Speciation and isolating barriers in a parasitoid wasp focusing on the role of reproductive isolation caused by endosymbionts

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Chapter 1: General Introduction

1.1 Hymenoptera

The order of Hymenoptera (predominantly bees, wasps, and ants) is one of the most species-rich, if not the most diverse in the animal kingdom (Forbes et al. 2018). One of the superfamilies contained within the Hymenoptera are the Chalcidoidea (chalcid wasps) which comprise over 20,000 described species (Noyes 2019) and up to 500,000 species in total according to some estimates (Heraty 2017). The majority of chalcid species are parasitoids, meaning that their larvae develop feeding on their hosts, killing them in the process (Godfray 1994; Heraty et al. 2013). The diversity of the Chalcidoidea has been shown to have been initially established by a high rate of speciation in the Paleogene (Cruaud et al. 2022). The frequency by which new hymenopteran and chalcid species are being discovered is very high, partially due to a high abundance of cryptic species and species complexes, continuously increasing the known diversity (Heimpel et al. 1997; Heraty et al. 2007; Desneux et al. 2009; Novković et al. 2011; Chesters et al. 2012; Aguiar et al. 2013; Fusu 2017; Haas et al. 2021). One of the reasons potentially explaining the suspected high speciation rate in Chalcidoidea is the frequent occurrence of sib-mating, i.e. the mating between brothers and sisters (Askew 1968). This limits the gene flow to individuals occurring in close quarters and prevents interbreeding between populations, promoting their independent evolution (Askew 1968; Malec et al. 2021). Their haplodiploidy contributes to a fast evolution as deleterious alleles are eliminated upon expression in the haploid males and are therefore not passed on to the next generation. In contrast, favourable alleles are likely to become fixed more quickly (Askew 1968).

1.2 The biological species concept

A variety of different concepts for the definition of species are in existence (see for example (Hey 2001; Coyne and Orr 2004) for a compilation of different species concepts). According to a recent survey, the biological species concept (BSC) (Mayr 1969) is the most widely used among researchers studying ecology and evolutionary processes (Stankowski and Ravinet 2021). It defines a species as consisting of populations which are able to reproduce amongst each other, but are separated from other groups of populations by complete (Mayr 1969) or very high reproductive isolation (Coyne and Orr 2004) preventing gene flow.

1.3 Species delimitation by barcodes

More recently, a new approach to species determination has become popular in taxonomy. Here, the barcode segment of the mitochondrial COI gene (Hebert et al. 2003b), supplemented with further data obtained from morphology, ecology, or nuclear genes, is central to the determination of species (Smith et al. 2005, 2006, 2008; Hajibabaei et al. 2006; Stahlhut et al. 2013). This method relies on the existence of a certain distance between intra- and interspecific variability in COI barcodes, known as the barcoding gap (Meyer and Paulay 2005). This divergence of COI sequences is used for the delimitation between species based on threshold distances between different species. Thresholds defining the boundaries of species,

potential species or other taxonomic units are set at 2% divergence in COI by many authors (e.g. (Strutzenberger et al. 2011; Stahlhut et al. 2013; Hubert and Hanner 2015; Kang et al. 2017; Fagan-Jeffries et al. 2018)). Similarly, the Barcode Index Number (BIN) system of the BOLD database used in studies practicing turbo-taxonomy (Meierotto et al. 2019; Sharkey et al. 2021) uses 2.2% COI difference as threshold to group specimens into operational taxonomic units (OTUs) (Ratnasingham and Hebert 2013). In extreme cases, species descriptions have been based solely on COI barcodes and included no noteworthy additional data, an approach termed turbo-taxonomy. This facilitates the description of high numbers of new species in relatively short periods of time (Butcher et al. 2012; Meierotto et al. 2019; Sharkey et al. 2021). Therefore, COI barcode-based investigations of biodiversity are preferentially used for groups which are especially species-rich, such as braconid wasps (Smith et al. 2008; Butcher et al. 2012; Fagan-Jeffries et al. 2018; Meierotto et al. 2019; Sharkey et al. 2021), Malagasy ants (Smith et al. 2005), Hymenoptera in general (Stahlhut et al. 2013), tachinid flies (Smith et al. 2006), and geometrid moths (Strutzenberger et al. 2011).

1.4 Speciation in Hymenoptera

The diversity of Hymenoptera and Chalcidoidea has been studied intensively (for example (Heimpel et al. 1997; Heraty et al. 2007; Desneux et al. 2009; Novković et al. 2011; Chesters et al. 2012; Aguiar et al. 2013; Fusu 2017; Haas et al. 2021)). In contrast, the factors playing a vital role in the evolution of this diversity, namely speciation and reproductive barriers, have been addressed in relatively few hymenopteran taxa, including *Nasonia* (Breeuwer and Werren 1990; Bordenstein et al. 2001), *Muscidifurax* (Legner 1969), *Encarsia* (Gebiola et al. 2016a, b), and *Bracon* (Heimpel et al. 1997). These studies are far outnumbered by studies approaching hymenopteran diversity with a focus on other aspects, such as phylogeny, and are generally lacking in numbers (K. König et al. 2019; Malec et al. 2021).

1.5 Speciation and reproductive isolation

If the BSC is utilized to define species, then the investigation of the speciation process, i.e. the split of one species into two or more different species, requires examining the barriers to gene flow arising during divergence and eventually leading to strong reproductive isolation in fully separated species (Turelli et al. 2001; Butlin et al. 2012; Butlin and Stankowski 2020). These barriers causing and upholding reproductive isolation act during different stages of reproduction. Specifically, they can occur before (premating) or after mating (postmating). Furthermore, they can be divided into pre- or postzygotic barriers, depending on whether they inhibit the formation of the zygote or act on the zygote after fertilization (Coyne 1992; Turelli et al. 2001; Coyne and Orr 2004).

Premating, prezygotic barriers include ecological as well as sexual isolation and act before mating can take place. They are followed by postmating, prezygotic barriers, which are in place after copulation has occurred, but before fertilization, such as the incompatibility of gametes. Finally, postmating, postzygotic barriers act after fertilization. They encompass detrimental effects on the fitness of hybrids, such as a decreased viability, behavioral and physiological sterility as well as a reduced fertility (Coyne and Orr 2004).

Apart from determining all barriers acting in a speciation process, identifying the barrier initializing speciation is an especially important question in investigating speciation processes (Turelli et al. 2001; Coyne and Orr 2004; Butlin et al. 2012).

1.6 Endosymbionts

Bacterial endosymbionts, i.e. bacteria which entertain close relationships with their hosts and live within them, have been reported from many arthropod species (Duron et al. 2008; Moran et al. 2008; Engelstädter and Hurst 2009). Their regular transmission mode is vertical (i.e. maternal) (Perlmutter and Bordenstein 2020), although horizontal transfers between species, including between hosts and parasitoids, appear to be common as well (Werren et al. 1995; Vavre et al. 1999; Dyson et al. 2002; Jaenike et al. 2007; Raychoudhury et al. 2009; Haselkorn et al. 2009; Majerus and Majerus 2010; Ahmed et al. 2016; Binetruy et al. 2019; Sanaei et al. 2021). Some endosymbionts confer beneficial effects on their hosts, such as defense against parasitoids (Xie et al. 2010, 2014), fungi (Łukasik et al. 2013), and nematodes (Jaenike et al. 2010b), or an improvement of their fitness, including fecundity increases (Weeks and Stouthamer 2004; Zhang et al. 2018; Xie et al. 2020). Other endosymbiont-induced effects on the host, including inducing cytoplasmic incompatibility (Yen and Barr 1971; Hunter et al. 2003; Zchori-Fein et al. 2004; Takano et al. 2017, 2021; Rosenwald et al. 2020), male-killing (MK) (Skinner 1985; Gherna et al. 1991; Werren et al. 1994; Hurst et al. 1999b, a; Majerus et al. 1999; Jiggins et al. 2000; von der Schulenburg et al. 2001; Lawson et al. 2001; Dyson et al. 2002; Tinsley and Majerus 2006; Simon et al. 2011), parthenogenesis (Stouthamer et al. 1993; Pijls et al. 1996; Arakaki et al. 2001; Zchori-Fein et al. 2001, 2004; Weeks and Breeuwer 2001; Hagimori et al. 2006; Kremer et al. 2009), and feminization (Rigaud et al. 1991; Weeks et al. 2001; Hiroki et al. 2004; Chigira and Miura 2005; Negri et al. 2006) manipulate the reproduction of the hosts. As these manipulations increase the proportion of infected females in the population, the spread of the predominantly maternally transmitted endosymbionts through the population is promoted (e.g. reviewed by (Perlmutter and Bordenstein 2020)).

Cytoplasmic incompatibility

Cytoplasmic incompatibility (CI), the most common mechanism of reproductive manipulation by endosymbionts (Shropshire et al. 2020), is the incompatibility between males infected with a specific bacterium and females not carrying the same bacterium, causing fertilization to fail after mating has occurred (Yen and Barr 1971). It occurs unidirectionally in crosses between infected males and uninfected females (Barr 1980; Hsiao and Hsiao 1985; Hoffmann et al. 1986) or bidirectionally between males and females infected with different bacterial strains (Yen and Barr 1971; Breeuwer and Werren 1990). In diploid organisms, complete CI prevents the formation of any offspring, whereas intended diploid offspring of haplodiploid organisms can develop as haploid offspring instead (e.g. (Ryan and Saul 1968)).

Wolbachia-induced CI has been reported for a plethora of organisms, including mosquitoes (Laven 1951; Yen and Barr 1971; Trpis et al. 1981), *Nasonia* wasps (Saul 1961; Breeuwer and Werren 1990; Bordenstein et al. 2001), spider mites (Breeuwer 1997), weevils (Hsiao and Hsiao 1985), *Tribolium confusum* (Wade

and Stevens 1985; O'Neill 1989), and between as well as within *Drosophila* species (Hoffmann et al. 1986; Hoffmann and Turelli 1988; Louis and Nigro 1989; Werren and Jaenike 1995).

Besides *Wolbachia*, known CI inducers are *Candidatus* Cardinium hertigii (Bacteroidetes) (Hunter et al. 2003; Zchori-Fein et al. 2004), hereafter referred to as *Cardinium*, *Candidatus* Mesenet longicola (Alphaproteobacteria) (Takano et al. 2017, 2021), and *Rickettsiella* (Gammaproteobacteria) (Rosenwald et al. 2020).

CI mechanism

In *Wolbachia*-induced CI, different cellular defects have been observed. Mechanisms comprise faulty incorporation of histones into the male pronucleus after fertilization as well as delays of the replication of the paternal DNA and of the nuclear envelope breakdown (Tram and Sullivan 2002; Landmann et al. 2009). Furthermore, the timely and correct condensation of the paternal chromosomes has been demonstrated to be impaired, leading them to form a chromatin mass (Breeuwer and Werren 1990; Reed and Werren 1995; Lassy and Karr 1996; Callaini et al. 1997; Bonneau et al. 2018). Their belated entry into mitosis and their incorrect segregation lead to aneuploid or haploid daughter nuclei sometimes connected by chromatin bridges (Lassy and Karr 1996; Callaini et al. 1997; Tram et al. 2006; Landmann et al. 2009). A similar phenotype has also been found in *Cardinium*-induced CI with the presumably paternal chromosomes showing a delayed entry into mitosis and a defective segregation as well as a formation of chromatin bridges (Gebiola et al. 2017).

Wolbachia-induced CI has been described as a modification-rescue-model (Werren 1997). Herein, *Wolbachia* in the males cause modifications to the sperm which render them incompatible with eggs in the females following copulation unless a compatible *Wolbachia* is present in the females to rescue the modifications (Werren 1997). The genetic basis of *Wolbachia*-induced CI was uncovered in recent years. Two adjoining genes often located on the prophage WO have been linked to the presence of CI. They are generally designated as *cifA* and *cifB* (cytoplasmic incompatibility factors A and B), and more specifically as *cinA* and *cinB* (CI-inducing nuclease) or *cidA* and *cidB* (CI-inducing DUB), depending on whether they encode nuclease domains or deubiquitylating enzymes (DUBs) (LePage et al. 2017; Beckmann et al. 2017; Shropshire and Bordenstein 2019). In some studies, a two-by-one model applies, wherein both *cifA* and *cifB* are required to be expressed in the males and the expression of *cifA* alone in the females is sufficient for rescue (Shropshire et al. 2018; Shropshire and Bordenstein 2019). In some studies, only the expression of *cifB* in the males was required to induce CI (Adams et al. 2021; Sun et al. 2022). Recently, it was shown that the transfer of CidB with the sperm to the eggs was crucial in inducing CI by negatively affecting replication of the paternal DNA whereas the binding to CidA eliminated this effect (Horard et al. 2022). In contrast, CifA and CifB were associated with sperm nuclei during spermatogenesis and spermiogenesis in the males and CifA occurred in the ovaries during oogenesis of the females whereas all Cifs were absent from the embryos (Kaur et al. 2022), suggesting variations in CI induction. Homologs of these apparently highly conserved genes have been found in different *Wolbachia* strains and their similarity as well as the absence of these genes in other *Wolbachia* fit the compatibility patterns observed in crossing experiments

(LePage et al. 2017; Beckmann et al. 2017). In support of a previous study which did not find any *Wolbachia* candidate genes for CI induction in the CI-inducing *Cardinium* (Penz et al. 2012), a recent study revealed that *cif* genes are absent in these endosymbionts (Lindsey et al. 2018). Obviously, CI has developed independently in *Wolbachia* and *Cardinium* (Penz et al. 2012). Nevertheless, as the cellular phenotype of CI induced by *Cardinium* is very similar to that of *Wolbachia*, the different genetic mechanisms in both genera might affect the same conserved targets in their hosts, leading to a similar outcome (Gebiola et al. 2017).

Endosymbionts and speciation

Due to their ubiquity and because their interactions with their hosts can influence reproduction of their hosts and affect gene flow between differentially infected host populations, bacterial symbionts are considered to have the potential to drive reproductive isolation in their hosts and therefore to contribute to speciation in different manners (reviewed by (Bordenstein 2003; Brucker and Bordenstein 2012; Shropshire and Bordenstein 2016)).

CI as barrier promoting speciation

Endosymbiont-induced CI has been considered as potential very fast reproductive barrier facilitating immediate isolation between differentially infected populations independently from changes in the hosts (Werren 1998; Wade 2001). Still, it has been considered unlikely to be the main cause for speciation events due to an observed incomplete barrier to gene flow, as both maternal transmission and CI itself are often imperfect, as well as due to the fact that a barrier based only on CI could be easily eliminated (Hurst and Schilthuizen 1998; Wade 2001). Nevertheless, Werren (1998) considered endosymbiont-CI to be able to promote speciation either by being strong and bidirectional or by being one of several barriers being incompletely bidirectional or unidirectional. Various studies have produced evidence for both scenarios. Bidirectional CI, albeit asymmetrical in strength, is the first and only barrier between two *Nasonia* species (Bordenstein et al. 2001). However, CI mostly contributes to speciation in conjunction with other barriers. For example, unidirectional CI works in opposite direction to higher sexual isolation in *Tribolium confusum* (Wade et al. 1995). Similarly, between the closely related species *D. recens*, which carries *Wolbachia*, and the uninfected *D. subquinaria*, CI presents a barrier in crosses between *D. recens* males and *D. subquinaria* females whereas sexual isolation acts in the opposite direction (Shoemaker et al. 1999; Jaenike et al. 2006). In addition, hybrid breakdown is present causing sterility of hybrid males (Shoemaker et al. 1999). Endosymbiont-induced CI is one of several barriers between *N. vitripennis* and *N. giraulti* (Breeuwer and Werren 1990). Similarly, CI inflicted by *Cardinium* causes reproductive isolation in one direction between *Encarsia suzannae* and *E. gennaroi* parasitoid wasps, while Bateson-Dobzhansky-Muller incompatibilities act in the other direction (Gebiola et al. 2016a).

Parthenogenesis

The induction of parthenogenesis is another potential way of endosymbiont-driven speciation. Individuals infected with the endosymbiont reproduce asexually, usually by producing only female offspring, which is

known as thelytoky (e.g. (Stouthamer et al. 1993; Zchori-Fein et al. 2004; Hagimori et al. 2006)). Prolonged endosymbiont-induced parthenogenesis has been shown to lead to the deterioration of traits associated with sexual reproduction, including the abilities to perform courtship behavior and produce sperm or the failure to undergo the same evolutionary changes in these traits as the uninfected, sexually reproducing populations (Pijls et al. 1996; Arakaki et al. 2000; Gottlieb and Zchori-Fein 2001; Adachi-Hagimori et al. 2011) (reviewed by (Bordenstein 2003; Brucker and Bordenstein 2012; Shropshire and Bordenstein 2016)). In both cases, reproduction between the parthenogenetic and non-parthenogenetic populations is prevented even after the former is cured, resulting in speciation, as has been observed for example in the genetically divergent populations of the parasitoid *Neochrysocharis formosa* (Adachi-Hagimori et al. 2011).

Assortative mating and sexual selection according to infection status

Besides influencing reproduction and gene flow in their hosts directly via reproductive manipulations, endosymbionts have also been shown to have an indirect impact, with infection status acting on mate choice, which can result in prezygotic/premating isolation (reviewed by (Beltran-Bech and Richard 2014; Shropshire and Bordenstein 2016)). Assortative mating according to infection status has been observed in several systems (Markov et al. 2009; Miller et al. 2010). In others, only uninfected females prefer uninfected males over infected ones and spatial separation of egg patches according to infection type promotes sib-mating and mating of individuals of the same infection type (Vala et al. 2004). A significant reduction of sexual isolation between *D. melanogaster* populations differentially infected with *Wolbachia* following treatment with tetracycline suggests a role of *Wolbachia* in sexual isolation (Koukou et al. 2006). Similarly, females of the uninfected *D. subquinaria* show a high sexual isolation against sympatric closely related *D. recens*, which are infected with *Wolbachia* inducing strong CI. In contrast, *D. subquinaria* females living in allopatry with *D. recens* do not show this sexual isolation (Jaenike et al. 2006). Indeed, it has been suggested that driving assortative mating instead of being a barrier in itself might be the more important role of CI in speciation (Hurst and Schilthuizen 1998).

1.7 *Lariophagus distinguendus*

Lariophagus distinguendus (Förster 1841) is a parasitic wasp and belongs to the family of Pteromalidae within the order Hymenoptera. Its larvae develop as ectoparasitoids on the outside of enclosed larvae and pupae of weevils and beetles, and kill their hosts during development (e.g. (Kaschef 1961; van den Assem 1971; Bellows 1985)). Many of the host species of *L. distinguendus* are pests on dry stored products (reviewed by (Niedermayer et al. 2016)). Notably, *Sitophilus granarius*, *S. oryzae*, *Rhyzopertha dominica*, *Stegobium paniceum*, *Gibbium psylloides*, *Lasioderma serricorne*, *Ptinus tectus*, *P. fur*, *Callosobruchus chinensis* and *C. maculatus* have been identified as host species (Hase 1919; Hüsing 1935; Kaschef 1955, 1959, 1961; Bellows 1985; Papadopoulou and Athanassiou 2004), see (Steidle and Schöller 1997) for a comprehensive list. However, wasp performance has been shown to differ between hosts as well as between wasp strains on the same host (Bellows 1985; Steidle and Schöller 2002; Steidle et al. 2006; Belda

and Riudavets 2012). Generally, due to the nature of its hosts, *L. distinguendus* can be used as biocontrol agent (Niedermayer et al. 2016).

L. distinguendus is ca 1-3 mm in length, has a black body with a metallic sheen, and parts of its legs and antennae are yellow-brownish legs (Hase 1919; van den Assem 1969). Its courtship follows a specific sequence of behaviors, starting with the males vibrating their wings ("wing fanning") and raising their abdomens upon perceiving the females. Next, the males mount the females and repeatedly perform distinct antennal and head movements above and next to the females' antennae ("antennal stroking"). If the females are receptive, they signal their receptivity by lowering their heads and antennae and opening their genital pouch. Following this signal, the males move backwards to the rear ends of the females and extend their abdomens below the females' abdomens to initiate copulation (Hase 1919; van den Assem 1969; Ruther et al. 2000). While the males mate with several females when given the chance, females are considered to be monandrous, meaning they mate only once (van den Assem 1969). After mating, the females search for suitable oviposition sites using the smell of the host substrate, especially infested substrate marked by host feces as cue (Steidle and Schöller 1997; Steidle 2000; Steiner et al. 2007; Benelli et al. 2013). Upon encountering host substrate, the females examine their hosts. To that end, they drum with their antennae on the structures containing the hosts, such as grains, and tap on the structure with their abdomens. Finally, they use their ovipositors to drill into the structure down to the hosts and insert their ovipositors to deposit the eggs on the outsides of the hosts (Hase 1924; van den Assem 1971; Steidle 2000). Usually, the females lay one egg per host, but more than one egg per host can occur (Gonen and Kugler 1970).

The sex of the deposited egg is determined by the females and depends on the size of the host (van den Assem 1971). Small hosts are used for males, whereas large hosts serve for the development of females, which require more resources (van den Assem 1971; Charnov et al. 1981; Simbolotti et al. 1987; van den Assem et al. 1989). Under normal conditions, more females than males are produced, with the proportion of males ranging from 17% to 35% (Hase 1919; van den Assem 1971; Charnov et al. 1981; Simbolotti et al. 1987; Ryoo et al. 1991a; Steiner et al. 2008; Belda and Riudavets 2012). Total offspring numbers per female are highly variable (e.g. (Kaschef 1954; Gonen and Kugler 1970)) and are strongly influenced by temperature (Niedermayer et al. 2013). The developmental time of the offspring is approximately 16 to 23, sometimes up to 40 days (Kaschef 1961; van den Assem 1969; Gonen and Kugler 1970), depending on temperature (Ryoo et al. 1991b), and is shorter in males by one day (Gonen and Kugler 1970; van den Assem 1971; Ryoo et al. 1991b; Steiner et al. 2008).

Regarding the longevity of *L. distinguendus*, different data has been obtained by various authors on different hosts, with females generally having a longer lifespan than males with 7 to 30 days as opposed to approximately 4 to 7 days (Kaschef 1954; Gonen and Kugler 1970; Ryoo et al. 1991a, b; Steidle and Schöller 2002; Steiner et al. 2008).

In recent years, *L. distinguendus* has been established as species complex containing at least two, albeit undescribed, species. According to their preferred host species, they are temporarily referred to as the

granary weevil (GW) species and the drugstore beetle (DB) species (K. König et al. 2019). In addition to this defining trait, they are characterized by different chromosome numbers (C. König et al. 2019). Males and females of the DB-species have 6 and 12 chromosomes, respectively, whereas the GW-species possesses 5 chromosomes in the males and 10 in the females (C. König et al. 2019). The GW-species also displays early learning by strongly increasing acceptance of drugstore beetles as hosts after coming into contact with them early in life, i.e. during development or as young adults. In contrast, the DB-species does not have this ability and always prefers drugstore beetles, which led to the hypothesis of the drugstore beetle being the ancestral host (König et al. 2015a). Furthermore, sexual isolation between single strains of each species has been shown to be high in crossings between females of the GW-species and males of the DB-species and complete in the opposite direction (K. König et al. 2019), which is likely caused by host-dependent variations in the male mandibular pheromones (König et al. 2015b). Weaker sexual isolation has been shown in crosses between strains of the DB-species (K. König et al. 2019). Cytoplasmic incompatibility induced by an unidentified endosymbiont is acting in the reverse direction to the complete sexual isolation (K. König et al. 2019). Preliminary evidence obtained in two Master's theses suggested this endosymbiont to be *Spiroplasma* (Krimmer 2015; Pollmann 2016).

1.8 Research questions of the thesis

In the following chapters, two main questions will be answered. First, the identity of the endosymbiont responsible for CI between DB-males and GW-females contributing to reproductive isolation between the two species will be confirmed. To this end, the presence of the known CI inducers and further endosymbiotic reproductive manipulators will be investigated within the *L. distinguendus* strain STU and the involvement of all bacteria comprising its microbiome will be excluded. Fluorescent in situ hybridization using specific probes on the ovaries of STU females will be used to investigate a potential for maternal transmission of the candidate and transfection of tetracycline-treated females will be conducted to confirm the link between CI and the candidate.

Then, the diversity within the *L. distinguendus* species complex will be addressed. The phylogenetic relationships between the currently known strains will be investigated using COI and nuclear genes. Furthermore, reproductive isolation in pairs of strains characterized by different genetic divergences will be studied with crossing experiments documenting the barriers sexual isolation as well as inviability, behavioral and physiological sterility, and reduced fertility for hybrids of both sexes. Thereby, species delimitation based on COI and the BSC will be conducted, comparing both methods. In addition, valuable insights into both the speciation process of *L. distinguendus* and potential species status of the investigated strains will be gained.

Chapter 2: Highly transmissible cytoplasmic incompatibility by the extracellular insect symbiont *Spiroplasma*[1]

2.1 Introduction

A number of maternally transmitted microorganisms in arthropods have evolved the remarkable ability to manipulate their hosts' reproduction, in order to increase the frequency of infected hosts (Moran et al. 2008; Engelstädter and Hurst 2009). The most common of these manipulations is cytoplasmic incompatibility (CI), whereby uninfected females produce few or no offspring upon mating with infected males (Werren 1997; Shropshire et al. 2020). As a result, infected females have a significant advantage over their uninfected counterparts and can rapidly spread and replace them in the population (Werren 1997; Shropshire et al. 2020).

In an important advance, the genetic basis of CI was recently discovered in the best studied CI microbe, the Alphaproteobacterium *Wolbachia pipientis,* and involves two linked genes, termed *cifA* and *cifB* (for cytoplasmic incompatibility factor). They operate in a manner similar to toxin-antitoxin systems, with one or both *cif* genes modifying or poisoning infected male sperm, and *cifA* alone rescuing incompatibility in the eggs of infected females (LePage et al. 2017; Beckmann et al. 2017; Adams et al. 2021). Without *cifA* rescue, paternally-derived chromosomes are destroyed, resulting in embryonic lethality (Beckmann et al. 2017) (or haploid male development in some species with haplodiploid sex determination (Vavre et al. 2001)).

There is great interest in using CI microbes to control arthropod pests and disease vectors. This can be done in two ways, both of which involve establishing a new, stable CI infection in the target host species (Brelsfoard and Dobson 2009). First, analogous to sterile insect techniques, large quantities of infected incompatible males can be released in the field, which will result in population crashes due to their mating with wild uninfected females (Brelsfoard and Dobson 2009). Alternatively, CI microbes can be used to spread desired traits when females and males infected with a novel CI microbe are released in the wild (Brelsfoard and Dobson 2009). This approach has been proven highly successful in reducing the prevalence of dengue virus in humans by releasing *Aedes aegypti* mosquitoes infected with a strain of the bacterial symbiont *Wolbachia* that causes both CI and suppresses viruses (Hoffmann et al. 2011; Utarini et al. 2021). *Wolbachia*-infected mosquitoes reach high frequencies due to CI, and this results in reduced dengue virus titer and transmission (Walker et al. 2011).

Wolbachia is by far the best known and most common CI-inducing microbe. It was first shown to cause CI in *Culex pipientis* mosquitoes in the 1970s, and has since been found to cause CI in at least 10 arthropod orders. In 2003, a second unrelated bacterial symbiont, *Candidatus* Cardinium hertigii (hereafter referred to as *Cardinium*), in the Bacteroidetes, was found to cause CI in a parasitic wasp (Hunter et al. 2003), with

[1] This chapter is based on Pollmann, M., Moore, L.D., Krimmer, E., D'Alvise, P., Hasselmann, M., Perlman, S.J., Ballinger, M.J., Steidle, J.L.M., and Gottlieb, Y. (2022). Highly transmissible cytoplasmic incompatibility by the extracellular insect symbiont *Spiroplasma*. iScience 25, 104335. 10.1016/j.isci.2022.104335.

later studies extending this phenomenon to mites (Gotoh et al. 2007), planthoppers (Nakamura et al. 2012), and thrips (Nguyen et al. 2017). Although *Cardinium*-induced CI also involves modification of male sperm and rescue in females, sequencing its genome revealed that it does not contain homologs of the *Wolbachia cif* genes (Penz et al. 2012), indicating independent evolution of CI. More recently, a new bacterium in the same family as *Wolbachia*, called *Candidatus* Mesenet longicola (hereafter referred to as *Mesenet*), and a strain of the Gammaproteobacterium *Rickettsiella*, were shown to cause CI in a beetle and a spider, respectively, but so far, little is known about them and the CI they induce (Takano et al. 2017, 2021; Rosenwald et al. 2020; Shropshire et al. 2020).

Here, the discovery of a surprising new CI microbe that infects *Lariophagus distinguendus* (Förster 1841) (Hymenoptera: Pteromalidae), a cosmopolitan parasitic wasp that is commonly used in biological control of beetle pests of stored products, is reported. Recently, CI was reported in *L. distinguendus*, marked by the absence of diploid female offspring in incompatible crosses (K. König et al. 2019). Interestingly, neither *Wolbachia* nor *Cardinium* were detected in wasps from the incompatible line (K. König et al. 2019).

Unexpectedly, CI in *L. distinguendus* is demonstrated to be caused by a maternally transmitted strain of *Spiroplasma,* a diverse lineage of microbes in the gram-positive Mollicutes that includes pathogens and commensals, as well as several vertically transmitted endosymbionts, and infects a wide range of invertebrates (Gasparich 2002; Regassa and Gasparich 2006). Some vertically transmitted *Spiroplasma* produce female-biased sex ratios as male-killers, while others protect their hosts against parasitic nematodes, wasps and pathogenic fungi (Gasparich 2002; Regassa and Gasparich 2006). *Spiroplasma*'s localization in the hemolymph throughout host life facilitates rapid and efficient transfer of heritable infections to new hosts in the laboratory (Anbutsu and Fukatsu 2011; Ballinger and Perlman 2019). Indeed, *Spiroplasma* and its CI phenotype could be transferred to uninfected wasps using microinjection. This easy transferability and broad host range promise new possibilities for research into CI and its applications.

2.2 Material and Methods

2.2.1 Study animals

The *L. distinguendus* strain dbSTU-D1 (STU), which was collected in households in 2007 in Stuttgart-Bad Cannstatt, Germany, was used for the experiments. The strain was reared on six-week-old larvae of *Stegobium paniceum* in koi pellets (Hikari Friend, Kamihata Fish Industry Group, Kyorin Corporation, Japan) in glass jars with a ventilated lid (diameter 12 cm, height 16 cm). Beetles were reared by placing about 1 g of newly emerged unsexed adult beetles (about 700 beetles) on 80 g koi pellets. After six weeks, freshly emerged *L. distinguendus* were placed on the infested pellets. The cultures were kept at 26 °C and 45% RH with a natural light:dark cycle. To obtain virgin wasps for the experiments, they were isolated in the pupal stage by dissecting the koi pellets and kept individually in 1.5 ml tubes until eclosion. An endosymbiont-free line, termed STU(-), was obtained by tetracycline treatment for at least three generations. Newly emerged wasps were placed in a Petri dish containing filter paper and a piece of cotton wool soaked in a solution of tetracycline (1 mg/ml) and sucrose (100 mg/ml). After 24 hours, wasps were

placed on host-infested substrate as described above. The loss of endosymbionts was confirmed by PCR (as described below) for randomly chosen individuals prior to using these lines in experiments.

2.2.2 Crossing experiments

1-day-old single virgin wasp males and females of the antibiotic and untreated lines were placed together to enable mating in all possible combinations. All pairs were subsequently transferred to host-infested substrate for oviposition, i.e. 5 g of koi pellets containing *S. paniceum* larvae. After four to five weeks, the number and sex of emerging offspring were recorded. Crossings without any offspring were excluded from the analysis.

2.2.3 Specific testing for bacteria

The wasps were tested for the presence of *Spiroplasma*, *Rickettsia*, *Arsenophonus, Mesenet*, and *Rickettsiella*. For the DNA extraction, two different methods were used. One method consisted of crushing single wasps in 10 µl of a lysis buffer (9.95 µl TE-buffer with 10 mM Tris, 0.5 mM EDTA, 3 mg molecular biology grade proteinase K (Sigma-Aldrich Products Ltd., Rehovot, Israel), and 0.5 µl IGEPAL®CA-630 (Sigma-Aldrich Products Ltd., Rehovot, Israel)) and the subsequent incubation of the mixture with another 30 µl or 90 µl of the same buffer at 65 °C for 15 minutes and 95 °C for 10 minutes using a heating block. Alternatively, wasp DNA was extracted using the nexttec™ 1$^{\text{-Step}}$ DNA Isolation Kit for Tissue & Cells (nexttec Biotechnologie GmbH, Hilgertshausen, Germany) following the corresponding protocol. The PCR conditions generally consisted of an initial denaturation step at 94 to 95 °C for 1 to 4 minutes, 35 cycles of 92 °C or 95 °C for 30 seconds, 30 seconds at the respective annealing temperatures of 55 to 58 °C and 30 to 60 seconds at 72 °C followed by a final extension at 72 °C for 5 minutes (see Table 2.1 for details). The reaction mix consisted either of 20 µl Promega GoTaq®Green Master Mix 2X (Promega, Madison WI, USA), with 4 µl of each primer and 10 µl of double distilled water per 2 µl sample, 5 µl Promega 5X Green GoTaq® Reaction Buffer with 0.5 µl 10mM dNTPs (Promega, Madison WI, USA), 1 µl of each primer, 16.375 µl double distilled water and 0.125 µl Promega GoTaq® G2 DNA Polymerase (Promega, Madison WI, USA) per 1 µl sample or 12.5 µl of ROTI®Pol TaqS Red-Mix (2x) (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) with 1 µl of each primer and 9.5 µl of double distilled water per 1 µl sample. The PCRs were performed using a Biometra professional Basic Thermocycler (Analytik Jena AG, Jena, Germany), a Biometra TGradient 96 Thermocycler (Analytik Jena AG, Jena, Germany) or a Techne® Prime Thermocycler (Cole-Parmer, Stone, UK). Gel electrophoresis was conducted on a 1 to 2% agarose gel using 5 µl peqgreen (VWR International GmbH, Darmstadt, Germany) per 100 ml TAE buffer or 5 to 10 µl RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, Inc.) for DNA staining and Norgen LowRanger 100 bp DNA (Norgen Biotek Corp., Canada) or an equimolar 100 bp ladder (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) as ladder. Of each sample, 5 to 10 µl were transferred to the gel. All PCR experiments in this study followed these procedures unless stated otherwise. The primers ApDnaAF1 (5'-ATT CTT CAG TAA AAA TGC TTG GA-3' (Fukatsu et al. 2001)) and ApDnaAR1 (5'-ACA CAT TTA CTT CAT GCT ATT GA-3' (Fukatsu et al. 2001)) were used to test for *Spiroplasma*, and RB-F (5'-GCT CAG

AAC GAA CGC TAT C-3' (Gottlieb et al. 2006)) and RB-R (5'-GAA GGA AAG CAT CTC TGC -3' (Gottlieb et al. 2006)), respectively, for *Rickettsia*. Ars23S-1(5'-CGT TTG ATG AAT TCA TAG TCA AA-3' (Thao and Baumann 2004)) and Ars23S-2 (5'-GGT CCT CCA GTT AGT GTT ACC CAA C-3' (Thao and Baumann 2004)) were used to test for *Arsenophonus*, L355F (5'-GCT ATG CCG CGT GAG TGA TT-3' (Takano et al. 2017)) and L749R (5'-ACA CAG AAA TAA AAA TTC CTA C-3' (Takano et al. 2017)) for *Mesenet* and RLA16SF1 (5'-CAG TAA ARR TTT CGG YCT TTA YGG G-3' (Duron et al. 2016)) and RLA16SR1 (5'-CAA ACC TAG TCA ACC ACC TAC ACG-3' (Duron et al. 2016)) for *Rickettsiella*.

Table 2.1. Primers and PCR conditions for PCRs used in this study.

Primer pair	Sequences	Target	PCR conditions	Reference
ApDnaAF1 ApDnaAR1	5'-ATT CTT CAG TAA AAA TGC TTG GA-3' 5'-ACA CAT TTA CTT CAT GCT ATT GA-3'	*Spiroplasma*	95 °C 4 min, 35 cycles 95 °C 30 s / 55 °C 30 s / 72 °C 1 min	(Fukatsu et al. 2001)
RB_F RB_R	5'-GCT CAG AAC GAA CGC TAT C-3' 5'-GAA GGA AAG CAT CTC TGC -3'	*Rickettsia*	95 °C 2 min, 35 cycles 92 °C 30 s / 58 °C 30 s / 72 °C 30 s, 72 °C 5 min	(Gottlieb et al. 2006)
Ars23S-1 Ars23S-2	5'-CGT TTG ATG AAT TCA TAG TCA AA-3' 5'-GGT CCT CCA GTT AGT GTT ACC CAA C-3'	*Arsenophonus*	95 °C 2 min, 35 cycles 95 °C 30 s / 58 °C 30 s / 72 °C 45 s , 72 °C 5 s	(Thao and Baumann 2004)
L355F L749R	5'-GCT ATG CCG CGT GAG TGA TT-3' 5'-ACA CAG AAA TAA AAA TTC CTA C-3'	*Mesenet*	95 °C 2 min, 35 cycles 95 °C 30 s / 58 °C 30 s / 72 °C 45 s , 72 °C 5 s	(Takano et al. 2017)
RLA16SF1 RLA16SR1	5'-CAG TAA ARR TTT CGG YCT TTA YGG G-3' 5'-CAA ACC TAG TCA ACC ACC TAC ACG-3'	*Rickettsiella*	95 °C 2 min, 35 cycles 95 °C 30 s / 56 °C 30 s / 72 °C 45 s , 72 °C 5 s	(Duron et al. 2016)
515F 926R	5'-GTG YCA GCM GCC GCG GTA A-3' 5'-CCG YCA ATT YMT TTR AGT TT-3'	V4-V5 variable regions of microbial 16S rDNA	See (Jiang et al. 2019)	(Walters et al. 2016)
27f 1492r	5'-AGA GTT TGA TCC TGG CTC AG-3' 5'-TAC CTT GTT ACG ACT T-3'	General bacteria	95 °C 5 min, 35 cycles 95 °C 30 s / 45 °C 30 s / 72 °C 1 min, 72 °C 10 min	(Weisburg et al. 1991) (Frank et al. 2008)
27f EnterobacterR	5'-AGA GTT TGA TCC TGG CTC AG-3' 5'-AGC GTC AGT CTT TGT CCA GGG-3'	*Enterobacter*	95 °C 5 min, 35 cycles 95 °C 30 s / 52 °C 30 s / 72 °C 45 s, 72 °C 5 min	(Weisburg et al. 1991) N/A
EnterobacterF EnterobacterR	5'-GAG GGT GCA AGC GTT AAT CGG-3' 5'-AGC GTC AGT CTT TGT CCA GGG-3'G-3'	*Enterobacter*	94-95 °C 5 min, 35 cycles 95 °C 30 s / 51 °C 40-45 s / 72 °C 1 min, 72 °C 5 min	N/A N/A
SP6 T7	5'-ATT TAG GTG ACA CTA TAG-3' 5'-TAATACGACTCACTATAGGG-3'	- (sequencing primers)	94 °C 5 min, 35 cycles 94 °C 30 s / 50 °C 15 s / 72°C 30 s, 72 °C 1 min	Microsynth Seqlab, Göttingen, Germany

2.2.4 Next generation sequencing of the microbiome

The whole microbiome of the STU strain was subjected to Next Generation Sequencing (16S rRNA amplicon sequencing) with five samples consisting of 50 females each, following the procedure described by (Dally et al. 2020). DNA extraction was conducted using the DNeasy® Blood and Tissue kit (QIAGEN, Germantown, MD, USA) according to the manufacturer's instructions. PCR was conducted with the primer pair 515F (5'-GTG YCA GCM GCC GCG GTA A-3')/926R (5'-CCG YCA ATT YMT TTR AGT TT-3'), targeting the V4-V5 variable regions of microbial 16S rDNA (Walters et al. 2016) to screen for microbes. Sequencing of the resulting amplicons was performed on an Illumina MiSeq sequencer (Illumina Inc., San Diego, CA) as described by (Jiang et al. 2019) using a MiSeq v3 flow cell (Illumina Inc., San Diego, CA). PCR, library preparation, and sequencing were performed at the University of Illinois at Chicago Sequencing Core (UICSQC).

The DADA2 pipeline, provided by the R package "dada2" (v. 1.14.0) (Callahan et al. 2016) was implemented to process the obtained sequences by trimming them and filtering those with poor quality ("filterAndTrim", maxEE=2, maxN=0 and trimleft=15). Error rate was estimated ("learnErrors", randomize set to "TRUE") and corrected sequences were inferred with the dada2 algorithm ("dada"). Complete sequences were assembled from the forward and reverse sequences via "mergePairs" and chimeras were identified and removed ("removeBimeraDenovo"). A count table containing the amplicon sequence variants (ASVs) and their respective counts was created ("makeSequenceTable"). The taxonomic matches of the ASVs were determined via the "assignTaxonomy" command (minimum bootstrap confidence value at 80%) using the SILVA non-redundant small subunit ribosomal RNA database (v.132) (Quast et al. 2013) as reference, and added to the table for further analysis.

2.2.5 Testing for Enterobacteriaceae

Tetracycline-treated and untreated STU females, as well as STU males of both CI and non-CI crossings were tested for the Enterobacteriaceae detected by NGS using the primers 27f (5'-AGA GTT TGA TCC TGG CTC AG-3' (Weisburg et al. 1991)), 1492r (5'-TAC CTT GTT ACG ACT T-3' (Frank et al. 2008)), EnterobacterF (5'-GAG GGT GCA AGC GTT AAT CGG-3'), and EnterobacterR (5'-AGC GTC AGT CTT TGT CCA GGG-3') (also see Table 2.1). A sample of a tetracycline-treated STU female subjected to PCR with the primer combination 27f/1492r was chosen for Sanger Sequencing and sent to Microsynth Seqlab (Göttingen, Germany) for this purpose. The sequence was processed with GENtle v. 1.9.4. (© by Magnus Manske, University of Cologne, released under GPL 2003) (Manske 2006).

2.2.6 Fluorescent *In-Situ* Hybridization

In order to test the presence of *Spiroplasma* in the ovaries of *L. distinguendus*, the ovipositor with attached ovaries of live females was removed in 1 x PBS using fine needles. Ovaries were kept in FAA (5% acetic acid, 5% formaldehyde, 90% absolute ethanol) for fixation for 1 to 3 days. The fixed ovaries were washed in 50% ethanol and successively incubated in fresh 50% ethanol, 80% ethanol and 100% ethanol for 15 minutes each. Afterwards the ovaries were air-dried for 10 minutes and subsequently moved to

hybridization buffer (20 mM pH 8.0 Tris-HCl, 0.9 M NaCl, 35% formamide, 0.01% SDS) pre-warmed at 46 °C for an incubation period of 15 minutes. The hybridization buffer was then replaced by pre-warmed hybridization buffer containing the fluorescent probes. The *Spiroplasma*-specific probe SPR-Cy3 (5'-Cy3-CCC ACC TTC CTC TAG CTT AC-3') and as controls the probe anti-sense Eub338-Cy3 (5'-Cy3-ACT CCT ACG GGA GGC AGC-3' (Amann et al. 1990)), as well as a no-probe approach were used. The samples were incubated overnight at 46 °C for hybridization. After incubation in 500 µl of pre-warmed washing buffer (20 mM Tris-HCl pH 8.0, 80 mM NaCl, 50 mM EDTA, 0.01% SDS) for 15 minutes, the samples were left in 1 ml of pre-warmed washing buffer for 30 minutes and subsequently washed twice with 1 x PBS. For mounting on a slide with 1 x PBS, the cuticles were removed from the ovaries, and a drop of mountant (glycerol, 1 x PBS, Hoechst staining) was added to the slide before it was covered with a coverslip that was fixed with nail polish. The slides were incubated in the dark for several minutes before being analyzed. Visual analysis was performed using a Zeiss LSM 700 confocal microscope (Carl Zeiss AG, Oberkochen, Germany) with an AxioCam HSm video camera (Carl Zeiss AG, Oberkochen, Germany) and Axiovision 4.6 software (Carl Zeiss AG, Oberkochen, Germany). ImageJ 1.x (Schneider et al. 2012) and the GNU Image Manipulation Program (GIMP, v. 2.10.24, © Spencer Kimball, Peter Mattis and the GIMP Development Team, www.gimp.org) were applied for processing.

2.2.7 Transinfection experiments

Transinfection experiments were performed to study if CI can be induced in an endosymbiont-free strain by infection with *Spiroplasma.* Hemolymph was transferred from infected female wasps (donor females) to uninfected STU(-) females (receiver females). Male offspring of receiver females was mated to endosymbiont-free females to check for CI between *Spiroplasma* positive males and negative females. The status of *Spiroplasma* infection was initially tested for in the hemolymph of infected females and subsequently in the receiver females of the endosymbiont-free strain STU(-) and their male offspring by PCR with the primers ApDnaAF1 and ApDnaAR1 as described above (also see Table 2.1) following DNA extraction with the nexttec™ 1$^{\text{-Step}}$ DNA Isolation Kit for Tissue & Cells (nexttec Biotechnologie GmbH, Hilgertshausen, Germany) for all samples except the hemolymph. Positive results were confirmed by Sanger Sequencing (Microsynth Seqlab, Göttingen, Germany).

2.2.8 Testing for *Spiroplasma* in the hemolymph

To confirm the presence of *Spiroplasma*, hemolymph was extracted from 20 STU females. The wasps were fixed on a double-sided adhesive tape to a slide and as much hemolymph as possible was removed from the abdomens with a drawn-out glass capillary mounted on a micromanipulator and pooled in 30 µl of TE buffer and 3 µl of proteinase K (nexttec™ 1$^{\text{-Step}}$ DNA Isolation Kit for Tissue & Cells; nexttec Biotechnologie GmbH, Hilgertshausen, Germany). This mixture was incubated at 55 °C for 30 minutes and 300 rpm and at 100 °C for 10 minutes for DNA extraction as described by (Holehouse et al. 2003). The presence of *Spiroplasma* was tested for with PCR conditions slightly modified from those specified before by using 40

cycles instead of 35 and by increasing the template volume to 2 µl while decreasing the volume of double distilled water in the PCR mixture by 1 µl accordingly.

2.2.9 Transfer of hemolymph

Donor and receiver females were newly hatched and virgin. Prior to the experiment, receiver females were cooled in a refrigerator at 5 °C to reduce their mobility. Hemolymph was extracted from a donor female as described above and subsequently injected with an Eppendorf FemtoJet® (Eppendorf AG, Hamburg, Germany) into the abdomen of a receiver female that was carefully held using a spring steel forceps. The receiver females were transferred to batches of 2 g koi pellets infested with larvae of *S. paniceum* as hosts for oviposition. Host batches were replaced every two to three days until the death of the wasps. After their death, the receiver females were either transferred to 100% ethanol and stored at -20 °C until DNA extraction, or immediately extracted. Because they remained unmated, they only produced male offspring. For the subsequent experiments, only male offspring of *Spiroplasma* positive receiver females were used.

2.2.10 Testing for CI with male offspring

Newly hatched male offspring of positive receiver females were mated to virgin, uninfected STU(-) females. After copulation, the males were killed in 2 ml tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany) containing 100% ethanol and kept at -20 °C until DNA extraction unless they were extracted immediately. All samples were subsequently checked for *Spiroplasma* infection. The uninfected STU(-) females were placed on 5 g koi pellets infested with larvae of *S. paniceum* as hosts for oviposition. After four weeks, the number and sex of the F2 offspring were recorded to study CI. Crosses without any offspring were excluded from the analysis.

2.2.11 Effect of *Spiroplasma* titer on CI level

In order to examine whether the strength of CI detected in matings between positive males and tetracycline-treated females was correlated to the *Spiroplasma* load in these males, quantitative PCR (qPCR) was conducted with all samples of mated *Spiroplasma*-positive F1 male offspring of receiver females. *DnaA*, amplified with the primers ApDnaAF1 and ApDnaAR1, was chosen as target gene.

qPCR standards

A PCR product of one of the studied samples was purified using the Thermo Scientific GeneJet Gel extraction and DNA Cleanup Micro Kit (Thermo Fisher Scientific, Waltham MA, USA). The DNA content was checked with an Eppendorf BioPhotometer® D30 (Eppendorf AG, Hamburg, Germany), and the purified PCR product was cloned into pGEM®-T Easy Vector (Promega, Madison WI, USA), whereby the ligation protocol was modified by using 0.5 µl pGEM®-T Easy Vector (50 ng), 1 µl DNA Ligase (3 U/µl), 2.5 µl 2X Rapid Ligation Buffer, and 2 µl PCR product. The ligation was incubated overnight at 16 °C. The ligation mix was then used to transform 45 µl of chemically competent *E. coli* JM109. The transformed bacteria were grown overnight at 37 °C and 250 rpm in 20 ml test tubes filled with 5 ml LB-medium containing 100 µg/ml ampicillin. Plasmid extraction was performed using the peqGold Plasmid Miniprep Kit I (VWR International GmbH, Darmstadt, Germany). The plasmid extract was amplified with 2.5 µl

DreamTaq™ Green Buffer (Thermo Fisher Scientific, Waltham MA, USA), 2.5 µl dNTPs, 0.125 DreamTaq™ Green DNA Polymerase (Thermo Fisher Scientific, Waltham MA, USA), 18.87 µl double distilled water, and 0.5 µl of each of the sequencing primers SP6/T7 (see Table 2.1 for details) and sent for Sanger Sequencing (Microsynth Seqlab, Göttingen, Germany) to confirm the *Spiroplasma dnaA* insert. The closest match in the NCBI database was *Spiroplasma* sp. Ozg *dnaA* gene (Accession number: AB586705.1) with 99% identity and 100% query coverage. The concentration of the plasmid extract was measured using a Qubit fluorometer (Thermo Fisher Scientific, Waltham MA, USA), and the original extract was serially diluted (1:10) to serve as standards for qPCR. The standard curve had a slope of -3.501 and a y intercept of 39.256 with a regression coefficient R2 of 0.991. PCR efficiency was determined to be approximately 90%. The range of reliable quantification was determined to be 103 to 1010 target molecules/µl DNA extract, with a Cq variation of 0.25 at the lower boundary.

qPCR

qPCR was conducted on a CFX96 Real Time System (Bio-Rad Laboratories, Inc.) with Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Carlsbad CA, USA), in reaction volumes of 10 µl, consisting of 0.8 µl template (DNA extract or standard), 5 µl 2x qPCR supermix, 0.2 µl 10mM forward primer, 0.2 µl 10 mM reverse primer, 0.2 µl ROX Reference Dye and 3.6 µl nuclease-free water. The annealing temperature of 52 °C was determined by gradient PCR prior to qPCR. A lower elongation temperature of 60 °C had to be used to allow amplification of the AT-rich template. The resulting cycling conditions were therefore: 50 °C for 2 minutes; 95 °C for 2 minutes; 35 cycles of 95 °C for 20 seconds, 52 °C for 20 seconds and 60 °C for 1 minute; followed by recording of the melting curve (50 to 95 °C in increments of 0.5°). Two analytic runs were conducted, each with technical duplicates of all samples and standards as well as four non-template controls. The measurements of each sample were averaged for further analyses after excluding outliers according to Grubbs's test (Grubbs 1950). *Spiroplasma dnaA* copy numbers were calculated by linear regression to the standard curve.

2.2.12 Statistical analyses

Statistical analyses were conducted using R v. 4.0.3 in RStudio v. 2022.07.1 (R Core Team 2020; RStudio Team 2022)) with the packages "multcomp" (Hothorn et al. 2008) and "car" (Fox and Weisberg 2019) in addition to the pre-installed packages. Statistical significance was assumed at $P < 0.05$, with $P < 0.01$ and $P < 0.001$ indicating high and very high significance, respectively. To test for CI in crossing experiments between tetracycline-treated and untreated STU individuals, the numbers of male and female F1 offspring as well as total F1 offspring were analyzed with generalized linear models, followed by Tukey tests (Tukey 1949), with negative binomial chosen for family as the best fit, since the data did not adhere to normal distribution. A 2 x 4 Fisher's Exact Test for Count Data (Taub 1979) was used to compare the rate of infection with Enterobacteriaceae between tetracycline-treated and untreated STU females as well as CI-inducing and non-CI inducing STU males due to low replicate numbers. For the comparisons of F2 female offspring numbers as well as F2 male offspring numbers between crosses of STU(-) females with *sDis*-negative and positive F1 male offspring of hemolymph-injected STU(-), Wilcoxon rank sum tests with

continuity correction (Wilcoxon 1945) were used due to the data being non-normally distributed. As normal distribution applied to the total F2 offspring numbers of these crosses, they were compared using a Welch Two Sample t-test (Welch 1947). The correlation between *dnaA* copy number determined by qPCR and the level of CI represented by proportion of female offspring (number of female F2 offspring divided by total number of F2 offspring) was assessed using Spearman's rank correlation (Spearman 1904).

2.3 Results

2.3.1 Bacteria induce CI in *L. distinguendus*

Tetracycline-treated and untreated females and males of the STU strain of *L. distinguendus* were crossed in all possible combinations and offspring production was compared. Crosses between tetracycline-treated females and untreated males produced significantly fewer daughters ($P < 1e\text{-}04$ *** in single comparisons with all other combinations, GLM, family = negative binomial, followed by Tukey test for multiple comparisons) and more sons ($P < 0.001$ *** in single comparisons with all other combinations, GLM, family = negative binomial, followed by Tukey test for multiple comparisons) with no difference in total offspring numbers ($P > 0.05$ n.s. in single comparisons with all other combinations, GLM, family = negative binomial, followed by Tukey test for multiple comparisons), which is indicative of the presence of CI-inducing bacteria (Figure 1, for full test statistics see Table A1). Increased male production is common in CI in hosts with haplodiploid sex determination, with incompatible fertilized embryos developing into males (Vavre et al. 2001).

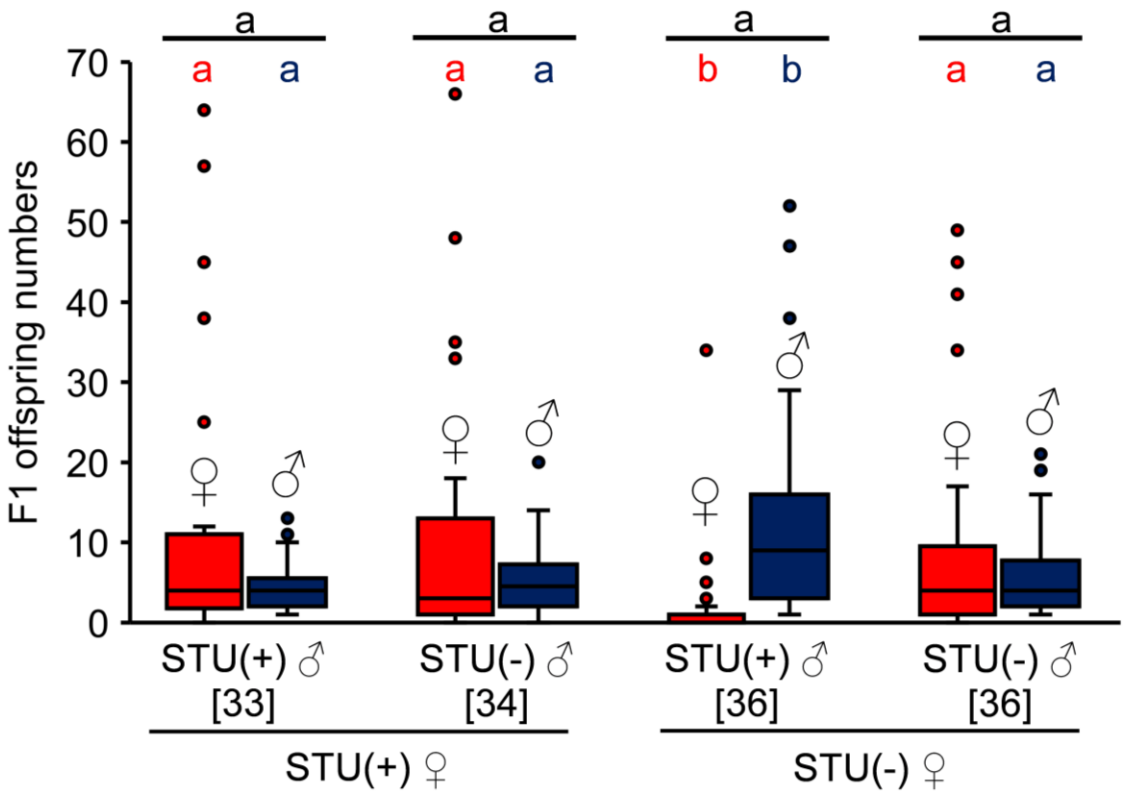

Figure 2.1. Numbers of F1 female (red) and male (blue) offspring of crosses between endosymbiont-carrying (+) and uninfected (-) females and males of the STU strain. Numbers of replicates (i.e. the numbers of crosses) are given in parentheses below the paternal male of each combination. Statistical significances among numbers of female offspring, numbers of male offspring, and total offspring numbers, respectively, were tested using GLMs (family = negative binomial) followed by Tukey tests for multiple comparisons, different lower case numbers (red: female offspring, blue: male offspring, black: total offspring) indicate statistical differences at $P < 0.05$ (see Table A1 for test statistics). Medians are represented by the middle horizontal lines, the 25% and 75% quartiles are shown as the lower and upper boundaries of the boxes, respectively. Minimum and maximum values within a range across 1.5 times the distances between the quartiles above the 75% quartile and below the 25% quartile are included in the whiskers, outliers outside of this range are shown as single points.

2.3.2 *Spiroplasma* is the only reproductive manipulator

Individual wasps were tested for endosymbionts known to act as reproductive manipulators using PCR. Previously, the STU strain was shown to not be infected with either *Wolbachia* or *Cardinium* (K. König et al. 2019). Here, *Rickettsia*, *Arsenophonus*, *Mesenet*, and *Rickettsiella*, as well as *Spiroplasma*, were also screened for. All tested STU females (n = 10) and 90% of the tested STU males (n = 10) were found to carry *Spiroplasma*, hereafter referred to as *sDistinguendus* (*sDis*); none of the other symbionts were present. In order to reveal a potential involvement of other bacteria, 16S rRNA amplicon sequencing of the whole bacterial community was used in five separate pooled samples of STU females. Overall, 12 amplicon sequence variants (ASVs) were found, of which only five were present in at least four of the five samples sequenced. One ASV was identified as *Spiroplasma* and was present in all samples with an average of 8258.4 total reads. Three ASVs were members of the Proteobacteria: a strain of *Yersinia* (4/5 samples,

average of 22.6 total reads), a strain of *Pseudomonas* (4/5 samples, an average of 20.6 total reads), and an unidentified member of the Enterobacteriaceae (5/5 samples, average of 8411.6 total reads). The role of the unknown Enterobacteriaceae in CI was dismissed as the rate of infection did not differ between tetracycline-treated (n = 5, 100% infection) and untreated (n = 4, 75% infection) STU females as well as CI-inducing (n = 4, 100% infection) and non-CI inducing (n = 3, 100% infection) STU males (2 x 4 Fisher's Exact Test for Count Data, *P* = 0.6875). A standard nucleotide BLASTn (Altschul et al. 1990) of the consensus sequence resulted in many close hits of the same rank (Table A2) and a comparison with only the NCBI rRNA/ITS database found *Enterobacter cancerogenus*, for which a resistance to tetracycline has been shown before (Zwenger et al. 2008), to be the best match (99% coverage, 99.53% identity, E value= 0.0, Accession Number NR_044977.1).

The final ASV was identified as *Enterococcus* (phylum Firmicutes) with an average of 138.6 total reads (see Table A3 for full ASV count table). As *sDis* was the dominant bacterium and only potential reproductive manipulator, it was set about characterizing it and establishing its role in CI.

2.3.3 *sDis* is present in the ovaries of *L. distinguendus*

To determine the presence of *sDis* in wasp ovaries, fluorescent in situ hybridization (FISH) was performed using the *Spiroplasma*-specific probe SPR and anti-sense and no-probe controls. Specific, localized signals were obtained with SPR in the ovaries of STU females (Figure 2).

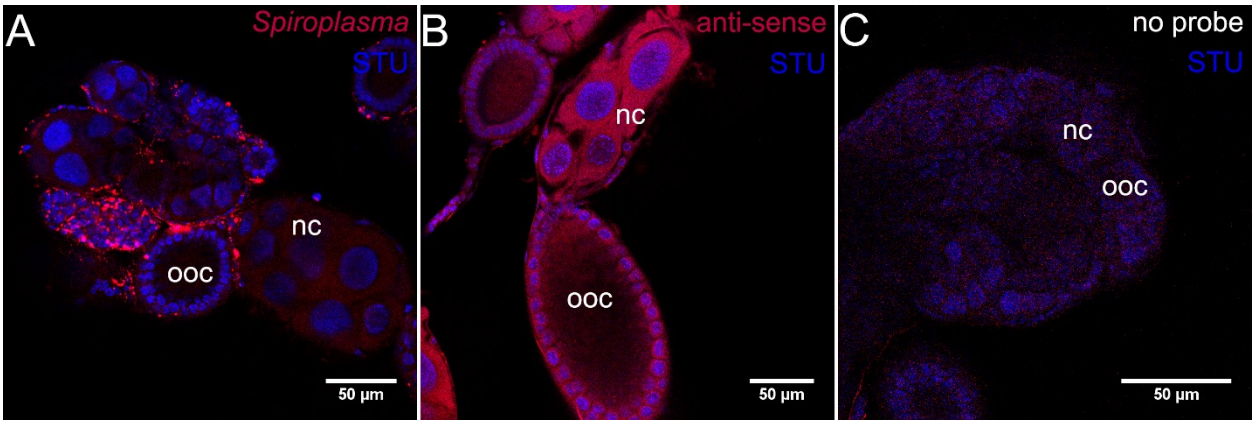

Figure 2.2. FISH images depicting the presence of *sDis* in the ovaries of *L. distinguendus* females of the strain STU. Blue: Cell nuclei stained with Hoechst DNA staining, Red: *Spiroplasma*-specific probe (SPR) or anti-sense probe with Cy3 fluorochrome, nc: nurse cells, ooc: oocyte. Scale bars = 50 µm. Brightness and contrast were set to "auto" in ImageJ 1.x for all images. A: specific, localized signals in STU ovaries hybridized with SPR. B: background fluorescence in STU ovaries with anti-sense probe. C: fluorescence of STU ovaries with no probe.

2.3.4 *sDis* can be transferred to a non-infected host and induce CI

To demonstrate that the CI phenotype is ultimately induced by *sDis*, a transinfection experiment was performed. As prerequisite, hemolymph from STU females was tested for infection with *sDis*. For the transinfection, hemolymph from STU females was injected into endosymbiont-free STU(-) females. Of these, 27 (69.2%) were positive for *sDis* at the end of the experiment. These females were consecutively

offered different batches of koi pellets infested with host beetle larvae to parasitize for two to three days each. The first three host batches parasitized by positive females produced *sDis*-negative offspring. Positive males started to emerge from the fourth batch and increased in proportion up to 100% in the last two batches (Figure 3 A). Eventually, 15 of the positive injected females (56%) were found to have *sDis*-positive F1 male offspring.

Newly hatched F1 male offspring of injected females were mated to endosymbiont-free STU(-) females and the presence of CI was inferred from the numbers of F2 females and F2 males. *sDis*-positive F1 males (n = 82) sired significantly F2 fewer females than uninfected F1 males (n = 36) (Wilcoxon rank sum test with continuity correction, W = 2718, *P* = 3.581e-13 ***; Figure 3 B), whereas the number of F2 male offspring was significantly higher in crosses with positive F1 males (Wilcoxon rank sum test with continuity correction, W = 839, *P* = 0.0001971 ***) and total F2 offspring number did not differ between the crosses (Welch two sample t-test, t = 1.341, *P* = 0.1853 n.s.) (Figure 3 B), recreating the CI phenotype shown by Figure 1. This demonstrated that CI was induced by *sDis* and that *sDis* could be transferred into previously uninfected wasps by injection of hemolymph from infected wasps, maintaining its CI-inducing effect.

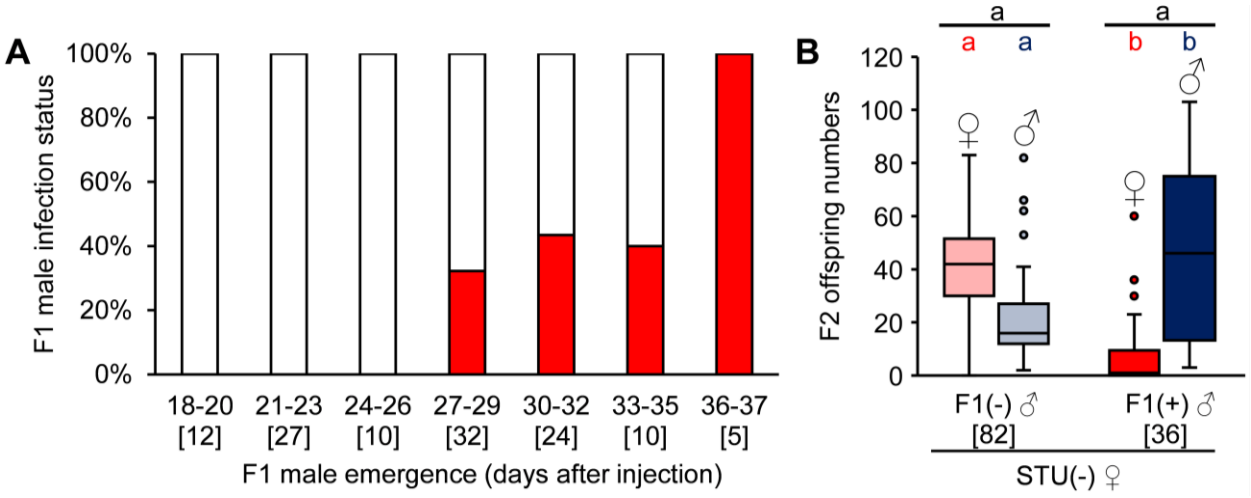

Figure 2.3. Hemolymph transfer of s*Dis* infection and CI. A: Proportion of *sDis*-positive F1 male offspring of injected females by day of emergence from consecutively parasitized host batches. Only males used for subsequent crossing experiments are shown. Numbers of replicates (numbers of males emerged in the respective timeframe) are given in parentheses below the bars. White part of the bars: *sDis*-negative males, red part of the bars: *sDis*-positive males. B: Numbers of F2 female (red) and male (blue) offspring of *sDis*-negative (-) and positive (+) F1 males and uninfected (-) STU females. Numbers of replicates (numbers of males of the given infection status used for this experiment) are given in parentheses below the bars; crosses without any offspring are excluded. Statistical significances between crosses were tested for F2 female offspring and F2 male offspring using Wilcoxon rank sum tests with continuity correction (W = 2718, *P* = 3.581e-13 for females, W = 839, *P* = 0.0001971 for males), and for F2 total offspring using a Welch two sample t-test (t = 1.341, *P* = 0.1853); different lower case numbers (red: female offspring, blue: male offspring, black: total offspring) indicate statistical differences at *P* < 0.05. The middle horizontal line shows the median, the lower boundary of the box indicates the 25% quartile, whereas the upper boundary represents the 75% quartile. The whiskers indicate minimum and maximum values within a range stretching from 1.5 times the distances between the quartiles above the 75% quartile and below the 25% quartile. Data outside of this range are outliers and are shown as single points.

In order to examine whether the strength of CI was influenced by *Spiroplasma* titer, qPCR was conducted on the DNA samples of mated *sDis*-positive F1 male offspring of receiver females using *dnaA* as a target gene. There was no correlation between *Spiroplasma* titer in F1 male wasps and proportion of female offspring (number of female F2 offspring divided by total number of F2 offspring) used as proxy for CI level (Spearman's rank correlation, n = 36, rho = 0.171, $P = 0.319$).

2.4 Discussion

In this study, it is demonstrated that the widespread symbiotic bacterium *Spiroplasma* causes cytoplasmic incompatibility in an insect host and show that the symbiont and phenotype can be efficiently transmitted by adult recipients following hemolymph microinjection. The benefits of understanding and applying CI have motivated decades of studies developing and optimizing protocols for symbiont transfer.

Prior to the present study, all CI-inducing bacteria have been primarily intracellular symbionts. There have been successful transfers of *Wolbachia* using hemolymph injection (Frydman 2007), but most transinfections of CI-inducing symbionts require challenging techniques and specialized equipment for transfer into very young recipient embryos, often with limited success (Zabalou et al. 2004; Xi et al. 2005; Walker et al. 2011; Duplouy et al. 2013; Hughes and Rasgon 2014). *Spiroplasma*'s ecology as a hemolymph-dwelling bacterium bypasses all of these challenges.

In this study system, CI *Spiroplasma* could be established by adult-to-adult microinjection, mirroring previous results showing high success of *Spiroplasma* transfer between fruit fly hosts using this method (Haselkorn and Jaenike 2015; Ballinger and Perlman 2019). The horizontal transfer experiments also suggest that the strength of CI in *Spiroplasma* is not related to overall bacterial titer, which also has positive implications for the success of transferring CI to new hosts.

As a new phenotype for *Spiroplasma*, this discovery presents exciting new avenues for basic and applied research in arthropod reproductive manipulation. Like other facultative symbionts, *Spiroplasma* has a broad host range that does not reflect host or symbiont phylogeny (Gasparich et al. 2004; Binetruy et al. 2019). As a member of the Ixodetis clade (Pollmann et al. 2022), closely related strains infect fruit flies, aphids, ticks, sawflies, scale insects, and spiders. Reproductive manipulations performed by members of this group can also affect many different arthropod hosts, as evident by the male-killing strains in butterflies and beetles (Hurst et al. 1999a; Majerus et al. 1999; Jiggins et al. 2000; Tinsley and Majerus 2006; Tabata et al. 2011) – and now a CI strain in wasps. This broad host manipulation range suggests a potential for the transfer and maintenance of *sDis* to diverse arthropod hosts of agricultural, medical, and basic research relevance that should be a focus of future studies. Beyond the Ixodetis clade, the host range of *Spiroplasma* is even broader, with strains infecting terrestrial and aquatic arthropods (Regassa and Gasparich 2006; Wang et al. 2011), jellyfish (Cortés-Lara et al. 2015), and sea cucumbers (He et al. 2018). If the ease of *Spiroplasma* transfer can be replicated for interspecific transfers of *sDis* as well, it will facilitate similar investigations of *sDis*'s reproductive effects in other insect hosts. Finally, a number of *Spiroplasma* strains,

including vertically transmitted male-killers, can be grown in cell-free media and genetically transformed (Masson et al. 2018, 2020), which has the potential to greatly facilitate the study and manipulation of CI.

The discovery that *Spiroplasma* can cause CI adds to a growing list of microbial symbionts, such as *Rickettsiella* and *Mesenet*, that join *Wolbachia* and *Cardinium* in the small club of CI microbes. As infection with multiple symbionts is common, this suggests that when observing CI, one should be careful not to assume that it is being caused by *Wolbachia*. However, it is possible to use differing susceptibility to antibiotics to link CI to a specific symbiont; for example, *Wolbachia* is highly susceptible to rifampicin, while *Spiroplasma* is resistant (Jaenike et al. 2010b).

It is interesting that closely related *Spiroplasma* Ixodetis clade strains cause male-killing and CI, as previous studies have demonstrated a close link between CI and male-killing in *Wolbachia*. For example, multiple *Wolbachia* CI-inducing strains have been shown to act as male-killers following introgression into different host genetic backgrounds (Sasaki et al. 2002, 2005; Jaenike 2007), suggesting a possible connection between CI and male-killing induced by *Spiroplasma* as well.

Chapter 3: New species based on the biological species concept within the complex of *Lariophagus distinguendus* (Hymenoptera, Chalcidoidea, Pteromalidae), a parasitoid of household pests[2]

3.1 Introduction

Lariophagus distinguendus (Förster) (Pteromalidae) is a pteromalid wasp that belongs to the superfamily Chalcidoidea within the Hymenoptera, which is an extremely speciose, if not the most speciose animal order (Forbes et al. 2018). The Chalcidoidea comprise more than 20,000 described species (Noyes 2019), and new species are discovered on a regular basis (Aguiar et al. 2013). This is partly due to a high abundance of cryptic species and the existence of numerous species complexes (e.g. (Heimpel et al. 1997; Heraty et al. 2007; Desneux et al. 2009; Chesters et al. 2012; Fusu 2017; K. König et al. 2019)). For the Chalcidoidea, a high speciation rate is hypothesized to be caused by a high abundance of sib-mating, which quickly restricts gene flow between populations, and by the haplodiploid mode of sex determination, which causes rapid elimination of deleterious alleles in the haploid males and a rapid selection of favorable gene combinations (Askew 1968; Malec et al. 2021).

L. distinguendus is a parasitoid of coleopteran larvae from at least 17 beetle species in six families (Niedermayer et al. 2016). Most of its hosts occur in grain stores and pantries of households and are pests on dry stored plant products like grain, corn, pasta, and dried. Therefore, *L. distinguendus* can be used as biological control agent against its hosts (Niedermayer and Steidle 2013; Niedermayer et al. 2016). Previous studies established the existence of at least two distinct cryptic species within *L. distinguendus*, which are almost indistinguishable morphologically (Wendt et al. 2014; K. König et al. 2019). These two species, which remain undescribed so far, were provisionally named GW-species and DB-species after their preferred hosts, the granary weevil *Sitophilus granarius* (Coleoptera: Curculionidae) L. and the drugstore beetle *Stegobium paniceum* (Coleoptera: Ptinidae) L., respectively. Barriers between the species are formed by differences in host and habitat preferences (K. König et al. 2015a, 2019), different numbers of chromosomes (C. König et al. 2019), sexual and postzygotic isolation as well as endosymbiont-induced cytoplasmic incompatibility (CI) caused by the bacterium *Spiroplasma* (K. König et al. 2015b, 2019; C. König et al. 2019; Gokhman et al. 2019, Pollmann et al. 2022). The bacterium *Wolbachia*, which is known to induce CI as reproductive barrier between *Nasonia* species (Breeuwer and Werren 1990; Bordenstein et al. 2001), was also found in the GW-species, but did not cause reproductive isolation (K. König et al. 2019). Recently, a larger number of *L. distinguendus* strains was collected in the area of Stuttgart in southern Germany, a largely urban area. Phylogenetic analysis of parts of the mitochondrial cytochrome C oxidase subunit I gene (COI) different to the barcode region of some of these strains indicates that all belong to the already established DB-species and share the same chromosome numbers (C. König et al. 2019). However, this analysis also revealed well-supported sub-clades, which could indicate that the DB-

[2] This chapter is based on Pollmann, M., Kuhn, D., König, C., Homolka, I., Paschke, S., Reinisch, R., Schmidt, A., Schwabe, N., Weber, J., Gottlieb, Y., and Steidle, J.L.M. (2023). New species based on the biological species concept within the complex of *Lariophagus distinguendus* (Hymenoptera, Chalcidoidea, Pteromalidae), a parasitoid of household pests. Ecology and Evolution 13, e10524. 10.1002/ece3.10524.

species might be in fact a complex of two or three distinct species. So far, no analysis of the barcode region of the mitochondrial cytochrome C oxidase subunit I gene (COI) sequences as well as of nuclear genes has been conducted for the majority of these new strains. In addition, there are no studies on reproductive isolation for these strains. Thus, it is unclear if more cryptic species are hidden within the DB-species.

Here, the hidden diversity in the *L. distinguendus* species complex was investigated by addressing the following two questions: Are there more distinct species among the *L. distinguendus* strains traceable (1) by genetic divergence and (2) based on the biological species concept? For species delimitation, phylogenetic trees based on the barcoding gene COI as well as five nuclear genes (Hebert et al. 2004; Smith et al. 2005, 2006; Ward et al. 2005; Hajibabaei et al. 2006) were reconstructed and potential species status according to the biological species concept (BSC) (Mayr 1969; Coyne and Orr 2004) was examined. To that end, pre- and postzygotic isolating barriers, i.e. barriers occurring before and after fertilization, in particular sexual isolation as well as hybrid viability, sterility, and reduced fertility (Coyne and Orr 2004) were investigated in strains representative of the different genetically determined clusters in crossing experiments.

The results facilitate a comparison between the heavily discussed approach of “turbo-taxonomy” (Butcher et al. 2012) for species delimitation based on a 2% divergence in COI (Meierotto et al. 2019; Sharkey et al. 2021) and the traditional BSC (Mayr 1969; Coyne and Orr 2004). In addition, because the divergence in COI in the studied strain pairs ranges along a gradient from 1.7% to 14%, this study also allows for drawing conclusions on the potential emergence of reproductive barriers during the speciation process in *L. distinguendus.*

3.2 Material and Methods

3.2.1 Studied insects

All studied individuals of *L. distinguendus* were reared at the Department of Chemical Ecology of the University of Hohenheim and by collaborators. Almost all wasp strains were collected in Germany, except for three strains originating from Great Britain, The Netherlands, and Denmark, respectively. Strains were named after their respective collection site and host species, i.e. larvae of either drugstore beetles or granary weevils (see Table 3.1 for details).

Table 3.1. *L. distinguendus* strains with host species and the localities the collection sites belong to. Strains with a preference for drugstore beetles are labelled with the prefix db, whereas the prefix gw designates strains preferring granary weevils. Consecutively numbered strains named for the same localities were collected at different collection sites within these localities.

Strain	Short name	Host	Locality of the collection site	used[c]
dbBIR-D1	BIR	*St. p.*[a]	Stuttgart-Birkach, Baden-Württemberg, Germany	+
dbBIR-D2	-	*St. p.*	Stuttgart-Birkach, Baden-Württemberg, Germany	-
dbBIR-D3	-	*St. p.*	Stuttgart-Birkach, Baden-Württemberg, Germany	-
dbBIR-D4	-	*St. p.*	Stuttgart-Birkach, Baden-Württemberg, Germany	-
dbBRU-D1	-	*St. p.*	Bruchsal, Baden-Württemberg, Germany	-
gwBYG-DK1		*S. g.*[b]	Bygholm, Horsens Kommune, Region Midtjylland, Denmark	-
dbCAN-D1	CAN	*St. p.*	Stuttgart-Bad Cannstatt, Baden-Württemberg, Germany	+
dbCAN-D2	-	*St. p.*	Stuttgart-Bad Cannstatt, Baden-Württemberg, Germany	-
dbFRI-D1	-	*St. p.*	Fritzlar, Hesse, Germany	-
dbLUD-D1	-	*St. p.*	Ludwigsburg, Baden-Württemberg, Germany	-
dbOBE-D1	-	*St. p.*	Stuttgart-Obertürkheim, Baden-Württemberg, Germany	-
dbOST-D1	OST	*St. p.*	Ostfildern, Baden-Württemberg, Germany	+
gwPFO-D1	PFO	*S. g.*	Pforzheim, Baden-Württemberg, Germany	+
dbPLI-D2	-	*St. p.*	Stuttgart-Plieningen, Baden-Württemberg, Germany	-
dbRAV-D1	-	*St. p.*	Ravensburg, Baden-Württemberg, Germany	-
gwSAC-D1	-	*S. g.*	Saxony, Germany	-
gwSAT-D1	SAT	*S. g.*	Satrup, Schleswig-Holstein, Germany	-
gwSIL-D1	-	*S. g.*	Stuttgart-Sillenbuch, Baden-Württemberg, Germany	-
gwSLO-GB1	-	*S. g.*	Slough, Berkshire, Great Britain	-
dbSTU-D1	STU	*St. p.*	Stuttgart-Bad Cannstatt, Baden-Württemberg, Germany	+
dbSTU-D3	-	*St. p.*	Stuttgart West, Baden-Württemberg, Germany	-
gwSWD-D1	-	*S. g.*	Schwieberdingen, Baden-Württemberg, Germany	-
dbVAI-D1	-	*St. p.*	Stuttgart-Vaihingen, Baden-Württemberg, Germany	-
dbVAI-D2	-	*St. p.*	Stuttgart-Vaihingen, Baden-Württemberg, Germany	-
dbVAI-D3	-	*St. p.*	Stuttgart-Vaihingen, Baden-Württemberg, Germany	-
dbVAI-D4	-	*St. p.*	Stuttgart-Vaihingen, Baden-Württemberg, Germany	-
dbWAG-N1	-	*St. p.*	Wageningen, Gelderland The Netherlands	-
dbWAN-D1	-	*St. p.*	Stuttgart-Wangen, Baden-Württemberg, Germany	-

[a] *St. paniceum;* [b] *S. granarius;* [c] + - used in crossing experiments

Both hosts were obtained from the Julius Kühn-Institut in Berlin. For the rearing of drugstore beetles, honey jars (diameter 12 cm, height 16 cm) with ventilated lids containing 80 g of koi pellets (Hikari Friend, Kamihata Fish Industry Group, Kyorin Corporation, Japan) were inoculated with approximately 1 g of beetles. Larvae suitable for parasitization were obtained after around six weeks at 26–27 °C, 45% RH and a natural L:D cycle determined by the light from outdoors. For granary weevil cultures, weevils were placed in honey jars containing 200 ml of wheat grains (*Tritium aestivum* L.) for oviposition and removed after one week. The wheat was moistened (1 ml water / 40 g wheat grains). To obtain four-week-old larvae, which are suitable as hosts, these cultures were kept at 25 °C and a L:D cycle of 16:8 for three weeks, and at 20 °C and a natural L:D cycle for one week. Then they were transferred to 15 °C. DB-strains were reared on drugstore beetle larvae in honey jars with koi pellets, except for the strains dbBIR-D1 (BIR) and dbWAG-N1, which were reared on drugstore beetle larvae infesting wheat grains in Petri dishes. GW-strains were reared on granary weevil larvae in Petri dishes containing wheat grains. All wasp strains were maintained

by transferring newly hatched wasps onto new host substrate in regular intervals and kept at 26 °C, a relative humidity of 45–50% and a natural L:D cycle.

Wasps used for crossing experiments were isolated prior to eclosion to ensure their virginity. Therefore, single infested wheat grains were separated in 1.5 ml Eppendorf tubes and wasps developing in koi pellets were removed as pupae by dissection and isolated in 1.5 ml Eppendorf tubes.

3.2.2 Antibiotic treatment

To remove endosymbionts, namely *Wolbachia* in the GW-strains (K. König et al. 2019) and *Spiroplasma* in dbSTU-D1 (STU) (Pollmann et al. 2022), which could impair the results of the crossing experiments and phylogenetic analyses, as described below, tetracycline-treated lines were generated for the strains gwPFO-D1 (PFO), gwSAT-D1 (SAT), gwBYG-DK1, gwSAC-D1, gwSLO-GB1, BIR, and STU. Wasps were placed in Petri dishes containing filter paper and a piece of cotton wool soaked with a solution of 10 mg tetracycline (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and 1 g sucrose per 10 ml water before being moved to their respective host substrate for oviposition after 24 hours. After three generations with antibiotic treatment, the elimination of endosymbionts was confirmed by polymerase chain reaction (PCR) as described below.

3.2.3 DNA extraction, gene amplification, and sequencing

Genomic DNA (gDNA) was extracted from individual wasps using the nexttec 1-step tissue & cells kit (nexttec Biotechnologie GmbH, Hilgertshausen, Germany) by following the manufacturer's instructions. All gDNA samples were stored at -20 °C until further processing. To amplify the five nuclear genes the following primer combinations were used: Carbamoyl phosphate synthase domain of the conserved ATPase Domain (CAD): CAD f (5'-CAG TTC GAT GAA GAG CGT AGG-3') / CAD r (5'-ATA GAC ACC CGA ACC TTT GAA GA-3') (Klopfstein et al. 2013), parts of the internal transcribed spacer 2 (ITS2): ITS2 f (5'-TGT GAA CTG CAG GAC ACA TG-3') / ITS2 r (5'-ATG CTT AAA TTT AGG GGG T-3') (Quicke et al. 2006), LOC100123206 (LOC1): HOG4652_10 f (5'-GGW TTT GGY TTT ATT CGT TG-3') / HOG4652_10 r (5'-YTC TTT ATT YCG YTT YAC TTG-3')), LOC100123909 (LOC2): HOG5134_01 f (5'-AGT AAA ATG GGT YTW ATG TC-3') / HOG5134_01 r (5'-STR TTC CAR TTW ACT CCR TA-3'), and LOC100117339 (LOC3): (HOG5592_08 f (5'-YAA YGA GGA CCA ATC GAG AT-3') / HOG5592_08 r (5'-GCA TWA CRA TAG ATC TYG CTT CTC-3') without sequencing tails (Hartig et al. 2012). To amplify the COI region of the mitochondrial region the primer combination LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') / HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3')) (Folmer et al. 1994) was used. Both CAD and ITS2 were amplified at 95 °C for 5 minutes followed by 40 cycles of 94 °C for 1 minute, 55 °C for 1 minute and 72 °C for 1.5 minutes and finally at 72 °C for 10 minutes. For amplification with the COI and W-Spec primers, PCR conditions consisted of an initial denaturation at 95 °C for 2 minutes, 32 cycles of 94 °C for 30 seconds, 49 °C for 45 seconds, and 72 °C for 1 minute, and a final elongation step at 72 °C for 1 minute. LOC3 was amplified using 94 °C for 4 minutes, 35 cycles of 94 °C for 1 minute, 49 °C for 1 minute, and 72 °C for 1.5 minutes, followed by 72 °C for 1 minute. Touchdown PCRs consisting of 4 minutes at

94 °C for initial elongation, 2 cycles of 94 °C for 1 minute, 52 °C for 1 minute, and 72 °C for 1.5 minutes, 2 cycles of 94 °C for 1 minute, 50 °C for 1 minute, and 72 °C for 1.5 minutes, 36 cycles of 94 °C for 1 minute, 48 °C for 1 minute, and 72 °C for 1.5 minutes and a final elongation step at 72 °C for 5 minutes, were conducted for LOC1 and LOC2.

All strains were tested for the endosymbionts *Wolbachia* and *Spiroplasma*, which had been detected within *L. distinguendus* strains before (K. König et al. 2019, Pollmann et al. 2022). To that end, they were submitted to PCR amplification with the specific primer pairs W-Specf (5'- CAT ACC TAT TCG AAG GGA TAG-3') / W-Specr (5'- AGC TTC GAG TGA AAC CAA TTC-3') (Werren and Windsor 2000) or *wsp* 81 F (5'-TGG TCC AAT AAG TGA TGA AGA AAC-3') / *wsp* 691 R (5'-AAA AAT TAA ACG CTA CTC CA-3') (Braig et al. 1998), with 95 °C for 2 minutes, 35 cycles of 92 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 30 seconds, followed by 72 °C for 5 minutes for *Wolbachia* and ApDnaAF1 (5'-ATT CTT CAG TAA AAA TGC TTG GA-3') and ApDnaAR1 (5'-ACA CAT TTA CTT CAT GCT ATT GA-3') (Fukatsu et al. 2001), with 95 °C for 4 minutes, 35 cycles of 95 °C for 30 seconds and 55 °C for 30 seconds, and a final elongation at 72 °C for 1 minute, for *Spiroplasma*, respectively.

PCRs were conducted either with 12.5 µl of ROTI®Pol TaqS Red-Mix (2x) (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), 1 µl of each primer, and 9.5 µl double distilled water per 1 µl template, with 5 µl Promega 5X Green GoTaq® Reaction Buffer, 2.5 µl 10mM dNTPs (Promega, Madison WI, USA), 1 µl of each primer, 14.3 µl double distilled water and 0.2 µl Promega GoTaq® G2 DNA Polymerase (Promega, Madison WI, USA) per 1 µl sample (or 2 µl sample with the amount of double distilled water reduced to 13.3 µl accordingly) or with 20 µl Promega GoTaq®Green Master Mix 2X (Promega, Madison WI, USA), 4 µl of each primer and 10 µl of double distilled water per 2 µl sample. A Techne® Prime thermal cycler (Cole-Parmer, Stone, UK), a Biometra TGradient 96 Thermocycler (Analytik Jena AG, Jena, Germany) or a Biometra professional Basic Thermocycler (Analytik Jena AG, Jena, Germany) were used. As mentioned above, all GW-strains (PFO, SAT, gwBYG-DK1, gwSAC-D1, and gwSLO-GB1) were infected with *Wolbachia* (K. König et al. 2019), which can also be accidentally amplified with the primer pair LCO1490 / HCO2198 (Magnacca and Brown 2012; Bleidorn and Henze 2021). Therefore, tetracycline-treated, endosymbiont-free wasps were used for the analysis. To confirm the absence of *Wolbachia*, these strains were amplified with the *Wolbachia*-specific primer pair W-Specf (5'- CAT ACC TAT TCG AAG GGA TAG-3') / W-Specr (5'- AGC TTC GAG TGA AAC CAA TTC-3') (Werren and Windsor 2000), as described above. Gel electrophoresis was conducted on a 1-2% agarose gel with either 5 µl of peqgreen (VWR International GmbH, Darmstadt, Germany) or 5 µl ROTI®-GelStain (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) per 100 ml as DNA markers and 10 µl of an equimolar 100 bp DNA ladder (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) or Norgen LowRanger 100 bp DNA (Norgen Biotek Corp., Canada) as ladders. 5 to 10 µl per PCR product were transferred to the gel for visualization. To check PCR results and obtain DNA sequences, the PCR products were sent to Microsynth Seqlab (Göttingen, Germany) or Macrogen Europe (Amsterdam, The Netherlands) for Sanger dideoxy sequencing.

3.2.4 Phylogenetic data analyses

Raw sequencing data was assembled, trimmed, and checked for indels using GENtle v. 1.9.4. (© by Magnus Manske, University of Cologne, released under GPL 2003) (Manske 2006). All sequences were compared to nucleotide sequences in GenBank (Benson et al. 2017) using BLAST (Altschul et al. 1990) to confirm that the correct organism had been amplified. Ambiguous positions were named according to IUPAC nomenclature. Subsequently, all sequences were aligned on nucleotide level using the multiple sequence alignment (MSA) program MAFFT v.7 (Katoh et al. 2019) with the L-INS-i algorithm. All sequences were transcribed into amino acids using Virtual Ribosome v. 2.0 (Wernersson 2006) with translation table 1 (standard genetic code) for the nuclear genes and translation table 5 (invertebrate mitochondrial) for COI in order to control for unexpected stop codons or gaps. In addition to the newly collected sequence data, further *L. distinguendus* sequence data obtained in previous studies (König et al. 2015a, C. König et al. 2019) published in GenBank (Benson et al. 2017), was added. Corresponding sequence data, retrieved from GenBank (Benson et al. 2017), of the outgroup species *Nasonia vitripennis* Wlk., a member of the same family as *L. distinguendus*, for COI and the nuclear genes, and *Eupelmus confusus* Al Khatib, which belongs to the same superfamily, for COI, but not the nuclear genes due to a lack of published sequences, was included as well (see Table A4 for overall characters and character types for all genes and Table A6 for Accession Numbers of all previously published sequences).

All nuclear sequences per individual were merged into one concatenated matrix with MEGA v. X (Kumar et al. 2018). Partition homogeneity was tested and confirmed ($P = 0.8$) using PAUP* version 4.0a (build 169) (Swofford 2003), and the nuclear genes were subsequently analyzed as matrix, whereas the barcode segment was analyzed separately. The appropriate partitioning schemes and nucleotide substitution models (see Table A5) were determined with ModelFinder (Kalyaanamoorthy et al. 2017) implemented in IQ-TREE v.1.6.12 (Nguyen et al. 2015), testing for available nuclear models. The edge-proportional partition model (−spp, (Chernomor et al. 2016)) was chosen to allow for partitions evolving at different velocities and the best models for each partition were determined by the Bayesian Information Criterion (Schwarz 1978). Standard settings were used for all other parameters. Phylogenetic trees were inferred using the maximum likelihood optimality criterion as implemented in IQ-TREE v.1.6.12 (Nguyen et al. 2015) with 1000 ultrafast bootstrap replicates (Hoang et al. 2018) and standard parameters. The best tree was determined by the best log-likelihood value. FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) was used to display the resulting phylogenetic trees along with the bootstrap support values and root them using the outgroup species. Uncorrected intra- and interspecific pairwise distances between the strains used for the crossing experiments were calculated using MEGA v. X (Kumar et al. 2018) with the pre-set parameters.

3.2.5 Crossing experiments

The strain pairs BIR x OST, BIR x STU, CAN x STU, BIR x SAT, and CAN x PFO were chosen to conduct the crossing experiments as they represent the DB-species and GW-species as well as the sub-clades within the DB-species and cover a gradient of genetic divergence (C. König et al. 2019). For each pair of strains, different reproductive barriers were studied in the four possible combinations, i.e. two interstrain

crossings, with females of one strain paired with males of the other strain and vice versa, and two intrastrain crossings, with females paired with males of their own strain, as controls. The intrastrain crossings were continuously conducted in parallel to the interstrain crossings at each stage to serve as control. As females of different strains have been shown to naturally differ in their acceptance of males as well as their fecundity (K. König et al. 2015a, 2019), data from the interstrain crosses were compared with data from intrastrain crosses with females of the same strain as controls. For crossing experiments between the strains STU and BIR, tetracycline-treated lines were used to investigate reproductive barriers not affected by endosymbionts.

To study sexual isolation, a minimum of 20 pairs were observed for 20 minutes or until mating had occurred. Afterwards, both wasps were transferred onto host-infested grains or pellets, regardless of whether copulation had occurred or not. The offered host species depended on the females in the crosses. If females originated from a GW-strain, 10 g of wheat grains infested with granary weevils were provided. Females of a DB-strain were offered either 10 g of wheat grains containing one drugstore beetle larva each or 5 g of koi pellets with multiple drugstore beetle larvae in each pellet. F1 offspring were counted after four to five weeks to assess the viability of hybrid offspring. To study sexual isolation of hybrid females, mating experiments were performed with pairs of virgin F1 females and parental-type males as described above. To study physiological sterility and reduced fertility of hybrid females, F1 females were allowed to hatch together with F1 males to enable mating and transferred to new batches of their respective hosts, as described in detail above, for oviposition. The occurrence and numbers of total F2 offspring served as parameters for the investigated barriers. Because males in *L. distinguendus* are haploid, hybrid males do not occur until the F2-generation. To study viability of hybrid males, F1 wasps were isolated prior to hatching as described above to prevent mating and transferred to new hosts for oviposition where they only produced male offspring. The viability of hybrid males was then assessed by comparing the offspring numbers of mated F1-females producing mainly female offspring, and virgin F1-females producing only male offspring. To study behavioral and physiological sterility as well as fecundity of hybrid males, they were backcrossed to parental-type females of both strains. Mating success served as parameter for behavioral sterility, and the occurrence and number of F3 female offspring of these crosses for sterility and reduced fertility, respectively.

3.2.6 Calculation of strength of reproductive isolation

The strength of the reproductive isolation (RI) was determined based on Sobel and Chen (2014):

$$\mathrm{RI}=1-2*\frac{(H)}{(\mathrm{H})+(\mathrm{C})}$$

H refers to data resulting from interstrain crossings and C to data from intrastrain crossings. The absolute contribution (AC) of a barrier to the reproductive isolation according to their position (n) within the sequence of all barriers considering existing restrictions of gene flow by barriers occurring earlier in the sequence was calculated following Ramsey, Bradshaw and Schemske (Ramsey et al. 2003):

$$\mathrm{AC}_{\mathrm{n}}=\mathrm{RI}_{\mathrm{n}}\left(1-\sum_{\mathrm{i}=1}^{\mathrm{n}-1}\mathrm{AC}_{\mathrm{i}}\right)$$

The total isolation T was calculated as the sum of the absolute contributions of all barriers m:

$T = \sum_{i=1}^{m} AC_i$

3.2.7 Crossing experiments testing cytoplasmic incompatibility

To test the effect of endosymbionts on hybridization, crossings of the strains BIR and STU were studied. STU has been shown to carry CI-inducing *Spiroplasma* (Pollmann et al. 2022), whereas BIR is uninfected. Untreated individuals of the strains BIR and STU were crossed and offspring numbers in the F1 generation were analyzed.

3.2.8 Sperm counts in hybrid males of CAN and STU

In addition to sexual isolation, a reduced fecundity in hybrid males was detected as very early barrier between the strains CAN and STU (see Fig. 3.10). To study the underlying reason for this barrier, virgin males

and females of these strains were crossed in all possible combinations as described above, resulting in two hybrid (CAN females x STU males, STU females x CAN males) and two control (CAN females x CAN males, STU females x STU males) crosses (n = 25 per combination, N = 100 in total). The resulting virgin F1 females were transferred to hosts to lay unfertilized male eggs. The resulting males were used for dissection of seminal vesicles or were backcrossed two to three days after emergence to one-day-old parental-type females according to the male strain of the original combination. After mating, females were kept isolated for one day to allow the sperm to move to the spermathecae. The seminal vesicles of the unmated F2 males and the spermathecae of the females from the backcrosses were dissected under a stereomicroscope (Stemi 2000, Carl Zeiss AG, Oberkochen, Germany) using fine needles and forceps in order to examine the amount of sperm produced and transferred to females during copulation following the procedure described by (Clark et al. 2010). Unmated F2 males (n = 100 per combination; N = 400 in total) were placed in a drop of Beadle-Ephrussi-Ringer's solution (7.5 mg NaCl, 0.35 mg KCl, 0.27 mg $CaCl_2$ per ml double distilled water), decapitated, and their aedeagi were removed along with the reproductive tissues, i.e. testes, seminal vesicles and male accessory glands, which were then transferred to 20 µl of double distilled water in a depression well on a microscope slide. One seminal vesicle per male was opened and the mixture of water and released sperm cells was transferred to a 0.2 ml Eppendorf tube to be vortexed for 30 seconds to facilitate sperm isolation. Afterwards, eight spots of this mix were applied onto a new microscope slide and left to air-dry for 24 hours after which they were washed with 95% denatured ethanol for fixation and again left to air-dry. To obtain sperm from spermathecae, females from the backcrosses (at least 30 per combination) were decapitated in a drop of Ringer's solution. The contents were removed from the abdomen and the spermathecae were transferred to 20 µl of double distilled water on a microscope slide with a depression well. After being isolated from the opened spermathecae, sperm was further processed as described above.

Three of the eight spots per slide were selected at random and the sperm were counted under a microscope (Axioskop 40, Carl Zeiss AG, Oberkochen, Germany) at 100 x magnification. Overall calculation was

conducted by multiplying the sum of sperm cells obtained from counting the selected spots by 20 for the total volume of the water of 20 µl and dividing the result by three to control for the three spots counted. Values obtained from seminal vesicles were doubled to obtain the number of sperm for one male.

3.2.9 Statistical analyses

All statistical analyses were conducted using R v. 4.0.3 in RStudio v. 2022.07.1 (R Core Team 2020; RStudio Team 2022) with the pre-installed packages as well as the packages multcomp (Hothorn et al. 2008) and car (Fox and Weisberg 2019). Significance was assumed at $P < 0.05$. Binomial data, i.e. occurrence data of copulation and female offspring in several generations, were analyzed using a Pearson's Chi-squared test (Pearson 1900) for comparisons amongst groups and for single comparisons following a significant result if the frequencies of all observations were greater than five. Sets of binomial data with the frequency of at least one observation below five were analyzed using a 2 x 4 and 2 x 6 Fisher's Exact Test for Count Data (Taub 1979), respectively, for group comparisons and 2 x 2 Fisher tests for single comparisons. Single comparisons within a group were followed by Bonferroni corrections (Miller 1981). Numerical data were analyzed with linear models when data were normally distributed and variances were homogenous. If this was not the case, generalized linear models with the family best representing the data were used. All models were followed by Tukey tests (Tukey 1949) for single comparisons. Comparisons of sperm counts between hybrid and non-hybrid males of crossings between CAN and STU were conducted using Wilcoxon rank sum tests (Wilcoxon 1945) with continuity correction for non-normally distributed data and Welch Two Sample t-tests (Welch 1947) if normal distribution applied.

3.3 Results

3.3.1 Phylogenetic tree reconstruction

The phylogenetic analyses are based on five concatenated nuclear genes and the barcode segment of COI covering 28 *L. distinguendus* strains (see Table A4 for overall characters and character types for all genes). The sequences of the five nuclear genes clustered into two well-supported distinct clades (average uncorrected pairwise distance between the clades: 0.0101), containing all strains collected on granary weevils and drugstore beetles, respectively (Figure 3.1 A). The inferred phylogenetic tree based on the COI gene shows three well-supported clades which are further referred to as clades A, B, and C (Figure 3.1 B). All strains with granary weevils as putative main host form clade C that is distinct from the strains with drugstore beetles as main host from which it differs by 13.28% (clade A) and 14.22% (clade B), respectively (Table A7). The latter can be further divided into the two clades A and B with an average pairwise distance of 7.17% (Figure 3.1 B, Table A7).

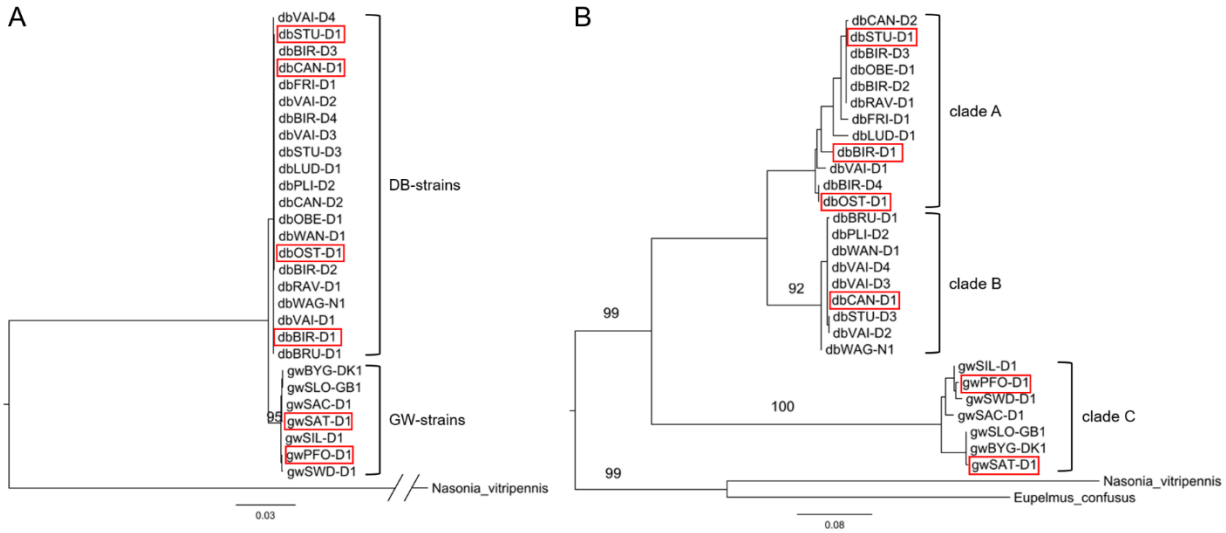

Figure 3.1. Phylogenetic trees of 28 *L. distinguendus* strains. A. Maximum likelihood phylogenetic tree inferred from the concatenated dataset comprising the five nuclear genes CAD, ITS2, and LOC 1–3. Numbers along branches represent ML bootstrap values; values below 90 have been omitted. Brackets indicate the division of strains into the two clades A and B representing strains collected on drugstore beetles (DB-strains) and granary weevils (GW-strains) as hosts, respectively. Strains in red rectangles are strains studied in crossing experiments. B. Maximum likelihood phylogenetic tree inferred from sequences of the COI gene. Numbers along branches represent ML bootstrap values; values below 90 and within clusters with low divergence have been omitted. Brackets indicate the division of the strains into the clades A, B, and C. Strains in red rectangles are strains studied in crossing experiments.

3.3.2 Occurrence of endosymbionts

All investigated *L. distinguendus* strains were tested for infections with *Spiroplasma* and *Wolbachia*. Most DB-strains were found to carry *Spiroplasma*, whereas the GW-strains were infected with *Wolbachia* and two of them, SWD and PFO, additionally were infected with *Spiroplasma*. The four DB-strains BIR (dbBIR-D1), dbBIR-D4, dbFRI-D1, and OST were negative for infections with either bacterium (Table 3.2).

Table 3.2. Status of infection with *Spiroplasma* and *Wolbachia* for all featured *L. distinguendus* strains.

Strain	Infection status
dbBIR-D1	None
dbBIR-D2	*Spiroplasma*
dbBIR-D3	*Spiroplasma*
dbBIR-D4	None
dbBRU-D1	*Spiroplasma*
gwBYG-DK1	*Wolbachia*
dbCAN-D1	*Spiroplasma*
dbCAN-D2	*Spiroplasma*
dbFRI-D1	None
dbLUD-D1	*Spiroplasma*
dbOBE-D1	*Spiroplasma*
dbOST-D1	None
gwPFO-D1[a]	*Wolbachia, Spiroplasma*
dbPLI-D2	*Spiroplasma*
dbRAV-D1	*Spiroplasma*
gwSAC-D1	*Wolbachia*
gwSAT-D1[a]	*Wolbachia*
gwSIL-D1	*Wolbachia*
gwSLO-GB1[a]	*Wolbachia*
dbSTU-D1[b]	*Spiroplasma*
dbSTU-D3	*Spiroplasma*
gwSWD-D1	*Wolbachia, Spiroplasma*
dbVAI-D1	*Spiroplasma*
dbVAI-D2	*Spiroplasma*
dbVAI-D3	*Spiroplasma*
dbVAI-D4	*Spiroplasma*
dbWAG-N1	*Spiroplasma*
dbWAN-D1	*Spiroplasma*

[a] K. König et al. (2019); [b] Pollmann et al. (2022)

3.3.3 Reproductive barriers

In the crossing experiments, reproductive barriers were studied within and between the clades with the strains dbBIR-D1 (“BIR”), dbOST-D1 (“OST”), dbSTU-D1 (“STU”) as representatives of clade A, dbCAN-D1 (“CAN") as representatives of clade B, and gwSAT-D1 (“SAT”) and gwPFO-D1 (“PFO”) as representatives of clade C.

Sexual isolation

In the hybrid combinations OST females x BIR males, STU females x BIR males, and STU females x CAN males, the occurrence of copulations was significantly decreased compared to the respective control combinations, indicating slight sexual isolation (see Tables A9-A11 for test statistics; Figure 3.2). In the reverse hybrid combinations, there was an increase in copulations in OST females x BIR males, and no significant differences to the controls in BIR females x STU males, and CAN females x STU males (see Tables A9-A11 for test statistics; Figure 3.2). In the interstrain combinations BIR x SAT and CAN x PFO, there were almost no copulations (see Tables A12-A13 for test statistics; Figure 3.2). Therefore, subsequent barriers in the latter combination could only be investigated for CAN males and PFO females.

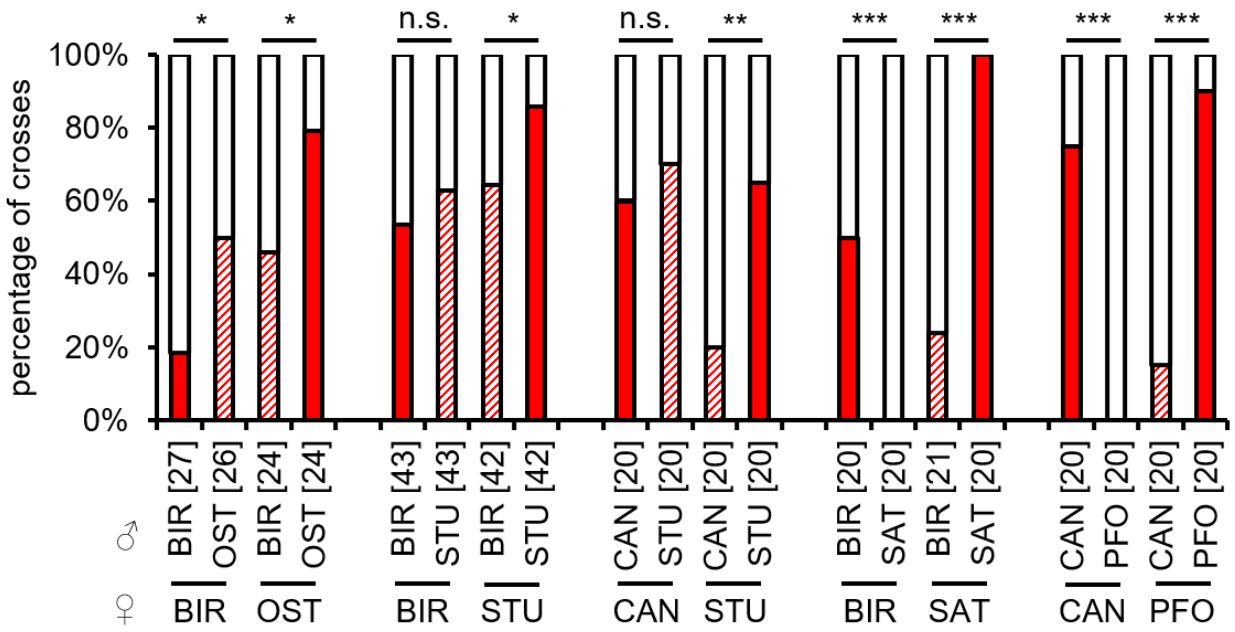

Figure 3.2. Occurrence of copulations [%] in intrastrain and interstrain crosses between females (lower strain designation) and males (upper strain designation) of several *L. distinguendus* strains. Colored parts of the bars: presence of copulation, filled: intrastrain, hatched: interstrain; white parts of the bars: absence of copulation. Numbers of replicates are given in parentheses above each crossing combination. n.s. $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, 2 x 2 Fisher’s Exact Tests for Count Data (for BIR x OST, CAN x STU, BIR x SAT, and CAN x PFO) and Pearson’s Chi-squared test (for BIR x STU) for single comparisons within a group (intrastrain vs. interstrain combination) (see Tables A9-A13 for full test statistics).

F1 female inviability

There were no significant differences in the number of F1 female offspring between intrastrain and interstrain crosses within each of the strain combinations (see Tables A14-A18 for test statistics; Figure 3.3). Thus, non-hybrid and hybrid F1 females are equally viable. In the crosses BIR females x SAT males and CAN females x PFO males, only a very small number of female wasps was available for testing. Most likely, this prevented significant results in these crosses.

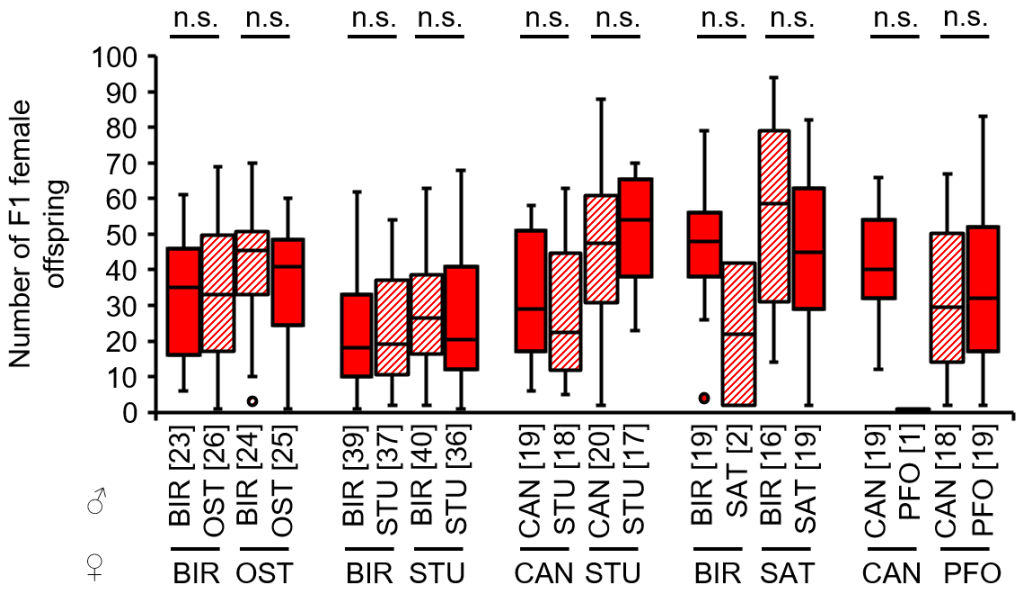

Figure 3.3. Numbers of female F1 offspring of intrastrain (filled boxes) and interstrain (hatched boxes) crosses of females (lower strain designation) and males (upper strain designation) of several *L. distinguendus* strains. Numbers of replicates are given in parentheses above each crossing combination. Only crosses with female offspring were considered. n.s. $P > 0.05$, BIR x OST: linear model, BIR x STU: GLM, family = quasipoisson, CAN x STU: linear model, BIR x SAT: GLM, family = quasipoisson, CAN x PFO: linear model, all models followed by Tukey tests for multiple comparisons (see Tables A14-A18 for full test statistics).

F1 female behavioral sterility

Sexual isolation of F1-hybrid females was only studied for the crosses of BIR x OST, BIR x STU, and CAN x STU (Figure 3.4). In the combinations BIR x OST and BIR x STU, no differences in the occurrence of copulations between F1 females and parental-type males were observed (see Tables A19-A20 for test statistics). In contrast, hybrid F1-females from the combination CAN x STU had a reduced number of copulations with non-hybrid males from the parental strains, demonstrating sexual isolation of these females (see Table A21 for test statistics; Figure 3.4).

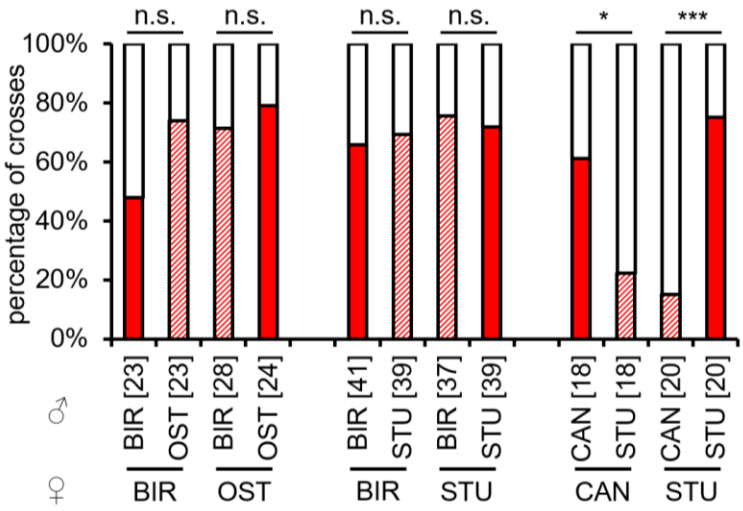

Figure 3.4. Occurrence of copulations [%] between non-hybrid and hybrid F1-females and parental type males from combinations of several *L. distinguendus* strains. Strain designations on the x-axis refer to the females (lower strain designation) and males (upper strain designation) of the parental cross from which the F1-females originated. Colored parts of the bars: presence of copulation, filled: intrastrain, non-hybrid, hatched: interstrain, hybrid; white parts of the bars: absence of copulation. Numbers of replicates are given in parentheses above each crossing combination. n.s. $P > 0.05$, * $P < 0.05$, *** $P < 0.001$, 2 x 2 Fisher's Exact Tests for Count Data (for BIR x OST and CAN x STU) and Pearson's Chi-squared tests (for BIR x STU) for single comparisons within a group (non-hybrid vs. hybrid combination) (see Tables A19-A21 for full test statistics).

F1 female physiological sterility

The occurrence of F2 offspring of F1 females mated to parental-type males did not differ between hybrids and non-hybrids, indicating the absence of physiological sterility in F1 hybrid females (see Tables A22-A26 for test statistics; Figure 3.5).

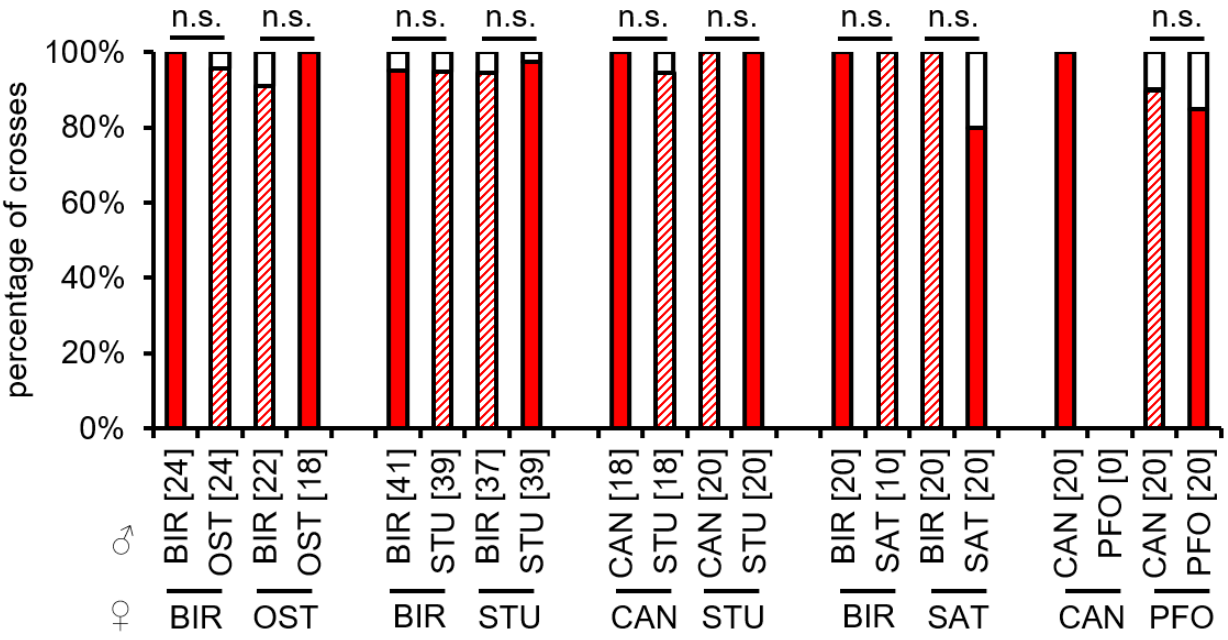

Figure 3.5. Occurrence of total F2 offspring [%] of non-hybrid and hybrid F1 females when crossed to parental-type males of several *L. distinguendus* strains. Strain designations on the x-axis refer to the females (lower strain designation) and males (upper strain designation) of the parental cross from which the F1-females originated. Colored parts of the bars: presence of offspring, filled: intrastrain, non-hybrid, hatched: interstrain, hybrid; white parts of the bars: absence of offspring. Numbers of replicates are given in parentheses above each crossing combination. n.s. $P > 0.05$, 2 x 2 Fisher's Exact Tests for Count Data for single comparisons within a group (non-hybrid vs. hybrid combination) (see Tables A22-A26 for full test statistics).

F1 female physiological reduced fertility

There were no significant differences in the number of total F2 offspring from non-hybrid and hybrid F1 females of the combinations BIR x OST, BIR x STU, CAN x STU, and BIR x SAT (see Tables A27-A30 for test statistics; Figure 3.6). In contrast, hybrid F1 females of the combination PFO females x CAN males had significantly reduced F2 total offspring numbers compared to non-hybrid control females (see Table A31 for full test statistics; Figure 3.6). No hybrid F1 females of the combination CAN females x PFO males were available.

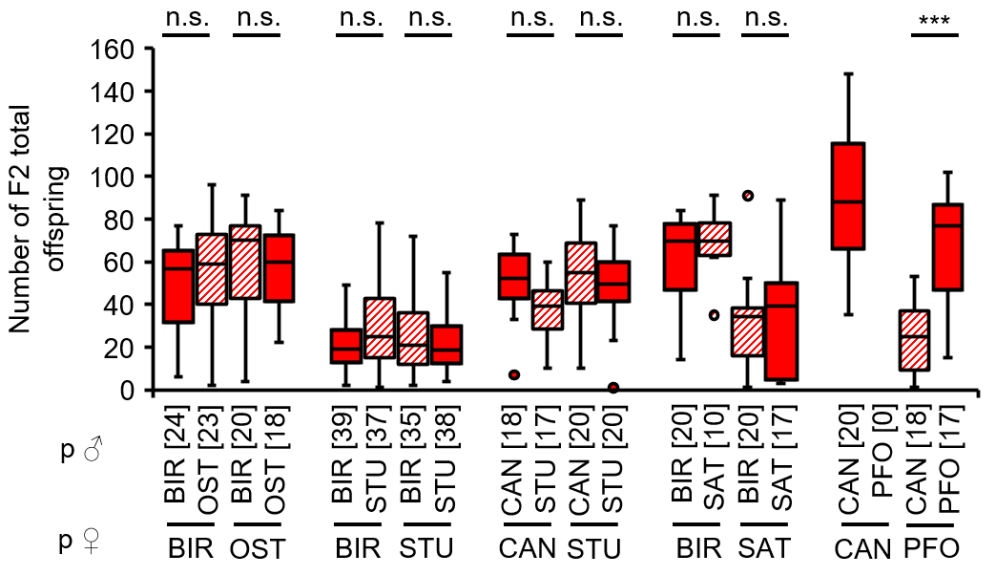

Figure 3.6. Total F2 offspring numbers of non-hybrid (filled boxes) and hybrid (hatched boxes) F1 females when crossed to parental-type males of several *L. distinguendus* strains. Strain designations on the x-axis refer to the females (lower strain designation) and males (upper strain designation) of the parental cross from which the F1-females originated. Numbers of replicates are given in parentheses above each crossing combination. Only crosses with offspring were considered. n.s. $P > 0.05$, *** $P < 0.001$, BIR x OST: GLM, family = quasipoisson, BIR x STU: GLM, family = quasipoisson, CAN x STU: linear model, BIR x SAT: GLM, family = quasipoisson, CAN x PFO: linear model, all models followed by Tukey tests for multiple comparisons (see Tables A27-A31 for full test statistics).

F2 male inviability

F2 male inviability was studied with the offspring of virgin non-hybrid and hybrid females, which produce only males. Combinations BIR x OST, BIR x STU, and CAN x STU did not show any significant differences in numbers of F2 male offspring between non-hybrid and hybrid F1 females and therefore no hybrid male inviability (see Tables A32-A34 for test statistics; Figure 3.7). In contrast, for the combination BIR x SAT, the numbers of hybrid F2 male offspring were significantly reduced compared to the control crosses, indicating hybrid male inviability (see Table A35 for test statistics; Figure 3.7). Likewise, there were less hybrid F2 male offspring from hybrid PFO x CAN females (see Table A36 for test statistics; Figure 3.7).

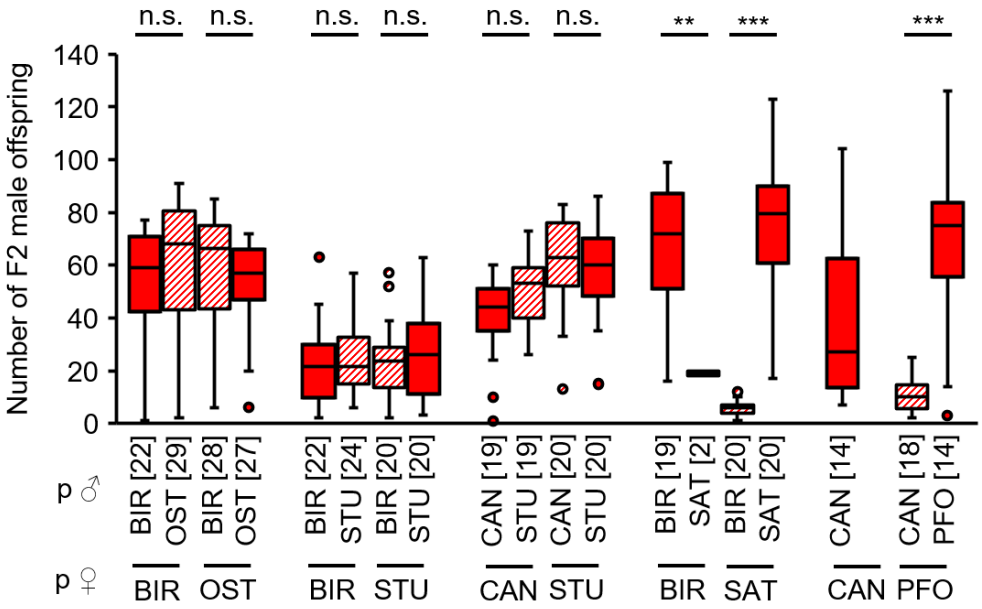

Figure 3.7. F2 male offspring numbers of virgin non-hybrid (filled boxes) and hybrid (hatched boxes) F1 females. Strain designations on the x-axis refer to the females (lower strain designation) and males (upper strain designation) of the parental cross from which the F1-females originated. Numbers of replicates are given in parentheses above each crossing combination. Only crosses in which females produced offspring were considered. n.s. $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$, BIR x OST: GLM, family = quasipoisson, BIR x STU: GLM, family = negative binomial, CAN x STU: GLM, family = quasipoisson, BIR x SAT: linear model, CAN x PFO: GLM, family =quasipoisson, all models followed by Tukey tests for multiple comparisons (see Tables A32-A36 for full test statistics).

F2 male behavioral sterility

The occurrence of copulations in backcrosses with parental-type females did not differ significantly between hybrid and non-hybrid F2 males from all strain combinations of BIR x OST, BIR x STU, and CAN x STU, demonstrating the absence of behavioral sterility. Likewise, there were no differences between hybrid SAT x BIR males (both combinations) crossed to SAT females, as well as hybrids of BIR females x SAT males crossed to BIR females, (see Tables A37-A39 for test statistics; Figure 3.8). Hybrid F2 males of SAT females x BIR males crossed to BIR females and the available crosses from PFO x CAN had much less copulations compared to the control males when backcrossed to both parental females (see Tables A40-A41 for test statistics; Figure 3.8).

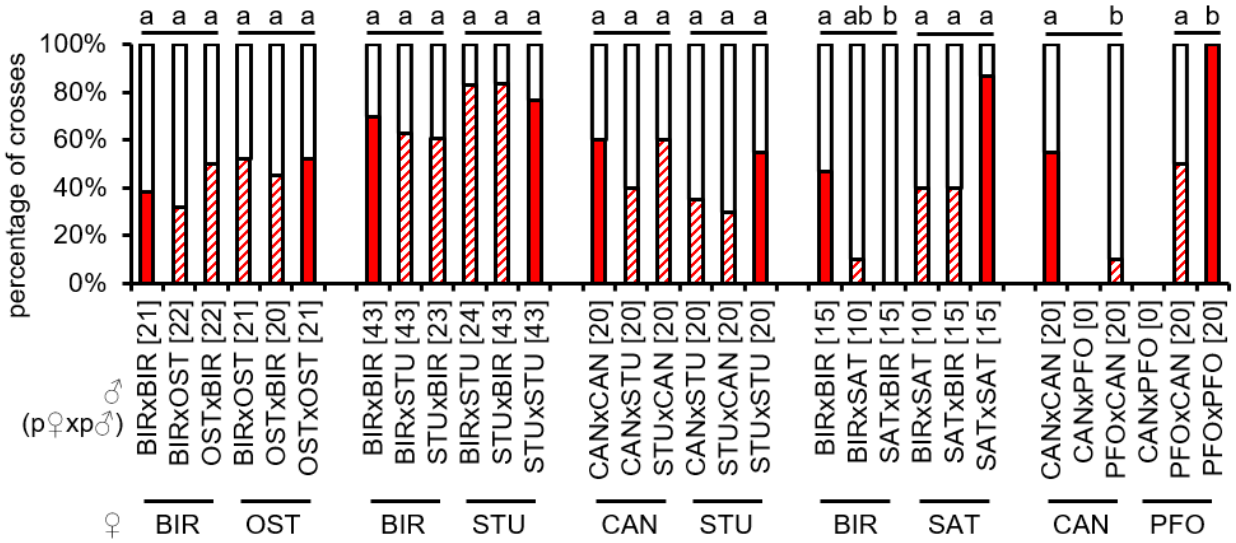

Figure 3.8. Occurrence of copulations [%] in backcrosses of F2 non-hybrid and hybrid males to parental-type females of several *L. distinguendus* strains. Strain designations on the x-axis refer to the parental-type females (lower strain designation) and the parental cross from which the F2 males originated (upper strain designation). Colored parts of the bars: presence of copulation, filled: non-hybrid, hatched: hybrid; white parts of the bars: absence of copulation. Numbers of replicates are given in parentheses above each crossing combination. Different lower-case letters indicate statistical differences, 2 x 3 Fisher's Exact Tests for Count Data (for BIR x STU and BIR x SAT), Pearson's Chi-squared tests (for BIR x OST and CAN x STU), and 2 x 2 Fisher's Exact Tests for Count Data (for CAN x PFO) for group comparisons (non-hybrid vs. hybrid males crossed to parental-type females of the same strain), 2 x 2 Fisher's Exact Tests for Count Data followed by Bonferroni correction for single comparisons after significant differences in group comparisons within a strain combination (see Tables A37-A41 for full test statistics).

F2 male physiological sterility

The percentage of crosses with female F3 offspring did not differ significantly in backcrosses of hybrid and non-hybrid F2 males to parental-type females for the strain combinations BIR x OST, BIR x STU, CAN x STU, and BIR x SAT. Obviously, these hybrid males are able to produce female offspring and are not physiologically sterile (see Tables A42-A44 for test statistics; Figure 3.9). However, the percentage of crosses with female offspring was significantly reduced as compared to controls in hybrid F2 males from the combination BIR females x SAT males crossed to BIR females, and of the parental cross PFO females x CAN males. Some of these hybrids are physiologically sterile (see Tables A45-A46 for test statistics; Figure 3.9).

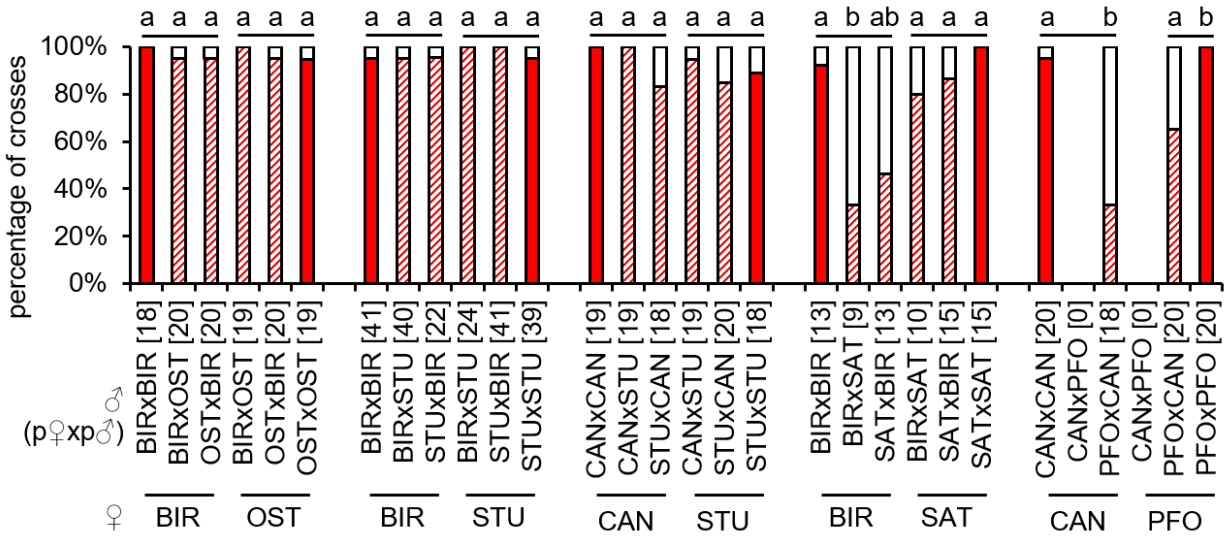

Figure 3.9. Occurrence of female F3 offspring [%] in backcrosses of non-hybrid and hybrid F2 males to parental-type females of several *L. distinguendus* strains. Strain designations on the x-axis refer to the parental-type females (lower strain designation) and the parental cross from which the F2 males originated (upper strain designation). Colored parts of the bars: presence of female offspring, filled: non-hybrid, hatched: hybrid; white parts of the bars: absence of female offspring. Numbers of replicates are given in parentheses above each crossing combination. Different lower-case letters indicate statistical differences, 2 x 3 Fisher's Exact Tests for Count Data (for BIR x OST, BIR x STU, CAN x STU, and BIR x SAT) and 2 x 2 Fisher's Exact Tests for Count Data (for CAN x PFO) for group comparisons (non-hybrid vs. hybrid males crossed to parental-type females of the same strain), 2 x 2 Fisher's Exact Tests for Count Data followed by Bonferroni correction for single comparisons after significant differences in group comparisons within a strain combination (see Tables A42-A46 for full test statistics).

F2 male reduced fertility or F3-female inviability

A reduced number of F3 female offspring numbers compared to controls, was observed in hybrid F2 males when backcrossed to parental-type females in all strain combinations of BIR x OST, and BIR x STU (see Tables A47-A48 for test statistics; Figure 3.10). In contrast, hybrid F2 males from several combinations of STU x CAN, BIR x SAT, and CAN x PFO sired significantly fewer F3 females than control F2 males. This likely indicates a reduced fertility of these hybrid males (see Tables A49-A51 for test statistics; Figure 3.10). However, the possibility that this result is in fact caused by an increased inviability of hybrid F3 females cannot be excluded.

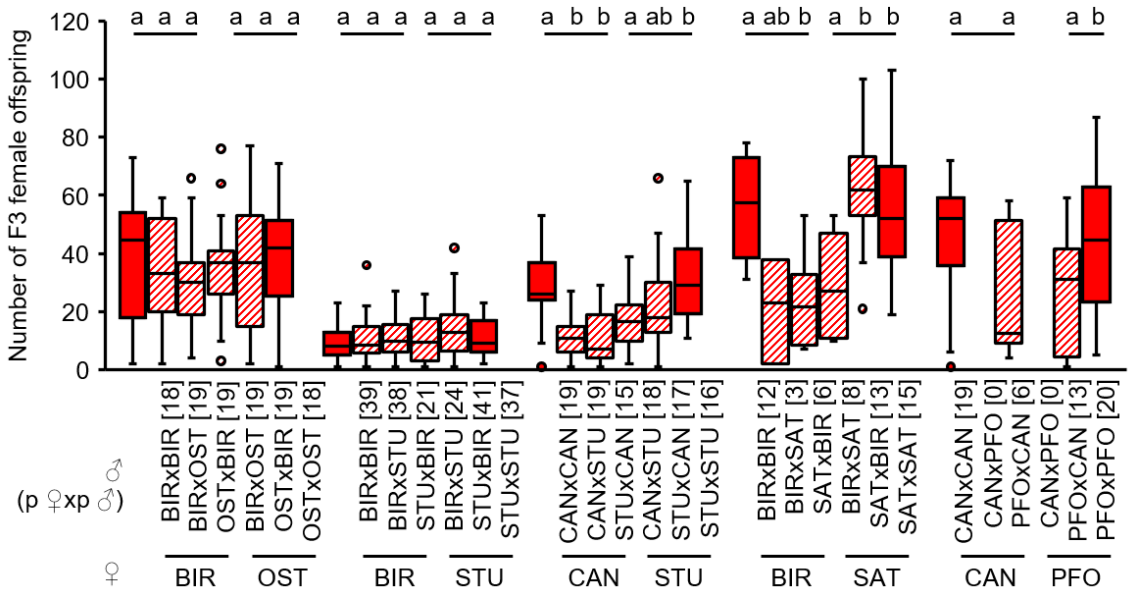

Figure 3.10. F3 female offspring numbers of non-hybrid (filled boxes) and hybrid (hatched boxes) F2 males when backcrossed to parental-type females of several *L. distinguendus* strains. Strain designations on the x-axis refer to the parental-type females (lower strain designation) and the parental cross from which the F2 males originated (upper strain designation). Numbers of replicates are given in parentheses above each crossing combination. Only crosses with female offspring were considered. Different lower-case letters indicate statistical differences, BIR x OST: linear model, BIR x STU: GLM, family = negative binomial, CAN x STU: GLM, family = quasipoisson, BIR x SAT: linear model, CAN x PFO: linear model, all models followed by Tukey tests for multiple comparisons (see Tables A47-A51 for full test statistics).

3.3.4 The role of CI due to endosymbionts in BIR x STU

To examine the role of CI as barrier between BIR and STU, the number of F1 female offspring was studied with wasps that were not treated with antibiotics, and one additional treatment with tetracycline-fed STU males. Thereby, a reduction in the occurrence of female offspring was observed in crosses between BIR females and STU males compared to the non-hybrid control combination and the hybrid combination with BIR females and tetracycline fed STU males. There was no reduction of female offspring when STU females were crossed to BIR males (see Tables A52-A53 for full test statistics; Table 3.3). This demonstrates the presence of CI as unilateral isolating barrier between BIR females and STU males.

Table 3.3. Numbers of crosses with and without female offspring and percentage of crosses with female offspring for all possible combinations with untreated individuals of the strain combination BIR x STU as well as the crossing combination BIR females x tetracycline-treated (-) STU males. Different lowercase letters indicate statistical difference at $P < 0.05$ (see Tables A52-A53 for test statistics).

crossing combination	n	crosses with female offspring	crosses without female offspring	% crosses with female offspring	statistical difference
BIR ♀ x BIR ♂	21	21	0	100	a
BIR ♀ x STU(-) ♂	39	39	0	100	a
BIR ♀ x STU ♂	40	19	21	47.5	b
STU ♀ x BIR ♂	38	33	5	86.8	a
STU ♀ x STU ♂[a]	33	29	4	87.9	a

[a] data re-used from Pollmann et al. (2022)

3.3.5 Isolation indices

For the strain combination BIR x OST, all isolation indices except for one were close to zero, indicating random gene flow (Table 3.4). Only for sexual isolation as barrier, significant indices of -0.44 and 0.27 were found for the combinations BIR females x OST males, and OST females x BIR males, respectively. Thus, interstrain matings occurred about 40% more often in the combination BIR females x OST males, and were about 30% less likely in the combination OST females x BIR males than intrastrain matings. Similarly, the isolation indices in the strain combination BIR x STU with wasps treated with antibiotics, i.e. in the absence of CI, diverged only little from zero in either direction, ranging from -0.14 to 0.14. There was only very slight, but significant sexual isolation in the combination STU females x BIR males. In contrast, with wasps which have not been treated with antibiotics, CI caused a hybrid female inviability index of 0.356. Regardless of CI, total isolation between BIR and STU was -0.28 and 0.14, respectively. Isolation indices in the combination CAN x STU were high for several barriers, i.e. for sexual isolation of the parental cross STU females x CAN males, for hybrid F1 females of both combinations, and with respect to reduced fertility of hybrid males. The combined barriers between these strains resulted in a total isolation of 0.8319, and 0.9651, respectively, indicating very high to near complete isolation (Table 3.4). For the strain combinations BIR x SAT and CAN x PFO, isolation indices, except for reduced fertility of hybrid females, were consistently ranging between intermediate and high values. Sexual isolation in these combinations was close to

complete, although the occurrence of females in the F1 generation suggests that copulation did occur in some crosses (Table 3.4). The resulting total isolation indices of 1.0 and 0.996 for BIR x SAT, and 1.0 and 0.9991 for CAN x PFO (Table 3.4) suggests the absence of gene flow between the strains.

Table 3.4. Strength of reproductive isolation per barrier for all crossing combinations given as indices ranging from -1 (outcrossing is favored), over 0 (random crossing) to 1 (complete isolation), sorted by % difference in the COI barcode. Indices which are based on significant differences between non-hybrid and hybrid crosses are given in bold.

Barrier	BIR ♀ x OST ♂	OST ♀x BIR ♂	BIR ♀ x STU ♂	STU ♀ x BIR ♂	CAN ♀ x STU ♂	STU ♀ x CAN ♂	BIR ♀ x SAT ♂	SAT ♀ x BIR ♂	CAN ♀ x PFO ♂	PFO ♀ x CAN ♂
% COI difference	1.7%		2.8%		7.2%		13.9%		14.0%	
Ecological Isolation			N/A[a]				Present		N/A	
Sexual Isolation parentals	-0.44	**0.27**	-0.08	**0.14**	-0.08	**0.53**	**1.0**	**0.62**	**1.0**	**0.71**
Inviability hybrid ♀ (without CI)	0.03	-0.05	-0.03	0.13	0.13	0.06	**0.37**	-0.13	(0.95)[b]	0.04
Inviability hybrid ♀ (with CI)[c]	-	-	**0.356**	0.006	-	-	-		-	
Behavioral sterility hybrid ♀	-0.19	0.08	-0.02	-0.03	**0.47**	**0.67**	Not studied		Not studied	
Physiological sterility hybrid ♀	0.02	0.04	0	0.01	0.03	0	0	-0.11	N/A	-0.03
Reduced fertility hybrid ♀	-0.02	-0.08	-0.14	-0.06	0.14	-0.05	0.0	0.06	N/A	**0.51**
Inviability hybrid ♂	-0.07	-0.08	0	0.05	-0.09	-0.02	**0.58**	**0.86**	N/A	**0.76**
Behavioral sterility hybrid ♂[d]	0.05	-0.03	0.01	0.01	0.21	0.15	**0.51**	**0.68**	N/A	**0.51**
Physiological sterility hybrid ♂	0.0	0.01	-0.01	-0.01	-0.02	0.06	**0.29**	**0.20**	N/A	**0.35**
Reduced fertility hybrid ♂	0.09	0.13	-0.01	-0.1	**0.34**	**0.40**	**0.37**	**0.23**	N/A	**0.40**
Total isolation (without CI)	-0.5276	0.2949	-0.2785	0.1436	**0.8319**	**0.9651**	**1.0**	**0.996**	**1.0**	**0.9991**
Total isolation (with CI)[c]	-	-	0.0860	0.1498	**-**	**-**	**-**	**-**	**-**	**-**

[a] N/A: not available; [b] n = 1; [c] only present in the crossing combination BIR x STU; [d] from behavioral sterility of hybrid males onward: means of indices are provided, calculated from data of backcrosses to both wildtype strain females.

3.3.6 Sperm counts

To investigate if the reduced fertility of hybrid males in the combination CAN x STU is due to a reduced number of sperm cells produced by the males and/or transferred to females during copulation, sperm counts in seminal vesicles and spermathecae were compared in hybrid and non-hybrid males and females after mating. The amount of sperm cells in the seminal vesicles of hybrid males of the parental cross STU females x CAN males were significantly lower than in those of the control cross. This was not the case in

hybrid males from the parental combination CAN females x STU males (see Table A54 for full statistics; Table 3.5). However, sperm retrieved from spermathecae did not differ in numbers between females mated to males of either combination and controls (see Table A54 for full statistics; Table 3.5).

Table 3.5. Sperm counts in seminal vesicles of unmated F2 males obtained and in spermathecae of females mated to F2 males. Pairwise statistical comparisons were conducted according to the origin strain of the males (*** $P < 0.001$, n.s. $P > 0.05$; see Table A54 for full statistics).

	parental cross of F2 ♂	♀	n	median	quartile 1	quartile 3	statistical difference
Seminal vesicles of males	CAN ♀ x CAN ♂	-	100	3163.335	2598.3325	3724.975	
	STU ♀ x CAN ♂	-	100	2363.33	1680.0025	3050	P = 0.00 ***
	CAN ♀ x STU ♂	-	100	1986.67	1383.3325	2461.6675	
	STU ♀ x STU ♂	-	100	1800	1301.67	2546.67	P = 0.58 n.s.
Spermathecae of females after copulation	CAN ♀ x CAN ♂	CAN	32	380	280	454.9975	
	STU ♀ x CAN ♂	CAN	34	300	214.9975	424.9975	P = 0.14 n.s.
	CAN ♀ x STU ♂	STU	32	433.33	310	596.67	
	STU ♀ x STU ♂	STU	31	393.33	266.67	576.665	P = 0.40 n.s.

3.4 Discussion

In this study, the diversity within the species complex of *L. distinguendus* was investigated by conducting phylogenetic tree inferences with fragments of five nuclear genes and the COI gene, and by studying reproductive isolation and isolation barriers according to the biological species concept (BSC) in crossing experiments to identify separate species within the complex.

3.4.1 *L. distinguendus* is split into three reproductively isolated clusters

The phylogenetic tree inference based on nuclear loci recognized two clades, which agree with the preference for either *S. granarius* or *S. paniceum* as hosts. They also match with *Stegobium* Clade I and *Sitophilus* Clade I from an earlier study which was based on the same nuclear genes, another COI gene, and only nine of the 28 *L. distinguendus* strains presented here (König et al. 2015a; C. König et al. 2019). In contrast, the newly reconstructed COI phylogenetic tree displays a more distinct topology and clusters into three well-supported clades. Of these, two comprise strains which were collected in households and/or with drugstore beetles as baits (clades A and B). From these, clade A largely equals the *Stegobium* Clade I from a previous study (C. König et al. 2019), while clade B consists mostly of strains which were collected only recently and was unknown. The third clade (clade C) comprises strains with granary weevils as preferred hosts and is identical to the *Sitophilus* Clade I (König et al. 2015a; C. König et al. 2019). Discordances between the resolution and topology of phylogenetic trees recovered from mitochondrial and

nuclear genes are common (e.g. (Gebiola et al. 2012; Hernández-López et al. 2012)) and can be caused by higher rates of evolution of mitochondrial DNA compared to nuclear DNA (Brown et al. 1979; Hubert and Hanner 2015), for example due to incomplete lineage sorting (Gebiola et al. 2012). This difference in evolutionary rates has been shown to be especially pronounced in Hymenoptera (Kaltenpoth et al. 2012). Alternatively, a high diversity in mtDNA can result from a high diversity in endosymbionts in arthropod populations even though the diversity in nuclear genes is low (Hurst and Jiggins 2005). This is because endosymbionts, such as *Wolbachia*, can link to specific mtDNA types, causing them to sweep through a population along with the endosymbionts (reviewed by (Hurst and Jiggins 2005)). In fact, different infections with endosymbiotic bacteria have been detected within the *L. distinguendus* species complex ((K. König et al. 2019, Pollmann et al. 2022) that could have influenced the results. Therefore, to study, whether clade B within the COI-tree is in fact separate from the other clades, reproductive isolation based on BSC was studied between clades A, B, and C. This confirmed the COI-tree topology presented here. The three COI clusters are reproductively isolated groups, with almost complete reproductive isolation between clade A (represented by strain STU) and clade B (represented by strain CAN), ranging from 0.83 to 0.97, and complete reproductive isolation between clade A (represented by strain BIR) and clade C (represented by strain SAT), and between clade B (represented by strain CAN) and clade C (represented by strain PFO). As the values of total reproductive isolation of 0.83 to 1.0 are considered to be “substantial reproductive isolation” (see (Coyne and Orr 2004)), these clades constitute separate species according to the BSC. While clades A and C have been shown to be reproductively isolated before (K. König et al. 2015a, 2019), here, it is also demonstrated that clade B, so far considered to belong to clade A based on a phylogeny gained from a single locus analyzed in a single strain (C. König et al. 2019), is in fact a new group reproductively isolated from clade A and clade C. Thus, the *L. distinguendus* species complex does not only comprise two (K. König et al. 2019), but at least three distinct species. An outlier clade, designated as *Stegobium* Clade II, which was detected in an earlier study (C. König et al. 2019), might constitute yet another separate species. However, this clade was not incorporated into the present study as the laboratory strains had been lost beforehand. As there are no discernible morphological differences, at least between clades A and C (Wendt et al. 2014), *L. distinguendus* seems to be another example for cryptic diversity. This is common in parasitic Hymenoptera and Chalcidoidea in particular, e.g. (Heraty et al. 2007; Hernández-López et al. 2012; Stahlhut et al. 2013; Kenyon et al. 2015; Darwell and Cook 2017), which supports the hypothesis that Hymenoptera are the most diverse order within the animal kingdom (Forbes et al. 2018). Remarkably, all species in the *L. distinguendus* complex, including the newly discovered cryptic species, occur in close contact to human habitations.

3.4.2 Results from barcode data do not agree with data based on the BSC

The BSC (Mayr 1969) defines species as populations which are readily interbreeding with each other, but not with other populations. Applied for species delimitation, it therefore requires the existence of complete (Mayr 1969) or very strong (Coyne and Orr 2004) reproductive isolation caused by sexual isolation, as well as partial or total inviability, sterility, and/or reduced fertility of hybrids (Coyne and Orr 2004) to justify the

assumption of separate species (Mayr 1969; Coyne and Orr 2004). Remarkably, while the BSC is the most prominent species concept in text books (Barton et al. 2007; Futuyma 2018) and predominantly used by researchers focusing on study areas such as ecology and evolution, it is rarely used in taxonomy and phylogenetics, the scientific fields where species are described (Stankowski and Ravinet 2021). In these fields, mostly molecular differences are used for species delimitation, sometimes combined with morphological and/or ecological data in an integrative taxonomy approach (Schlick-Steiner et al. 2010). Thereby, species are often separated based on the divergence of the barcode segment (Hebert et al. 2004; Smith et al. 2005, 2006; Ward et al. 2005; Hajibabaei et al. 2006), i.e. a gap between intraspecific variance and interspecific distance, referred to as barcode gap (Hebert et al. 2004; Meyer and Paulay 2005). Although different methods have been suggested to set the threshold for the barcode gap (e.g. (Hebert et al. 2004)), a threshold of 2% difference in COI is often used to support the assumption of separate species, especially for parasitoid wasps (Stahlhut et al. 2013; Smith et al. 2013; Fernández-Flores et al. 2013) (e.g. (Smith et al. 2013; Kang et al. 2017; Fagan-Jeffries et al. 2018; Meierotto et al. 2019; Sharkey et al. 2021), reviewed by (Hubert and Hanner 2015)). Likewise, the BIN system used by the BOLD database uses a 2.2% threshold of COI difference (Ratnasingham and Hebert 2013). This strategy was successful to recover taxonomies which were established based on other traits like morphology and ecology in different groups of organisms and to reveal cryptic diversities within the studied groups (Hebert et al. 2004; Smith et al. 2005, 2006; Ward et al. 2005; Hajibabaei et al. 2006). In extreme cases, up to hundreds of presumed new species were described largely based on barcodes, which has been termed turbo-taxonomy (Butcher et al. 2012; Meierotto et al. 2019; Sharkey et al. 2021).

Comparing this method of species delimitation to the results of the crossing experiments with *L. distinguendus* reveals that the strictly set thresholds commonly used for species delimitation based on barcodes, specifically thresholds of 2% or 2.2% COI divergence (Smith et al. 2013; Ratnasingham and Hebert 2013; Kang et al. 2017; Fagan-Jeffries et al. 2018; Meierotto et al. 2019; Sharkey et al. 2021) do not match the species determined based on the BSC. If a COI difference of 2% or 2.2% would be accepted as sufficient to declare two populations as distinct species, the strains BIR and STU, which differ in COI by 2.8%, would have to be considered as separate species. However, as there is only little reproductive isolation between BIR females and STU males, this hypothesis must be rejected. In contrast, the threshold for species delimitation in *L. distinguendus* presumably lies well above the 2% and 2.2% COI difference, as the least divergent species pair with substantial reproductive isolation has a COI difference of 7.2% (CAN x STU). Therefore, these results support the criticism towards relying on universally fixed barcoding gaps for species delimitation and species description (Meyer and Paulay 2005; DeSalle 2006; Wiemers and Fiedler 2007; Zamani et al. 2021, 2022; Ahrens et al. 2021; Meier et al. 2022). Because the divergence and threshold values for species delimitation are likely to be taxon-specific (Hebert et al. 2003a; Huang et al. 2008; Phillips et al. 2022; Gadawski et al. 2022), based on the results presented here, a case could be made for calibrating species delimitation thresholds based on barcoding and morphology by studying reproductive barriers according to the BSC in suitable related species. This should be particularly easy in

parasitoids, many species of which are bred in laboratories as they can be used as biological control agents (Waage and Hassell 1982; Smith 1996; Quicke 1997; Ovruski and Schliserman 2012; Gabarra et al. 2015; Postali Parra and Coelho 2019; Wang et al. 2019; Martel et al. 2019; Ibouh et al. 2019; Cherif et al. 2021), making them easily accessible for such experiments.

3.4.3 Emergence of reproductive barriers during the process of speciation in *L. distinguendus*

While the process of speciation and the order of emergence of reproductive barriers was intensively studied in recent years for a large number of taxa (Coyne and Orr 1989; Mendelson et al. 2007; Fitzpatrick et al. 2009; Xue et al. 2009; Seehausen et al. 2014), only very few studies have dealt with speciation in hymenopterans. Because the strains of *L. distinguendus* represent different stages within the continuum from closely related populations over incipient to distinct species, they can provide valuable insight into the emergence of reproductive barriers during speciation in parasitoid hymenopterans.

Sexual isolation

The results indicate that sexual isolation, albeit weak and unilateral, emerges as first barrier between closely related strains without obvious ecological separation, such as the use of different hosts, and increases in strength during the process of separation. This agrees with other data on reproductive barriers in *L. distinguendus* (K. König et al. 2019), and with the recent study on reproductive barriers within a population of *N. vitripennis* (Malec et al. 2021), where slight sexual isolation was found between closely related populations and even within a population of the same species. As in *N. vitripennis*, sexual isolation in *L. distinguendus* is most likely caused by a mate choice decision of the female, which do not accept males with diverging mandibular pheromones that are applied on the female antennae during courtship (Ruther and Hammerl 2014; König et al. 2015b). The findings that sexual isolation as reproductive barrier seems to precede ecological isolation, challenge the view that sexual selection is only a by-product of natural selection and should be dropped as unique speciation mechanism (Ritchie 2007; Weissing et al. 2011; Safran et al. 2013; Scordato et al. 2014; Rundle and Rowe 2018). In *N. vitripennis*, evidence suggested that inbreeding between sisters and brothers, which is common in Chalcidoidea and also occurs in *L. distinguendus*, might lead to sexual isolation by genetic drift (Uyeda et al. 2009) and promote speciation similar to geographic barriers in allopatric populations, as hypothesized by Askew (Askew 1968; Malec et al. 2021). However, detailed studies on the ecology of the strains studied here, such as the thorough investigation of host choice and host use, are required to answer the question if sexual isolation precedes ecological isolation in *L. distinguendus.*

Cytoplasmic incompatibility (CI)

Following sexual isolation, cytoplasmic incompatibility (CI), the incompatibility of sperm and egg (Yen and Barr 1971), was identified as second barrier between the closely related strains BIR and STU. Generally, CI can occur in arthropods in crossings between uninfected females and males with specific endosymbionts (Barr 1980; Breeuwer and Werren 1990). In haplodiploids like *L. distinguendus* it appears as a reduction in the number or the complete absence of female offspring (Ryan and Saul 1968). Endosymbionts which are

able to induce CI are *Wolbachia* (Yen and Barr 1971), *Candidatus* Cardinium hertigii (Hunter et al. 2003), a new bacterium in the same family as *Wolbachia*, called *Candidatus* Mesenet longicola (Takano et al. 2017, 2021), and a strain of the Gammaproteobacterium *Rickettsiella* (Rosenwald et al. 2020). For *L. distinguendus,* it was shown that the STU strain carries the endosymbiont *Spiroplasma*, named *sDis*, which also induces CI (Pollmann et al. 2022). This represents a novel phenotype for *Spiroplasma*. In the present work, it is demonstrated that the occurrence of female offspring is reduced in crosses between BIR females and STU males, and can be rescued in crosses between tetracycline-treated individuals. This suggests that *sDis* in STU is also able to induce CI in crosses with females of the BIR strain, which does not carry *Spiroplasma*. Among the barriers between these strains, CI was by far the strongest, albeit unilateral. It is unclear if this could drive the separation of these strains along with slight sexual isolation and mark the initiation of speciation, because these barriers do not cause strong reproductive isolation, as demonstrated by a total isolation of -0.28 and 0.14. Unidirectional CI is generally considered to be insufficient to drive speciation by itself because it is often incomplete, transmission is imperfect, and it can only be a barrier in one direction (Hurst and Schilthuizen 1998; Wade 2001; Telschow et al. 2007). However, it can represent the first and only barrier when acting bidirectionally as in *N. longicornis* and *N. girault*i (Bordenstein et al. 2001), or in conjunction with other barriers as in *Encarsia*, *Drosophila* (Shoemaker et al. 1999; Gebiola et al. 2016a), and between clade A and clade C of *L. distinguendus* where unidirectional CI is only one of several barriers (K. König et al. 2019). In fact, this latter CI also seems to be caused by *Spiroplasma*, because the strain RAV of clade A, which carries the respective CI inducer, is infected with *Spiroplasma* as found in this study. *Spiroplasma* might therefore be a factor in the divergence between clades A and C as well as between strains of the clade A. Despite the *Spiroplasma* infections of other strains used for crossing experiments, no further endosymbiont-induced effects, specifically CI between untreated individuals were observed. As demonstrated before (K. König et al. 2019), *Wolbachia* did not influence their hosts' reproduction.

Other postzygotic barriers

Behavioral sterility in hybrid females and a reduction in fertility of hybrid males were found between CAN (clade B) and STU (clade A), which are separated by 7.2% in COI, as well as in the more divergent strain pairs of BIR (clade A) x SAT (clade C) and CAN (clade B) x PFO (clade C), respectively. Apparently, they are the next barriers to emerge after sexual isolation and CI. Nothing is known about behavioral sterility of hybrid females in *L. distinguendus*. In *Nasonia,* significant behavioral sterility was only observed between *N. vitripennis* and *N. longicornis* (Beukeboom et al. 2015), but not between closer related species (Raychoudhury et al. 2010). A reduced fertility in hybrid males between CAN and STU was examined more closely by investigating sperm. This revealed a unidirectional reduction in sperm numbers in seminal vesicles of F2 hybrid male offspring, but no differences in the amount of sperm transferred to the spermathecae of females between hybrid and non-hybrid males. Thus, it is unlikely that less sperm transferred to the females caused the reduction of female offspring of hybrid males. In *Nasonia*, hybrid physiological sterility has been linked to cytonuclear incompatibilities with dominance effects (Koevoets et

al. 2012; Beukeboom et al. 2015), with negative effects depending on the ploidy level, rather than sex (Beukeboom et al. 2015). However, unlike in *Lariophagus*, neither sperm motility nor the production of females were impaired in *Nasonia* (Clark et al. 2010), suggesting different kinds of sperm impairment in both genera.

The last barriers to appear were identified between the strain combinations with the highest COI differences, BIR x SAT, and CAN x PFO, respectively. They consisted of inviability, behavioral sterility, and physiological sterility of hybrid males, and unidirectional reduction in the fertility of hybrid females, with the latter occurring only in the strain pair CAN x PFO. Therefore, these last barriers mostly affected hybrid males but not hybrid females. This points to Haldane's rule (Haldane 1922), which states that postzygotic barriers, i.e. negative effects on hybrids, appear first in the heterogametic sex. So far, numerous taxa have been found to obey Haldane's rule (see recent reviews by (Schilthuizen et al. 2011; Delph and Demuth 2016)), making it an evolutionary pattern that seems to be almost universal, at least in the animal kingdom. However, these studies have mostly focused on diploid organisms with chromosomal sex determination, while haplodiploid organisms have been largely neglected. A recent review on Haldane's rule does not mention haplodiploids at all (Delph and Demuth 2016). Phillips and Edmands (2012) hypothesize that because postzygotic isolation evolves more slowly in taxa with small X-chromosomes as compared to taxa with large X-chromosomes (Turelli and Begun 1997), postzygotic isolation should evolve even slower in taxa without heteromorphic sex chromosomes like haplodiploids. In support of this idea, a meta-analysis done by Lima (2014) found that taxa without sex chromosomes evolve lower levels of postzygotic isolation at a similar level of genetic divergence than taxa with sex chromosomes. However, Lima did not include taxa with haplodiploid sex determination either (Lima 2014). In contrast to these ideas, Breeuwer and Werren (1995) and Koevoets and Beukeboom (2009) pointed to the fact that the basic mechanism of Haldane's rule, the stronger effect of DM incompatibilities on the heterogametic sex, also applies to males of haplodiploids, where the whole genome is hemizygous. This hypothesis is supported by the observation that intrinsic postzygotic barriers in *L. distinguendus* mostly affected hybrid males, but not hybrid females.

3.4.4 Conclusion

The cryptic diversity within the *L. distinguendus* species complex, a parasitoid which also occurs in human households, was investigated by inferring phylogenetic trees based on the COI gene and five nuclear genes as well as crossing experiments. Previous results that strains collected on drugstore beetles and strains from granary weevils belong to different, albeit undescribed, species according to the BSC (K. König et al. 2019) were confirmed. In addition, a third clade, which also was collected on drugstore beetle strains and which can be considered a separate species based on the reproductive isolation from the other two species, was identified. Remarkably, although many of the strains were collected from the same area and with the same hosts as baits, they were genetically distinct enough as to belong to different clades. This discovery highlights that cryptic biodiversity also exists in close proximity to humans but remains largely undetected.

The crossing experiments with a variety of strains along a gradient of relatedness indicate that reproductive isolation in *L. distinguendus* might have evolved from weak and unilateral sexual isolation, over behavioral

sterility in hybrid females and reduced fertility of hybrid males, to strong sexual isolation and strong intrinsic postzygotic isolation (inviability, behavioral sterility, physiological sterility) affecting hybrid males. This supports the hypothesis that Haldane's rule also applies to Hymenoptera. In addition, the finding of CI caused by the endosymbiotic bacterium *Spiroplasma* between two closely related strains raises the question if speciation in *L. distinguendus* might be initiated by bacterial infestation, similar to the related *Nasonia*. Finally, barcoding by itself was found not to be suitable for species delimitation in *L. distinguendus*. It results in the separation of strains, which are not reproductively isolated according to the BSC. Therefore, using data on reproductive isolation from crossing experiments with suitable candidate species for calibration to determine taxon-specific thresholds that can then be used for species delimitation would be advisable. Thereby, it should be possible to reconcile the BSC, which is employed by most scientists studying ecology and evolution, and the species concepts based on molecular and morphological data, which are used by most taxonomists for species delimitation and species description.

Chapter 4: General Discussion

In earlier studies, the pteromalid parasitoid wasp *L. distinguendus* was used as a model organism for speciation processes in Hymenoptera (K. König et al. 2015b, a, 2019; C. König et al. 2019; Gokhman et al. 2019), one of the most species rich orders within the animal kingdom. These studies revealed *L. distinguendus* as a complex of at least two species, which are separated by several barriers, including sexual and ecological isolation, different karyology and endosymbiont-induced CI. In this thesis, I studied the emergence of barriers during the process of separation in *L. distinguendus* and identified the bacterium responsible for CI.

4.1 CI is induced by *Spiroplasma*

CI in *L. distinguendus* was first detected in crosses between males of the RAV strain and females of the PFO strain. This CI was shown to be caused by a bacterial endosymbiont which was neither *Wolbachia* nor *Cardinium* (K. König et al. 2019). Initial experiments suggested this endosymbiont to be *Spiroplasma* (Krimmer 2015; Pollmann 2016). Therefore, this study analyzed the strain STU, a close relative to RAV, to conclusively identify a candidate bacterium for the CI, again revealing *Spiroplasma*. This strain of *Spiroplasma* was termed *sDistinguendus* (*sDis*) upon its discovery. Subsequently, *sDis* was confirmed as CI-inducer for the first time by excluding other candidates, confirming its localization in the ovaries of the infected strain, which strongly suggests maternal transmission, and by re-establishing *sDis* and CI in a cured strain by hemolymph microinjection. The ease of transferring *sDis* promises to be a valuable tool in the further investigation of *sDis* and the interactions with its wasp host as well as for applicative uses such as biocontrol.

4.1.1 First case of CI induction by *Spiroplasma*

In the absence of any other candidate, the CI detected in crosses of infected males and uninfected females within the *L. distinguendus* strain STU was found to be induced by *Spiroplasma*, subsequently termed *sDis.* As the discovery of *Spiroplasma* in nearly all tested STU individuals suggests a very high prevalence in the STU strain, it is likely that the CI detected in crosses between untreated STU males and BIR females is also due to *sDis*. The presence of *sDis* in the ovaries of STU females suggests that it is maternally transmitted within *L. distinguendus*. *Spiroplasma* (Mollicutes) are motile, wall-less bacteria visually characterized by a helical shape and small size (Davis et al. 1972; Davis and Worley 1973). The known *Spiroplasma* isolates cluster into four distinct clades (Gasparich et al. 2004). They have been shown to employ a wide variety of life strategies and infect organisms across many taxa, inhabiting different tissues both extra- and intracellularly, including the hemolymph. They maintain various relationships with their hosts (Gasparich 2002; Regassa and Gasparich 2006), facilitated by their high evolutionary rates and the resulting adaptive potential (Gerth et al. 2021). Some *Spiroplasma* defend their hosts against parasitism of pathogens (Xie et al. 2010, 2014; Jaenike et al. 2010b; Paredes et al. 2016), others maintain commensal relationships with their hosts (Ota et al. 1979; Clark 1982; McCoy et al. 1982; Clark et al. 1982), and a third group acts as pathogen (Davis et al. 1972; Saglio et al. 1973; Tully et al. 1976; Mouches et al. 1982; Clark

et al. 1985; Wang et al. 2004, 2005; Nunan et al. 2004; Aquilino et al. 2015). Importantly, several *Spiroplasma* strains from the poulsonii clade as well as from the Ixodetis clade induce MK in their *Drosophila*, lepidopteran, coleopteran, and hemipteran hosts (Poulson and Sakaguchi 1961; Williamson et al. 1999; Majerus et al. 1999; Jiggins et al. 2000; Montenegro et al. 2005; Tinsley and Majerus 2006; Tabata et al. 2011; Sanada-Morimura et al. 2013). *sDis* is a member of the Ixodetis clade (Pollmann et al. 2022). CI, however, had not been shown to be induced by *Spiroplasma* before.

Different reproductive manipulations caused by the same endosymbionts have been demonstrated when they were transferred to new hosts or host backgrounds (e.g. (Fujii et al. 2001; Sasaki et al. 2002, 2005; Jaenike 2007)). Similarly, suppression or imperfect expression of one phenotype has been shown to result in the appearance of another, which had previously been concealed. For instance, a shift from male-killing to CI was reported by Hornett et al. (2008) and Kraaijeveld et al. (2011). In this case, *Wolbachia* as the causative agent of both the “original” and the “hidden” phenotype must be equipped with the means to induce both phenotypes, suggesting the underlying mechanism to be linked. In fact, the cellular mechanism described to cause male-killing induced by *Spiroplasma poulsonii* in *Drosophila willistoni* does bear similarities to the cytological characteristics of both *Wolbachia*- and *Cardinium*-induced CI, including incorrect segregation of chromosomes to the poles, aneuploid and polyploid daughter nuclei and chromatin bridges (Counce and Poulson 1962; Lassy and Karr 1996; Callaini et al. 1997; Tram et al. 2006; Landmann et al. 2009; Gebiola et al. 2017). *sDis* inducing CI might be another example of an endosymbiont being able to cause different reproductive manipulations. Therefore, future studies will have to address the ability of *sDis* to induce MK in other organisms and a potential connection between its MK and CI phenotypes via the respective cellular and genetic basis.

4.1.2 CI mechanism

The cellular mechanism of CI induced by *sDis* has not been studied and is unknown so far. Interestingly, CI in *Wolbachia* and *Cardinium* seems to be caused by similar cytological processes despite different responsible genes, suggesting convergent evolution to affect the same targets in the hosts (Gebiola et al. 2017). As this phenotype in *Wolbachia* and *Cardinium* also shares characteristics described for *Spiroplasma*-induced male-killing (Counce and Poulson 1962), it is conceivable that the CI induced by *sDis* also relies on influencing these same host targets.

The genome of *sDis* does not contain *cif* genes or their homologues responsible for *Wolbachia*-induced CI (Pollmann et al. 2022), which were not found in CI-inducing *Cardinium* either (Lindsey et al. 2018). As in *Cardinium* (Penz et al. 2012), this suggests that CI in *Spiroplasma* has evolved independently. The male-killing factors *spaid* from *Spiroplasma poulsonii* in *D. melanogaster*, which is located on a plasmid and encodes for a protein with ankyrin repeats and an OTU deubiquitinase domain (Harumoto and Lemaitre 2018; Masson et al. 2018), and the unrelated *wmk* from *Wolbachia,* a transcriptional regulator (Perlmutter et al. 2019), were absent as well (Pollmann et al. 2022).

Although no definitive candidate gene underlying *sDis*-CI was identified, several potentially involved genes have been revealed (Pollmann et al. 2022). These include genes encoding eukaryotic high mobility (HMG)

box DNA-binding proteins which were also present in the genome of the MK-*Spiroplasma* from *Danaus chrysippus* (Martin et al. 2020) and in an Ixodetis-clade *Spiroplasma* within *Adalia bipunctata* and *A. decempunctata* without a MK phenotype (Archer et al. 2023). Interestingly, as part of the sperm chromatin, HMG box proteins have vital functions during spermatogenesis, as demonstrated in *Drosophila* (Rathke et al. 2010; Doyen et al. 2015). This might suggest a connection to the CI phenotype of *Wolbachia* and *Cardinium* seen after the transfer of sperm to the females.

In addition, the *sDis* genome contains OTU-like cysteine proteases and ankyrin repeats, the former of which were also identified in the non-MK ladybeetle *Spiroplasma* (Archer et al. 2023). Both are also encoded by *spaid* (Harumoto and Lemaitre 2018), suggesting a possible genetic connection between the CI and MK phenotypes of *Spiroplasma.*

4.1.3 CI type

There are two types of CI described for haplodiploids with regards to the fate of the affected offspring. If the intended diploid female offspring die as a result of CI, as has been observed for example in *Leptopilina heterotoma*, *Pachycrepoideus dubius*, *Trichopria drosophilae*, *N. giraulti* and *N. longicornis*, it is called female mortality (FM) (Vavre et al. 2000; Bordenstein et al. 2003). In contrast, in the male development type (MD), diploid offspring which are supposed to become females develop into haploid males, which occurs in crosses between infected and uninfected *Nasonia vitripennis* strains (Breeuwer and Werren 1993; Vavre et al. 2001). Several studies reported CI types being intermediate between FM and MD (Vavre et al. 2001; Mouton et al. 2005; Nguyen et al. 2017), including interspecies crosses between *N. vitripennis* males and *N. giraulti* females (Bordenstein et al. 2003). In this study, CI in crosses between infected males and uninfected females of the *L. distinguendus* strain STU was found to be of the male development type, since the amount of male offspring in the incompatible cross was significantly increased whereas the total offspring number remained the same.

4.1.4 Transfection and its potential

sDis could be transferred from infected STU females to tetracycline-treated STU females via the microinjection of hemolymph, re-introducing *sDis* infection into the cured wasp line and recovering CI in the male offspring of the injected females in crosses with uninfected females. This primarily served to confirm CI as novel phenotype of *sDis*, but the success of this experiment also suggests promising opportunities for application.

Artificially transferring bacteria to new hosts via injection is widely used as a tool in studying endosymbionts, their phenotypes, and their interactions with hosts (e.g. (Sasaki and Ishikawa 2000; Anbutsu and Fukatsu 2003; Xie et al. 2010), reviewed by (Hughes and Rasgon 2014)). There have been successful transfers of both *Spiroplasma* and *Wolbachia* to adult *Drosophila* using hemolymph injections (Frydman 2007; Xie et al. 2010). Nevertheless, embryonic microinjection with material obtained from the original hosts, such as ooplasm (Sasaki and Ishikawa 2000) or homogenized body parts (Tinsley and Majerus 2007), is the most commonly applied method to transfer bacteria, specifically *Wolbachia*, between hosts. This method is highly

laborious, time-consuming and prone to failure because of the predominantly intracellular occurrence of *Wolbachia* (reviewed by (Hughes and Rasgon 2014)).

Such transfers of bacteria into new hosts and the establishment of stable and CI-inducing infections are also required for applicative uses, as CI-inducing bacteria, specifically *Wolbachia*, have attracted interest as biocontrol agents against arthropod pests and disease vectors (Brelsfoard and Dobson 2009). Specifically, populations of such arthropods can be reduced by releasing males whose infections with bacteria render them incompatible to the local uninfected females, causing matings to result in few or no offspring and reducing the population size (Brelsfoard and Dobson 2009), which is referred to as incompatible insect technique (Boller et al. 1976). This strategy is showing promise for the control of several vector as well as pest species (Zabalou et al. 2004; Atyame et al. 2011; Beebe et al. 2021). Furthermore, *Wolbachia* transferred from *D. melanogaster* has been shown to suppress titers and transmission of dengue virus in *Aedes aegypti* and *A. albopictus* (Walker et al. 2011; Blagrove et al. 2012). Due to CI facilitating both *Wolbachia* and its dengue-suppressing trait spreading quickly through the uninfected target populations of *A. aegypti* to a stable high prevalence by inducing CI, the dengue prevalence in human habitats was reduced in field trials (Hoffmann et al. 2011, 2014). Hence, this method is a promising strategy to control not only dengue virus, but also other mosquito-borne pathogens, such as Zika virus, Chikungunya virus, and yellow fever virus, on which *Wolbachia* infection has been shown to have an inhibiting effect as well (van den Hurk et al. 2012; Aliota et al. 2016b, a; Ferreira et al. 2020). As these methods require *Wolbachia* to be newly established in their target organisms, they are restricted by the difficulties of isolating and transferring *Wolbachia* to the new hosts. The CI induced by *sDis* is the only CI reliably transferrable via adult-to-adult microinjection, which is an easy and fast method of transfer as well as a new tool to study endosymbiont-induced CI. Furthermore, it could make biocontrol efforts easier, should *sDis* and its CI also prove to be easily transferred to arthropods relevant for biocontrol.

4.1.5 CI strength

sDis-induced CI proved to be incomplete in crosses between STU males and uninfected females of STU or BIR as well as between untreated STU males and tetracycline-treated STU females, as some female offspring were present. Likewise, in experiments transfecting *sDis* from infected to tetracycline-treated females of the *L. distinguendus* strain STU, F1 males that had inherited *sDis* from their re-infected mothers expressed incomplete CI.

There are various explanations for differences in the presence as well as the strength of phenotypes induced by endosymbionts between individuals. For example, endosymbiont density has been shown to play a role either by endosymbiont titer having to surpass a specific threshold for the phenotype to be expressed or by the phenotype increasing in strength with increasing endosymbiont density (Breeuwer and Werren 1993; Boyle et al. 1993; Noda et al. 2001; Anbutsu and Fukatsu 2003; Jaenike 2009; Kraaijeveld et al. 2011; Bordenstein and Bordenstein 2011; Haselkorn et al. 2013). Alternatively, a recent study found the transcript level of *cifB* to be correlated to the level of *Wolbachia*-induced CI (Shropshire et al. 2022). Furthermore, host age, often linked to changes in endosymbiont titer, has also been shown to be linked to

the strength of the expressed phenotype, with the phenotype either weakening (Hoffmann et al. 1986; Noda et al. 2001; Reynolds and Hoffmann 2002) or increasing (Anbutsu and Fukatsu 2003; Kageyama et al. 2007) with host age. Endosymbiont presence and titer in the developing sperm are also presumed to be important for CI occurrence and levels (Bressac and Rousset 1993; Poinsot et al. 1998; Clark et al. 2003; Doremus et al. 2020), where strength of CI and the duration of specific developmental stages were shown to be positively correlated (Bordenstein and Bordenstein 2011; Perlman et al. 2014; Doremus et al. 2020; Shropshire et al. 2022), whereas another study found the opposite for the relationship between *Wolbachia*-induced CI and developmental time (Yamada et al. 2007). Regarding *L. distinguendus*, no effect of the overall *sDis* titer on CI level was found in crosses between male offspring of STU females injected with *sDis* and tetracycline-treated STU females. Neither male age nor development time of the males are likely to have played a role in the different CI levels as the crosses were conducted with males roughly the same age which had developed at the same constant temperatures and therefore for the same amount of time. While it is conceivable that the total amount of *sDis* in the developing sperm and the proportion of infected sperm as well as the transcript levels of the CI-inducing gene(s) varied between *L. distinguendus* males expressing different levels of *sDis*-induced CI, none of these parameters have been examined and a conclusion in this regard can therefore not be made. As all males originated from the same strain, a role of host factors suppressing CI in some males, but not in others, seems unlikely.

4.2 Diversity within the *L. distinguendus* species complex

In previous studies, *L. distinguendus* was revealed to consist of at least two separate species separated by a number of barriers and distinguished by their genetic divergence, but not morphology (Wendt et al. 2014). These species were named DB-species and GW-species, respectively (K. König et al. 2015a, 2019; C. König et al. 2019), after their favored hosts, the drugstore beetle *S. paniceum* and the granary weevil *S. granarius*. In the present study, all available *L. distinguendus* strains were subjected to genetic analyses using the barcode segment of COI and five nuclear genes to study the genetic diversity within *L. distinguendus*. Furthermore, five pairs of *L. distinguendus* strains representing different levels of genetic differentiation were selected to investigate isolation barriers both within the DB-species and between the DB- and GW-species by conducting crossing experiments. Herein, the sexual isolation between the parental pairs as well as the inviability, behavioral sterility, physiological sterility, and reduced fertility of female and male hybrids were evaluated.

At least one more species, which was previously considered to be part of the DB-species, but was shown to be almost completely reproductively isolated from both the GW-species and other DB-strains, was discovered. This increased the total number of species within the *L. distinguendus* species complex to a minimum of three.

4.2.1 Genetic differentiation within *L. distinguendus*

Previous genetic studies on the *L. distinguendus* species complex found two separate clades corresponding to the GW- and DB-species (König et al. 2015a; C. König et al. 2019), as did a maximum-likelihood

phylogenetic tree inferred from five nuclear genes for the newly investigated *L. distinguendus* strains. Two distinct clades were recovered, in accord with the host preferences of the respective strains and corresponding to *Stegobium* Clade I and *Sitophilus* Clade I, i.e. the two species proposed by previous studies (K. König et al. 2015a, 2019; C. König et al. 2019). However, the phylogenetic tree constructed based on COI, which is generally more divergent, especially in Hymenoptera (e.g. (Gebiola et al. 2012; Kaltenpoth et al. 2012)), showed the studied strains to cluster into three groups. One encompasses the GW-strains (clade C), matching the GW-species and *Sitophilus* Clade I (König et al. 2015a; C. König et al. 2019), respectively, and two contain the DB-strains. While one of the DB-clusters (clade A) is largely identical to the previously identified DB-species or *Stegobium* Clade I (König et al. 2015a; C. König et al. 2019), the other cluster (clade B) consisted predominantly of strains that had not been studied before and therefore had been overlooked.

4.2.2 Genetic data combined with RI

The data from the crossing experiments served to evaluate the genetic clusters with respect to the biological species concept (BSC) based on their reproductive isolation. As a rule, the number of barriers and the resulting reproductive isolation between two strains increased with their genetic distance. The strain pair BIR x SAT, representing clade A and clade C, was completely reproductively isolated. This reaffirms previous results (K. König et al. 2015a, 2019) that they are separate species. Likewise, reproductive isolation was complete between the strains CAN and PFO as representatives of clade B and clade C, respectively. As CAN and STU (clade A) were also separated by very high reproductive isolation, the division of the *L. distinguendus* strains into three distinct clusters as obtained by the phylogeny inferred from COI was validated by the criterion for species status according to the BSC (Mayr 1969; Coyne and Orr 2004). In contrast, the crosses of the combinations BIR x OST and BIR x STU which all belong to clade A, i.e. the DB-species, did not reveal reproductive isolation strong enough to warrant the consideration of them not belonging to the same species. These results imply that the cluster of strains currently designated as clade B is in fact a separate species. This increases the number of currently known species within the *L. distinguendus* species complex to three. Provided that the new species cannot be distinguished visually from the others due to a lack of morphological differences as shown for the previously known species (Wendt et al. 2014), *L. distinguendus*, originally assumed to be a single species, encompasses a surprising amount of cryptic diversity, as discovered for multiple taxa within the Hymenoptera (e.g. (Heraty et al. 2007; Hernández-López et al. 2012; Stahlhut et al. 2013; Kenyon et al. 2015; Darwell and Cook 2017)).

4.2.3 Continuum of divergence

The strain pairs of *L. distinguendus* featured in the crossing experiments demonstrated reproductive isolation ranging from non-existent to complete. They thus form a continuum of divergence from two closely related strains belonging to the same species readily interbreeding with each other with no negative results on fitness to strains representing separate species with strong or complete isolation preventing gene flow.

This proves that the *L. distinguendus* species complex is highly suitable for the study of divergence and speciation processes.

4.2.4 Evaluation of COI for species delimitation

These results also suggest that in *L. distinguendus*, the thresholds of COI divergence often used for species delimitation in approaches based only or predominantly on barcoding, i.e. a divergence in COI of 2% (e.g. (Smith et al. 2013; Kang et al. 2017; Fagan-Jeffries et al. 2018; Meierotto et al. 2019; Sharkey et al. 2021)) or 2.2%, as used for the BIN system of the BOLD database (Ratnasingham and Hebert 2013), do not conform to the species boundaries set by the BSC. Specifically, the strains BIR and STU are merely weakly reproductively isolated based on the BSC but show a COI divergence of 2.8%, and therefore would be considered true species based on the 2% and 2.2% thresholds. From the studied combination of strains, the genetically least divergent pair of strains confirmed as separate species based on their RI is CAN x STU, which showed a COI difference of 7.2%. The broadly assumed thresholds for species delimitation therefore are not applicable in *L. distinguendus*. Interestingly, other authors have equally reported a failure of barcoding to reliably identify separate species due to the absence of the barcoding gap (Wiemers and Fiedler 2007) and a re-examination of species delimited based on BINs alone (Sharkey et al. 2021) using more thorough analyses demonstrated these species delimitations to be incorrect (Meier et al. 2022). Obviously, the use of only COI barcoding data for species description is not sufficient. Therefore, the lack of consideration of other data in approaches relying almost exclusively on differences in COI barcodes to define species and the use of pre-set thresholds for species delimitation, as practiced for example by (Meierotto et al. 2019; Sharkey et al. 2021) elicited heavy criticism by different authors (Zamani et al. 2021, 2022; Ahrens et al. 2021; Meier et al. 2022). In line with these authors, I suggest to further validate and question the use of these thresholds and the reliance on COI barcoding as sole criterion for species delimitation and consider the inclusion of appropriate additional criteria, such as reproductive isolation, to verify the results obtained with COI wherever possible.

4.2.5 Order of the reproductive barriers

Isolation barriers present within the *L. distinguendus* species complex were identified as prezygotic, intrinsic postzygotic, and endosymbiont-induced CI. If the speciation process in *L. distinguendus* can be assumed to consistently follow the same pattern, then conclusions can be drawn about the order in which the isolation barriers emerge during speciation.

Sexual isolation

The first barrier to arise in the speciation process of *L. distinguendus*, recognizable due to it being present in all studied crosses, was sexual isolation. Whereas it was very weak and unidirectional in the crosses between closely related strains, it both gained in strength and became bidirectional in the crosses between more genetically distant strains. This result matches a previous study which focused on different crossing combinations within *L. distinguendus* and concluded that the initial barrier in the speciation process in *L. distinguendus* was either prezygotic isolation, i.e. sexual or ecological isolation, or CI (K. König et al.

2019). There, it was argued that because of the absence of sexual isolation in the GW-species it was impossible to determine which of these barriers emerged first (K. König et al. 2019). The present work still cannot shed light onto the start of speciation within the GW-strains of *L. distinguendus* or between the GW- and DB-species. However, the discovery of sexual isolation, albeit slight and unidirectional, between closely related strains of the DB-species, BIR and OST, in this thesis, and RAV and STU in the study by K. König et al. (2019), suggest that the very first barrier driving separation between these strains is sexual isolation. In addition, there is very strong sexual isolation between the two almost completely isolated strains STU and CAN, which also share the same host preference and are therefore presumably not ecologically separated. This suggests that sexual isolation can be the very first barrier driving separation, which agrees with a recent study in another parasitoid wasp, *N. vitripennis*, which also found sexual isolation to act as first barrier in the divergence (Malec et al. 2021), as well as with investigations into other hymenopterans, including the genera *Cotesia* and *Encarsia*, showing sexual isolation to be the first or one of the first barriers between species (Bredlau and Kester 2015; Gebiola et al. 2016a).

In all strain pairs studied in this thesis, sexual isolation was asymmetric. The occurrence of copulation was reduced in one, but not the other direction, in the three more closely related pairs of strains, i.e. BIR x OST, BIR x STU, and CAN x STU. In the further diverged pairs of strains, sexual isolation was complete between females of the DB-strains BIR and CAN and males of the GW-strains SAT and PFO, whereas some copulations were present in the reverse crosses. This asymmetry was also observed in previous studies of other strain combinations of *L. distinguendus* (K. König et al. 2019) and in crosses of other study systems, such as *Encarsia* (Gebiola et al. 2016a).

Cytoplasmic incompatibility

Besides sexual isolation, unidirectional CI was found in the combination BIR x STU, likely induced by *sDis* in STU. It is stronger than sexual isolation and acts in the opposite direction; however, the order in which the two barriers developed is unclear. Similar situations have been demonstrated between two species of *Encarsia* wasps as well between two *Drosophila* species, with CI acting as one of several barriers, including sexual isolation (Shoemaker et al. 1999; Gebiola et al. 2016a).

Behavioral sterility of hybrid females and reduced fertility of hybrid males

Reduced fertility of hybrid males seems to be the next barrier in the speciation process, as it appeared in three of the investigated strains pairs, namely STU x CAN, BIR x SAT, and CAN x PFO. To investigate if the reduced fertility observed in the hybrid males of the strain combination CAN x STU is caused by a reduced amount of sperm, its amount was determined in the seminal vesicles in hybrid and wildtype F2 males and the spermathecae of their female mating partners. Interestingly, the amount of sperm was significantly reduced in male hybrids of the crossing combination STU f x CAN m. Similarly, reduced counts of mature sperm have been found in the *N. vitripennis*-*N. giraulti* species pair (Clark et al. 2010). However, in *L. distinguendus*, the amount of sperm transferred to the spermathecae of the females did not differ

compared to wildtype males. Thus, the reduced fertility of the hybrid males is not caused by a lack of sperm, but must have other causes.

The presence of behavioral sterility of hybrid females in the cross STU x CAN suggests that this barrier might arise at the same stage as reduced fertility of hybrid males as first effect on female hybrids. This is similar to the behavioral sterility which precedes physiological sterility of female hybrids between *N. vitripennis* and *N. longicornis* (Beukeboom et al. 2015). Unfortunately, no data exist on behavioral sterility in the two strain pairs with the highest divergence, BIR x SAT and CAN x PFO. Therefore, it is currently unclear how this barrier might have evolved in the entire species complex of *L. distinguendus*.

Behavioral sterility, inviability, and physiological sterility of hybrid males

These barriers are followed by behavioral sterility, inviability, and physiological sterility of hybrid males, all of which occurred in the strain pairs BIR x SAT and CAN x PFO. Behavioral sterility in hybrid males has been attributed to either males being unable to perform courtship properly or because they are not accepted by the females (Noor 1997; Clark et al. 2010). In the strain pair BIR x SAT, only the matings between BIR females and the hybrid males descending from the parental cross SAT females x BIR males were reduced compared to the control cross. As these hybrid males were accepted by the SAT females as well as non-hybrid SAT males, the reduced proportion of matings in this combination does not seem to be caused by an inability of these males to court, as found in other study systems (Breeuwer and Werren 1995; Noor 1997; Clark et al. 2010; Koevoets et al. 2012), but rather because they are not attractive to the BIR females.

Reduced fertility, inviability and physiological sterility of hybrid females

While the last barrier to appear was a reduced fertility of hybrid females in the strain pair CAN x PFO, inviability and physiological sterility of hybrid females were not found as barriers in the studied strains. Therefore, they either emerge at an even later stage of separation or they do not appear at all between separate species of *L. distinguendus*. As female inviability was among the first barriers present in other divergence processes in Hymenoptera such as in the genera *Encarsia* and *Cotesia* (Bredlau and Kester 2015; Gebiola et al. 2016a), the order of barriers reported here for *L. distinguendus* clearly does not apply for Hymenoptera in general.

4.2.6 Haldane's rule

Most of the postzygotic barriers, i.e. reduced fertility, behavioral sterility, inviability, and physiological sterility, were negatively affecting the fitness of hybrid males, whereas only two barriers, sexual isolation and reduced fertility of F1 females, had an adverse impact on hybrid females. This pattern suggests that *L. distinguendus* adheres to Haldane's rule, which states that if one sex is negatively affected by a reduction in fitness following hybridization, it will be the heterozygous sex (Haldane 1922). Haplodiploid organisms have been theorized by Koevoets and Beukeboom (2009) to comply with this rule, with haploid males as heterozygous sex. Later this idea has been experimentally confirmed in *Nasonia* by the same authors (Koevoets et al. 2012; Beukeboom et al. 2015). In fact, Haldane's rule is expected to come into effect faster in haplodiploids than in diploids, as any incompatibilities would immediately be expressed in the haploid

hybrids, with the resulting reproductive isolation being comparatively stronger as well (Koevoets and Beukeboom 2009; Koevoets et al. 2012). It has been noted that the fitness loss in haploid hybrids predominantly results from the ploidy level, with very little effect of sex (Beukeboom et al. 2015).

4.2.7 Underlying causes for these barriers

Hybrid incompatibilities result from Dobzhansky-Muller incompatibilities (Dobzhansky 1936; Muller 1942), which are defined as incompatibilities resulting from incompatible alleles in diverging populations or species evolving independently. This is assumed to lead to defects in hybrids which express these incompatible alleles (Dobzhansky 1936; Muller 1942; Orr 1995). For example, in crosses within the *Nasonia* genus, hybrid male inviability and physiological sterility have been attributed to incompatibilities between cytoplasmic and nuclear genes and, to a lesser extent, nuclear-nuclear incompatibilities adhering to this model (Breeuwer and Werren 1995; Gadau et al. 1999; Niehuis et al. 2008; Koevoets and Beukeboom 2009; Beukeboom et al. 2015).

Hybrid lethality in crosses between *N. vitripennis* and *N. giraulti* has been hypothesized to be connected to differences in the microbiota of the two species potentially causing incompatibilities between the microbiota and the wasp genomes (Brucker and Bordenstein 2013). The differences in the gut bacteria of *L. distinguendus* have not been analyzed, but especially in crosses between wasps raised on different hosts, i.e. DB- and GW-strains, their existence is likely. Incompatibilities between microbiota and genomes might therefore also be involved in hybrid fitness losses in *L. distinguendus*.

4.3 The role of endosymbionts in the speciation process of *L. distinguendus*

Endosymbiont distribution among *L. distinguendus* strains

Within the *L. distinguendus* species complex, infections with both *Wolbachia* and *Spiroplasma* have been detected, with infection status varying between the major groups. Most strains belonging to the DB-clades are carrying *Spiroplasma*, except for three which are uninfected. In contrast, the strains from the GW-clade are all infected with *Wolbachia* and the strains PFO and SWD also harbor *Spiroplasma*. This pattern of infections could suggest a role of endosymbionts in the separation of these clades. While no effect on the hosts has been found for *Wolbachia* (K. König et al. 2019), the *Spiroplasma* isolated from STU, termed *sDis*, has been shown to induce CI in this strain. It is also likely to be the cause of CI between STU males and the naturally uninfected females from BIR, which are genetically distinct based on COI. In addition, unidirectional CI induced by a then-unknown endosymbiont has been shown before to act between RAV males and PFO females (K. König et al. 2019). Because the present work demonstrates RAV to be infected with *Spiroplasma*, because RAV does not carry any other known CI inducers like *Wolbachia* and *Cardinium* (K. König et al. 2019), and because of RAV is closely related to STU, it is likely that the *Spiroplasma* present in RAV is inducing unidirectional CI with PFO, like *sDis* in STU.

Apart from *Spiroplasma* in STU and RAV, it is unknown whether the *Spiroplasma* infection in the other strains of the DB-clades are causing CI as well or if they have any effect on their hosts' reproduction at all. If CI is a phenotype shared among the *Spiroplasma* occurring in all of the DB-strains, then it could have

been one factor involved in the separation of DB- and GW-species. Although unidirectional CI is considered unlikely to be the sole driver of these speciation processes (Hurst and Schilthuizen 1998; Wade 2001), it has been shown to act in concert with other isolating barriers in the divergence of *Encarsia* and *Drosophila* species pairs (Shoemaker et al. 1999; Gebiola et al. 2016a).

4.4 Variations in infection status and endosymbiont-induced phenotype between strains

Curiously, CI in crosses between untreated PFO females and infected RAV males was perfect whereas it was absent in crosses between tetracycline-treated females and untreated PFO males (K. König et al. 2019) despite the presence of *Spiroplasma* in some of the tested PFO individuals. Therefore, the *Spiroplasma* in PFO might be unable to induce CI or rescue CI induced by the *Spiroplasma* in RAV. Furthermore, no CI occurred in crosses between CAN and PFO, suggesting that either the *Spiroplasma* in CAN was unable to induce CI or the *Spiroplasma* in both strains were compatible, enabling the *Spiroplasma* in PFO to rescue the CI. Such variability in the ability of phenotype induction by strains of the same endosymbiont in the same or closely related host species has for example also been demonstrated in *D. simulans* and *T. urticae*, from which both CI-inducing and non-CI-inducing strains of *Wolbachia* have been isolated (Hoffmann et al. 1986; Vala et al. 2002).

Alternatively, endosymbiont-induced phenotypes can be lost or reduced for example due to suppressors in the host organisms and the loss and mutation of the underlying genes (e.g. (Hornett et al. 2006; Jaenike 2007; Koehncke et al. 2009; Majerus and Majerus 2010; Funkhouser-Jones et al. 2018; Martinez et al. 2021; Wybouw et al. 2022)).

While the endosymbionts predominantly occur as single infections, some *L. distinguendus* strains, namely PFO and SWD, are infected with both *Spiroplasma* and *Wolbachia*. Few studies have so far investigated the interactions between the two endosymbionts and their results differ from each other. In *D. melanogaster* artificially infected with *Spiroplasma* and *Wolbachia*, *Wolbachia* density is negatively affected by *Spiroplasma* during pupa and young adult stages, with no discernible reciprocal effect (Goto et al. 2006). Conversely, in *Tetranychus truncatus*, *Spiroplasma* seems to be suppressed by the presence of *Wolbachia* (Yang et al. 2020). No negative effect on each other was detected in *D. neotestacea*, but them being commonly transmitted together and being positively associated instead led the authors to speculate about a future mutualism (Jaenike et al. 2010a). In line with the results from the former two studies, infections with *Spiroplasma* and *Wolbachia* being almost perfectly mutually exclusive in *L. distinguendus* suggests that they might not be able to coexist in this system for longer periods of time. However, from the current results, no assumptions can be made as to the real nature of this relationship.

Some strains contained in the DB-species are not infected with *Spiroplasma* despite being closely related to *Spiroplasma*-carrying strains and occurring at nearby locations at time of capture. The absence of *Spiroplasma* in some wasp strains could be explained by each *L. distinguendus* strain barring the few uninfected ones undergoing a separate infection event or by horizontal transmission from one or few initially infected strains to many, but not all, uninfected strains. A more likely explanation for these uninfected strains

is that a common ancestor of all DB-strains, or possibly of all *L. distinguendus* strains, seeing that some GW-strains are also infected, was infected with *Spiroplasma*. The strains then diversified, potentially owing to *Spiroplasma* at least in part, and *Spiroplasma* was lost in some of the strains. On an evolutionary time scale, infections with endosymbionts are expected to be lost eventually following events such as the evolution of resistance in the hosts or the loss of their phenotype due to losses of or mutations in the genes responsible (Koehncke et al. 2009; Bailly-Bechet et al. 2017; Martinez et al. 2021). Furthermore, environmental factors are known to eliminate endosymbiont infections. Increases or decreases in temperature relative to the respective normal temperatures have been demonstrated to negatively affect or even eliminate endosymbiont infections as well as the induced phenotypes in numerous study systems (for example (Pijls et al. 1996; Hurst et al. 2000; Arakaki et al. 2001; Weeks et al. 2001; Montenegro and Klaczko 2004; Anbutsu et al. 2008; Osaka et al. 2008). As the temperatures shown to eliminate *Spiroplasma* infections in *Drosophila* species, namely 15 °C to 18 °C as the lower and 28 °C as the upper deviation from measured optimal temperatures (Montenegro and Klaczko 2004; Anbutsu et al. 2008; Osaka et al. 2008) occur in their natural habitats, single *L. distinguendus* strains might have lost their *Spiroplasma* infections due to exposure to such temperatures while the majority of strains retained their infections. Furthermore, naturally occurring antibiotics have been shown to eliminate endosymbiont infections (Stevens and Wicklow 1992), although it is unknown if *L. distinguendus* could be cured upon encountering such antibiotics since they do not feed on substrate potentially containing antibiotics, but on coleopteran larvae.

4.5 Conclusion

In conclusion, CI found in crosses between tetracycline-treated and untreated individuals of the *L. distinguendus* strain STU was found to be caused by *Spiroplasma*, termed *sDis*. Importantly, this study is the first to show *Spiroplasma* causing CI, by excluding all other candidates present in STU and demonstrating the potential of maternal transmission with the presence of *sDis* in the ovaries of STU. Using microinjection of the hemolymph from infected females, both *sDis* and its CI phenotype could be transferred to uninfected females, which not only strengthens the line of evidence connecting *sDis* to the observed CI, but also provides a fast and easy method potentially useful for future applicative purposes as shown for CI induced by *Wolbachia.* No link between the strength of CI and *sDis* titer was found.

Furthermore, *L. distinguendus* is in the process of ongoing speciation. The present work showcases several strain pairs along a gradient of genetic distances which show reproductive isolation ranging from non-existent to complete, representing a continuum of divergence. Reproductive isolation between a DB-strain belonging to a newly identified COI cluster and representatives of the two established species demonstrates a third distinct species to exist within the *L. distinguendus* species complex, an example for surprising cryptic diversity. COI, specifically the threshold distances of 2% and 2.2% used in many studies to delimitate species, was found to not be applicable for species delimitation in *L. distinguendus* and a critical evaluation of the use of such set thresholds in general and for each taxon that is being investigated for diversity seems advisable.

CI induced by *Spiroplasma* was shown to be involved in the divergence of several strain pairs. While the presence of CI between two strains within the same DB-species shows divergence within this species to be linked to CI, differences in the occurrence of *Spiroplasma* coinciding with the boundary between two of the *L. distinguendus* species suggest CI to have played a role within the entire speciation process of the currently recognized *L. distinguendus* species.

Summary

Hymenoptera are one of the most speciose animal taxa, containing superfamilies like the Chalcidoidea which themselves are extremely species-rich, presumably caused by a high rate of speciation. The investigation of evolutionary processes is predominantly based on the biological species concept (BSC), which defines species as groups of interbreeding individuals which are reproductively isolated from individuals of other groups. As an alternative approach, species are delimitated by predetermined threshold distances in the so-called barcode segment of the mitochondrial COI gene. In Hymenoptera and Chalcidoidea, the diversity has been studied extensively whereas speciation processes and reproductive barriers remain highly understudied. The delimitation of species according to the BSC requires the investigation of reproductive barriers and the resulting impairment of gene flow between target groups, i.e. populations or species. These barriers act before mating (premating), such as sexual isolation, after mating but before fertilization (postmating, prezygotic) or after fertilization (postzygotic), like hybrid sterility and inviability.

Many arthropods are infected with endosymbiotic bacteria, some of which have been shown to manipulate their hosts' reproduction via several mechanisms including cytoplasmic incompatibility (CI). It occurs between males carrying the endosymbiont and uninfected females and results in the reduction or absence of diploid offspring. So far, it is known for *Wolbachia*, *Candidatus* Cardinium hertigii (*Cardinium*), *Candidatus* Mesenet longicola, and *Rickettsiella*. Due to their ability to interfere with the reproduction of their hosts, endosymbionts have been suggested to be potential drivers of their hosts' speciation processes.

Lariophagus distinguendus is a parasitoid wasp which uses larvae of multiple coleopteran species as hosts. As its host species are pests in different environments, *L. distinguendus* serves as agent in biocontrol against them. In past studies, two distinct species of *L. distinguendus*, called the DB- and the GW-species according to their preferred host species the drugstore beetle and the granary weevil, respectively, have been discovered. They are separated by genetic divergence, different host preferences, different chromosome numbers, sexual isolation, and postzygotic barriers. As additional barrier, unidirectional CI caused by an unknown bacterial agent has been shown to act between GW-females and DB-males.

Here, the nature of the CI inducer in crosses between untreated males and tetracycline-treated females of the *L. distinguendus* strain STU, a member of the DB-species, was investigated. Additionally, diversity and speciation processes were investigated within the *L. distinguendus* species complex by constructing phylogenetic trees with COI and nuclear genes as well as by conducting crossing experiments with different strain combinations.

The bacterium *Spiroplasma*, termed *sDistinguendus* (*sDis*) for its *L. distinguendus* host, was identified to be the causative agent of this CI. Despite being known for multiple types of interactions with various hosts, *Spiroplasma* had not been demonstrated to cause CI before. None of the other bacteria identified within STU were associated with CI and none of the known CI inducers and other bacteria manipulating reproduction were found. The potential for maternal transmission was shown by the presence of *sDis* in the ovaries of STU females. Transferring *sDis* and CI from infected to uninfected STU females via hemolymph

microinjection solidified the connection between CI and *sDis*. The simplicity of this transfection technique could prove to be valuable for applicable purposes in future. CI strength varying between male offspring of the injected females was not linked to the titer of *sDis*.

All available *L. distinguendus* strains were analyzed with the barcode segment of COI as well as five nuclear genes to infer their phylogenetic relationships. Furthermore, five pairs of strains covering a gradient of genetic divergence were selected for crossing experiments investigating their reproductive isolation. The nuclear genes recovered two clusters identical to the previously known DB- and GW-species, whereas three distinct clusters were found based on barcoding. These three clusters were revealed as separate species due to very high to complete reproductive isolation in crossing experiments between strains representing each of them. Threshold values of COI distances commonly used for species delimitation relying predominantly on barcodes, i.e. 2% to 2.2%, were shown to be not applicable for *L. distinguendus* due to the divergence between strains of the same species exceeding these thresholds. This calls the faith in these pre-set thresholds and the barcode-only approach to species delimitation into question and suggests the consideration of additional data along with those obtained by barcoding for species delimitation. Reproductive isolation between the strains was found to span a continuum of divergence from no reproductive isolation and low genetic divergence to complete isolation and high genetic divergence. The first barrier, present in all strain pairs, was sexual isolation, ranging from weak and unidirectional to complete and near complete in both directions. In the untreated cross between BIR females and STU males, *sDis*-induced CI was present along with sexual isolation. The reduced fertility of hybrid males was the next barrier to appear in the three more distantly related strain pairs along with behavioral sterility of hybrid females in the one strain pair among them where it was measured. These barriers were followed by the behavioral sterility, inviability, and physiological sterility of hybrid males and as last barrier by the reduced fertility of hybrid females. Due to the majority of barriers affecting hybrid males, but not females, speciation in *L. distinguendus* follows Haldane's rule, stating that a fitness loss present in the hybrids of only one sex will be affecting the heterogametic sex, i.e. the males in *L. distinguendus*.

In conclusion, the CI between infected males and uninfected females of *L. distinguendus* was found to be caused by *Spiroplasma*, termed *sDis*, marking the first time for CI to be demonstrated as phenotype of *Spiroplasma*. It was also one reproductive barrier present in crossings of different *L. distinguendus* strains, apparently contributing to their divergence. The diversity within the *L. distinguendus* species complex was shown to be greater than detected before, as seen by the discovery of a third species. The studied pairs of strains covered a wide range of both genetic divergence and strength of reproductive isolation, suggesting *L. distinguendus* to be currently in the process of ongoing speciation. The commonly used threshold distances in COI were not applicable for species delimitation in *L. distinguendus*, challenging their unconditional use.

Zusammenfassung

Hymenoptera sind eines der artenreichsten Taxa im Tierreich und enthalten Superfamilien wie die Chalcidoidea, die selbst ebenfalls sehr artenreich sind, was vermutlich durch eine hohe Artbildungsrate verursacht wird. Die Untersuchung evolutionärer Prozesse beruht hauptsächlich auf dem biologischen Artkonzept, welches Arten als Gruppen von Individuen definiert, die sich untereinander paaren, aber von den Individuen aus anderen Gruppen reproduktiv isoliert sind. Bei einer alternativen Methode werden Arten durch festgelegte Schwellenwerte in der Divergenz des sogenannten Barcodeabschnittes des mitochondrialen COI-Gens abgegrenzt. Die Diversität der Hymenoptera und Chalcidoidea wurde umfangreich analysiert, wohingegen Artbildungsprozesse und reproduktive Barrieren bisher unzureichend untersucht wurden. Für die Artabgrenzung entsprechend des biologischen Artkonzepts wird die Untersuchung von reproduktiven Barrieren und der resultierenden Einschränkung des Genflusses zwischen Zielgruppen, also Populationen oder Arten, benötigt. Diese Barrieren wirken vor der Paarung („premating“), wie z.B. sexuelle Isolation, nach der Paarung, aber vor der Befruchtung („postmating“, präzygotisch) oder nach der Befruchtung (postzygotisch), wie z.B. Sterilität und eine reduzierte Lebensfähigkeit der Hybride.

Viele Arthropoden sind mit endosymbiontischen Bakterien infiziert, von denen einige die Fortpflanzung ihrer Wirte durch verschiedene Mechanismen wie zytoplasmatische Inkompatibilität („cytoplasmic incompatibility“, CI) manipulieren können. Diese tritt zwischen Männchen, die den Endosymbionten tragen und nichtinfizierten Weibchen auf und führt zu einer Reduzierung oder dem Ausbleiben von diploiden Nachkommen. Bis jetzt wurde sie bei *Wolbachia*, *Candidatus* Cardinium hertigii (*Cardinium*), *Candidatus* Mesenet longicola und *Rickettsiella* gezeigt. Aufgrund ihrer Fähigkeit, in die Fortpflanzung ihrer Wirte eingreifen zu können, wurde vorgeschlagen, dass Endosymbionten potentiell die Artbildungsprozesse ihrer Wirte antreiben könnten.

Lariophagus distinguendus ist eine parasitoide Wespe, die die Larven verschiedener Käferarten als Wirte nutzt. Da ihre Wirte Schädlinge in verschiedenen Umgebungen sind, wird *L. distinguendus* in der biologischen Schädlingsbekämpfung gegen sie eingesetzt. In früheren Untersuchungen wurden innerhalb von *L. distinguendus* zwei getrennte Arten entdeckt, die als DB- bzw. GW-Art nach ihren bevorzugten Wirten, dem Brotkäfer („drugstore beetle“) und dem Kornkäfer („granary weevil“) benannt wurden. Sie sind durch eine genetische Divergenz, verschiedene Wirtspräferenzen, unterschiedliche Chromosomenzahlen, sexuelle Isolation und postzygotische Barrieren voneinander getrennt. Einseitige CI, die durch ein unbekanntes Bakterium ausgelöst wurde, wurde als zusätzliche Barriere zwischen GW-Weibchen und DB-Männchen gefunden.

In dieser Arbeit wurde der Auslöser der CI in Kreuzungen zwischen unbehandelten Männchen und Tetrazyklin-behandelten Weibchen des STU-Stammes von *L. distinguendus*, der zu der DB-Art gehört, untersucht. Außerdem wurden die Diversität und die Artbildung innerhalb des Artkomplexes von *L. distinguendus* weiter untersucht, indem Stammbäume mit COI und mit nuklearen Genen erstellt wurden und mit verschiedenen Kombinationen aus Stämmen Kreuzungsversuche durchgeführt wurden.

Das Bakterium *Spiroplasma*, das nach seinem Wirt *L. distinguendus sDistinguendus* (*sDis*) genannt wurde, wurde als Verursacher der CI identifiziert. Obwohl es für verschiedene Arten der Interaktion mit unterschiedlichen Wirten bekannt war, war bisher noch nicht gezeigt worden, dass *Spiroplasma* CI verursacht. Keines der anderen Bakterien, die in STU vorhanden waren, war mit CI assoziiert und keines der bekannten CI-auslösenden Bakterien und anderen Bakterien, die für reproduktive Manipulationen bekannt sind, wurde in STU gefunden. Das Vorkommen von *sDis* in den Ovarien von STU-Weibchen zeigte das Potential für maternale Transmission. Durch den Transfer von *sDis* und CI von infizierten zu nichtinfizierten STU-Weibchen mittels Hämolymph-Mikroinjektion wurde die Verbindung zwischen CI und *sDis* bestätigt. Die Unkompliziertheit dieser Transfektionsmethode könnte zukünftig für angewandte Zwecke wertvoll sein. Unterschiede in der Stärke der CI zwischen männlichen Nachkommen der injizierten Weibchen hingen nicht mit dem Titer von *sDis* zusammen.

Alle verfügbaren *L. distinguendus* Stämme wurden mit dem Barcodesegment von COI und fünf nuklearen Genen analysiert, um ihre phylogenetischen Beziehungen zu ermitteln. Außerdem wurden fünf Paare von Stämmen, die einen Gradienten genetischer Divergenz abdecken, ausgewählt, um mit Kreuzungsversuchen ihre reproduktive Isolation zu untersuchen. Mit den nuklearen Genen wurden zwei Cluster erstellt, die mit den bereits bekannten DB- und GW-Arten übereinstimmten, während durch das Barcoding drei Cluster gefunden wurden. Diese drei Cluster stellten sich als separate Arten heraus, da Kreuzungsversuche sehr hohe bis vollständige reproduktive Isolation zwischen den Stämmen, die sie repräsentierten, zeigten. Die Schwellenwerte der Unterschiede in COI, die bei Artabgrenzungen, die hauptsächlich auf Barcodes beruhen, häufig verwendet werden, nämlich 2% bis 2.2%, waren bei *L. distinguendus* nicht geeignet, da der Unterschied zwischen zwei Stämmen derselben Art diese Werte überstieg. Dieses Ergebnis stellt das Vertrauen in diese festgelegten Schwellenwerte und die alleine auf dem Barcoding basierende Vorgehensweise für Artabgrenzungen in Frage und legt die Berücksichtigung zusätzlicher Daten zu denen des Barcodings für die Artabgrenzung nahe. Die reproduktive Isolation zwischen den Stämmen umfasste ein Kontinuum an Divergenz von keiner reproduktiven Isolation und geringen genetischen Unterschieden bis hin zu vollständiger Isolation und hohen genetischen Unterschieden. Die erste Barriere, die in allen Paaren von Stämmen vorhanden war, war sexuelle Isolation, die von schwach und einseitig zu vollständig bzw. fast vollständig in beiden Richtungen reichte. In den Kreuzungen zwischen unbehandelten BIR-Weibchen und STU-Männchen trat neben sexueller Isolation auch durch *sDis* ausgelöste CI auf. Die reduzierte Fertilität hybrider Männchen, die in den drei Paaren von Stämmen mit der höchsten genetischen Distanz auftrat, bildete die nächste Barriere zusammen mit einer Verhaltenssterilität in dem einen dieser drei Paare, in dem sie untersucht wurde. Es folgten Verhaltenssterilität, reduzierte Lebensfähigkeit und physiologische Sterilität in hybriden Männchen. Die zuletzt auftretende Barriere war die reduzierte Fertilität der hybriden Weibchen. Da die meisten Barrieren die hybriden Männchen, aber nicht die Weibchen betrafen, folgt die Artbildung in *L. distinguendus* „Haldane's rule", welche besagt, dass, wenn ein Fitnessverlust in den Hybriden von nur einem Geschlecht auftritt, dieses das heterogametische Geschlecht ist, was in *L. distinguendus* die Männchen sind.

Schlussfolgernd kann festgehalten werden, dass die CI zwischen infizierten Männchen und nichtinfizierten Weibchen von *L. distinguendus* durch *Spiroplasma*, genannt *sDis*, ausgelöst wird. Dies ist das erste Mal, dass CI als Phänotyp von *Spiroplasma* entdeckt wurde. CI war außerdem eine Barriere in Kreuzungen zwischen verschiedenen Stämmen von *L. distinguendus*, womit sie zu deren Auseinanderentwicklung beiträgt. Es wurde gezeigt, dass die Diversität im Artkomplex von *L. distinguendus* größer ist als zuvor bekannt war, da eine dritte zugehörige Art entdeckt wurde. Die untersuchten Paare von Stämmen deckten ein breites Spektrum von genetischer Divergenz sowie Stärke von reproduktiver Isolation ab, was darauf hindeutet, dass *L. distinguendus* sich aktuell in einem Prozess steter Artbildung befindet. Die häufig genutzten Schwellenwerte der COI Unterschiede waren für die Artabgrenzung von *L. distinguendus* nicht geeignet, was deren uneingeschränkte Benutzung hinterfragen lässt.

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Eidesstattliche Versicherung über die eigenständig erbrachte Leistung

gemäß § 18 Absatz 3 Satz 5 der Promotionsordnung der Universität Hohenheim für die Fakultäten Agrar-, Natur- sowie Wirtschafts- und Sozialwissenschaften

1. Bei der eingereichten Dissertation zum Thema
Speciation and isolating barriers in a parasitoid wasp focusing on the role of reproductive isolation caused by endosymbionts
handelt es sich um meine eigenständig erbrachte Leistung.

2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.

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Hohenheim, den 19.06.2023 — M. Pollmann

Ort, Datum — Unterschrift

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Curriculum Vitae

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2013-2016	Masterstudiengang Biologie, Universität Hohenheim (Abschluss mit der Note 1.2)
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Jan-Feb 2015	Forschungspraktikum am Naturkundemuseum Stuttgart, Sektion Ornithologie
Jul-Aug 2015	Forschungsaufenthalt im Labor von Prof. Yuval Gottlieb an der Robert H. Smith Faculty of Agriculture, Food and Environment der Hebrew University of Jerusalem in Rehovot im Rahmen der Masterarbeit
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Nov 2015-Feb 2016	Studentische Hilfskraft im Humboldt reloaded-Projekt „Und ab geht die Post! Ermittlung von Transportbedingungen und Eliminierung eventueller

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Apr 2015-Nov 2018	Aufsicht und Führungen am Zoologischen und Tiermedizinischen Museum Hohenheim
Jul 2016-Aug 2017	Wissenschaftliche Hilfskraft am Fachgebiet für Chemische Ökologie der Universität Hohenheim
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Weitere wissenschaftliche Aktivitäten

Betreuung von Humboldt reloaded-Projekten

2016-2017	Leonie Zott: „Darwins blinder Fleck - Die Rolle von Endosymbionten bei der Artbildung“
2017-2018	Simon Behrends, Lorenz Donndorf und Julian Wagner: „Auf Darwins Spuren: Wie neue Insektenarten entstehen“

Betreuung von Forschungspraktika

2021	Ulrich Higl: Experimenteller Transfer von *Spiroplasma* von *Lariophagus distinguendus* in *Nasonia vitripennis*

Betreuung von Bachelorarbeiten

2016	Irmela Homolka: „Prä- und postzygotische Barrieren bei der Lagererzwespe *Lariophagus distinguendus*“
2018	Denise Kuhn: „Reproduktive Isolation zwischen zwei Stämmen der Lagererzwespe *Lariophagus distinguendus*“
2019	Natascha Traub: „Cytoplasmatische Inkompatibilität bei der parasitischen Erzwespe *Lariophagus distinguendus*“
	Noa Schwabe: „Reproduktive Isolationsbarrieren bei zwei Stämmen der Lagererzwespe *Lariophagus distinguendus*“
	Ronja Reinisch: „Grad der reproduktiven Isolation zwischen zwei Stämmen der Lagererzwespe (*Lariophagus distinguendus*)“
2021	Lena-Maria Käppeler: „Untersuchung zur Auslösung von Cytoplasmatischer Inkompatibilität durch *Spiroplasma* bei einem Stamm der Lagererzwespe *Lariophagus distinguendus*“
2022	Anna Schmidt: „Reproduktive Isolationsbarrieren bei zwei Stämmen der

Lagererzwespe *Lariophagus distinguendus*“

Asude Demir und Timon Grum: „Experimental transfer of *Spiroplasma* between two *Lariophagus distinguendus* strains“

2023 Isabell Litvinov: „Auslösung von Cytoplasmatischer Inkompatibilität durch *Spiroplasma* bei der Lagererzwespe *Lariophagus distinguendus*“

Marina Geiselmann: „Experimenteller Transfer von *Spiroplasma* in einen nichtinfizierten *Lariophagus distinguendus* Stamm“ (Arbeitstitel)

Betreuung von Masterarbeiten

2018 Johanna Schaal: „How one species splits into two: Isolation barriers in the parasitic wasp *Lariophagus distinguendus*”

2021 Denise Kuhn: „Fertility of hybrid males as postzygotic barrier in two strains of *Lariophagus distinguendus*”

Lea von Berg: „Transfection experiments with *Spiroplasma* in different strains of *Lariophagus distinguendus*”

2022 Ronja Reinisch: „Resistance to *Spiroplasma*-induced CI in a strain of *Lariophagus distinguendus*”

Lena-Maria Käppeler: „Transfektionsexperimente bei einem Stamm der Erzwespe *Lariophagus distinguendus*“

2023 Natascha Traub: „Untersuchungen zur Resistenz der Lagererzwespe *Lariophagus distinguendus* gegen die von *Spiroplasma* ausgelöste Zytoplasmatische Inkompatibilität“

Tagungsteilnahmen

Poster

2015 Entomologentagung der Deutschen Gesellschaft für allgemeine und angewandte Entomologie e.V., Frankfurt am Main
„Chromosomes of the *L. distinguendus* complex”

Jahrestagung der Deutschen Zoologischen Gesellschaft e.V., Graz, Österreich
„Chromosomes of the *Lariophagus distinguendus* complex“

2017 Jahrestagung der Deutschen Zoologischen Gesellschaft e.V., Bielefeld
„Prezygotic and postzygotic barriers between strains of the parasitic wasp *Lariophagus distinguendus*”

2018 Münster Evolution Meeting, Münster
“Lake Malawi in the pantry: Surprising diversity of cryptic species in a parasitoid of household pests”

	Joint Congress on Evolutionary Biology, Montpellier, Frankreich “Reproductive barriers in a parasitic wasp”

Vorträge

2016	Hymenopterologen-Tagung Stuttgart “The role of endosymbionts in the reproductive isolation of *Lariophagus distinguendus* (Förster 1841)”
2018	Meeting of the Entomological Society of Israel, Rehovot, Israel “The role of endosymbionts in the speciation of a parasitic wasp”
	Hymenopterologen-Tagung Stuttgart “New kid on the block– a new addition to the ranks of CI-inducing bacteria promotes speciation in a parasitic wasp”
2020	Online Symbiosis Seminar “Speciation and cytoplasmic incompatibility in *Lariophagus distinguendus*: a novel phenotype for *Spiroplasma*”
2021	Virtuelle Jahrestagung der Deutschen Zoologischen Gesellschaft e.V. “Speciation and cytoplasmic incompatibility in *Lariophagus distinguendus*: a novel phenotype for *Spiroplasma*”
2022	International Symbiosis Society Congress, Lyon, Frankreich “Cytoplasmic incompatibility in *Lariophagus distinguendus* – a novel phenotype for *Spiroplasma*”
	Jahrestagung der Deutschen Zoologischen Gesellschaft e.V., Bonn “Cryptic diversity in our backyards: The case of a parasitoid wasp”
	Hymenopteren-Tagung Stuttgart “Cryptic diversity in a parasitoid wasp”

Preise

2022	Student travel award für den International Symbiosis Society Congress in Lyon unterstützt durch die Gordon and Betty Moore Foundation

Hohenheim, den 19.06.2023

Appendix

Table A1. Results of the generalized linear model (number of female offspring ~ crossing, family= negative binomial) comparing the number of F1 female offspring, the generalized linear model (number of male offspring ~ crossing, family= negative binomial) comparing the number of F1 male offspring, and the generalized linear model (number of total offspring ~ crossing, family= negative binomial) comparing the total number of F1 offspring in crossings between tetracycline-treated (-) and untreated, infected (+) males and females of the *L. distinguendus* strain STU. The reference contained in the intercept is the crossing STU(+) ♀ x STU(+) ♂.

Number of F1 female offspring

	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	2.47984	0.25919	9.568	< 2e-16 ***
STU(+) ♀ x STU(-) ♂	-0.04778	0.36401	-0.131	0.896
STU(-) ♀ x STU(+) ♂	-1.82926	0.37540	-4.873	1.1e-06 ***
STU(-) ♀ x STU(-) ♂	-0.23442	0.35969	-0.652	0.515

Results of the Tukey test for multiple comparisons

		Estimate	Std. Error	z value	Pr(>|z|)
STU(+) ♀ x STU(+) ♂	STU(+) ♀ x STU(-) ♂	-0.04778	0.36401	-0.131	0.999
	STU(-) ♀ x STU(+) ♂	-1.82926	0.37540	-4.873	< 1e-04 ***
	STU(-) ♀ x STU(-) ♂	-0.23442	0.35969	-0.652	0.915
STU(+) ♀ x STU(-) ♂	STU(-) ♀ x STU(+) ♂	-1.78148	0.37292	-4.777	< 1e-04 ***
	STU(-) ♀ x STU(-) ♂	-0.18664	0.35710	-0.523	0.954
STU(-) ♀ x STU(+) ♂	STU(-) ♀ x STU(-) ♂	1.59484	0.36870	4.326	< 1e-04 ***

Number of F1 male offspring

	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	1.55974	0.15165	10.285	< 2e-16 ***
STU(+) ♀ x STU(-) ♂	0.06719	0.21194	0.317	0.751
STU(-) ♀ x STU(+) ♂	0.98143	0.20710	4.881	1.06e-06 ***
STU(-) ♀ x STU(-) ♂	0.22738	0.20710	1.098	0.272

Results of the Tukey test for multiple comparisons

		Estimate	Std. Error	z value	Pr(>|z|)
STU(+) ♀ x STU(+) ♂	STU(+) ♀ x STU(-) ♂	0.06719	0.21194	0.317	0.989
	STU(-) ♀ x STU(+) ♂	0.98143	0.20107	4.881	< 0.001 ***
	STU(-) ♀ x STU(-) ♂	0.22738	0.20710	1.098	0.691
STU(+) ♀ x STU(-) ♂	STU(-) ♀ x STU(+) ♂	0.91423	0.19837	4.609	< 0.001 ***
	STU(-) ♀ x STU(-) ♂	0.16019	0.20448	0.783	0.862
STU(-) ♀ x STU(+) ♂	STU(-) ♀ x STU(-) ♂	-0.75405	0.19320	-3.903	< 0.001 ***

Total F1 offspring number				
	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	2.81523	0.17327	16.248	< 2e-16 ***
STU(+) ♀ x STU(-) ♂	-0.01365	0.24328	-0.056	0.955
STU(-) ♀ x STU(+) ♂	-0.13344	0.24037	-0.555	0.579
STU(-) ♀ x STU(-) ♂	-0.07978	0.24017	-0.322	0.740

Results of the Tukey test for multiple comparisons					
		Estimate	Std. Error	z value	Pr(>|z|)
STU(+) ♀ x STU(+) ♂	STU(+) ♀ x STU(-) ♂	-0.01365	0.24328	-0.056	1.000
	STU(-) ♀ x STU(+) ♂	-0.13344	0.24037	-0.555	0.945
	STU(-) ♀ x STU(-) ♂	-0.07978	0.24017	-0.322	0.987
STU(+) ♀ x STU(-) ♂	STU(-) ♀ x STU(+) ♂	-0.11979	0.23858	-0.502	0.959
	STU(-) ♀ x STU(-) ♂	-0.06613	0.23837	-0.277	0.993
STU(-) ♀ x STU(+) ♂	STU(-) ♀ x STU(-) ♂	0.05367	0.23541	0.228	0.996

n.s. $P > 0.05$, *** $P < 0.001$

Table A2. Closest BLASTn matches to the 16S rRNA sequence of the Enterobacteriaceae of the *L. distinguendus* strain STU.

Description	Max Score	Total Score	Query Cover	E value	Per. ident	Accession number
Enterobacter bugandensis strain XM29	1936	1936	100 %	0.0	99.72 %	MT023405.1
Enterobacter bugandensis strain XY7	1936	1936	100 %	0.0	99.72 %	MN709172.1
Enterobacter cancerogenus strain MiY-F	1936	15352	100 %	0.0	99.72 %	CP045769.1
Enterobacter hormaechei strain C44	1936	15208	100 %	0.0	99.72 %	LC484731.1
Enterobacter sp. CSCRZ6.1	1936	1936	100 %	0.0	99.72 %	LC484731.1
Enterobacter cancerogenus strain PJHBT1	1936	1936	100 %	0.0	99.72 %	MN203707.1
Enterobacter bugandensis strain CH6 16S	1936	1936	100 %	0.0	99.72 %	MN069628.1
Enterobacter cancerogenus strain PJH CE2	1936	1936	100 %	0.0	99.72 %	MK889234.1
Enterobacter bugandensis strain TJ6 16S	1936	1936	100 %	0.0	99.72 %	MK836418.1
Bacterium strain BS0452	1936	1936	100 %	0.0	99.72 %	MK823640.1
Enterobacter bugandensis strain 220	1936	14787	100 %	0.0	99.72 %	CP039453.1
Enterobacter cloacae strain CPO 4.14C	1936	1936	100 %	0.0	99.72 %	MF666755.1
Enterobacter cloacae strain CPO 4.12C	1936	1936	100 %	0.0	99.72 %	MF666753.1
Enterobacter bugandensis isolate EB-247	1936	15347	100 %	0.0	99.72 %	LT992502.1
Enterobacter cancerogenus strain CR-Eb1	1936	15265	100 %	0.0	99.72 %	CP025225.1
Enterobacter cancerogenus strain HPBBIH4 16S ribosomal RNA gene, partial sequence	1936	15265	100 %	0.0	99.72 %	KU605688.1
Enterobacter cloacae isolate MBRL1077	1936	15380	100 %	0.0	99.72 %	CP014280.1
Enterobacter cancerogenus strain RD_MOSAA_01	1936	1936	100 %	0.0	99.72 %	KU597513.1
Endophytic bacterium SV768	1936	1936	100 %	0.0	99.72 %	KP757637.1
Enterobacter cancerogenus strain AS1-131a	1936	1936	100 %	0.0	99.72 %	KF580847.1
Enterobacter sp. strain CEMTC_4006	1936	1936	100 %	0.0	99.72 %	MW672449.1
Enterobacter sp. strain CEMTC_4000	1936	1936	100 %	0.0	99.72 %	MW672448.1
Uncultured bacterium clone BD14735	1936	1936	100 %	0.0	99.72 %	JQ188835.1
Enterobacter sp. NCCP-755	1936	1936	100 %	0.0	99.72 %	AB715352.1
Enterobacter cancerogenus strain Gm13	1936	1936	100 %	0.0	99.72 %	MN826170.1
Enterobacter cloacae strain SN19	1936	1936	100 %	0.0	99.72 %	JQ904624.1
Enterobacter sp. strain ES1	1936	1936	100 %	0.0	99.72 %	MW131452.1
Enterobacter sp. PR3	1936	1936	100 %	0.0	99.72 %	GU086160.1
Enterobacter sp. PR1	1936	1936	100 %	0.0	99.72 %	GU086158.1
Enterobacter sp. 2B2A	1936	1936	100 %	0.0	99.72 %	EU693574.1
Uncultured bacterium clone 4.3	1936	1936	100 %	0.0	99.72 %	EF179826.1
Enterobacter aerogenes strain HC050612-1	1936	1936	100 %	0.0	99.72 %	EU047701.1

Table A3. ASV count table from the NGS analysis of the microbiome of the *L. distinguendus* strain STU.

Total # of reads	Average # of reads	Class	Order	Family	Genus	Species
42058	8411.6	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA	N/A
41292	8258.4	Mollicutes	Entomoplasmatales	Spiroplasmataceae	*Spiroplasma*	secondary
693	138.6	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	N/A
113	22.6	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Yersinia*	N/A
103	20.6	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	N/A
12	2.4	Bacilli	Bacillales	Bacillaceae	NA	N/A
11	2.2	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	N/A
10	2	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Rubellimicrobium*	N/A
6	1.2	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	N/A
4	0.8	Actinobacteria	Micrococcales	Micrococcaceae	NA	N/A
4	0.8	Actinobacteria	Micrococcales	Brevibacteriaceae	*Brevibacterium*	N/A
2	0.4	Bacteroidia	Flavobacteriales	Flavobacteriaceae	*Flavobacterium*	resistens

Table A4. Numbers of overall characters and character types for all genes.

Gene	Characters (of aligned sequences)	Constant characters	Parsimony-informative characters	Singletons
COI	652	466	129	57
CAD	550	452	5	93
ITS2	449	359	8	82
LOC 100123206	432	378	3	51
LOC 100123909	511	449	6	56
LOC 100117339	550	454	4	92
Sum of all genes	3144	2558	155	401

Table A5. Partitions and best-fit substitution models for all genes.

Partitions		Substitution models
COI		
Subset 1	1st codon position	TrN+F+G4
Subset 2	2nd codon position	TrN+F+G4
Subset 3	3rd codon position	F81+F
Concatenation of nuclear genes		
Subset 1	CAD Exon 3rd codon position	K2P
Subset 2	CAD Exon 1st codon position	F81+F
	LOC 100123206 Exon 1st codon position	
	LOC 100123909 Exon 1st codon position	
	LOC 100117339 Exon 1st codon position	
Subset 3	CAD Exon 2nd codon position	F81+F
	LOC 100123206 Exon 2nd codon position	
	LOC 100123909 Exon 2nd codon position	
	LOC 100117339 Exon 2nd codon position	
Subset 4	CAD Intron	TPM3+F
	LOC 100123206 Intron1	
	LOC 100123206 Intron2	
	LOC 100123909 Intron1	
	LOC 100117339 Intron1	
	LOC 100117339 Intron2	
	LOC 100117339 Intron3	
Subset 5	LOC 100123206 Exon 3rd codon position	HKY+F
	LOC 100123909 Exon 3rd codon position	
	LOC 100123909 Intron2	
	LOC 100117339 Exon 3rd codon position	
Subset 6	ITS2	JC+I

Table A6. Accession numbers of sequences retrieved from GenBank used in this study. Sequences in bold were generated by König et al. (2015a).

Strain	COI (barcode segment)	ITS2	CAD	LOC100123206	LOC100123909	LOC100117339
dbBIR-D1	-	**KJ923919**	**KJ867408**	**KJ923863**	**KJ923883**	**KJ923901**
gwBYG-DK1	-	**KJ923905**	**KJ867400**	**KJ923856**	**KJ923875**	**KJ923893**
gwPFO-D1	-	**KJ923903**	**KJ867392**	**KJ923849**	**KJ923867**	**KJ923885**
dbRAV-D1	-	**KJ923913**	**KJ867404**	**KJ923859**	**KJ923879**	**KJ923897**
gwSAC-D1	-	**KJ923912**	**KJ867396**	**KJ923852**	**KJ923871**	**KJ923889**
gwSAT-D1	-	**KJ923908**	**KJ867394**	**KJ923851**	**KJ923869**	**KJ923887**
gwSLO-GB1	-	**KJ923910**	**KJ867398**	**KJ923854**	**KJ923873**	**KJ923891**
dbSTU-D1	-	**KJ923915**	**KJ867402**	**KJ923866**	**KJ923877**	**KJ923895**
dbWAG-N1	-	**KJ923917**	**KJ867406**	**KJ923861**	**KJ923881**	**KJ923899**
Nasonia vitripennis	EU746537	**KJ923923**	KC213163	**KJ923921**	XM_001607631	**KJ923922**
Eupelmus confusus	KJ018429	-	-	-	-	-

T**able A7.** Uncorrected p-distances of the barcode segment of COI for all *L. distinguendus* strains used for crossing experiments and mean p-distances between the clades based on all strains. Distances for strain combinations in bold.

							clade	
clade	strain	STU	OST	BIR	CAN	PFO	A	B
A	STU							
	OST	0.022						
	BIR	**0.028**	**0.017**					
B	CAN	**0.072**	0.068	0.074			0.0717	
C	PFO	0.131	0.132	0.140	**0.140**		0.1328	0.1422
	SAT	0.126	0.132	**0.139**	0.143	0.031		

Table A8: GenBank accession numbers of all sequences generated in this study.

Strain	COI (barcode segment)	ITS2	CAD	LOC100123206	LOC100123909	LOC100117339
dbBIR-D1	OQ933721	-	-	-	-	-
dbBIR-D2	OQ933722	OQ938963	OQ939843	OQ939881	OQ939900	OQ939862
dbBIR-D3	OQ933723	OQ938964	OQ939844	OQ939882	OQ939901	OQ939863
dbBIR-D4	OQ933724	OQ938965	OQ939845	OQ939883	OQ939902	OQ939864
dbBRU-D1	OQ933725	OQ938966	OQ939846	OQ939884	OQ939903	OQ939865
gwBYG-DK1	OQ933726	-	-	-	-	-
dbCAN-D1	OQ933727	OQ938967	OQ939847	OQ939885	OQ939904	OQ939866
dbCAN-D2	OQ933728	OQ938968	OQ939848	OQ939886	OQ939905	OQ939867
dbFRI-D1	OQ933729	OQ938969	OQ939849	OQ939887	OQ939906	OQ939868
dbLUD-D1	OQ933730	OQ938970	OQ939850	OQ939888	OQ939907	OQ939869
dbOBE-D1	OQ933731	OQ938971	OQ939851	OQ939889	OQ939908	OQ939870
dbOST-D1	OQ933732	OQ938972	OQ939852	OQ939890	OQ939909	OQ939871
gwPFO-D1	OQ933733	-	-	-	-	-
dbPLI-D2	OQ933734	OQ938973	OQ939853	OQ939891	OQ939910	OQ939872
dbRAV-D1	OQ933735	-	-	-	-	-
gwSAC-D1	OQ933736	-	-	-	-	-
gwSAT-D1	OQ933737	-	-	-	-	-
gwSIL-D1	OQ933738	OQ938974	OQ939854	OQ939892	OQ939917	OQ939873
gwSLO-GB1	OQ933739	-	-	-	-	-
dbSTU-D1	OQ933740	-	-	-	-	-
dbSTU-D3	OQ933741	OQ938975	OQ939855	OQ939893	OQ939911	OQ939874
gwSWD-D1	OQ933742	OQ938976	OQ939856	OQ939894	OQ939918	OQ939875
dbVAI-D1	OQ933743	OQ938977	OQ939857	OQ939895	OQ939912	OQ939876
dbVAI-D2	OQ933744	OQ938978	OQ939858	OQ939896	OQ939913	OQ939877
dbVAI-D3	OQ933745	OQ938979	OQ939859	OQ939897	OQ939914	OQ939878
dbVAI-D4	OQ933746	OQ938980	OQ939860	OQ939898	OQ939915	OQ939879
dbWAG-N1	OQ933747	-	-	-	-	-
dbWAN-D1	OQ933748	OQ938981	OQ939861	OQ939899	OQ939916	OQ939880

Statistical analyses

Sexual isolation

Table A9. Comparison of the occurrence of copulation in crosses between males and females of the *L. distinguendus* strains BIR and OST. Comparisons were made using the 2 x 2 Fisher's Exact test for Count Data.

		P value
BIR ♀ x BIR ♂	BIR ♀ x OST ♂	0.02141 *
OST ♀ x BIR ♂	OST ♀ x OST ♂	0.03551 *

* $P < 0.05$

Table A10. Comparison of the occurrence of copulation in crosses between males and females of the *L. distinguendus* strains BIR and STU. A Pearson's Chi-squared test was conducted for the comparisons.

		P value
BIR ♀ x BIR ♂	BIR ♀ x STU ♂	0.512 n.s.
STU ♀ x BIR ♂	STU ♀ x STU ♂	0.04382 *

n.s. $P > 0.05$, * $P < 0.05$

Table A11. Comparison of the occurrence of copulation in crosses between males and females of the *L. distinguendus* strains CAN and STU. Comparisons were made using the 2 x 2 Fisher's Exact test for Count Data.

		P value
CAN ♀ x CAN ♂	CAN ♀ x STU ♂	0.7411
STU ♀ x CAN ♂	STU ♀ x STU ♂	0.00953 **

n.s. $P > 0.05$, ** $P < 0.01$

Table A12. Comparison of the occurrence of copulation in crosses between males and females of the *L. distinguendus* strains BIR and SAT. Comparisons were made using the 2 x 2 Fisher's Exact test for Count Data.

		P value
BIR ♀ x BIR ♂	BIR ♀ x SAT ♂	0.0004359 ***
SAT ♀ x BIR ♂	SAT ♀ x SAT ♂	2.444e-07 ***

*** $P < 0.001$

Table A13. Comparison of the occurrence of copulation in crosses between males and females of the *L. distinguendus* strains CAN and PFO. Comparisons were made using the 2 x 2 Fisher's Exact test for Count Data.

		P value
CAN ♀ x CAN ♂	CAN ♀ x PFO ♂	7.709e-07 ***
PFO ♀ x CAN ♂	PFO ♀ x PFO ♂	3.358e-06 ***

*** $P < 0.001$

Female inviability

Table A14. Results of the linear model (number of female offspring ~ crossing) comparing the number of F1 female offspring in intra- and interstrain crossings between males and females of the *L. distinguendus* strains BIR and OST and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing BIR ♀ x BIR ♂.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	31.348	3.717	8.435	3.88e-13***
BIR ♀ x OST ♂	2.075	5.102	0.407	0.6851
OST ♀ x BIR ♂	10.152	5.201	1.952	0.0539
OST ♀ x OST ♂	3.012	5.150	0.585	0.5600

Tukey test for multiple comparisons

		Estimate	Std. Error	z value	Pr(>|t|)
BIR ♀ x BIR ♂	BIR ♀ x OST ♂	2.0753	5.1021	0.407	0.977
	OST ♀ x BIR ♂	10.1522	5.2010	1.952	0.214
	OST ♀ x OST ♂	3.0122	5.1498	0.585	0.936
BIR ♀ x OST ♂	OST ♀ x BIR ♂	8.0769	5.0454	1.601	0.383
	OST ♀ x OST ♂	0.9369	4.9927	0.188	0.998
OST ♀ x BIR ♂	OST ♀ x OST ♂	-7.1400	5.0936	-1.402	0.501

n.s. $P > 0.05$, *** $P < 0.001$

Table A15. Results of the generalized linear model (number of female offspring ~ crossing, family= quasipoisson) comparing the number of F1 female offspring in intra- and interstrain crossings between males and females of the *L. distinguendus* strains BIR and STU and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing BIR ♀ x BIR ♂.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	3.10837	0.10847	28.657	<2e-16 ***
BIR ♀ x STU ♂	0.04459	0.15371	0.290	0.772
STU ♀ x BIR ♂	0.18931	0.14580	1.298	0.196
STU ♀ x STU ♂	0.12265	0.15179	0.808	0.420

Tukey test for multiple comparisons

		Estimate	Std. Error	z value	Pr(>|z|)
BIR ♀ x BIR ♂	BIR ♀ x STU ♂	0.04459	0.15371	0.290	0.991
	STU ♀ x BIR ♂	0.18931	0.14580	1.298	0.564
	STU ♀ x STU ♂	0.12265	0.15179	0.808	0.851
BIR ♀ x STU ♂	STU ♀ x BIR ♂	0.14472	0.14613	0.990	0.755
	STU ♀ x STU ♂	0.07806	0.15210	0.513	0.956
STU ♀ x BIR ♂	STU ♀ x STU ♂	-0.06666	0.14411	-0.463	0.967

n.s. $p > 0.05$, *** $p < 0.001$

Table A16. Results of the linear model (number of female offspring ~ crossing) comparing the number of F1 female offspring in intra- and interstrain crossings between males and females of the *L. distinguendus* strains CAN and STU and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing CAN ♀ x CAN ♂.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	31.474	4.044	7.782	4.6e-11 ***
CAN ♀ x STU ♂	-4.363	5.798	-0.752	0.45434
STU ♀ x CAN ♂	14.726	5.647	2.608	0.01114 *
STU ♀ x STU ♂	19.820	5.885	3.368	0.00123 **

Tukey test for multiple comparisons

		Estimate	Std. Error	z value	Pr(>|t|)
CAN ♀ x CAN ♂	CAN ♀ x STU ♂	-4.363	5.798	-0.752	0.87529
	STU ♀ x CAN ♂	14.726	5.647	2.608	0.05296
	STU ♀ x STU ♂	19.820	5.885	3.368	0.00699 **
CAN ♀ x STU ♂	STU ♀ x CAN ♂	19.089	5.727	3.333	0.00737 **
	STU ♀ x STU ♂	24.183	5.962	4.056	< 0.001 ***
STU ♀ x CAN ♂	STU ♀ x STU ♂	5.094	5.815	0.876	0.81719

n.s. $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

T**able A17.** Results of the generalized linear model (number of female offspring ~ crossing, family= quasipoisson) comparing the number of F1 female offspring in intra- and interstrain crossings between males and females of the *L. distinguendus* strains BIR and SAT and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing BIR ♀ x BIR ♂.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	3.84791	0.11181	34.416	< 2e-16 ***
BIR ♀ x SAT ♂	-0.75686	0.51540	-1.469	0.148
SAT ♀ x BIR ♂	0.16170	0.15852	1.020	0.312
SAT ♀ x SAT ♂	-0.02961	0.15930	-0.186	0.853

Tukey test for multiple comparisons

		Estimate	Std. Error	z value	Pr(>|z|)
BIR ♀ x BIR ♂	BIR ♀ x SAT ♂	-0.75686	0.51540	-1.469	0.428
	SAT ♀ x BIR ♂	0.16170	0.15852	1.020	0.717
	SAT ♀ x SAT ♂	-0.02961	0.15930	-0.186	0.997
BIR ♀ x SAT ♂	SAT ♀ x BIR ♂	0.91856	0.51552	1.782	0.257
	SAT ♀ x SAT ♂	0.72725	0.51576	1.410	0.464
SAT ♀ x BIR ♂	SAT ♀ x SAT ♂	-0.19131	0.15970	-1.198	0.602

n.s. $P > 0.05$, *** $P < 0.001$

Table A18. Results of the linear model (number of female offspring ~ crossing) comparing the number of F1 female offspring in intra- and interstrain crossings between males and females of the *L. distinguendus* strains CAN and PFO and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing CAN ♀ x CAN ♂.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	41.421	4.583	9.038	2.55e-12 ***
CAN ♀ x PFO ♂	-40.421	20.495	-1.972	0.0538
PFO ♀ x CAN ♂	-9.199	6.570	-1.400	0.1673
PFO ♀ x PFO ♂	-5.474	6.481	-0.845	0.4021

Tukey test for multiple comparisons

		Estimate	Std. Error	t value	Pr(>|t|)
CAN ♀ x CAN ♂	CAN ♀ x PFO ♂	-40.421	20.495	-1.972	0.191
	PFO ♀ x CAN ♂	-9.199	6.570	-1.400	0.478
	PFO ♀ x PFO ♂	-5.474	6.481	-0.845	0.818
CAN ♀ x PFO ♂	PFO ♀ x CAN ♂	31.222	20.523	1.521	0.405
	PFO ♀ x PFO ♂	34.947	20.495	1.705	0.306
PFO ♀ x CAN ♂	PFO ♀ x PFO ♂	3.725	6.570	0.567	0.936

n.s. $P > 0.05$, *** $P < 0.001$

Female behavioral sterility

Table A19. Comparison of the occurrence of copulation in crosses between males and F1 female offspring of the *L. distinguendus* strains BIR and OST. Comparisons were made using the 2 x 2 Fisher's Exact test for Count Data.

		P value
BIR ♀ x BIR ♂	BIR ♀ x OST ♂	0.1299 n.s.
OST ♀ x BIR ♂	OST ♀ x OST ♂	0.749 n.s.

n.s. $P > 0.05$

Table A20. Comparison of the occurrence of copulation in crosses between males and F1 female offspring of the *L. distinguendus* strains BIR and STU. A Pearson's Chi-squared test was conducted for the comparison.

		P value
BIR ♀ x BIR ♂	BIR ♀ x STU ♂	0.9334 n.s.
STU ♀ x BIR ♂	STU ♀ x STU ♂	0.9018 n.s.

n.s. $P > 0.05$

Table A21. Comparison of the occurrence of copulation in crosses between males and F1 female offspring of the *L. distinguendus* strains CAN and STU. Comparisons were made using the 2 x 2 Fisher's Exact test for Count Data.

		P value
CAN ♀ x CAN ♂	CAN ♀ x STU ♂	0.04091 *
STU ♀ x CAN ♂	STU ♀ x STU ♂	0.0003284 ***

* $P < 0.05$, *** $P < 0.001$

Female physiological sterility

Table A22. Comparison of the occurrence of F2 offspring in crosses between males and F1 female offspring of the *L. distinguendus* strains BIR and OST. Comparisons were made using the 2 x 2 Fisher's Exact test for Count Data.

		P value
BIR ♀ x BIR ♂	BIR ♀ x OST ♂	1 n.s.
OST ♀ x BIR ♂	OST ♀ x OST ♂	0.4923 n.s.

n.s. $P > 0.05$

Table A23. Comparison of the occurrence of F2 offspring in crosses between males and F1 female offspring of the *L. distinguendus* strains BIR and STU. Comparisons were made using the 2 x 2 Fisher's Exact test for Count Data.

		P value
BIR ♀ x BIR ♂	BIR ♀ x STU ♂	1 n.s.
STU ♀ x BIR ♂	STU ♀ x STU ♂	0.61 n.s.

n.s. $P > 0.05$

Table A24. Comparison of the occurrence of F2 offspring in crosses between males and F1 female offspring of the *L. distinguendus* strains CAN and STU. Comparisons were made using the 2 x 2 Fisher's Exact test for Count Data.

		P value
CAN ♀ x CAN ♂	CAN ♀ x STU ♂	1 n.s.
STU ♀ x CAN ♂	STU ♀ x STU ♂	1 n.s.

n.s. $P > 0.05$

Table A25. Comparison of the occurrence of F2 offspring in crosses between males and F1 female offspring of the *L. distinguendus* strains BIR and SAT. Comparisons were made using the 2 x 2 Fisher's Exact test for Count Data.

		P value
BIR ♀ x BIR ♂	BIR ♀ x SAT ♂	1 n.s.
SAT ♀ x BIR ♂	SAT ♀ x SAT ♂	0.106 n.s.

n.s. $P > 0.05$

Table A26. Comparison of the occurrence of F2 offspring in crosses between males and F1 female offspring of the *L. distinguendus* strains CAN and PFO. Comparisons were made using the 2 x 2 Fisher's Exact test for Count Data.

		P value
CAN ♀ x CAN ♂	CAN ♀ x PFO ♂	N/A
PFO ♀ x CAN ♂	PFO ♀ x PFO ♂	1 n.s.

n.s. $P > 0.05$

Female physiological reduced fertility

Table A27. Results of the generalized linear model (total offspring number ~ crossing, family=quasipoisson) comparing the total number of F2 offspring of mated F1 female offspring of intra- and interstrain crossings between the *L. distinguendus* strains BIR and OST and wildtype F1 males and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing BIR ♀ x BIR ♂.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	3.89776	0.08478	45.978	<2e-16 ***
BIR ♀ x OST ♂	0.13147	0.11731	1.121	0.2657
OST ♀ x BIR ♂	0.20819	0.11912	1.748	0.0843
OST ♀ x OST ♂	0.14041	0.12456	1.127	0.2629

Tukey test for multiple comparisons

		Estimate	Std. Error	z value	Pr(>|t|)
BIR ♀ x BIR ♂	BIR ♀ x OST ♂	0.13147	0.13956	0.942	0.782
	OST ♀ x BIR ♂	0.20819	0.14455	1.440	0.474
	OST ♀ x OST ♂	0.14041	0.14905	0.942	0.782
BIR ♀ x OST ♂	OST ♀ x BIR ♂	0.07672	0.14561	0.527	0.953
	OST ♀ x OST ♂	0.00894	0.15008	0.060	1.000
OST ♀ x BIR ♂	OST ♀ x OST ♂	-0.06778	0.15473	-0.438	0.972

n.s. $P > 0.05$, *** $P < 0.001$

Table A28. Results of the generalized linear model (total offspring number ~ crossing, family=quasipoisson) comparing the total number of F2 offspring of mated F1 female offspring of intra- and interstrain crossings between the *L. distinguendus* strains BIR and STU and wildtype F1 males and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing BIR ♀ x BIR ♂.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	3.0875	0.1038	29.739	<2e-16 ***
BIR ♀ x STU ♂	0.2844	0.1390	2.046	0.0426 *
STU ♀ x BIR ♂	0.1023	0.1470	0.696	0.4875
STU ♀ x STU ♂	-0.0318	0.1490	-0.213	0.8313

Tukey test for multiple comparisons

		Estimate	Std. Error	z value	Pr(>|z|)
BIR ♀ x BIR ♂	BIR ♀ x STU ♂	0.2844	0.1390	2.046	0.171
	STU ♀ x BIR ♂	0.1023	0.1470	0.696	0.899
	STU ♀ x STU ♂	-0.0318	0.1490	-0.213	0.997
BIR ♀ x STU ♂	STU ♀ x BIR ♂	-0.1821	0.1393	-1.307	0.558
	STU ♀ x STU ♂	-0.3162	0.1413	-2.238	0.113
STU ♀ x BIR ♂	STU ♀ x STU ♂	-0.1341	0.1492	-0.899	0.805

n.s. $P > 0.05$, * $P < 0.05$, *** $P < 0.001$

Table A29. Results of the linear model (total offspring number ~ crossing) comparing the total number of F2 offspring of mated F1 female offspring of intra- and interstrain crossings between the *L. distinguendus* strains CAN and STU and wildtype F1 males and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing CAN ♀ x CAN ♂.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	50.611	3.971	12.744	<2e-16 ***
CAN ♀ x STU ♂	-12.729	5.698	-2.234	0.0286 *
STU ♀ x CAN ♂	3.889	5.474	0.710	0.4798
STU ♀ x STU ♂	-1.661	5.474	-0.303	0.7624

Tukey test for multiple comparisons

		Estimate	Std. Error	z value	Pr(>|t|)
CAN ♀ x CAN ♂	CAN ♀ x STU ♂	-12.729	5.698	-2.234	0.1241
	STU ♀ x CAN ♂	3.889	5.474	0.710	0.8926
	STU ♀ x STU ♂	-1.661	5.474	-0.303	0.9902
CAN ♀ x STU ♂	STU ♀ x CAN ♂	16.618	5.558	2.990	0.0198 *
	STU ♀ x STU ♂	11.068	5.558	1.991	0.2010
STU ♀ x CAN ♂	STU ♀ x STU ♂	-5.550	5.328	-1.042	0.7256

n.s. $P > 0.05$, * $P < 0.05$, *** $P < 0.001$

Table A30. Results of the generalized linear model (total offspring number ~ crossing, family=quasipoisson) comparing the total number of F2 offspring of mated F1 female offspring of intra- and interstrain crossings between the *L. distinguendus* strains BIR and SAT and wildtype F1 males and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing BIR ♀ x BIR ♂.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	4.1207	0.1030	39.992	< 2e-16 ***
BIR ♀ x SAT ♂	0.1105	0.1721	0.642	0.523068
SAT ♀ x BIR ♂	-0.6643	0.1768	-3.758	0.000377 ***
SAT ♀ x SAT ♂	-0.6642	0.1868	-3.556	0.000721 ***

Tukey test for multiple comparisons

		Estimate	Std. Error	z value	Pr(>|z|)
BIR ♀ x BIR ♂	BIR ♀ x SAT ♂	0.1105419	0.1721275	0.642	0.91747
	SAT ♀ x BIR ♂	-0.6643452	0.1767686	-3.758	< 0.001 ***
	SAT ♀ x SAT ♂	-0.6641596	0.1867706	-3.556	0.00212 **
BIR ♀ x SAT ♂	SAT ♀ x BIR ♂	-0.7748871	0.1991022	-3.892	< 0.001 ***
	SAT ♀ x SAT ♂	-0.7747015	0.2080332	-3.724	0.00117 **
SAT ♀ x BIR ♂	SAT ♀ x SAT ♂	0.0001855	0.2118893	0.001	1.00000

n.s. $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table A31. Results of the linear model (total offspring number ~ crossing) comparing the total number of F2 offspring of mated F1 female offspring of intra- and interstrain crossings between the *L. distinguendus* strains CAN and PFO and wildtype F1 males and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing CAN ♀ x CAN ♂.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	88.650	5.756	15.400	< 2e-16 ***
CAN ♀ x PFO ♂	N/A due to absence of mating in parental generation			
PFO ♀ x CAN ♂	-65.650	8.364	-7.849	2.2e-10 ***
PFO ♀ x PFO ♂	-20.238	8.492	-2.383	0.0209 *

Tukey test for multiple comparisons

		Estimate	Std. Error	t value	Pr(>|t|)
CAN ♀ x CAN ♂	PFO ♀ x CAN ♂	-65.650	8.364	-7.849	<0.001 ***
	PFO ♀ x PFO ♂	-20.238	8.492	-2.383	0.0534
PFO ♀ x CAN ♂	PFO ♀ x PFO ♂	45.412	8.706	5.216	<0.001 ***

n.s. $P > 0.05$, * $P < 0.05$, *** $P < 0.001$

Male inviability

Table A32. Results of the generalized linear model (number of male offspring ~ crossing, family=quasipoisson) comparing the number of F2 male offspring of virgin F1 female offspring of intra- and interstrain crossings between the *L. distinguendus* strains BIR and OST and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing BIR ♀ x BIR ♂.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	3.95124	0.08761	45.102	<2e-16 ***
BIR ♀ x OST ♂	0.13849	0.11289	1.227	0.223
OST ♀ x BIR ♂	0.13173	0.11385	1.157	0.250
OST ♀ x OST ♂	0.01625	0.11759	0.138	0.890

Tukey test for multiple comparisons

		Estimate	Std. Error	z value	Pr(>|t|)
BIR ♀ x BIR ♂	BIR ♀ x OST ♂	0.138493	0.112891	1.227	0.609
	OST ♀ x BIR ♂	0.131727	0.113848	1.157	0.653
	OST ♀ x OST ♂	0.016249	0.117593	0.138	0.999
BIR ♀ x OST ♂	OST ♀ x BIR ♂	-0.006766	0.101763	-0.066	1.000
	OST ♀ x OST ♂	-0.122244	0.105937	-1.154	0.655
OST ♀ x BIR ♂	OST ♀ x OST ♂	-0.115478	0.106955	-1.080	0.701

n.s. $P > 0.05$, *** $P < 0.001$

Table A33. Results of the generalized linear model (number of male offspring ~ crossing, family= negative binomial) comparing the number of F2 male offspring of virgin F1 female offspring of intra- and interstrain crossings between the *L. distinguendus* strains BIR and STU and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing BIR ♀ x BIR ♂.

	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	3.0744	0.1406	21.872	<2e-16 ***
BIR ♀ x STU ♂	0.1361	0.1940	0.702	0.483
STU ♀ x BIR ♂	0.1465	0.2029	0.722	0.470
STU ♀ x STU ♂	0.1952	0.2027	0.963	0.336

Tukey test for multiple comparisons

		Estimate	Std. Error	z value	Pr(>|z|)
BIR ♀ x BIR ♂	BIR ♀ x STU ♂	0.13613	0.19397	0.702	0.896
	STU ♀ x BIR ♂	0.14650	0.20292	0.722	0.888
	STU ♀ x STU ♂	0.19519	0.20269	0.963	0.770
BIR ♀ x STU ♂	STU ♀ x BIR ♂	0.01037	0.19821	0.052	1.000
	STU ♀ x STU ♂	0.05906	0.19797	0.298	0.991
STU ♀ x BIR ♂	STU ♀ x STU ♂	0.04870	0.20675	0.236	0.995

n.s. $P > 0.05$, *** $P < 0.001$

Table A34. Results of the generalized linear model (number of male offspring ~ crossing, family= quasipoisson) comparing the number of F2 male offspring of virgin F1 female offspring of intra- and interstrain crossings between the *L. distinguendus* strains CAN and STU and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing CAN ♀ x CAN ♂.

	Estimate	Std. Error	z value	Pr(>|t|)
Intercept	3.68492	0.07845	46.969	< 2e-16 ***
CAN ♀ x STU ♂	0.23653	0.10495	2.254	0.02717 *
STU ♀ x CAN ♂	0.43655	0.09967	4.380	3.85e-05 ***
STU ♀ x STU ♂	0.38837	0.10060	3.860	0.00024 ***

Tukey test for multiple comparisons

		Estimate	Std. Error	z value	Pr(>|z|)
CAN ♀ x CAN ♂	CAN ♀ x STU ♂	0.23653	0.10495	2.254	0.109
	STU ♀ x CAN ♂	0.43655	0.09967	4.380	<0.001 ***
	STU ♀ x STU ♂	0.38837	0.10060	3.860	<0.001 ***
CAN ♀ x STU ♂	STU ♀ x CAN ♂	0.20002	0.09294	2.152	0.136
	STU ♀ x STU ♂	0.15184	0.09394	1.616	0.368
STU ♀ x CAN ♂	STU ♀ x STU ♂	-0.04818	0.08800	-0.548	0.947

n.s. $P > 0.05$, * $P < 0.05$, *** $P < 0.001$

Table A35. Results of the linear model (number of male offspring ~ crossing) comparing the number of F2 male offspring of virgin F1 female offspring of intra- and interstrain crossings between the *L. distinguendus* strains BIR and SAT and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing BIR ♀ x BIR ♂.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	69.105	4.390	15.743	< 2e-16 ***
BIR ♀ x SAT ♂	-50.105	14.224	-3.523	0.000849 ***
SAT ♀ x BIR ♂	-63.455	6.130	-10.352	1.04e-14 ***
SAT ♀ x SAT ♂	5.345	6.130	0.872	0.386895

Tukey test for multiple comparisons

		Estimate	Std. Error	t value	Pr(>|t|)
BIR ♀ x BIR ♂	BIR ♀ x SAT ♂	-50.105	14.224	-3.523	0.00399 **
	SAT ♀ x BIR ♂	-63.455	6.130	-10.352	< 0.001 ***
	SAT ♀ x SAT ♂	5.345	6.130	0.872	0.80782
BIR ♀ x SAT ♂	SAT ♀ x BIR ♂	-13.350	14.190	-0.941	0.76990
	SAT ♀ x SAT ♂	55.450	14.190	3.908	0.00125 **
SAT ♀ x BIR ♂	SAT ♀ x SAT ♂	68.800	6.051	11.371	< 0.001 ***

n.s. $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table A36. Results of the generalized linear model (number of male offspring ~ crossing, family=quasipoisson) comparing the number of F2 male offspring of virgin F1 female offspring of intra- and interstrain crossings between the *L. distinguendus* strains CAN and PFO and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing CAN ♀ x CAN ♂.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	3.6763	0.1593	23.08	< 2e-16 ***
CAN ♀ x PFO ♂	N/A due to absence of mating in parental generation			
PFO ♀ x CAN ♂	-1.3196	0.3150	-4.19	0.000136 ***
PFO ♀ x PFO ♂	0.5464	0.2001	2.73	0.009149 **

Tukey test for multiple comparisons

		Estimate	Std. Error	z value	Pr(>|z|)
CAN ♀ x CAN ♂	PFO ♀ x CAN ♂	-1.3196	0.3150	-4.190	<0.001 ***
	PFO ♀ x PFO ♂	0.5464	0.2001	2.730	0.0168 *
PFO ♀ x CAN ♂	PFO ♀ x PFO ♂	1.8660	0.2975	6.271	<0.001 ***

** $P < 0.01$, *** $P < 0.001$

Male behavioral sterility

Table A37. Comparison of the occurrence of copulation in crosses between F2 male offspring of crosses between the *L. distinguendus* strains BIR and OST and wildtype females. A Pearson's Chi-squared test was conducted for each group comparison.

	P value
Group comparison BIR females	0.4579 n.s.
Group comparison OST females	0.8628 n.s.

n.s. $P > 0.05$

Table A38. Comparison of the occurrence of copulation in crosses between F2 male offspring of crosses between the *L. distinguendus* strains BIR and STU and wildtype females. A 2 x 3 Fisher's Exact test for Count Data was conducted for each group comparison.

	P value
Group comparison BIR females	0.7322 n.s.
Group comparison STU females	0.7782 n.s.

n.s. $P > 0.05$

Table A39. Comparison of the occurrence of copulation in crosses between F2 male offspring of crosses between the *L. distinguendus* strains CAN and STU and wildtype females. A Pearson's Chi-squared test was conducted for each group comparison.

	P value
Group comparison CAN females	0.3425 n.s.
Group comparison STU females	0.2326 n.s.

n.s. $P > 0.05$

Table A40. Comparison of the occurrence of copulation in crosses between F2 male offspring of crosses between the *L. distinguendus* strains BIR and SAT and wildtype females. A 2 x 3 Fisher's Exact test for Count Data was conducted for each group comparison. Single comparisons were made using the 2 x 2 Fisher's Exact test for Count Data followed by Bonferroni corrections.

		P value	Significance level after Bonferroni correction (for single comparisons)
Group comparison BIR females		0.003076	**
BIR ♀ x (BIRxBIR) ♂	BIR ♀ x (BIRxSAT) ♂	0.08754	n.s.
	BIR ♀ x (SATxBIR) ♂	0.006322	*
BIR ♀ x (BIRxSAT) ♂	BIR ♀ x (SATxBIR) ♂	0.4	n.s.
Group comparison SAT females		0.0175	*
SAT ♀ x (BIRxSAT) ♂	SAT ♀ x (SATxBIR) ♂	1	n.s.
	SAT ♀ x (SATxSAT) ♂	0.02804	n.s.
SAT ♀ x (SATxBIR) ♂	SAT ♀ x (SATxSAT) ♂	0.02094	n.s.

n.s. $P > 0.05$, * $P < 0.05$, ** $P < 0.01$

Table A41. Comparison of the occurrence of copulation in crosses between F2 male offspring of crosses between the *L. distinguendus* strains CAN and PFO and wildtype females. A 2 x 2 Fisher's Exact test for Count Data was conducted for each comparison.

		P value
CAN ♀ x (CANxCAN) ♂	CAN ♀ x (PFOxCAN) ♂	0.005736 **
PFO ♀ x (PFOxCAN) ♂	PFO ♀ x (PFOxPFO) ♂	0.0004359 ***

** $P < 0.01$, *** $P < 0.001$

Male physiological sterility

Table A42. Comparison of the occurrence of female F3 offspring in crosses between F2 male offspring of crosses between the *L. distinguendus* strains BIR and OST and wildtype females. A 2 x 3 Fisher's Exact test for Count Data was conducted for each group comparison.

	P value
Group comparison BIR females	1 n.s.
Group comparison OST females	1 n.s.

n.s. $P > 0.05$

Table A43. Comparison of the occurrence of female F3 offspring in crosses between F2 male offspring of crosses between the *L. distinguendus* strains BIR and STU and wildtype females. A 2 x 3 Fisher's Exact test for Count Data was conducted for each group comparison.

	P value
Group comparison BIR females	1 n.s.
Group comparison STU females	0.1899 n.s.

n.s. $P > 0.05$

Table A44. Comparison of the occurrence of female F3 offspring in crosses between F2 male offspring of crosses between the *L. distinguendus* strains CAN and STU and wildtype females. A 2 x 3 Fisher's Exact test for Count Data was conducted for each group comparison. Single comparisons were made using the 2 x 2 Fisher's Exact test for Count Data followed by Bonferroni corrections.

		P value	Significance level after Bonferroni correction (for single comparisons)
Group comparison CAN females		0.02944	*
CAN ♀ x (CANxCAN) ♂	CAN ♀ x (CANxSTU) ♂	1	n.s.
	CAN ♀ x (STUxCAN) ♂	0.105	n.s.
CAN ♀ x (CANxSTU) ♂	CAN ♀ x (STUxCAN) ♂	0.105	n.s.
Group comparison STU females		0.7663	n.s.

n.s. $P > 0.05$, * $P < 0.05$

Table A45. Comparison of the occurrence of female F3 offspring in crosses between F2 male offspring of crosses between the *L. distinguendus* strains BIR and SAT and wildtype females. A 2 x 3 Fisher's Exact test for Count Data was conducted for each group comparison. Single comparisons were made using the 2 x 2 Fisher's Exact test for Count Data followed by Bonferroni corrections.

		P value	Significance level after Bonferroni correction (for single comparisons)
Group comparison BIR females		0.007166	**
BIR ♀ x (BIRxBIR) ♂	BIR ♀ x (BIRxSAT) ♂	0.006614	*
	BIR ♀ x (SATxBIR) ♂	0.03021	n.s.
BIR ♀ x (BIRxSAT) ♂	BIR ♀ x (SATxBIR) ♂	0.674	n.s.
Group comparison SAT females		0.2745	n.s.

n.s. $P > 0.05$, * $P < 0.05$, ** $P < 0.01$

Table A46. Comparison of the occurrence of female F3 offspring in crosses between F2 male offspring of crosses between the *L. distinguendus* strains CAN and PFO and wildtype females. Comparisons were made using the 2 x 2 Fisher's Exact test for Count Data.

		P value
CAN ♀ x (CANxCAN) ♂	CAN ♀ x (PFOxCAN) ♂	8.446e-05 ***
PFO ♀ x (PFOxCAN) ♂	PFO ♀ x (PFOxPFO) ♂	0.008316 **

** $P < 0.01$, *** $P < 0.001$

Male physiological reduced fertility

Table A47. Results of the linear model (number of female offspring ~ crossing) comparing the number of F3 female offspring of F2 male offspring of intra- and interstrain crossings between the *L. distinguendus* strains BIR and OST and wildtype females and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing BIR ♀ x (BIRxBIR) ♂.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	38.056	4.568	8.331	3.1e-13 ***
BIR ♀ x (BIRxOST) ♂	-3.898	6.375	-0.611	0.542
BIR ♀ x (OSTxBIR) ♂	-7.213	6.375	-1.132	0.260
OST ♀ x (BIRxOST) ♂	-2.266	6.375	-0.355	0.723
OST ♀ x (OSTxBIR) ♂	-1.371	6.375	-0.215	0.830
OST ♀ x (OSTxOST) ♂	-1.056	6.460	-0.163	0.871

Tukey test for multiple comparisons

		Estimate	Std. Error	z value	Pr(>|t|)
BIR ♀ x (BIRxBIR) ♂	BIR ♀ x (BIRxOST) ♂	-3.8977	6.3747	-0.611	0.990
	BIR ♀ x (OSTxBIR) ♂	-7.2135	6.3747	-1.132	0.867
	OST ♀ x (BIRxOST) ♂	-2.2661	6.3747	-0.355	0.999
	OST ♀ x (OSTxBIR) ♂	-1.3713	6.3747	-0.215	1.000
	OST ♀ x (OSTxOST) ♂	-1.0556	6.4603	-0.163	1.000
BIR ♀ x (BIRxOST) ♂	BIR ♀ x (OSTxBIR) ♂	-3.3158	6.2880	-0.527	0.995
	OST ♀ x (BIRxOST) ♂	1.6316	6.2880	0.259	1.000
	OST ♀ x (OSTxBIR) ♂	2.5263	6.2880	0.402	0.999
	OST ♀ x (OSTxOST) ♂	2.8421	6.3747	0.446	0.998
BIR ♀ x (OSTxBIR) ♂	OST ♀ x (BIRxOST) ♂	4.9474	6.2880	0.787	0.969
	OST ♀ x (OSTxBIR) ♂	5.8421	6.2880	0.929	0.938
	OST ♀ x (OSTxOST) ♂	6.1579	6.3747	0.966	0.928
OST ♀ x (BIRxOST) ♂	OST ♀ x (OSTxBIR) ♂	0.8947	6.2880	0.142	1.000
	OST ♀ x (OSTxOST) ♂	1.2105	6.3747	0.190	1.000
OST ♀ x (OSTxBIR) ♂	OST ♀ x (OSTxOST) ♂	0.3158	6.3747	0.050	1.000

n.s. $P > 0.05$, *** $P < 0.001$

Table A48. Results of the generalized linear model (number of female offspring ~ crossing, family= negative binomial) comparing the number of F3 female offspring of F2 male offspring of intra- and interstrain crossings between the *L. distinguendus* strains BIR and STU and wildtype females and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing BIR ♀ x (BIRxBIR) ♂.

	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	2.2029	0.1082	20.359	< 2e-16 ***
BIR ♀ x (BIRxSTU) ♂	0.1205	0.1530	0.788	0.43075
BIR ♀ x (STUxBIR) ♂	0.1597	0.1808	0.884	0.37685
STU ♀ x (BIRxSTU) ♂	0.1988	0.1729	1.149	0.25038
STU ♀ x (STUxBIR) ♂	0.4221	0.1480	2.851	0.00435 **
STU ♀ x (STUxSTU) ♂	0.2240	0.1531	1.463	0.14345

Tukey test for multiple comparisons

		Estimate	Std. Error	z value	Pr(>|z|)
BIR ♀ x (BIRxBIR) ♂	BIR ♀ x (BIRxSTU) ♂	0.12051	0.15295	0.788	0.969
	BIR ♀ x (STUxBIR) ♂	0.15974	0.18076	0.884	0.950
	STU ♀ x (BIRxSTU) ♂	0.19877	0.17293	1.149	0.859
	STU ♀ x (STUxBIR) ♂	0.42212	0.14805	2.851	0.049 *
	STU ♀ x (STUxSTU) ♂	0.22405	0.15313	1.463	0.685
BIR ♀ x (BIRxSTU) ♂	BIR ♀ x (STUxBIR) ♂	0.03923	0.18070	0.217	1.000
	STU ♀ x (BIRxSTU) ♂	0.07826	0.17287	0.453	0.998
	STU ♀ x (STUxBIR) ♂	0.30160	0.14797	2.038	0.318
	STU ♀ x (STUxSTU) ♂	0.10353	0.15306	0.676	0.984
BIR ♀ x (STUxBIR) ♂	STU ♀ x (BIRxSTU) ♂	0.03903	0.19790	0.197	1.000
	STU ♀ x (STUxBIR) ♂	0.26237	0.17657	1.486	0.671
	STU ♀ x (STUxSTU) ♂	0.06430	0.18086	0.356	0.999
STU ♀ x (BIRxSTU) ♂	STU ♀ x (STUxBIR) ♂	0.22335	0.16854	1.325	0.769
	STU ♀ x (STUxSTU) ♂	0.02528	0.17303	0.146	1.000
STU ♀ x (STUxBIR) ♂	STU ♀ x (STUxSTU) ♂	-0.19807	0.14816	-1.337	0.762

n.s. $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table A49. Results of the generalized linear model (number of female offspring ~ crossing, family= quasipoisson) comparing the number of F3 female offspring of F2 male offspring of intra- and interstrain crossings between the *L. distinguendus* strains CAN and STU and wildtype females and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing CAN ♀ x (CANxCAN) ♂.

	Estimate	Std. Error	z value	Pr(>|t|)
Intercept	3.37092	0.11436	29.477	< 2e-16 ***
CAN ♀ x (CANxSTU) ♂	-0.87728	0.21100	-4.158	6.89e-05 ***
CAN ♀ x (STUxCAN) ♂	-1.02272	0.24319	-4.205	5.77e-05 ***
STU ♀ x (CANxSTU) ♂	-0.51190	0.19003	-2.694	0.00831 **
STU ♀ x (STUxCAN) ♂	-0.26660	0.17933	-1.487	0.14033
STU ♀ x (STUxSTU) ♂	0.08105	0.16553	0.490	0.62547

Tukey test for multiple comparisons

		Estimate	Std. Error	z value	Pr(>|z|)
CAN ♀ x (CANxCAN) ♂	CAN ♀ x (CANxSTU) ♂	-0.87728	0.21100	-4.158	<0.001 ***
	CAN ♀ x (STUxCAN) ♂	-1.02272	0.24319	-4.205	<0.001 ***
	STU ♀ x (CANxSTU) ♂	-0.51190	0.19003	-2.694	0.0737
	STU ♀ x (STUxCAN) ♂	-0.26660	0.17933	-1.487	0.6657
	STU ♀ x (STUxSTU) ♂	0.08105	0.16553	0.490	0.9964
CAN ♀ x (CANxSTU) ♂	CAN ♀ x (STUxCAN) ♂	-0.14544	0.27840	-0.522	0.9951
	STU ♀ x (CANxSTU) ♂	0.36538	0.23340	1.565	0.6137
	STU ♀ x (STUxCAN) ♂	0.61068	0.22478	2.717	0.0694
	STU ♀ x (STUxSTU) ♂	0.95833	0.21393	4.480	<0.001 ***
CAN ♀ x (STUxCAN) ♂	STU ♀ x (CANxSTU) ♂	0.51083	0.26286	1.943	0.3676
	STU ♀ x (STUxCAN) ♂	0.75613	0.25524	2.962	0.0350 *
	STU ♀ x (STUxSTU) ♂	1.10377	0.24573	4.492	<0.001 ***
STU ♀ x (CANxSTU) ♂	STU ♀ x (STUxCAN) ♂	0.24530	0.20522	1.195	0.8348
	STU ♀ x (STUxSTU) ♂	0.59295	0.19327	3.068	0.0255 *
STU ♀ x (STUxCAN) ♂	STU ♀ x (STUxSTU) ♂	0.34765	0.18276	1.902	0.3926

n.s. $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table A50. Results of the linear model (number of female offspring ~ crossing) comparing the number of F3 female offspring of F2 male offspring of intra- and interstrain crossings between the *L. distinguendus* strains BIR and SAT and wildtype females and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing BIR ♀ x (BIRxBIR) ♂.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	55.583	5.571	9.978	1.39e-13 ***
BIR ♀ x (BIRxSAT) ♂	-34.583	12.457	-2.776	0.00767 **
BIR ♀ x (SATxBIR) ♂	-32.583	9.649	-3.377	0.00141 **
SAT ♀ x (BIRxSAT) ♂	-27.333	8.808	-3.103	0.00312 **
SAT ♀ x (SATxBIR) ♂	6.340	7.725	0.821	0.41567
SAT ♀ x (SATxSAT) ♂	1.283	7.474	0.172	0.86435

Tukey test for multiple comparisons

		Estimate	Std. Error	t value	Pr(>|t|)
BIR ♀ x (BIRxBIR) ♂	BIR ♀ x (BIRxSAT) ♂	-34.583	12.457	-2.776	0.07405
	BIR ♀ x (SATxBIR) ♂	-32.583	9.649	-3.377	0.01578 *
	SAT ♀ x (BIRxSAT) ♂	-27.333	8.808	-3.103	0.03291 *
	SAT ♀ x (SATxBIR) ♂	6.340	7.725	0.821	0.96040
	SAT ♀ x (SATxSAT) ♂	1.283	7.474	0.172	0.99998
BIR ♀ x (BIRxSAT) ♂	BIR ♀ x (SATxBIR) ♂	2.000	13.646	0.147	0.99999
	SAT ♀ x (BIRxSAT) ♂	7.250	13.065	0.555	0.99304
	SAT ♀ x (SATxBIR) ♂	40.923	12.361	3.311	0.01899 *
	SAT ♀ x (SATxSAT) ♂	35.867	12.205	2.939	0.04982 *
BIR ♀ x (SATxBIR) ♂	SAT ♀ x (BIRxSAT) ♂	5.250	10.422	0.504	0.99556
	SAT ♀ x (SATxBIR) ♂	38.923	9.524	4.087	0.00190 **
	SAT ♀ x (SATxSAT) ♂	33.867	9.322	3.633	0.00757 **
SAT ♀ x (BIRxSAT) ♂	SAT ♀ x (SATxBIR) ♂	33.673	8.672	3.883	0.00358 **
	SAT ♀ x (SATxSAT) ♂	28.617	8.449	3.387	0.01533 *
SAT ♀ x (SATxBIR) ♂	SAT ♀ x (SATxSAT) ♂	-5.056	7.313	-0.691	0.98112

n.s. $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table A51. Results of the linear model (number of female offspring ~ crossing) comparing the number of F3 female offspring of F2 male offspring of intra- and interstrain crossings between the *L. distinguendus* strains CAN and PFO and wildtype females and the subsequent Tukey test for multiple comparisons. The reference contained in the intercept is the crossing CAN ♀ x (CANxCAN) ♂.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	45.52632	4.98624	9.130	1.55e-12 ***
CAN ♀ x (PFOxCAN) ♂	-21.02632	10.17812	-2.066	0.0437 *
PFO ♀ x (PFOxCAN) ♂	-20.52632	7.82306	-2.624	0.0113 *
PFO ♀ x (PFOxPFO) ♂	0.07368	6.96291	0.011	0.9916

Tukey test for multiple comparisons

		Estimate	Std. Error	t value	Pr(>|t|)
CAN ♀ x (CANxCAN) ♂	CAN ♀ x (PFOxCAN) ♂	-21.02632	10.17812	-2.066	0.1730
	PFO ♀ x (PFOxCAN) ♂	-20.52632	7.82306	-2.624	0.0513
	PFO ♀ x (PFOxPFO) ♂	0.07368	6.96291	0.011	1.0000
CAN ♀ x (PFOxCAN) ♂	PFO ♀ x (PFOxCAN) ♂	0.50000	10.72703	0.047	1.0000
	PFO ♀ x (PFOxPFO) ♂	21.10000	10.11687	2.086	0.1666
PFO ♀ x (PFOxCAN) ♂	PFO ♀ x (PFOxPFO) ♂	-20.60000	7.74320	-2.660	0.0469 *

n.s. $P > 0.05$, * $P < 0.05$, *** $P < 0.001$

Cytoplasmic incompatibility

Table A52. Comparison of the occurrence of F1 female offspring in crosses between females and males of the *Spiroplasma*-carrying STU(+) and potentially endosymbiont-infected BIR(+) strains using a 2 x 4 Fisher's Exact Test for Count Data for the group comparison and 2 x 2 Fisher's Exact Test for Count Data followed by Bonferroni correction for single comparisons.

		P value	Significance level after Bonferroni correction
Group comparison		1.197e-06	***
BIR(+) ♀ x BIR(+) ♂	BIR(+) ♀ x STU(+)	1.193e-05	***
	STU(+) ♀ x BIR(+) ♂	0.1498	n.s.
	STU(+)♀x STU(+)♂	0.1483	n.s.
BIR(+) ♀ x STU(+)	STU(+) ♀ x BIR(+) ♂	0.0002763	**
	STU(+)♀x STU(+)♂	0.0004133	**
STU(+) ♀ x BIR(+) ♂	STU(+)♀x STU(+)♂	1	n.s.

n.s. $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table A53. Comparison of the occurrence of F1 female offspring in crosses between untreated females and males of the strains STU(+) and BIR(+) strains in the combinations STU females x STU males, STU females x BIR males, BIR females x BIR males and untreated BIR(+) females with tetracycline-treated STU(-) males using a 2 x 4 Fisher's Exact Test for Count Data for the group comparison.

		P value	Significance level after Bonferroni correction
Group comparison		0.02489	*
BIR(+) ♀ x BIR(+) ♂	BIR(+) ♀ x STUtet(-) ♂	1	n.s.
	STU(+) ♀ x BIR(+) ♂	0.1498	n.s.
	STU(+)♀x STU(+)♂	0.1483	n.s.
BIR(+) ♀ x STUtet(-) ♂	STU(+) ♀ x BIR(+) ♂	0.0254	n.s.
	STU(+)♀x STU(+)♂	0.03977	n.s.
STU(+) ♀ x BIR(+) ♂	STU(+)♀x STU(+)♂	1	n.s.

n.s. $P > 0.05$, * $P < 0.05$

Sperm count

Table A54. Statistical analyses of sperm counts in seminal vesicles and spermathecae of F2 males and females crossed to F2 males in crosses between CAN and STU females and males.

	Statistics
Seminal vesicles	
Parental cross of tested F2 males	
CAN ♀ x CAN ♂	*P* = 3.821e-08 ***
	W = 7251
STU ♀ x CAN ♂	Wilcoxon ranked sum test
CAN ♀ x STU ♂	*P* = 0.5808 n.s.
	W = 5226.5
STU ♀ x STU ♂	Wilcoxon ranked sum test
Spermathecae	
Tested females x parental cross of tested F2 males	
CAN ♀ x (CAN ♀ x CAN ♂) ♂	*P* = 0.1365 n.s.
	W = 660.5
CAN ♀ x (STU ♀ x CAN ♂) ♂	Wilcoxon ranked sum test
STU ♀ x (CAN ♀ x STU ♂) ♂	*P* = 0.4026 n.s.
	t = -0.84294
STU ♀ x (STU ♀ x STU ♂) ♂	Welch two sample t-test

n.s. $P > 0.05$, *** $P < 0.001$