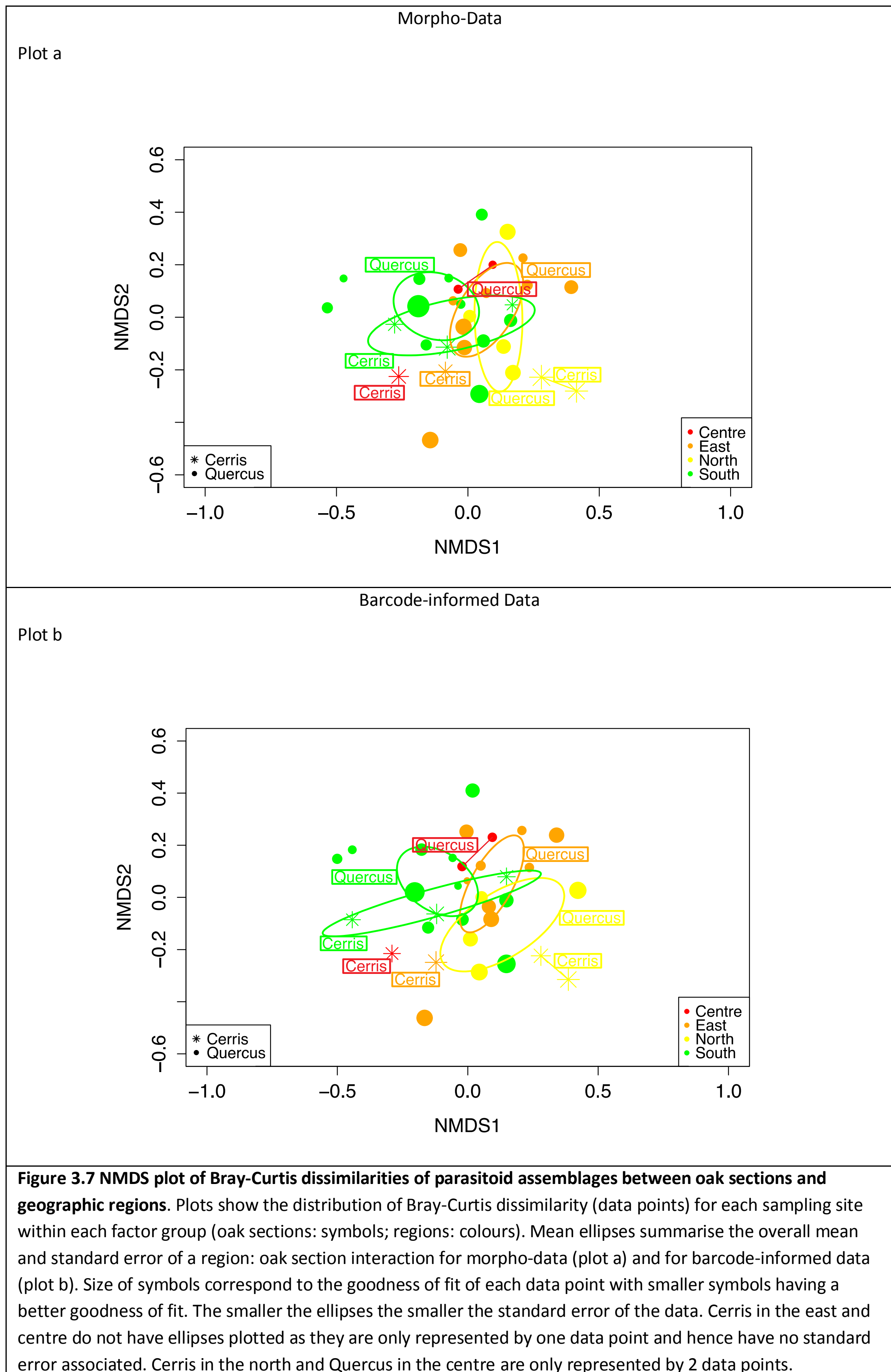

Figure 3.7 NMDS plot of Bray-Curtis dissimilarities of parasitoid assemblages between oak sections and geographic regions. Plots show the distribution of Bray-Curtis dissimilarity (data points) for each sampling site within each factor group (oak sections: symbols; regions: colours). Mean ellipses summarise the overall mean and standard error of a region: oak section interaction for morpho-data (plot a) and for barcode-informed data (plot b). Size of symbols correspond to the goodness of fit of each data point with smaller symbols having a better goodness of fit. The smaller the ellipses the smaller the standard error of the data. Cerris in the east and centre do not have ellipses plotted as they are only represented by one data point and hence have no standard error associated. Cerris in the north and Quercus in the centre are only represented by 2 data points.

Table 3.6 Parasitoid species contributing most to variation in the region: oak section interaction for morpho-data (left) and barcode-informed data (right).

Contributing Species	Morpho-Data			Barcode-informed Data		
	Deviance	Proportion Contributed to overall Deviance	Cumulative Contribution	Deviance	Proportion Contributed to overall Deviance	Cumulative Contribution
Sycophila variegata	11.52	0.07	0.07	11.52	0.06	0.06
Megastigmus dorsalis spA	-	-	-	8.88	0.04	0.1
Mesopolobus fasciiventris	8.39	0.05	0.12	8.39	0.04	0.14
Mesopolobus tibialis	8.19	0.05	0.16	8.19	0.04	0.18
Torymus cyaneus sp3	-	-	-	7.92	0.04	0.22
Aprostocetus aethiops	7.46	0.04	0.21	7.46	0.04	0.26
Mesopolobus amaenus	7.35	0.04	0.25	7.35	0.04	0.29
Eupelmus annulatus	7.22	0.04	0.29	7.22	0.04	0.33
Ormyrus nitidulus	6.28	0.04	0.33	6.28	0.03	0.36
Pediobius rotundatus	6.08	0.04	0.36	6.08	0.03	0.39
Mesopolobus tarsatus	5.54	0.03	0.39	5.54	0.03	0.42
Cecidostiba fungosa	5.4	0.03	0.43	5.4	0.03	0.44
Megastigmus dorsalis	5.35	0.03	0.46	-	-	-
Aprostocetus cerricola	5.29	0.03	0.49	5.29	0.03	0.47
Mesopolobus dubius	5.27	0.03	0.52	5.27	0.03	0.49
Torymus auratus	-	-	-	5.13	0.03	0.52

3.4.2 Comparison of Parasitoid Assemblages Associated with *D. kuriphilus* and with Native Oak Gall Wasps

Analyses of the impact of host plant genera (oaks *versus* chestnut) are summarised in table 3.7. Both morpho and barcode-informed data show significant differences in species richness between regions (Table 3.7; morpho: df=3, dev=106.39, p=0.017, barcode-informed: df=3, dev= 268.25, p=0.001, Figure 3.8) and between oak and chestnut (Table 3.5; morpho: df=1, dev= 187.96, p<0.001, barcode-informed: df =1, dev=162.86, p=0.015, Figure 3.9). There was no significant region by plant interaction in either dataset.

Effective species richness differed significantly between regions in the barcode-informed data (df=3, dev=75.652, p=0.001, Figure 3.8), but not in the morpho-data (Table 3.5). Both datasets showed a significant difference between oak and chestnut (morpho: df=1, dev=114.09, p<0.001, barcode-informed: df=1, dev=83.59, p<0.001, Figure 3.9). There was no significant region by plant interaction in either dataset.

Table 3.7 GLMs and mvGLMs for oak and chestnut parasitoid assemblages. Analyses comparing species richness, effective species richness and species composition between regions on oak and chestnut, between host plant genera across regions and a region: oak and chestnut interaction. Interaction terms are not shown if their introduction caused no significant change in deviance. If introduction of interaction between terms did explain the data better, main effects are not shown.

Response Variable	Explanatory Variable	DF	Morpho-Data					Barcode-informed Data				
			Deviance	Res. DF	Res. Dev.	F	p-value	Deviance	Res. DF	Res. Dev.	F	p-value
Species Richness	Region	3	106.39	79	466.81	3.595	0.017	268.25	79	728.3	5.67	0.001
	Oak v Chestnut	1	187.96	81	385.24	23.467	<0.001	162.86	81	833.7	6.23	0.014
	Interaction Deviance Change	3	40.687	75	287.86	2.346	0.08	62.53	75	571.82	1.58	0.2
Effective Species Richness	Region	3	17.638	79	327.07	1.42	0.243	75.65	79	401	4.97	0.003
	Oak v Chestnut	1	114.09	81	230.61	40.074	<0.001	83.59	81	393.1	17.23	<0.001
	Interaction Deviance Change	3	28.449	75	309.05	1.27	0.291	36.6	75	408.7	1.26	0.29
Similarity Species Composition	Interaction Deviance Change	3	224.6	75	-	-	0.001	240.6	75	-	-	0.001
	Region	3	427	79	-	-	0.001	567	79	-	-	0.001
	Region:Oak v Chestnut	4	462.9	75	-	-	0.001	548.3	75	-	-	0.001

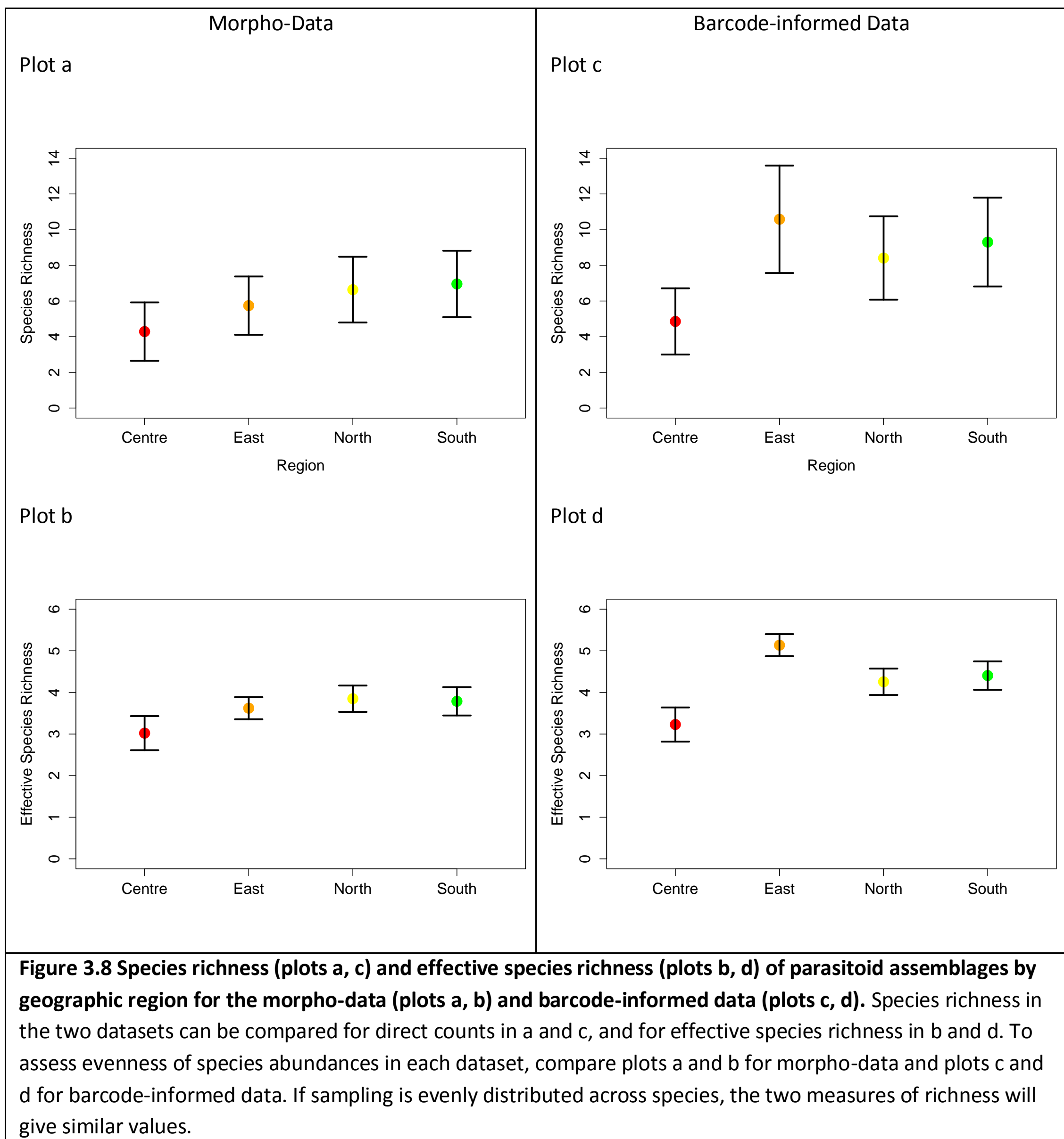

Figure 3.8 Species richness (plots a, c) and effective species richness (plots b, d) of parasitoid assemblages by geographic region for the morpho-data (plots a, b) and barcode-informed data (plots c, d). Species richness in the two datasets can be compared for direct counts in a and c, and for effective species richness in b and d. To assess evenness of species abundances in each dataset, compare plots a and b for morpho-data and plots c and d for barcode-informed data. If sampling is evenly distributed across species, the two measures of richness will give similar values.

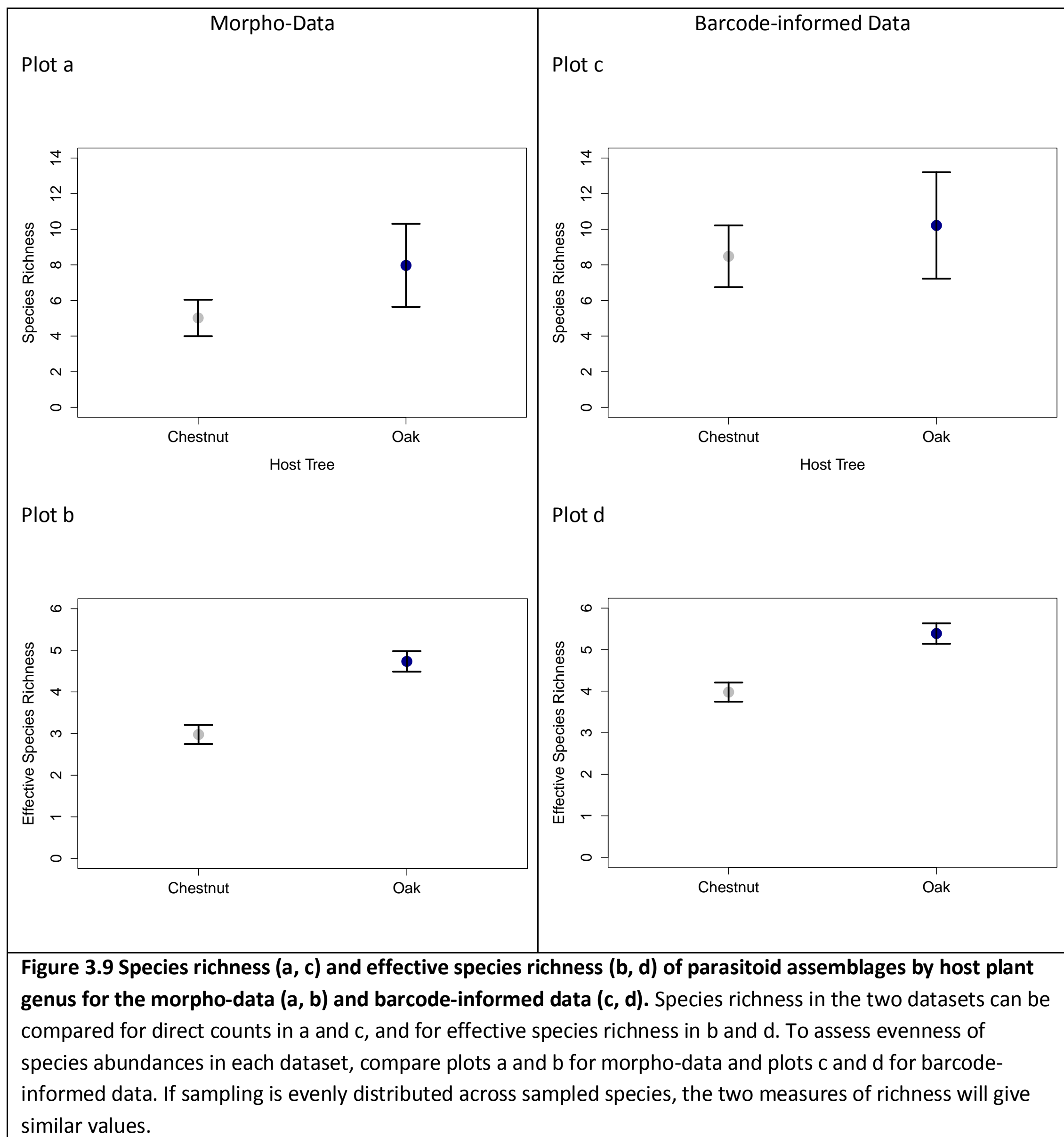

Figure 3.9 Species richness (a, c) and effective species richness (b, d) of parasitoid assemblages by host plant genus for the morpho-data (a, b) and barcode-informed data (c, d). Species richness in the two datasets can be compared for direct counts in a and c, and for effective species richness in b and d. To assess evenness of species abundances in each dataset, compare plots a and b for morpho-data and plots c and d for barcode-informed data. If sampling is evenly distributed across sampled species, the two measures of richness will give similar values.

Both morpho and barcode-informed datasets showed significant variation in parasitoid species composition between host plant genera within regions (Table 3.7, Figure 3.10, morpho: df=4, dev=462.9, p=0.001, barcode-informed: df=3, dev=548.3, p=0.001). Examination of figure 3.10 shows that parasitoid species composition differs between oak and chestnut in most regions, with the exception of the Central region in morpho-data, for which no significant difference is apparent. For both datasets, differences between oak and chestnut are more pronounced for northern sites than elsewhere. The parasitoid species that most contribute to the differentiation between oak and chestnut assemblages in the different regions are listed in table 3.8.

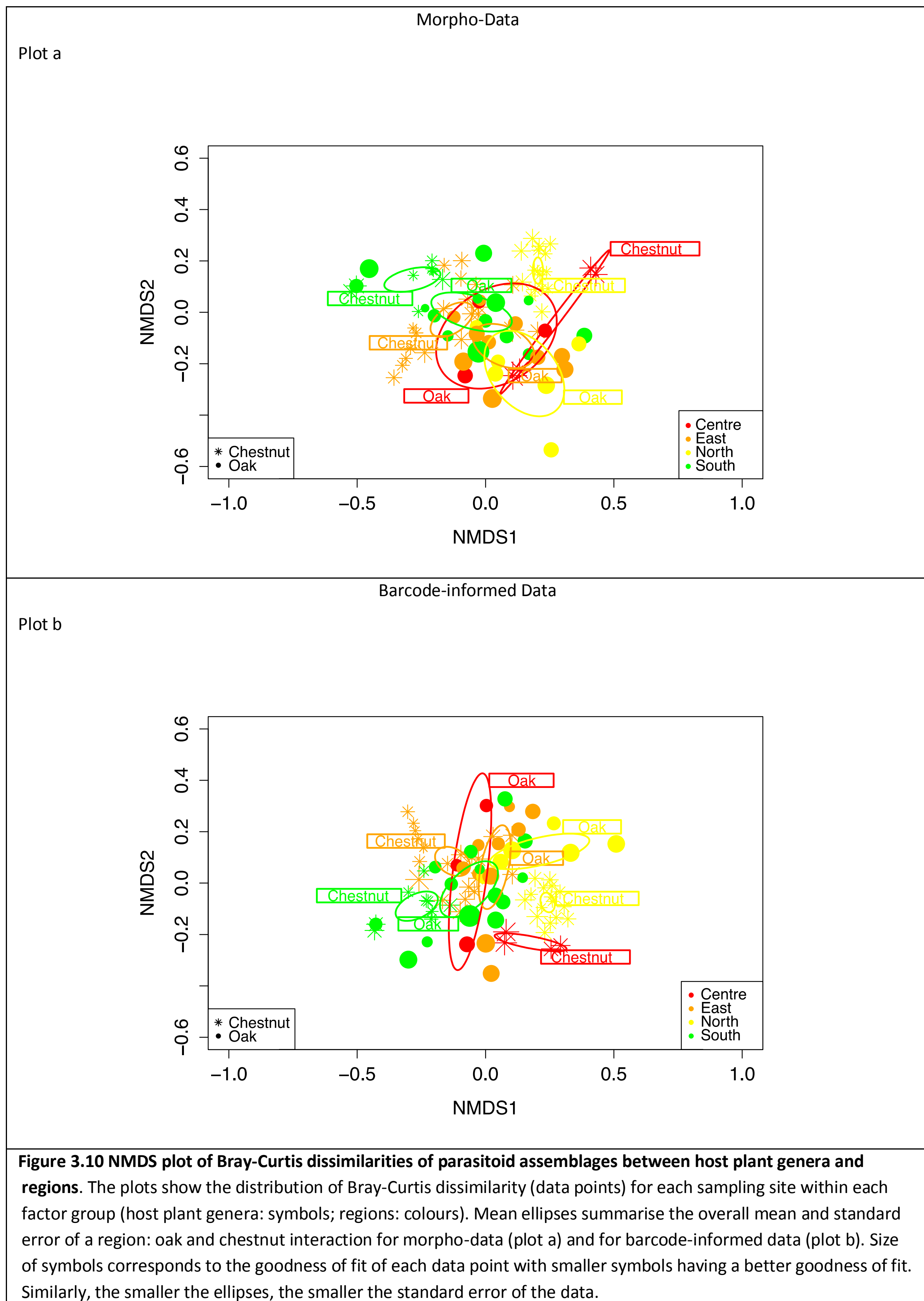

Figure 3.10 NMDS plot of Bray-Curtis dissimilarities of parasitoid assemblages between host plant genera and regions. The plots show the distribution of Bray-Curtis dissimilarity (data points) for each sampling site within each factor group (host plant genera: symbols; regions: colours). Mean ellipses summarise the overall mean and standard error of a region: oak and chestnut interaction for morpho-data (plot a) and for barcode-informed data (plot b). Size of symbols corresponds to the goodness of fit of each data point with smaller symbols having a better goodness of fit. Similarly, the smaller the ellipses, the smaller the standard error of the data.

Table 3.8 Parasitoid species contributing most to regional variation in the difference between oak and chestnut communities within regions for morpho-data (left) and barcode-informed data (right). **Eupelmus urozonus* and *Torymus cyaneus* are repeated in the "Contributing Species" list to reflect their respective contribution to the morpho and barcode-informed datasets.

Contributing Species	Morpho-Data			Barcode-informed Data		
	Deviance	Proportion Contributed to overall Deviance	Cumulative Contribution	Deviance	Proportion Contributing to overall Deviance	Cumulative Contribution
Megastigmus dorsalis	35.62	0.08	0.08	-	-	-
Eupelmus urozonus	34.37	0.08	0.16	-	-	-
Megastigmus dorsalis spB	-	-	-	53.67	0.09	0.09
Eupelmus fulvipes	-	-	-	35.59	0.06	0.16
Eurytoma pistacina	30.02	0.07	0.23	30.13	0.05	0.21
Torymus flavipes	25.67	0.06	0.29	-	-	-
Torymus cyaneus	24.22	0.06	0.35	-	-	-
Eupelmus annulatus	23.55	0.06	0.41	25.02	0.04	0.25
Mesopolobus tibialis	23.07	0.05	0.46	23.07	0.04	0.3
Eupelmus rostratus	21.38	0.05	0.51	21.38	0.04	0.33
*Eupelmus urozonus**	-	-	-	20.02	0.04	0.37
Mesopolobus sericeus	-	-	-	18.51	0.03	0.4
Torymus flavipes sp1	-	-	-	17.4	0.03	0.43
Megastigmus dorsalis spA	-	-	-	16.69	0.03	0.46
*Torymus cyaneus**	-	-	-	14.6	0.03	0.49
Torymus cyaneus sp3	-	-	-	13.23	0.02	0.51

Table 3.8 shows a striking difference in contributing species to the differentiation between oak and chestnut when comparing morpho-data with barcode-informed data. This is in contrast to the most important species seen to contribute to the differentiation between section Quercus and section Cerris oaks in table 3.6. While more species contribute less to the differentiation between oak and chestnut in the barcode-informed dataset (12 taxa rather than 8 for the morpho-data) 6 out of those 12 taxa have only been identified by means of barcoding. This indicates that barcoding the 4 focal species informed the parasitoid community on chestnut quite strikingly by adding more contributing species and, as a result, changing the importance of some contributing species such as *Megastigmus dorsalis* (versus *M. dorsalis* sp A and B in the barcode-informed dataset) and *Eupelmus urozonus* (second most contributing species in the morpho-dataset versus 7th most contributing species in the barcode-informed dataset.

3.4.3 Temporal Patterns in Assembly of the *D. kuriphilus* Parasitoid Community

Temporal data were available only for Northern Italy. My first question was whether data for sites in Northern Italy (summed across years in each site) were similar enough in assemblage composition to allow analysis of temporal patterns, even though not all sites were sampled in all years. Both morpho-species and barcode-informed data sets showed no significant between-site differences (Table 3.9), so I proceeded with the temporal analysis. I then fitted a linear regression of species richness/effective species richness for both datasets across years. Neither richness measure showed a significant linear relationship in either dataset, with no trend towards increasing species richness over time. Fitting year as a categorical factor also revealed no significant differences. Both datasets, however, showed significant differences in species composition between years (morpho: df=1, dev=80.45, p=0.008, molecular: df=1, dev=95.26, p=0.017, Figure 3.11). Plotting of community composition in multi-dimensional NMDS space shows that changes between years do not follow an obvious trajectory. The species contributing most to community differentiation across years in the north of Italy (Table 3.10) are very similar in both the morpho and barcode-informed datasets.

Table 3.9. GLM and mvGLM analyses of the species richness and composition of *D. kuriphilus* parasitoid assemblages over the period 2006-2011 in Northern Italy. For species richness and effective species richness I first compared sites. Where there were no significant differences between sites, I first fitted year as a linear term (Year Lin). If there was no linear year effect, I then fitted year as a categorical variable (Year Cat). This process did not apply for species composition as the mvGLM treats year as a categorical variable.

Response Variable	Explanatory Variable	DF	Morpho-Data					Barcode-informed Data				
			Deviance	Res. DF	Res. Dev.	F	p-value	Deviance	Res. DF	Res. Dev.	F	p-value
Species Richness	Site	2	93.979	12	93.557	2.049	0.171	125.72	12	138.21	1.5334	0.2552
	Year Lin	1	58.53	13	129	2.89	0.113	84.029	13	179.89	2.8085	0.1176
	Year Cat	5	104.31	9	83.229	0.799	0.577	148.38	9	115.54	0.7714	0.5935
Effective Species Richness	Site	2	76.673	12	76.176	1.331	0.301	91.955	12	98.381	1.0586	0.3772
	Year Lin	1	42.522	13	110.33	1.883	0.193	58.478	13	131.86	2.0774	0.1731
	Year Cat	5	19.256	9	43.394	0.799	0.577	23.033	9	51.511	0.8049	0.5737
Compostion	Year Cat	1	80.45	13	-	-	0.008	95.26	13	-	-	0.017

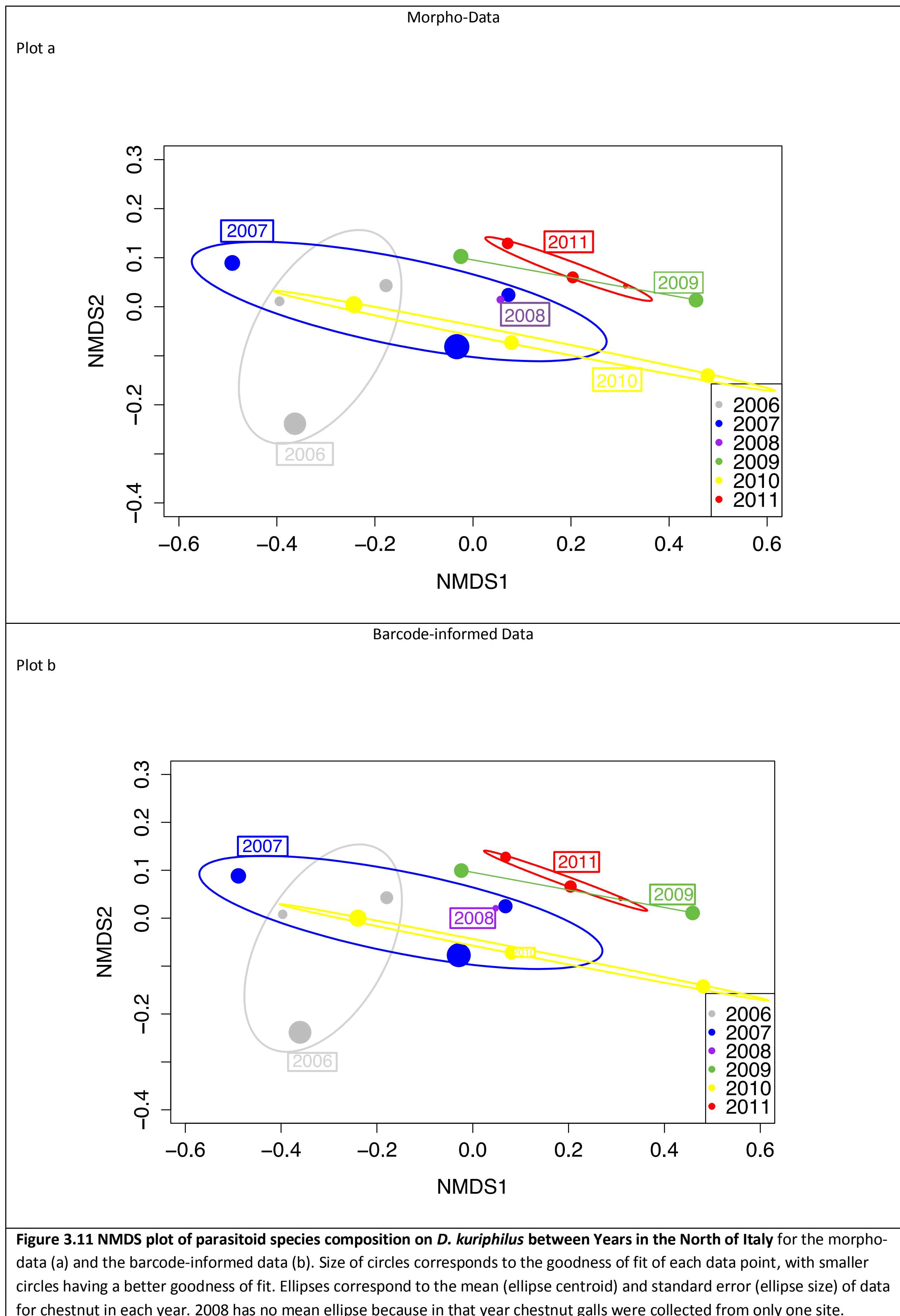

Figure 3.11 NMDS plot of parasitoid species composition on *D. kuriphilus* between Years in the North of Italy for the morpho-data (a) and the barcode-informed data (b). Size of circles corresponds to the goodness of fit of each data point, with smaller circles having a better goodness of fit. Ellipses correspond to the mean (ellipse centroid) and standard error (ellipse size) of data for chestnut in each year. 2008 has no mean ellipse because in that year chestnut galls were collected from only one site.

Table 3.10 Most contributing parasitoid species to variation in composition of the *D. kuriphilus* community between years

Contributing Species	Morpho-Data			Barcode-informed Data		
	Deviance	**Proportion Contributed to overall Deviance**	**Cumulative Contribution**	**Deviance**	**Proportion Contributing to overall Deviance**	**Cumulative Contribution**
Eupelmus annulatus	13.28	0.17	0.17	14.32	0.15	0.15
Eupelmus rostratus	9.25	0.11	0.28	9.25	0.1	0.25
Torymus cyaneus	9.11	0.11	0.39	9.11	0.1	0.34
Mesopolobus fasciiventris	8.37	0.1	0.5	8.37	0.09	0.43
Sycophila variegata	-	-	-	6.66	0.07	0.5

Chapter 4 – Discussion

4.1 How DNA Barcoding Affects Interpretation of my Data

I identified 51 morpho-species attacking oak and chestnut across my sampling range from Sicily via Switzerland to Croatia. Barcoding of four focal taxa increased the 51 morpho-species to 66 barcode-informed taxa, an increase in species richness of almost 30%. This increase comes as no surprise as growing numbers of papers show that morphologically conserved groups (such as many chalcid and other parasitoid taxa) harbour cryptic lineages when DNA sequence data are analysed. This is particularly apparent for insects (Bickford et al 2007, Pfenninger & Schwenk 2007) including gall wasp parasitoids (e.g. Nicholls et al 2010a&b, Kaartinen et al 2010, Alkhatib et al 2014) and in parasitoids in other communities (e.g. Smith et al 2006, 2007, 2011).

Barcoding of the 4 focal parasitoid species resulted in some informative differential allocation of resultant taxa between sampling regions and between host plant genera. Examples include differential regional abundance of the MOTUs *M. dorsalis* spA and spB that resulted from splitting of morpho-*Megastigmus dorsalis*, where *M. dorsalis* spA and spB are split approximately 50/50 in the East and South but only spA or spB are represented in the Centre and North respectively. The differential allocation of barcode-informed *Eupelmus* taxa (primarily *E. urozonus* and *E. fulvipes*) resulted in splitting of morpho-*Eupelmus urozonus* between the two major MOTUs on chestnut but not so much on oak. Barcode-based re-allocation of morpho-*Eurytoma brunniventris* and morpho-*Torymus flavipes* individuals revealed molecularly identified parasitoid species in *D. kuriphilus* galls that cannot have originated from oak cynipid gall communities, and are the first record of parasitoids from cynipid galls on herbs (the parasitoid *Eurytoma hypochoeridis* attacks the cynipids *Phanacis hypochoeridis*, *Timaspis lampsanae* and *T. phoenixopodos*, which gall Asteraceae) and brambles (*Torymus rubi,* usually a parasitoid of *Diastrophus rubi* galls on *Rubus fruticosus,* Rosaceae) (see Askew et al 2006). Barcoding of morpho-*T. flavipes* also revealed one misidentified individual of the released bio-control agent *T. sinensis*. This individual was reared from *D. kuriphilus* galls and therefore does not indicate a host shift by *T. sinensis* onto other cynipid hosts, but shows the value of DNA barcoding in monitoring spread of *T. sinensis* across Europe. *Torymus sinensis* may remain undetected in the local community without monitoring wider associations and corroborating morphological identification with DNA barcoding.

The above findings suggested that barcoding could influence the outcome of statistical analyses of regional and plant-associated patterns. However, statistical analyses were not majorly affected by adding barcode information to my morpho-dataset. Comparison of results in table 4.1 shows that analyses of the two datasets produced similar significant results, with differences primarily in significance levels for specific explanatory variables. There was no trend in which dataset produced stronger significance. The two datasets supported different significant explanatory variables in only two analyses.

Table 4.1 Comparison of significance of patterns in the morpho-species and barcode-informed datasets. Significance levels, indicated by asterisks, are as follows: *=p<0.05, **=p<0.01, ***=p<0.001, NS=p>0.05

Comparison	Response variable	Explanatory variable	Model type	Table	Morpho-Data	Barcode-informed Data
Between oak sections	Species richness	Region	GLM	3.5	*	NS
	Species composition	Region: Oak Section	mvGLM	3.5	**	**
Between oak and chestnut	Species richness	Oak v Chestnut	GLM	3.7	***	*
	Species richness	Region	GLM	3.7	*	***
	Effective species richness	Oak v Chestnut	GLM	3.7	***	***
	Effective species richness	Region	GLM	3.7	NS	**
	Species composition	Region: Oak v Chestnut	mvGLM	3.7	***	***
Between years in *D. kuriphilus*	Species composition	Year	mvGLM	3.9	**	*

The two largely similar outcomes had some noteworthy differences. The significant difference in species richness between oak sections has broken down with the addition of data informed by barcodes while effective species richness remains insignificant. The decrease in difference between the two oak sections is also illustrated when comparing the percentage of parasitoid species found to attack Quercus (reduced from the morpho-data) and those found to attack Cerris (increased from the morpho-data). Together this suggests that the addition of MOTUs has contributed to a more even spread of parasitoid species richness between oak sections (see table 3.3) and regions (see table 3.4). Despite a reduction in already low structure of species richness and effective species richness, species composition still varies significantly between oak sections in different regions (region:oak section interaction). The species that contribute to these differentiations remain largely the same as in the morpho-dataset as well as contributing to about the same amount of deviance (15 species contributing to 52% of the deviance between oak sections and regions rather than 13 species contributing to 51.82% of the deviance, listed in table 3.6).

4.2 The Parasitoids Attacking *D. kuriphilus* across Europe: A Comparison

My study represents the largest rearing of parasitoids from *D. kuriphilus*, and the widest geographic scope of sampling, to date. Table 4.2 summarises species presence/absence for my data and nine other studies to compare my findings with previous research on *D. kuriphilus*-associated communities in Europe and its native range in China.

Table 4.2 Parasitoids reared from *D. kuriphilus* galls in my data, in Europe in general and in *D. kuriphilus*' country of origin, China. Presence of a given parasitoid species is indicated by an X. Species that are private to a given publication are highlit in yellow.   *Guo et al 1997 report that 28 species were associated with *D. kuriphilus* in China but only quote 10 in their publication

FAMILY	PARASITOID GENUS	PARASITOID SPECIES	My Data	CHINA			EUROPE					
			BARCODE-INFORMED	GUO ET AL 1997 *	MURAKAMI 1981	MURAKAMI ET AL 1980	AEBI ET AL 2007 (Europe)	QUACCHIA ET AL 2013 (North Italy)	MATOSEVIC ET AL 2013 (Croatia)	PANZAVOLTA ET AL 2013 (Central Italy)	PALMERI ET AL 2014 (Central Italy)	KOS ET AL 2015 (Slovenia)
	Eurytoma	*adleriae*						X				
	Eurytoma	*brunniventris*	X		X	X	X	X	X	X		X
	Eurytoma	*hypochoeridis*	X									
	Eurytoma	*pistacina*	X				X	X	X			X
	Eurytoma	*concinna*		X								
Eurytomidae	*Eurytoma*	*setigera*		X	X	X						
	Sycophila	*biguttata*	X				X	X	X			X
	Sycophila	*binotata*									X	
	Sycophila	*flavicollis*									X	X
	Sycophila	*variegata*	X	X	X	X	X	X	X			X
	Sycophila	*iracemae*					X	X				
	Megastigmus	*nipponicus*		X	X	X						
	Megastigmus	*maculipennis*		X	X	X						
	Megastigmus	*dorsalis*					X		X	X	X	X
	Megastigmus	*dorsalis spA*	X					X				
	Megastigmus	*dorsalis spB*	X					X				
	Megastigmus	*synophri*	X									
	Torymus	*cerri*	X									
	Torymus	*cyaneus*	X									
Torymidae	*Torymus*	*affinis*	X									
	Torymus	*auratus*	X				X	X	X	X	X	X
	Torymus	*cerri*	X									
	Torymus	*flavipes*					X	X	X	X		X
	Torymus	*flavipes sp1*	X									
	Torymus	*rubi*	X									
	Torymus	*cyaneus sp3*	X									
	Torymus	*formosus*	X									X
	Torymus	*geranii*	X	X	X	X			X			X

	Torymus	*nobilis*	X									
	Torymus	*scutellaris*	X				X	X				
	Torymus	*sinensis*	X	X			X					
Ormyridae	*Ormyrus*	*nitidulus*						X			X	
	Ormyrus	*pomaceus*	X				X	X	X		X	X
	Ormyrus	*punctiger*		X	X	X						
Pteromalidae	*Cecidostiba*	*semifascia*										X
	Cyrtoptyx	*robustus*	X									
	Mesopolobus	*albitarsus*										X
	Mesopolobus	*amaenus*						X	X		X	
	Mesopolobus	*dubius*	X						X			
	Mesopolobus	*fasciiventris*	X							X	X	X
	Mesopolobus	*lichtensteinii*	X									
	Mesopolobus	*mediterraneus*	X				X	X				
	Mesopolobus	*sericeus*	X				X	X	X		X	X
	Mesopolobus	*tarsatus*					X	X		X	X	X
	Mesopolobus	*tibialis*	X					X	X	X	X	X
	Ormocerus	*latus*	X									
Eupelmidae	*Eupelmus*	*annulatus*	X					X	X	X	X	X
	Eupelmus	*rostratus*	X									
	Eupelmus	*splendens*	X					X				X
	Eupelmus	*spongipartus*						X				
	Eupelmus	*urozonus*	X	X	X	X	X	X	X	X	X	X
	Eupelmus	*fulvipes*	X					X				
	Macroneura	*vesicularis*	X								X	X
Eulophidae	*Aulogymnus*	*arsames*						X				
	Aulogymnus	*skianeuros*	X									X
	Aprostocetus	*aethiops*										X
	Aprostocetus	*biorrhizae*										X
	Aprostocetus	*glandicola*										X
	Baryscapus	*pallidae*					X	X				
	Pediobius	*chilaspis*						X				
	Pediobius	*saulius*						X				X

Examination of Table 4.2 shows a large overlap in parasitoid species reared from *D. kuriphilus* throughout Europe. I reared a total of 37 barcode-informed Linnean taxa from *D. kuriphilus* as well as two MOTUs that remain unidentified. This estimate of species richness is higher than, but of similar magnitude, to numbers of taxa reared by previous studies. For example, Quacchia et al (2013) reared 27 parasitoid taxa in Northern Italy, using a combined morphological and barcoding approach. Matosevic et al (2013) (Croatia) and Kos et al (2015) (Slovenia) (both sampling sites in the Eastern region of my study) reared totals of 15 and 27 morpho-species respectively. In central Italy Panzavolta et al (2013) and Palmeri et al (2014) both reared 9 and 14 morpho-species respectively. These estimates are also broadly compatible with species richness of 28 morpho-species recorded from China's Shandong Province by Guo et al (1997).

To some degree, variation in species richness between studies may reflect inclusion of DNA barcoding in the two studies that yielded among the highest species richness estimates (this study and Quacchia et al 2013), and reliance on morphological identification in the remainder. Particularly noteworthy is that the high richness recorded in Slovenia by Kos et al (2015) may well be an underestimate of the true species richness due to under-splitting of morpho-species. Another reason for differing estimates of species richness may be due to between-study variation in sampling effort (Quacchia et al (2013): 415,224 galls from 2006-2010, Matosevic et al (2013): 20,598 galls in 2011&2012, Kos et al (2015): 49,774 galls from 2010-2013, Panzavolta et al (2013): 1588 galls in June 2009, Palmeri et al (2014): ~3700 galls in 2011, Guo: unknown, my study: 28,368 galls from 2011-2013). Lastly, variation in species richness may be a true reflection of between-sampling-region differentiation for the different studies.

Noting the similarities between parasitoid assemblages on *D. kuriphilus* we can see that some of the major generalists that were noteworthy in my study are dominating the species pool on *D. kuriphilus* in Europe in general and even in its home range in China (e.g. *Eupelmus urozonus*, *Eurytoma brunniventris*). Guo et al (1997) state that 28 parasitoid species are known to associate with *D. kuriphilus* in China. Unfortunately only the names of the 10 most important parasitoid species are mentioned in their publication (one of which only receives a short hand and remains unknown here). *Eurytoma concinna*, *Megastigmus nipponicus* and *M. maculipennis* are not known to occur in Europe. *Eurytoma setigera* (suggested to be *E. pistacina*, Askew et al 2013), *Ormyrus punctiger* (now *O. pomaceus*, Askew et al 2013), *Sycophila variegata* and *Torymus geranii* are all known oak cynipid gall parasitoids in Europe and have been reared from *D. kuriphilus* galls here as well as China. Two further species recorded in China *Eupelmus urozonus* and *Eurytoma brunniventris* are also known oak cynipid gall parasitoids reared from *D. kuriphilus* in Europe and are 2 of my 4 focal species,

discussed further below. *Megastigmus dorsalis*, *Torymus auratus*, *T. flavipes*, *Mesopolobus sericeus*, *M. tarsatus*, *M. tibialis* and *Eupelmus annulatus* were all commonly associated with *D. kuriphilus* in Europe but we do not know if these species associate with the invader in its native range.

While this study recorded 39 parasitoid taxa reared from *D. kuriphilus* galls the combined number of parasitoid species encountered on *D. kuriphilus* in Europe is now 57. I have sampled 14 Linnean species of which the association with *D. kuriphilus* was not previously known in Europe or China. Nine of these 14 species were identified morphologically, this included *Eupelmus splendens*, the first cynipid gall parasitoid that is very likely to have jumped to *D. kuriphilus* from a local non-oak source. Five species were only identified by corroborating identification with DNA barcodes. These included *Torymus affinis*, previously mis-identified to *T. flavipes*, *Torymus flavipes* sp1 and *T. cyaneus* sp3 (both cryptic species within their respective morpho-species complex) and two further parasitoid species that confirm recruitment of cynipid gall parasitoids from local sources other than oak galls. *Eurytoma hypochoeridis* and *Torymus rubi* are further discussed in section 4.3.

Discrimination among alternative community assembly hypotheses for *D. kuriphilus* parasitoids depended on whether parasitoid assemblages differed among possible source communities, either on different plant groups or in different locations.
In sections 4.3 and 4.4 I discuss evidence for variation across plant groups and between locations respectively. I then bring this information together in section 4.5 to assess which community assembly hypothesis is best supported by my data.

4.3 Parasitoid Recruitment to Different Host Plant Assemblages in Europe

Of the 43 parasitoid taxa reared from oak cynipid galls the majority were collected from section Quercus oaks with 19 species collected only from section Quercus, 23 species collected on both sections and 1 species collected only on section Cerris oaks. In contrast to previous publications (Bailey et al 2009, Askew 1961) I find no differentiation between the parasitoid assemblages associated with section Quercus *s.s.* and section Cerris oaks. Since I find no significant differences in parasitoid assembly between these two host oak sections I am not able to consider host oak section associated origins of parasitoids attacking *D. kuriphilus* statistically.

Some parasitoid species attacking *D. kuriphilus* could still be allocated to likely source communities simply by considering their biology. Four species in Table 3.1 are particularly noteworthy. *Megastigmus synophri*, *Torymus cerri* and *Eupelmus rostratus* have,

prior to this study, been exclusively recorded on section Cerris oaks (Askew et al 2013). This strongly suggests that these species must have been recruited to *D. kuriphilus* from section Cerris oaks, even if not represented in this study. *Megastigmus synophri* is dependent on 3 cynipid oak gall wasp species: *Andricus glutinosus*, *Aphelonyx cerricola* (both gall inducers) and *Synophrus politus* (an inquiline gall wasp) are tightly associated with section Cerris oaks. *Megastigmus synophri* is therefore a prime example of a specialist parasitoid that has been recruited to the new invasive host from section Cerris trees. *Eupelmus rostratus*, which in this study has only been reared from *D. kuriphilus* in the North of Italy has previously been recorded in Hungary, Austria and Romania. It is known to attack 7 gall inducing cynipid wasps and 1 inquiline cynipid wasp and has only been encountered on *Quercus cerris* although some of the 7 host gall wasp species are known to gall section Quercus host trees as well (Askew et al 2013). *Torymus cerri* is another example of a specialist recruiting to *D. kuriphilus*. Prior to being encountered on the invasive cynipid host it has been reared from 3 gall wasp hosts including 1 inducing cynipid wasp , *Andricus singularis* and 2 inquiline cynipid wasps, *Synophrus olivieri* and *S. politus*. Before this study *Torymus cerri* has also only been recorded from *Quercus cerris* associated galls (Askew 1961, Sellenschlo & Wall 1984, Gyorfi 1962, deGraham & Gijswijt 1998, Askew et al 2013). In my work, it has been collected from *D. kuriphilus* on chestnut as well as from *Andricus curvator* and *A. testacipes* on *Quercus robur*, a section Quercus *s.s.* species. This may be due to two alternative explanations. Firstly, *T. cerri* is encountered very rarely in the field (see Askew et al 2013) and may simply not have been observed on section Quercus *s.s.* before. Alternatively, the two male individuals encountered on *Quercus* may have been mis-identified. Morphologically, *T. cerri* males are very similar to *T. notatus* which is a much more common *Tormyus* species, encountered on both oak sections across Europe. The specimens collected have been carefully checked before deciding on the calling of morpho-species and these individuals would be great candidates for molecular identification.

Cynipid gall inducers other than the oak gallers include the Aylacini, which gall herbs, the Pediaspidini, galling Acer and the Diplolepidini, that gall plants in the Rosaceae family (Askew et al 2006). Communities associated with the gall wasp tribes have so far been considered unlikely to contribute to the recruitment of parasitoid enemies to the chestnut gall wasp (Aebi et al 2007). They are less likely to encounter and contribute to the parasitoid assembly to *D. kuriphilus* because they are less common, more specialised and less species rich. This is, nevertheless, possible and is evidenced by the 3 parasitoid species. *Eupelmus splendens* (1 known gall wasp host), *Eurytoma hypochoeridis* (3 known gall wasp hosts) and *Torymus rubi* (6 known gall wasp hosts) were not collected in their native environment in this study but were subsequently reared from *D. kuriphilus*. *Eupelmus splendens* attacks the

cynipid *Pediaspis aceris* on sycamore trees (*Acer monspessulanum*, *A. opalus* and *A. pseudoplatanus*) (Askew et al 2006). *Eurytoma hypochoeridis* is a parasitoid of cynipid herb gallers on Asteraceae while *Torymus rubi* is a parasitoid of bramble gallers on Rosaceae (Askew et al 2006). These findings suggest that even though oak gall cynipid parasitoids are the dominating source of predators to *D. kuriphilus*, they are by all means not the only source. Furthermore, even though oak and chestnut are more closely related from a phylogenetic point of view, we have confirmed here that more distantly related plant groups such as the Rosaceae, Asteraceae and Sapindaceae can also provide sources for parasitoid recruitment to *D. kuriphilus*.

4.4 Parasitoid Recruitment to Different Regions in Europe

In my oak associated data I observed an apparent decrease in species richness from Eastern sites via North Italy to Central Italy. The same pattern is apparent for parasitoid species richness on *D. kuriphilus* (see table 4.2) in previous work and in my own study. A decline in parasitoid species richness from East/North southwards corresponds to the Out-of-Anatolia hypothesis, long discussed in European gall wasp ecology, as explaining both patterns in species richness and in within-species genetic diversity during dispersal from an Anatolian centre of origin (Rokas et al 2003a, Atkinson et al 2007).

Interestingly, I found highest oak-associated parasitoid richness in the South. While 69% of all parasitoids reared on oak in my study were represented in the East, the South harboured 77% of all species encountered on oak in this study. Of particular interest are the proportions of parasitoids shared between oak and chestnut in the respective regions. The more parasitoid species are represented in a given region (out of the full set of parasitoid species reared) the more parasitoids are shared between oak and chestnut. This is compatible with local recruitment with weak environmental filters and where the availability of recruits is a more likely limiting factor than ability to exploit the new host.

The high parasitoid richness in Sicily and the Balkans may be due to a more conducive environment. Both regions contain extensive mixed forests of oak and chestnut in warm, Mediterranean climates while both Northern and Central Italy are more mountainous areas with less oak cover and a particularly arid environment in Central Italy. This may be similar to inferences of environmental impact on community composition described by Tack et al (2010).

While roughly even numbers of parasitoids attacked oak cynipid galls in 4, 3, 2, or 1 region, more widely distributed parasitoids were more represented in the *D. kuriphilus*

community, with almost 90% of parasitoids reared from the invader occurring in all 4 sampling regions.

4.5 Community Assembly Hypotheses Compared

By far most parasitoids collected on *D. kuriphilus* are known to attack oak cynipid galls in Europe and all are known to be associated with cynipid gall wasps (Cynipidae). This excludes all other parasitoid sources and allows us to reject Random Shift (*sensu* Hubbell 2001) of parasitoids to *D. kuriphilus*. Although parasitoids are recruited from a wider range of plant gall wasp hosts than previously recognised.

There was no evidence that the number of parasitoid species recruiting to *D. kuriphilus* decreases with increasing distance from the North, its origin of invasion, arguing against community assembly involving Host Tracking (sensu Poulin 1999). Furthermore 14 out of 37 parasitoids collected from *D. kuriphilus* galls in this study have not previously been encountered in the North of Italy, where collections over time have been most extensive. This again is compatible with local recruitment of these species rather than tracking of the new invasive host from its centre of origin.

Several observations support Local Recruitment (Weiher & Keddy 1999). Oak and chestnut are more similar to each other in parasitoid species composition space than are regions (see figure 3.10). This is because oak and chestnut share parasitoid assemblages in a given region. In other words, parasitoids in a given region recruit locally to a new host. This is also evident when comparing the number of parasitoid species shared between oak and chestnut in a given region (mentioned in section 4.4 above). Lastly, almost 50% of all parasitoid species reared from oak cynipid galls were also recorded from *D. kuriphilus* at some point in time or space, suggesting not only local recruitment but also weak ecological filtering.

4.6 The recruitment of parasitoids to *D. kuriphilus* over time

Chestnut associated parasitoid assemblages vary significantly in species composition throughout the collection period of 2006-2011 in the North of Italy but do not change significantly in species richness or effective species richness. The change in species composition does not, however, follow a linear upwards trend as one might expect with establishment of local parasitoids on *D. kuriphilus* over time. The change in species composition rather seems stochastic (see figure 3.11). A stochastic community pattern over time has also been reported in native oak gall parasitoid assemblies, with high variation in relative abundance of specific species within and across sites. It would be interesting to have

comparable datasets for parasitoid communities on *D. kuriphilus* and on sympatric native oak galls over time, for which one expectation might be an initial increase in species richness on *D. kuriphilus* after arrival, followed by community dynamics that track native parasitoid assemblages. Unfortunately, no data are available from the introduction of *D. kuriphilus* (1996-2005) to investigate such a scenario.

4.7 An Overview of Community Assembly: Do General Rules Apply to *D. kuriphilus* Recruitment?

This study shows several similarities with previous research on invasion biology in general and gall wasp range expansions in particular. Cornell and Hawkins (1993) predicted that invasive hosts attacked by the same parasitoid genera in their native and invasive ranges would accumulate parasitoid species more rapidly than hosts that are attacked by very different natural enemy communities in the two parts of their range. This is also the case for *D. kuriphilus* in Europe, where many of the parasitoid species recorded in China also attack *D. kuriphilus*. They also state that host species supporting a rich (or poor) parasitoid community in their native range also support a rich (or poor) community in their invasive range (though often reduced). I believe this is not the case for *D. kuriphilus* as in this survey 37 out of 66 recorded parasitoids representing 6 families were found on *D. kuriphilus* (Table 3.1). This means that more than half of the species encountered on oak were also encountered on *D. kuriphilus*, and further, these represented all families also found on oak during this study. Twenty-eight species in 12 families are known to attack it in China (Guo et al 1997). Overall, *D. kuriphilus* has accumulated 57 species in 6 families in Europe since investigation of the galls began in 2006 (Quacchia et al 2013), which is almost double the number of species known to attack the invader in its native range. The caveat here is that there have been extremely limited rearing studies in *D. kuriphilus*' native range compared with Europe. One possibility might be, seeing that many of the parasitoid families attacking the invader in its native and invader range are shared, that parasitoid families are more species rich in Europe and a wider variety of potential predators are available. It would be of interest to compare communities between *D. kuriphilus'* native range, Europe and other invaded areas such as Japan and the USA although less is known of parasitoid communities (and particularly in other possible cynipid hosts) in these invaded areas (Rieske 2007). Cornell and Hawkins (1993) found that a reduced parasitoid assemblage on *D. kuriphilus* held true in comparison to China but this was based on early recruitment (Murakami et al 1977) and recent research in Japan has focussed on hybridisation effects between *T. sinensis*,

the biological control agent of *D. kuriphilus* and local relatives rather than community composition centred on the invader (Yara 2006, Yara et al 2007 & 2010).

Tack et al (2010), in a combination of common garden, reciprocal transplant and field observation studies found that, generally, location is a better predictor of herbivore insect community structure than genotype of the host plant or an interaction between the two effects. Although this was notably not the case for gallers as a specific guild within their study (7 out of 9 of the investigated gallers were Cynipid gall wasps), they found that spatial processes are a better explanation for variation in species richness with better connectivity between trees resulting in higher species richness of insect herbivores. The same may be the case for parasitoids centred around insect herbivores, and in this case, *D. kuriphilus* community assembly could be affected by differently connected oak stands as has been put forward anecdotally by Italian collaborators to explain the different attack rates of parasitoids between the north and south of Italy where more parasitoids are recorded per gall in Sicily (one of the youngest sites of invasion) than in north Italy (the origin of invasion).

Aebi et al 2006 suggested that some parasitoids (not at that time reared from *D. kuriphilus*) are likely to jump onto the invader due to high polyphagy and their association with oak gall wasps. These included *Torymus geranii (*Torymidae*)*, *Ormyrus nitidulus (*Ormyridae*)*, *Cecidostiba semifascia, C. fungosa, Mesopolobus amaenus, Cyrtoptyx robustus* (all Pteromalidae) and *Eupelmus annulatus* (Eupelmidae) whose association with *D. kuriphilus* in Europe has now been established. *Caenacis lauta* (Pteromalidae) and *Baryscapus berhidanus* (Eulophidae) have not yet been associated with the invader but are generally less abundant than the previously mentioned parasitoids. Aebi et al 2006 also suggested that recruitment of inquiline gall wasps to *D. kuriphilus* in future could facilitate recruitment of additional parasitoid species, as observed for the invading oak gall wasp *Andricus quercuscalicis* after its arrival in the UK (Schönrogge et al 1995, 1996a&b, 2006). There is now evidence to support inquiline recruitment to *D. kuriphilus* in Greece (James Nicholls, pers. comm.) and although the community of parasitoids on *D. kuriphilus* has been growing rapidly without the association of inquilines to *D. kuriphilus*, another wave of parasitoids may be able to now exploit the galls of the invader.

Schönrogge et al (1995, 1996a&b, 2006) found parasitoid recruitment on *A. quercuscalicsis* to be very slow at first, with this invader's galls remaining virtually parasitoid free for approximately 40 years before recruiting local parasitoids. They suggested that this may likely be due to presence in *A. quercuscalicis* of a specialised gall structure thought to act as parasitoid-defense (a hollow air space around the larval chamber) and gall induction on a novel site on the host tree (acorn). Morphological defense against parasitoids is common in

oak gall morphologies, though *D. kuriphilus* lacks many obvious defenses (woody exteriors, spikes, false chambers and additional air spaces within the gall, resinous excretions from the gall, Bailey et al 2009). The only possible defense of more mature galls is a relatively thick external wall, requiring a relatively long ovipositor to reach the larvae within. The influence of gall morphology on parasitoid attack rates in *D. kuriphilus* would be a valuable subject for future study.

Overall, parasitoid attack rates on *D. kuriphilus* remain low despite the high richness of parasitoid species known to associate with it and I was not able to observe an increase in parasitoid attack over time (see figure 3.11). This reduces the chance of identifying native parasitoids for biological control of *D. kuriphilus* as suggested by Aebi et al (2006). Introduction of *T. sinensis* as a biological control agent from the native range of *D. kuriphilus* may well have been the most suitable step towards managing *D. kuriphilus* in Europe despite criticism over how *T. sinensis* was introduced (Gibbs et al 2011).

4.8 Limitations and Further Research

Some of the statistical methods and explanatory variables in my analyses were specifically required to control for variation in sample size across gall types. While some galls were represented by very large samples, I was only able to sample small numbers of other types (see Chapter 2). This wide variation prevented use of gall wasp species as an explanatory factor in predicting associations between parasitoids attacking *D. kuriphilus* and specific oak gall types. It would be interesting to see if the morphology of a gall (on oak, rose, etc) in which a parasitoid develops predicts its preference for *D. kuriphilus*. This is of interest because previous work has shown relationships between gall morphology and parasitism (see Stone & Cook 1998, Schönrogge et al 1999, Stone & Schönrogge 2003, Bailey et al 2009). The small numbers of galls collected for most gall wasp species limit my ability to infer relationships for rarely sampled ecological traits. For example, I was unable to sample section Cerris oak galls in some sites within regions because no trees were available to sample or because sampled trees did not produce any emerging insects.

Resources and time placed strong limits on sampling effort in this project. Full sampling of all locations and both spring and autumn gall generations was only possible for one year (2011) and all other sampling schemes were limited to a subset of sites and/or seasons. This means that robust conclusions can only be made for a very short time period and may not be extrapolated beyond the surveying time. In addition, *D. kuriphilus* is only attacked by local parasitoids at a very low rate despite many species seemingly being ready to exploit the invader. Hence, the number of parasitoids obtained remains quite limited

despite intense collection efforts of *D. kuriphilus* galls. Longer surveying periods and higher collection efforts are expected to improve analyses drastically. Furthermore, an increased collection effort would allow for a wider surveying method including galls on other host plants such as Rosaceae, Asteraceae and *Acer* spp.. *Rosa* spp.-associated galls were originally included in the sampling scheme for this study but proved difficult to find. While very small numbers of parasitoids in my study are inferred to have originated from non-oak hosts, these provide particularly interesting examples of rare parasitoid host shifts between very different plant groups.

Analyses could have been improved by further increasing taxonomic resolution. Due to lack of time and resources, I focussed my barcoding efforts on the most abundant and potentially problematic morpho-species. I looked only at a subset of 4 morpho-species thought to contain cryptic taxa based on previous research. Status of other morpho-species in the community has not been extensively studied. Extension of barcoding to include all species, even where only represented by 1 or a few individuals, would increase confidence in sample allocation to taxa, including those individuals that could only be identified morphologically to genus due to poor sample quality. In particular, it would be worth checking for cryptic taxa in any of the parasitoid species that are abundant in oak and chestnut communities, whose reallocation among MOTUs could influence community patterns (Table 3.1). Potential candidates for future research include *Eurytoma pistacina, Sycophila biguttata, Ormyrus pomaceus, Mesopolobus tibialis, Mesopolobus sericeus, Eupelmus annulatus*. Among these candidates, at least three cryptic or sibling species are suspected in *Ormyrus pomaceus* (Hernández Nieves 2007, Gomez et al 2017) and preliminary findings strongly suggest crypsis in all of the mentioned morpho-species.

While identification to MOTUs was adequate for my analyses, it limits comparison of my results to data that may exist for MOTUs not matched to a Linnean species. Generation of an improved set of reference barcodes from a wider panel of candidate species would improve identification of MOTUs to species, and hence ability to use existing biological information.

In conclusion, I have found the arrival of *D. kuriphilus* in Europe to have inadvertently created a natural experiment with vast possibilities for study to further understand the impact, implications and processes involved in invasion biology.

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Appendix A – Raw Data

Table S2.1 - The data collected on *D. kuriphilus* associated parasitoids is provided in electronic form on the accompanying disc

Table S2.2 - The data collected on oak associated parasitoids is provided in electronic form on the accompanying disc

Appendix B - Methods

Box S2.1 Fieldwork Protocol detailing materials, collection, rearing, dissection, and identification of oak gall inhabitants during sampling from spring 2011 until spring 2013.

MATERIALS
(NB.: * Items listed elsewhere previously)

~Travel & Accomodation~
Sleeping Bag
Sleeping Matt
4 Person Tent

~Fieldwork~
Telescopic Tree Cutters
Secateurs
Zip Lock Bags (2L)
Paper Labels
Pencils
Permanent Marker (black sharpie)
Notebook

~Sorting & Potting~
Plastic Vials (various sizes ~5ml -250ml)
*Paper Labels
*Pencils
Fine Mesh Cotton Cloth
Rubber Bands
Sterile Moss (Autumm rearings only, Veterinary Grade available in Pet Shops)
Small Ziploc bags (somewhere between .5 and 1L, Autumn rearings only)

~Rearing, Dissecting & Identifying~
Dissection Kit (soft tweezers, thin tweezers, sharpening stone, scalpel, scissors, fine paint brush)
Microscope
Microscope light(s)
Magnifying glass
Glass Wells (2x)
Paper Towels
Alcohol (100% ethanol)
Alcohol Tubes
Tube Boxes
Cooling box (and several in the autumn)
Cooling cartridges

COLLECTIONS

Notes of Tree and Location

1) For each surveyed tree at least three pictures should be taken before surveying (for morphological reference so make sure these pictures are not blurry).

- The tree trunk (especially structure and shape, hence taking the picture upwards so that the branching into the canopy can be seen as well is particularly useful)
- Leaf top-side and
- Leaf under-side (to record shape and morphology) as well as
- Acorns, in case there are any present

USE THE NOTEBOOK TO RECORD ANY ADDITIONAL INFORMATION USEFUL FOR IDENTIFICATION

Use a digital camera that logs pictures as a running total (you can change this in the settings) rather than

starting to count from ‘1’ every time pictures get taken off the storage.

2) To collect galls from this tree use a 2L Ziploc bag and mark the bag with a permanent marker as follows:

- Picture Numbers: The numbers of the pictures taken for said tree are recorded within the camera (make sure that the numbers logged by the camera are set to a ‘running total’ i.e. that the camera does not reset picture numbers every time that old pictures are deleted). Record the numbers of the taken pictures on the Ziploc bag.
- Date: Record the date on which you are collecting from this tree on the Ziploc bag
- Location: Record the Country, Place and Co-ordinates of the location from which you are collecting and add any additional information that makes identification of the location by third parties easier
- Oak ID: If possible to species if not at least to section.

YOU CAN USE YOUR NOTEBOOK FOR RECORDING CO-ORDINATES AND ADDITIONAL INFORMATION ABOUT THE LOCATION this way you only have to write it down once for each location
IF IT’S WET OUT IN THE FIELD MAKE SURE YOU RECORD THE ABOVE INFORMATION WRITTEN IN PENCIL ON A PIECE OF PAPER THAT GOES INTO THE ZIPLOC BAG because permanent markers are not permanent when used in the wet.

3) For each surveyed tree, some intact leaves should be collected to act as a morphological reference (as well as potential molecular identification later in the study). If the leaves have varying morphology throughout the canopy, try to take this into consideration by taking several leaves of varying morphology. If morphology is broadly uniform take at least two leaves to act as upper and underside reference. The leaves can initially be collected in the marked Ziploc bag together with the galls from the same tree.

Gall Sampling

1) The canopy of each tree should be assessed as broadly as possible (at least on three sides of the tree) and from the bottom of the canopy to as far up as possible (use telescopic tree cutters if necessary, see figure 1 below as a guide)

- This ensures that as many gall types as possible are being collected throughout the survey as some galls can be found exclusively towards the top of the canopy while others prefer the centre or the periphery of the canopy

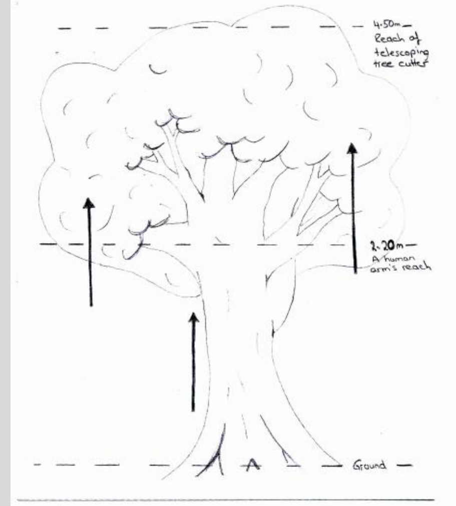

Figure 1. A tree diagram depicting the sampling area that should be covered for each tree facilitating as inclusive a sampling strategy as possible.

2) Survey only fresh shoots from this year's growth. A fresh shoot can be identified as follows (Figure 2):

- Locate a leaf node (where it attaches to the twig) and follow the twig from which the leaf is growing all the way to the next scar. This is where the shoot has started growing this season and all leaves growing from it belong to this season's growth and should be surveyed for galls. Galls on older branches should not be collected
- unless looking fresh and lacking emergence holes.
-

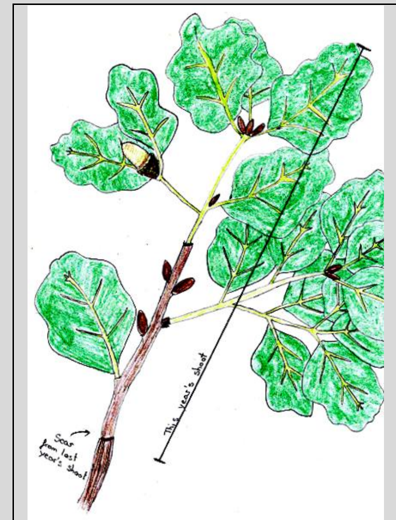

Figure 2. A diagram indicating the growth of the current year's shoot (distance line) and resulting shoot scar (arrow).

3) Collect any galls you find in your marked 2L Ziploc bag. This associates the collections with the correct tree.

4) Also collect catkins or acorns associated with the tree if possible. If there are none on the tree but on the ground collect a few but place them in a separate bag within your collection bag. Mark the separate bag as 'FALLEN'. This way they will not be associated with any particular tree but only with the location from which they were collected.

REARINGS

Sorting & Storing Galls

Once back from working in the field all galls must be potted individually so that their information is as

independent from each other a
s possible

1) Sort the tree bags by location, collector and number respectively. This makes recording of the data more swift.

2) For each bag, sort the galls by species and identify the leaves that should be used for morphological reference

- Place the reference leaves in a herbarium to dry
- Write next to them in pencil the date, location and the picture numbers of the tree from which they were taken

3) Place each individual gall in a plastic vial, appropriate to its size

4) Add a label to the inside of the vial, clearly stating

- The rearing number associated with the gall
- The species of gall wasp it was most likely produced from (see picture guide and gall species reference available within the group to identify the species morphologically)
- The location from which it was collected
- The oak species from which it was collected and
- The date on which it was collected

5) The same data as above should simultaneously be logged either in a notebook or in a backed up excel spreadsheet. In addition to the data above the spreadsheet should also be used to record the picture numbers of the tree associated with each gall, whether the gall is a sexual or asexual generation gall, who collected it and any additional information about species status of the gall and/or how it was collected (such as fallen catkins)

6) Seal the vials by placing the appropriate size cotton material over them and sealing it shut with a rubber band wrapped around it tightly

7) Place the labeled vials into collection trays where they can be stored securely while also allowing enough air to get to them. In the case of travelling with the specimens it is particularly useful to have trays which are a little bit taller than the vials you use so you can stack trays without having to worry about them toppling on the move.

CATKINS, ACORNS AND OTHER GALL SPECIES THAT ARE PARTICULARLY SMALL OR NUMEROUS CAN ALSO BE PLACED IN MASS REARINGS (in the case of N. albipes, N. numismalis and N. quercusbaccarum of the asexual generation, for example, 9 galls are reared individually and the rest of galls on the same tree are collected in mass rearings of up to 20 individuals allowing both efficient potting and a maximum amount of independent data)

Insect Emergences

You want to regularly check your pots of galls for emerged individuals (especially in the spring), this way you can estimate more accurately when they have emerged as well as preventing your samples from drying out or

moulding before you get to them. Rubber bands might rip as they dry and need to be replaced as soon as possible to prevent any missing of samples or insects getting away. In the case of the European Dryocosmus Fieldwork, vials were checked approximately every 4 days in spring and every 7 days in autumn. You check galls by closely looking at the interior of each vial, looking out for any small insects.

1) You will need your boxes with alcohol tubes and use one tube per plastic vial in which you find any insects.

2) If you find any live insects

- Make sure they are not too lively for you to catch them.
- If they are flying too fast you can place them in a cooling box or fridge for a couple of minutes before catching them, this slows them down for a minute or two.
- Label an alcohol tube with the corresponding vial number. You do this by labelling the lid with a permanent marker and labelling the inside with a piece of paper and pencil. Leave the alcohol tube opened next to you.
- Open the plastic vial slightly but keep it as closed as possible by keeping at least one finger over the cotton lid and hence keeping it firmly on the vial. This will give you the opportunity to quickly close the top of the vial in case an insect is trying to get away.
- Use the fine paint brush, dowsed in alcohol to catch the insects. This works best tabbing an insect on the back with the brush held flat, then twist the brush upwards together with the insect which should get stuck to the brush (if it isn't moving and struggling too much).
- With the insect stuck to the brush quickly move it into the alcohol tube.
- Repeat this with as many insects as are in the vial. You can place insects of the same gall/vial in the same alcohol tube until identifying later on.

3) If you find only dead insects in a given vial you don't need to worry about speed as much as with live specimens

- Label an alcohol tube with the corresponding vial number. You do this by labeling the lid with a permanent marker and labeling the inside with a piece of paper and pencil. Leave the alcohol tube opened next to you.
- Open the plastic vial
- Use the fine paint brush, dowsed in alcohol to collect the insects. Usually all you need to do is slightly tab the insect with the tip of the moist paint brush and they will stick to the brush. If the brush is too wet they may start sticking to the vial instead so make sure the brush is only moist.

4) If you find mouldy galls you need to get to dissecting (below). If there is only some mould, it can often be enough to make sure that the vial has enough opportunity to dry out, by placing it in the top tray for example where it gets a lot of air.

DISSECTION

You may need to dissect some galls because of a number of reasons:

- They are going too mouldy (especially in the spring)
- You have trouble identifying them and internal morphology is a useful identifier (see gall book guide)

- One particular gall is the only one you found and you don’t want to lose the one individual you may find inside

1) Make a separate spreadsheet and call it an appropriate name (such as ‘Dissected Individuals’)

2) Record the same information as you did in the rearing spreadsheet and include the date you dissected the gall

3) Label an alcohol tube with the corresponding vial number. You do this by labeling the lid with a permanent marker and labeling the inside with a piece of paper and pencil. Leave the alcohol tube opened next to you.

4) When dissecting a gall you have to be very careful because they are often a shape that doesn’t allow for a lot of grip

- Place the gall under the microscope and put it to the lowest possible magnification for you to see the gall well but also your fingers if possible
- Hold the gall firmly with your thin tweezers (they are particularly good if they have a riffled tip for more grip
- Use a scalpel to slowly chip away at the gall. Often the gall chamber is in the centre of the gall but this is especially not the case for multilocular galls and additionally chamber walls are often hardened and if you take a scalpel directly to them you will harm the insect within beyond recognition. This is why it is especially important to take your time and be careful
- Slice off the gall with the scalpel until you get to the chamber and keep slicing until you have an opening in the chamber.
- Then try to gently and carefully squeeze the gall with the tweezers to brake it open further
- Once the chamber is broken open enough you can pry it apart with either two tweezers or by holding the gall in one had and pulling it open with the tweezers in the other hand. What ever it is that makes you feel like you have more control over the gall.
- Place whatever insects or larvae you find inside in the labeled alcohol tube

IDENTIFICATION

Identifying in the field works pretty much the same as identifying in the lab so long as you remembered to bring everything you need for identifying. Classification booklets for parasitoids(down to species), inducers and inquilines (down to genus) and larvae (down to family) are available within the group. You will need glass wells, alcohol, soft tweezers, a pipette, paper towels and the magnifying glass.

Box S2.2. PCR Recipes & Conditions

Gene: Cytochrome *b*

Primer:
CB1
Sequence:
CB1: 5’ TAT GTA CTA CCA TGA GGA CAA ATA TC 3’
Reverse complement: GAT ATT TGT CCT CAT GGT AGT ACA TA
Primer:
CB2
CB2: 5’ ATT ACA CCT CCT AAT TTA TTA GGA AT 3’
Reverse complement: AT TCC TAA TAA ATT AGG AGG TGT AAT
Primer:
CP2
Sequence:
CP2: 5’ CTA ATG CAA TAA CTC CTC C 3’
Reverse complement: GGA GGA GTT ATT GCA TTA G

PCR conditions:
A touchdown program was used, which starts at a higher temperature to make the initial cycles more specific and then cycles down sequentially to facilitate annealing for remaining cycles.

94°C 2 minutes
94°C 30 seconds
60°C 30 seconds
decrease by 1°C every cycle
72°C 40 seconds
Go to step 2, repeat 9x
94°C 30 seconds
50°C 30 seconds
72°C 40 seconds
Go to step 6, repeat 30x
72°C 5 minutes
10°C for ever
END

PCR mix:
This mix is used for both primer pairs.
Final magnesium concentration: 2mM

For one 20µl reaction add:
autoclaved MilliQ water 15.04µl
10x PCR buffer 2µl
$MgCl_2$ (50mM) 0.8µl
primer CB1 (20µM) 0.3µl
primer CP2 **AND** CB2 (20µM) 0.3µl **each**
dNTPs (each 25mM) 0.16µl
Taq (Bioline 5U/µl) 0.1µl
DNA extract 1.0µl

... continued on next page

Gene: Cytochrome Oxidase subunit I
Primers: COI_pF1, COI_pF2, COI_2413d
Fragment length: 698 base pairs in chalcid parasitoids (756 bp including primers).
This fragment overlaps the 3' end of the Folmer fragment by 458 bp and avoids the poly T present in many parasitoid species. These primers are designed specifically for chalcid parasitoids and have been tested extensively.

Primer:
COI_pF1
Primer sequence:
COI_pF1: 5’ AGG RGY YCC WGA TAT AGC WTT YCC 3’
Reverse complement: GGR AAW GCT ATA TCW GGR RCY CCT

Primer:
COI_pF2
Primer sequence:
COI_pF2: 5’ ACC IGT DAT RAT RGG DGG ITT YGG DAA TT 3’
Reverse complement: AAT THC CRA ANC CHC CYA TYA THA CNG GT

Primer:
COI_2413d
Primer sequence:
COI_2413d: 5’ GCT ADY CAI CTA AAA ATY TTR ATW CCD GT 3’
Reverse complement: ACH GGW ATY AAR ATT TTT AGN TGR HTA GC

Variable sites:
I = Inosine
Y = T or C
R = A or G
D = A or G or T
H = A or C or T
W = A or T
N = A or C or G or T

PCR conditions:

94°C	2 minutes
94°C	30 seconds
45°C	1 minute
72°C	1 minute
Go to step 2, repeat 4x	
94°C	30 seconds
50°C	1 minute
72°C	1 minute
Go to step 6, repeat 34x	
72°C	5 minutes
10°C	for ever
END	

PCR mix:
Final magnesium concentration: 1.5mM
note also that the primer volume is less than our other mtDNA PCR mixes

For one 20µl reaction add:
autoclaved MilliQ water 16.2µl

10x PCR buffer	2µl	
$MgCl_2$ (50mM)	0.6µl	
primer pF1 **OR** pF2 (20µM)		0.2µl
primer 2413d (20µM)	0.2µl	
dNTPs (each 25mM)		0.1µl
Taq (Bioline 5U/µl)		0.1µl
DNA extract		0.6µl

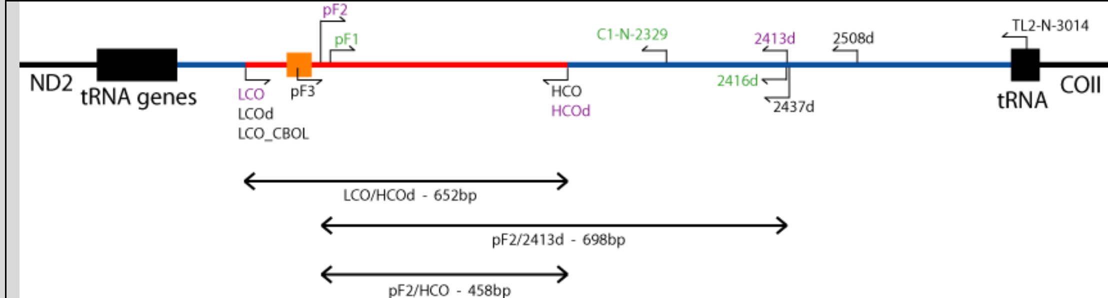

Illustration of the COI region with primers available for sequencing in chalcidoids. The illustration includes (amongst others) from left to right forward primer pF2, forward primer pF1 reverse primer 2413d. The blue line indicates the COI region. The red line indicates the Folmer region (with available primers LCO(d, CBOL) and HCO(d) creating a 652 bp fragment in chalcids. The orange box indicates the poly-T region present in chalcidoid parsitoids. Purple primers are preferred primers for amplification work. Green primers are alternative primers that are known to work well. Black primers are variably working primers that are available as alternatives. The overlap of used primers pF2 and pF1 is visible using this illustration.

Appendix C - Results

Table S3.1 List of reared parasitoids across regions and host trees including MOTU re-allocation of the four focal species. Data collected on all host tree types from 2011 until 2013. Parasitoids in the table are grouped by family. Parasitoids that could only be identified to genus are at the end of the table. Decimal numbers are a result of re-allocating species identity as a proportion of the morphological identity of individuals using barcode information as described in the methods section.

	Region	EAST Slovenia & Croatia			NORTH North Italy & Switzerland			CENTRE Central Italy			SOUTH Sicily		
	Host Plant	**Castanea**	**Cerris**	**Quercus**	**Castanea**	**Cerris**	**Quercus**	**Castanea**	**Cerris**	**Quercus**	**Castanea**	**Cerris**	**Quercus**
	Galls Reared	11761	249	1113	8000	25	336	3000	70	266	5607	383	1859
	Species Recorded	25	14	31	18	6	23	8	6	8	14	18	37
Parasitoid Species	**Family**												
Eurytoma brunniventris	Eurytomidae	15		40.61			8.8			24.5		17	57.86
E. hypochoeridis	Eurytomidae	25											
Eurytoma MOTU0068	Eurytomidae		1										
E. pistacina	Eurytomidae			13	558	2	4.2						25
Sycophila biguttata	Eurytomidae	27	25	2	17		7	5	9		24	40	21
S. binotata	Eurytomidae		2				1					3	
S. variegata	Eurytomidae	5			40			5	3			53	17
Megastigmus dorsalis spA	Torymidae	45.42		8.44						23		3	56
M. dorsalis spB	Torymidae	42.58		9.5	1782		4					3	14
M. stigmatizans	Torymidae			1.06									
M. synophri	Torymidae				1								
Torymus cerri	Torymidae						2	1					
T. cyaneus	Torymidae		4		27								
T. affinis	Torymidae										9.58		
T. auratus	Torymidae	31.32		6			18				3		292.55
T. flavipes sp1	Torymidae	486.94		40.95			12			12	354.28		71.71
T. rubi	Torymidae	8.77											
T cyaneus sp3	Torymidae	39.48		1.05					1		19.15	26	5.74
T. formosus	Torymidae	1											
T. geranii	Torymidae	42.71					1						6

Table 4.3 continued on next page…

T. nobilis	Torymidae	1											5
T. scutellaris	Torymidae	1											
T. sinensis	Torymidae				3								
Ormyrus nitidulus	Ormyridae		2	1									10
O. pomaceus	Ormyridae	165	13				7				1		6
Cecidostiba atra	Pteromalidae			3									3
C. fungosa	Pteromalidae		68	18		5	2					10	26
C. ilicina	Pteromalidae												2
C. saportai	Pteromalidae			2		1							
C. semifascia	Pteromalidae			34			2						7
Cyrtoptyx robustus	Pteromalidae										5		
Hobbya stenonota	Pteromalidae			6									66
Mesopolobus albitarsus	Pteromalidae		3	7			1			1			3
M. amaenus	Pteromalidae		3	7					2	1		1	1
M. dubius	Pteromalidae	6			1		1		4		3		24
M. fasciiventris	Pteromalidae	4		4	13								26
M. fucipes	Pteromalidae			2			1						
M. lichtensteinii	Pteromalidae			1							2		
M. mediterraneus	Pteromalidae			7							1		5
M. sericeus	Pteromalidae	25		21			1				36		24
M. tarsatus	Pteromalidae											1	5
Me. tibialis	Pteromalidae	142	1	6	2					4	376	2	23
M. xanthocerus	Pteromalidae		4	1		1	3						17
Ormocerus latus	Pteromalidae			1				1					
Eupelmus annulatus	Eupelmidae	12		4.86	554.95	1	5	130		1	2	3	102
E. rostratus	Eupelmidae				141								
E. splendens	Eupelmidae				1			83					
E. urozonus	Eupelmidae	21	2	11.14	171.79		2	2			1	3.75	13
E. fulvipes	Eupelmidae	63			314.95		1	1					
Eupelmus MOTU0008	Eupelmidae				14.32								
Eupelmus MOTU0015	Eupelmidae											1.25	
Macroneura vesicularis	Eupelmidae	1											
Aulogymnus arsames	Eulophidae						1						
A. eudereschus	Eulophidae											1	1
A. gallarum	Eulophidae		10			2	38						42
A. skianeuros	Eulophidae	1	7	5	180		1					1	1

Table 4.3 continued on next page.

A. testaceoviridis	Eulophidae											2	
A. trilineatus	Eulophidae												4
Aprostocetus aethiops	Eulophidae			3					1				
A. cerricola	Eulophidae			4									
Baryscapus pallidae	Eulophidae			1									2
Pediobius rotundatus	Eulophidae									2		7	27
unidentified MOTUs													
Eurytoma MOTU0013	Family Status uncertain				6								
Eurytoma MOTU0035													5.79
Eurytoma MOTU0047													5.79
Torymus MOTU0012		8.77											
Samples IDed to genus only													
Sycophila sp	Eurytomidae			11			11			3			32
Megastigmus sp	Tormyridae	1											
Torymus sp	Tormyridae	4		17			5				1		
Ormyrus sp	Ormyridae			22									
Cecidostiba sp	Pteromalidae			18						11			20
Mesopolobus sp	Pteromalidae						1	2		6			
Eupelmus sp	Eupelmidae						1	2			3		
Aulogymnus sp	Eulophidae									1			
Aprostocetus sp	Eulophidae		1										
Baryscapus sp	Eulophidae			8									
Pediobius sp	Eulophidae												1

Figure S3.2 - *Eupelmus urozonus* NJ tree

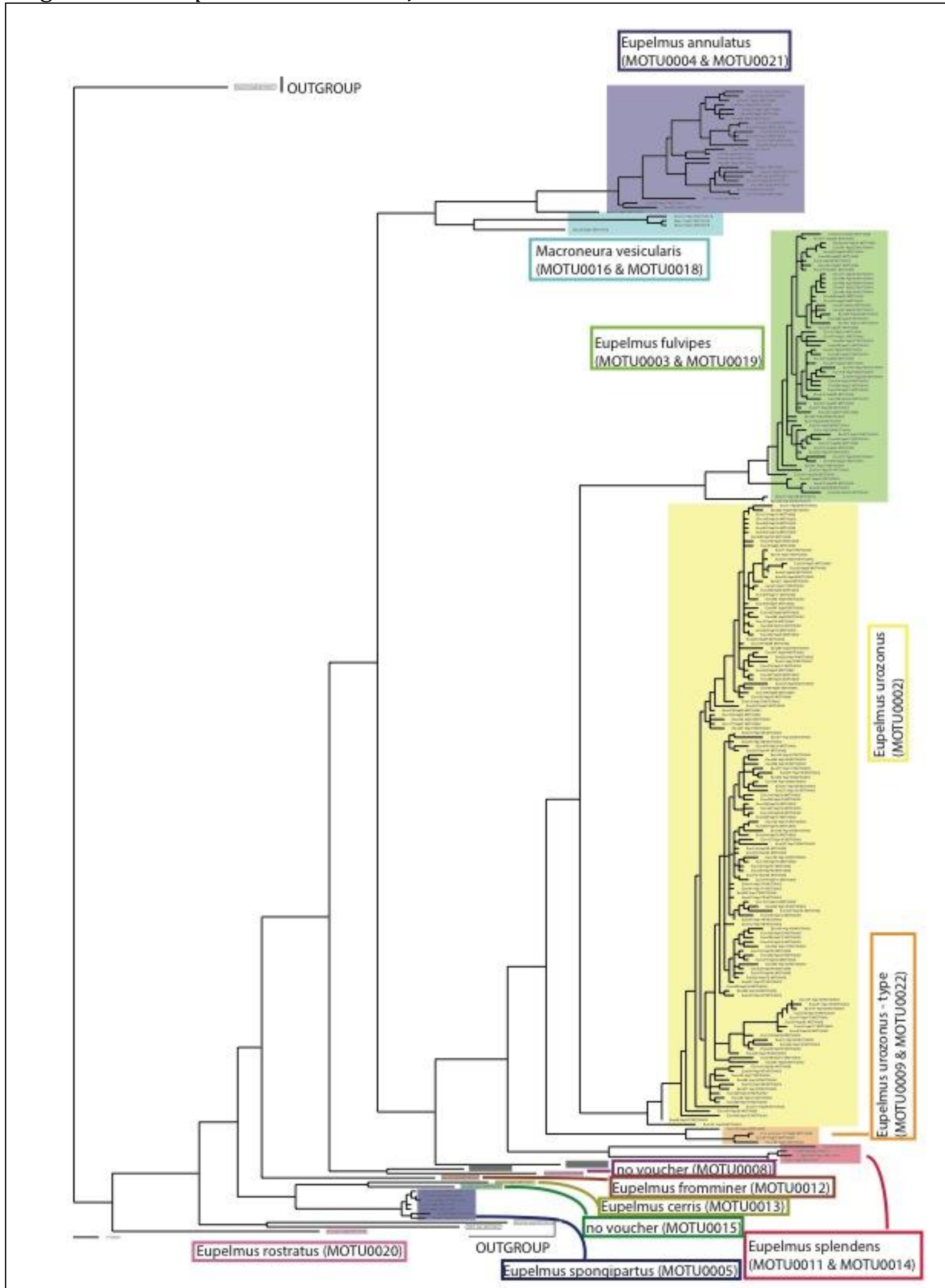

Figure S3.2 Illustration of *Eupelmus* NJ tree. Individual sequences have been collapsed to haplotypes to facilitate visual inspection of the tree. Each haplotype in the tree is identified by haplotype identity, MOTU identity as well as the name of one of the representatives from each haplotype. Clades have been named after each MOTU they represent as well as the reference sample the given clade groups with. This tree serves to indicate relationships between MOTUs as well as serving as a quality check for the MOTU cut-off.

Figure S3.3 - *Eurytoma brunniventris* NJ tree

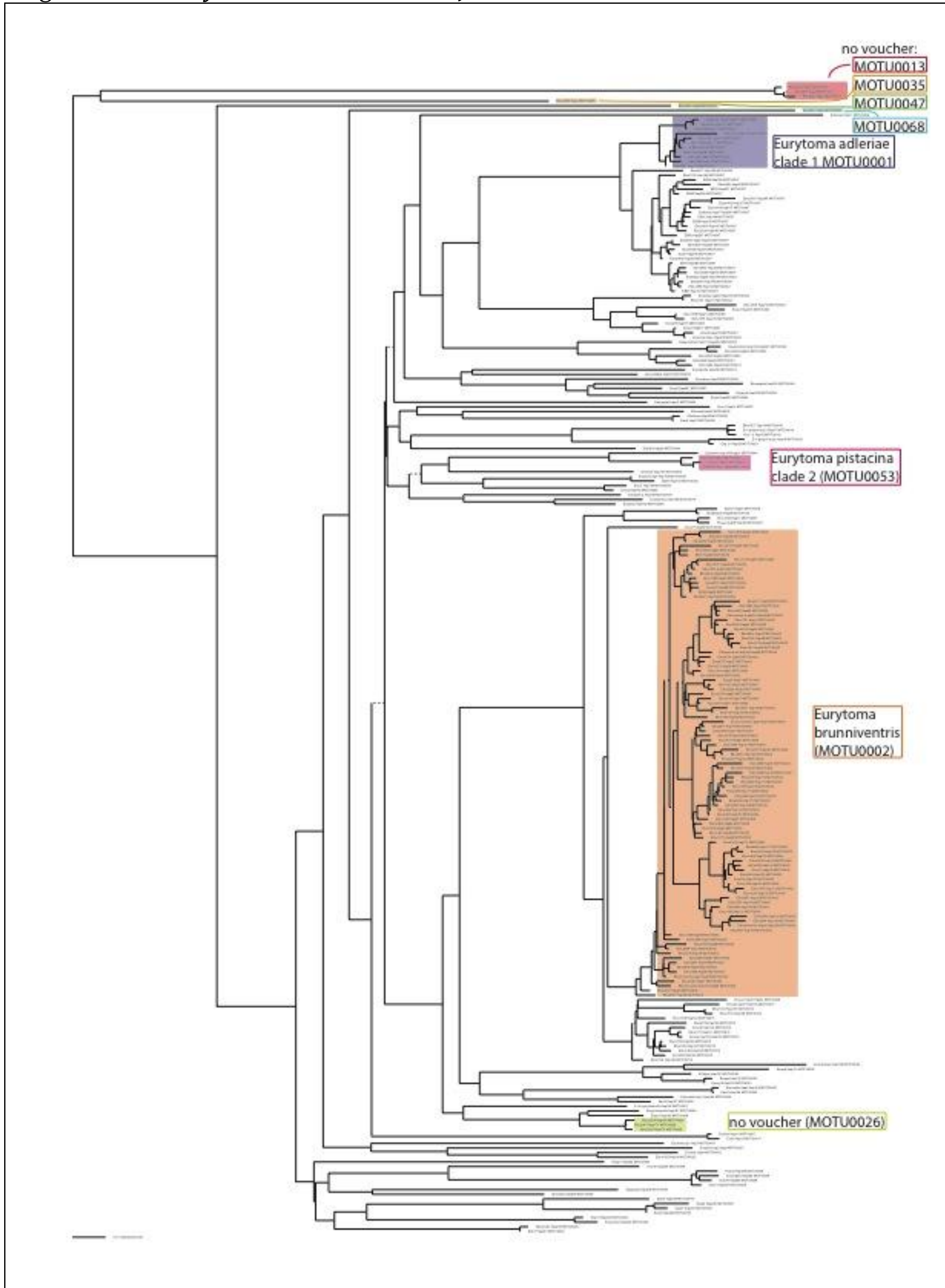

Figure S3.3 Illustration of *Eurytoma* NJ tree. Individual sequences have been collapsed to haplotypes to facilitate visual inspection of the tree. Each haplotype in the tree is identified by haplotype identity, MOTU identity as well as the name of one of the representatives from each haplotype. Clades have been named only for clades which were represented in molecular analyses of this chapter to facilitate ease of viewing. Clades are named after each MOTU they represent as well as the reference sample that the given clade groups with. This tree serves to indicate relationships between MOTUs as well as serving as a quality check for the MOTU cut-off.

Figure S3.4 - *Megastigmus dorsalis* NJ tree

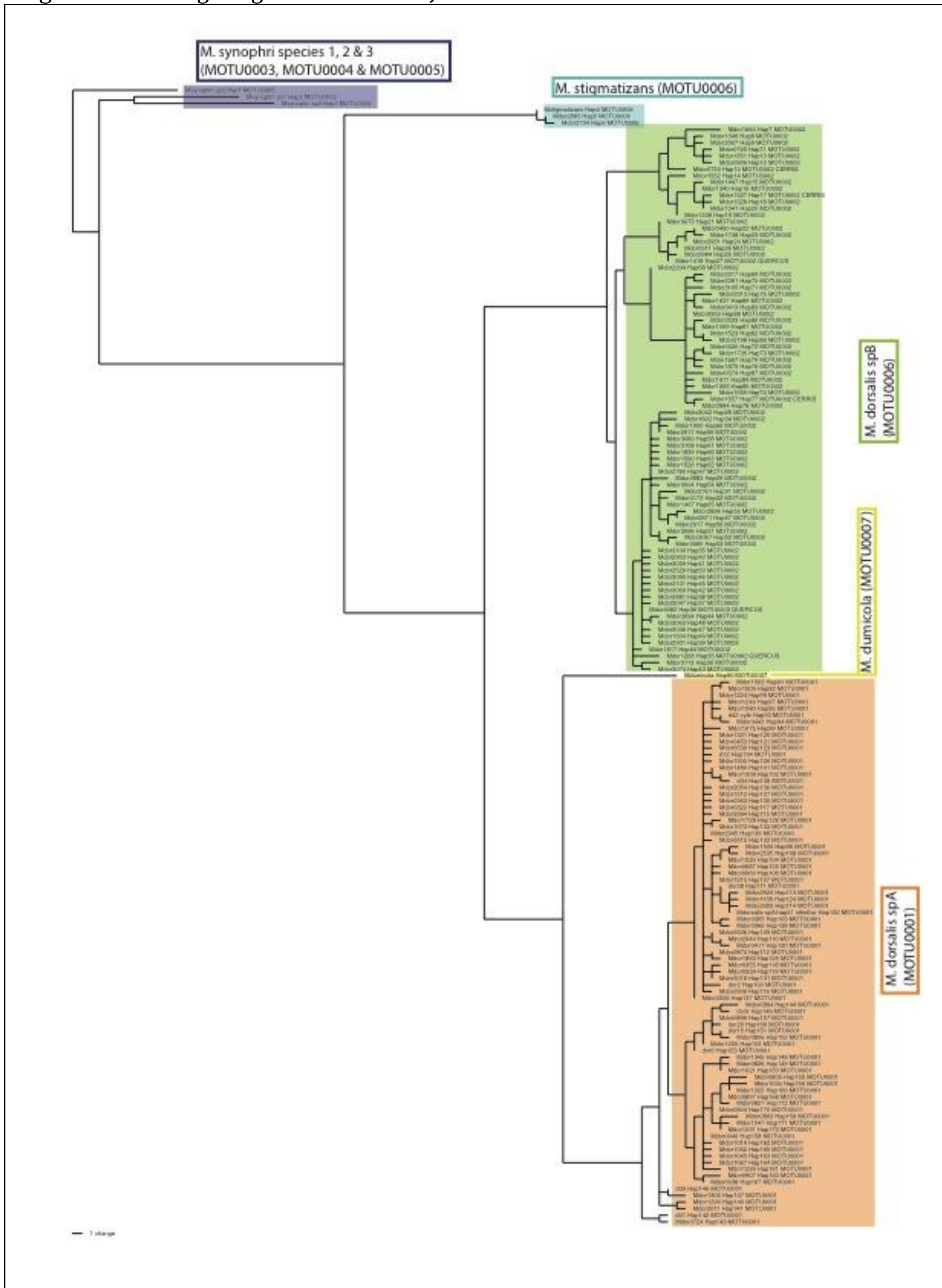

Figure S3.4 Illustration of *Megastigmus* NJ tree. Individual sequences have been collapsed to haplotypes to facilitate visual inspection of the tree. Each haplotype in the tree is identified by haplotype identity, MOTU identity as well as the name of one of the representatives from each haplotype. Clades have been named after each MOTU they represent as well as the reference sample that the given clade groups with. This tree serves to indicate relationships between MOTUs as well as serving as a quality check for the MOTU cut-off.

Figure S3.5 - *Torymus flavipes* NJ tree

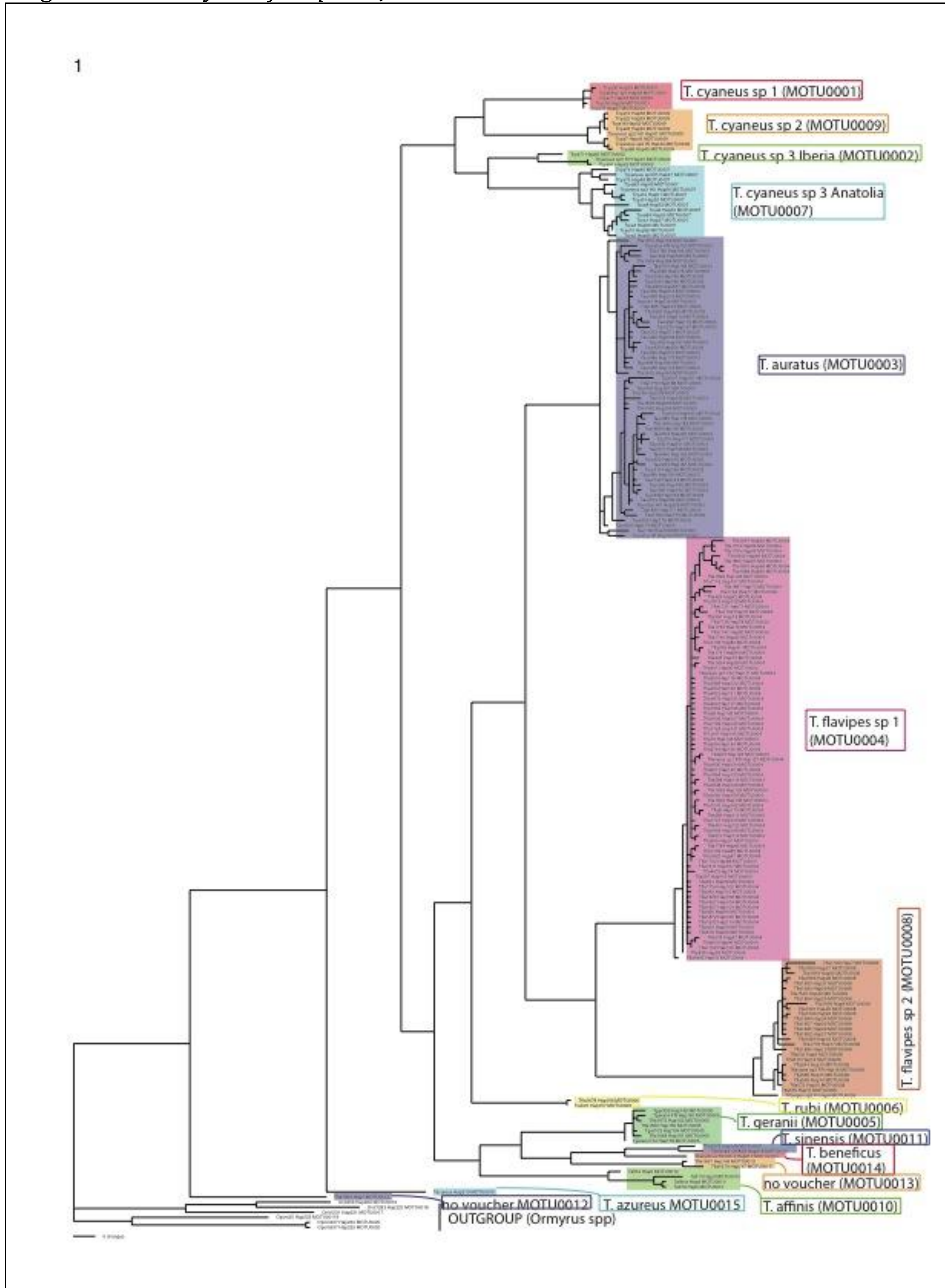

Figure S3.5 Illustration of Torymus NJ tree. Individual sequences have been collapsed to haplotypes to facilitate visual inspection of the tree. Each haplotype in the tree is identified by haplotype identity, MOTU identity as well as the name of one of the representatives from each haplotype. Clades have been named after each MOTU they represent as well as the reference sample that the given clade groups with. This tree serves to indicate relationships between MOTUs as well as serving as a quality check for the MOTU cut-off.

Appendix D – Additional Information

The following species have been recently renamed and may be in discordance in other literature to what they are named in this thesis:

Species Name in this thesis	Official Species Name
Eurytoma pistacina	*Eurytoma pistaciae*
Megastigmus dorsalis	*Bootanomyia dorsalis*
M. stigmatizans	*B. stigmatizans*
M. synophri	*B. synophri*
Eupelmus rostratus	*Reikosiella rostrata*
Macroneura vesicularis	*Eupelmus vesicularis*