Geographic Mosaic of Coevolution in *Drosophila*-parasitoid Interactions

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General Introduction

Defined as reciprocal evolutionary change between interacting species driven by natural selection, coevolution is the key ecological process governing interactions, organizing communities, and shaping biodiversity (Thompson 1999a, b). The importance of coevolution is gaining more and more recognition in ecological and evolutionary studies, and coevolutionary research is gaining in momentum, especially in the last two decades.

Much of evolution is coevolution, as most species interact with multiple other species. These interactions, furthermore, differ in type and intensity throughout the species range, resulting in selective pressures in some populations but not in others, leading to genetic differentiation between populations. This dynamic coevolutionary process can produce geographic mosaics over timescales sometimes as short as thousands or hundreds of years (Thompson 2009). To explore this coevolutionary phenomenon, ecology, genetics and phylogenetics are brought together in a framework termed the geographic mosaic theory of coevolution (Thompson 2005).

The geographic mosaic theory of coevolution is built on a tripartite hypothesis: 1) interspecific interactions are subjected to a selection mosaic that favors different evolutionary trajectories in different populations; 2) there are coevolutionary hot spots in which reciprocal selection is actually occurring, while in cold spots it is not; and 3) there is a continual geographic remixing of the range of coevolving traits, that results from the selection mosaic, coevolutionary hot spots, gene flow, random genetic drift and local extinction of populations (Thompson 1999a). This theory also gives rise to three ecological predictions: 1) populations will differ in the traits shaped by an interaction; 2) traits of interacting species will be well matched in some communities while

mismatched in others, leading in some cases to local maladaptation; and 3) there will be few species-level coevolved traits (Thompson 1999a).

Ongoing local coevolutionary adaptation creates the basic template for the geographic mosaic theory of coevolution. However, local adaptation and rapid evolution have long been considered unusual, rare events, irrelevant for the understanding of ecological dynamics. Natural selection was considered weak within populations and most populations were considered as being in equilibrium genetic states, reinforcing the overall view that evolution is a slow, sustained, directional change in species. As more and more evidence accumulates to solidify the case of rapid evolution within natural populations, Thompson (2005) argues that nonequilibrium states are probably more common in local populations than equilibrium states, with short term dynamics in nonequilibrium states potentially producing large changes in population-genetic structures over short periods of time. Furthermore, evolution is heritable genetic change within populations, with nothing implying that this change has to be directional. Thus, most of the evolution and coevolution represent local and geographic processes that continually shift populations in one and then the other direction, in an ever changing environment. Much of the evolution is therefore an ecological process that may or may not result in long term change. The resulting geographic mosaic of coevolution links species across regions, and allows pairs and groups of species to continue to interact across millennia (Thompson 2005).

The geographic mosaic theory of coevolution unifies the evolutionary and ecological processes, and stimulates multidisciplinary research. Obtaining different forms of evidence for coevolution involves ecological observations and experiments, studying the phylogeny of interacting species, focusing on phylogeographic patterns

through molecular studies to evaluate population structure and gene flow, and exploring the distribution of coevolutionary hotspots and patterns of trait remixing (Thompson 2005). Linking the studies of comparative phylogeography with coevolution remains a major challenge in coevolutionary research (Thompson 2005).

Different aspects of this theory are explored using various organisms: the floral parasite moth *Greya politella* and its hosts (Thompson 1997), the garter snake *Thamnophis sirtalis* and the newt *Taricha granulosa* (Brodie et al. 2002), Galapagos finches, *Geospiza* spp. (Grant & Grant 2002), the Trinidadian guppy *Poecilia reticulata* (Reznick et al 2001), the Soapberry bug *Jadera haematoloma* feeding on fruit seeds (Caroll et al. 1997), crossbills, *Loxia* spp., the red squirrel *Tamiasciurus hudsonicus* and the lodgepole pine *Pinus contorta* (Benkman et al. 2001) and the human malaria *Plasmodium spp*. and its mosquito vectors *Anopheles* spp. (Niaré et al. 2002). One of the major models for testing the hypothesis of this theory, however, is the coevolutionary interaction between *Drosophila* and their parasitoids.

The term `parasitoid` (Reuter 1913) has been coined to describe the lifestyle of 10-20% of insect species, mainly Hymonoptera, Diptera and Coleoptera, where the successful development of parasites leads inevitably to the death of the host (Fleury et al. 2009). This tight link between interacting parties makes the host-parasitoid system exceptionally suited for the analyses of fundamental ecological and evolutionary processes. Within this group, *Drosophila* and their hymenopteran larval parasitoids arose as model organisms for studying host resistance, parasitoid virulence, the genetic basis and variation of fitness traits, coevolutionary dynamics and local adaptation (Fleury et al. 2009). These parasitoid wasps are koinobiont solitary endoparasitoids that oviposit eggs in the hemocele of host larvae. Wasp larvae hatch inside the drosophilid

host, and progressively consume host tissue until they become third instar, when they turn into ectoparasitoids and consume the host pupae. Methamorphosis occurs inside the host puparium, and adult wasps emerge after about three weeks or later, depending on species and environmental factors. To avoid death by parasitoids, host larvae engage in various strategies, including mounting of an immune response that can lead to successful encapsulation and the death of the parasitoid egg. Wasps, on the other hand, can overcome this response by injecting immune-suppressive factors into the host, or evolving eggs that adhere to host tissue. The term `resistance` refers to the ability of the host to survive an attack by the parasitoid, while `virulence` points to the ability of the parasitoid to defeat host defenses (Kraaijeveld & Godfray 1999).

The majority of the available studies, related to the geographic mosaic of coevolution, focus on *Drosophila melanogaster* and its major parasitoids *Asobara tabida* and *Leptopilina boulardi* (Kraaijeveld & Godfray 1999, Dupas et al. 2003, Pannebakker et al. 2008). Various other communities of drosophilid flies and their parasitoids and their geographic variation, are yet to be explored. This thesis focuses on the *Drosophila* – parasitoid communities in Asia, which, due to their high diversity and distribution across many barriers potentially restricting gene flow, i.e. islands and mountain chains, represent excellent subject for testing the geographic mosaic theory of coevolution.

Chapter I explores the geographic and ecological differentiation in three common fruit-feeding species of *Drosophila*, *D. albomicans*, *D. takahashii* and *D. bipectinata*, and their larval parasitoid *Leptopilina ryukyuensis*, across the islands of the Ryukyu archipelago and Taiwan. This is achieved through studying the phylogeography of these species and unraveling their respective histories in this region,

and by looking into the differentiation in host resistance and parasitoid virulence in these potential host-parasitoid interactions among different islands to check for potential local adaptation and coevolutionary mosaics. Chapter II focuses on *D. bipectinata* widening the geographic scale to most of Asia and Pacific, to encompass the whole range of distribution of this species. Susceptibility of this fly to the parasitoid *L. victoriae*, accessed across a matrix of host-parasitoid populations, captures the geographic mosaic of coevolution in host resistance and parasitoid virulence. Finally, chapter III explores the genetic mechanisms behind the coevolutionary mosaic of *D. bipectinata* and *L. victoriae*, by crossing selected isofemale fly lines and two parasitoid strains. The broader implications of these results related to the coevolution of *Drosophila*-parasitoid interactions are discussed.

Chapter I

Phylogeography and interaction patterns of three common drosophilid species *D. albomicans*, *D. takahashii* and *D. bipectinata*, and a *Drosophila* larval parasitoid *Leptopilina ryukyuensis* in the Ryukyu archipelago and Taiwan

I-1. Introduction

Phylogeographic studies provide the context for understanding the size of `tiles` within the geographic mosaic, and help interpret genetic differentiation among interacting species (Thompson 2005). In any particular interaction, geographic differentiation may arise as a result of differential coevolutionary selection, or mirror the shared history of phylogeographic differentiation. In the second case, many co-occuring species are driven in the same direction by climate and geography. If a trait, for example, shows a strong regional pattern, but there is little or no phylogeographic structure among populations/regions, selection rather than random genetic drift is assumed to be responsible for shaping these regional differences. Phylogeography thus gives a crucial template for differentiating between the effect of gene flow, hybridization, random genetic drift and natural selection in creating the geographic mosaic of coevolution (Thompson 2005).

Host-parasitoid interactions have been the focus of many phylogeographic studies. Althoff and Thompson (1999) are among the pioneers comparing the geographical structure of parasitoids and their hosts using neutral genetic markers. Their

study of the parasitoid *Agathis thompsoni* and the host moth *Greya subalba* showed incongruent patterns of geographic structure in the two species, indicating that the geographical scale at which the interaction evolves may differ for each species involved.

Thereafter some studies have similarly found no relationship between the host and parasitoid phylogeographic structures. Baer et al. (2004) failed to find host-associated lineages in *Diaeretiella rapae* in the Palearctic and Nearctic regions. Althoff (2008) discovered that *Eusandalum* parasitoids are genetically structured based on geographic distance rather than their yucca moth host species, or the *Yucca* plant. Laurin-Lemay et al. (2013) found that the populations of the bruchid parasitoid *Horismenus depressus* are highly structured, unlike its homogenized *Acanthoscelides* beetle host.

Other studies, however, indicate that there are strong links between the parasitoid and host geographical structures. Hayward and Stone (2006) found a similar phylogeographic distribution pattern for both the parasitoid *Megastigmus stigmatizans* and its host oak gall wasp *Andricus kollari*, with parasitoids pursuing their hosts from the Iberian refugia across Europe. Nicholls et al. (2010) further studied the phylogeography and criptic speciation in the two *Megastigmus* species complexes in Western Palearctic, finding stable ancestral populations in the Middle East, and rapidly expanding populations in Europe, a pattern congruent among trophic levels, supporting the Host-tracking hypothesis for community evolution.

Finally, some studies unraveled complex structures in host-parasitoid communities, e.g. the phylogeography of *Pediobius saulinus*, a parasitoid of the horse-chestnut leafminer in Europe, revealed at least five highly differentiated

parasitoid complexes, differing in degrees of host specialization (Hernandez-Lopez *et al*. 2011). However, none of previous comparative host-parasitoid phylogeographic studies focused on drosophilid flies and their parasitoids, in spite of the fact that *Drosophila*-parasitoid interactions are model systems in the host-parasitoid ceovolutionary studies.

There is a lot of potential for studying comparative geographic structures of *Drosophilia* parasitoids in relation to their hosts, especially because many studies have already addressed the phylogeography of different *Drosophila* species. Brito et al. (2002) explored the phylogeography of *D. buzzatii* in Brazil. Wilder and Hollocher (2003) inferred the recent radiation of endemic Caribbean *Drosophila* of the *D. dunni* subgroup. Brehm et al. (2004) studied the *Drosophila subobscura* phylogeography in the Iberian peninsula and North Atlantic islands. Hurtado et al. (2004) compared three sympatric cactophilic species from the Sonoran Desert, *D. patchea*, *D. mettleri* and *D. nigrospiracula*. Reed at al. (2007) defined genetic relationships among populations of sister species of D. *mojavensis* and *D. arizonae*. He et al. (2007) found two genetically significantly diverged lineages of *D. lacertosa* in East Asia with potential cryptic speciation. Similarly, Moraes et al. (2009) discovered two divergent subclades in *D. gouveai* in eastern Brazil. Mirol et al. (2007) studied phylogeographic patterns of *D. montana* from USA, Canada and Finland, and found clear genetic differentiation between these populations. On the other hand Schiffer et al. (2007) discovered no genetic structure in an Austrailian rainforest species, *D. birchii*. Finally, Mirol et al. (2008) inferred a recent demographic expansion in *D. virilis* from a small but stable refugial population.

In this paper we concentrate on three common fruit-feeding species of

Drosophila and their larval parasitoid *Leptopilina ryukyuensis*, across the Ryukyu archipelago and Taiwan. The Ryukyu archipelago is a long chain of approximately 150 subtropical islands stretching 1200 km between the Kyushu island of Japan and Taiwan. The Cenozoic land configuration of this area is considered to have varied extensively and temporarily, involving more than one period of land-bridge connections in various combinations with adjacent land masses, with several hypotheses about the land configuration and the closing of the Tokara gap between the Amami and Osumi island groups, and the Kerama gap separating the Okinawa group and the Miyako/Yaeyama island group.

Islands in the Ryukyu chain show differing drosophilid community structures. Thirty-seven drosophilid species were recorded in the Kume island (Kondo & Kimura 2008) and 95 species in the Iriomote island (Okada 1965, Hirai et al. 2000, Chen & Toda 2001, Itoh et al. 2003, Chen & Aotsuka 2003, 2004). The three species chosen for this study are among the most commonly collected species in the region. *Leptopilina ryukyuensis* is among the most common *Drosophila* larval parasitoids reported from this area, along with *L. pacifica*, *Asobara japonica*, *A. pleuralis*, and *Ganaspis xanthopoda* (Mitsui et al. 2007, Novković et al. 2012).

Drosophila albomicans is a species belonging to the *nasuta* species subgroup of the *immigrans* species group. It is mainly distributed in Southeast Asia, from north-eastern India in the west, through Thailand, China and Taiwan to the Ryukyu islands in the east (Ohsako et al. 1994). mtDNA restriction fragments length polymorphism (RFLP) revealed a remarkable polymorphism in *D. albomicans* populations (Wang et al. 1994). Additionally, *D. albomicans* is reported to have recently expanded northward to the Japanese mainland. The mainland Japanese population is

suggested to have originated in Taiwan, based on the studies of chromosomal arrangements and allozymes (Ohsako et al. 1994). In the Ryukyu islands, *D. albomicans* is present throughout the year, peaking in summer to early autumn, and decreasing in numbers in winter (Hirai et al. 2000, Kondo & Kimura 2008, Novković et al. 2012). This species was mainly collected from the forest floor in Kume island (Kondo & Kimura 2008), and approximately 90% of all Iriomote-island samples came from the forest (Novković et al. 2012). In another survey from this island, however, about one third of the *D. albomicans* samples were collected in domestic areas (Hirai et al. 2000).

Drosophila takahashii is a species belonging to the *takahashii* species subgroup of the *melanogaster* species group. This species is distributed throughout southern Japan and Taiwan (Kimura et al. 1994), and was recorded in high-altitude in India (Parkash et al. 2012). In the Ryukyus, this species is most abundant in winter and spring, and drastically plunges in numbers during summer (Kondo & Kimura 2008, Novković at al. 2012). Hirai et al. (2000) did, however, record a summer peak in abundance. *D. takahashii* can mainly be found in open lands (Kondo & Kimura 2008) and domestic areas (Hirai et al. 2000, Novković et al. 2012).

Drosophila bipectinata, belongs to the *D. bipectinata* species complex, inside the *ananassae* subgroup which belongs to the *melanogaster* species group. *D. bipectinata* is the most widely distributed of the three fly species used in this study, ranging from India, Thailand, Borneo, Philipines and Japan, across New Guinea to Samoa in the Pacific Ocean (Kopp & Barmina 2005). In Ryukyus it is recorded from summer to early winter (Hirai et al. 2000, Kondo & Kimura 2008, Novković et al. 2012). *D. bipectinata* is sampled frequently from open lands and domestic areas (Hirai et al. 2000, Kondo & Kimura 2008, Novković et al. 2012).

Leptopilina is, along with *Asobara*, the best known *Drosophila* parasitoid genus. *L. ryukyuensis* is a larval parasitoid species in the *heterotoma* group, with a reported distribution in Japanese Ryukyu islands, Taiwan and Indonesia (Novković et al. 2011). In previous studies it was reported as *L. victoriae* (Mitsui et al. 2007), but these species have since been shown to be genetically divergent and completely reproductively isolated (Novković et al. 2011).

This study had two main aims, 1) to determine the phylogeography of the three *Drosophila* species and the parasitoid *L. ryukyuensis* in the Ryukyu archipelago and Taiwan, and 2) to explore the differentiation in host resistance and parasitoid virulence in these potential host-parasitoid interactions between different islands. Mitochondrial COI and autosomal *Gpdh* partial sequences are used to establish geographic patterns. Host-parasitoid differentiation is then tested via host acceptance and host suitability experiments. Finally, the implications of the observed patterns are discussed.

I-2. Material and Methods

I-2-1. Sample collection and strains

Flies and wasps were collected from 5 localities in the Ryukyu archipelago and Taiwan, using banana baited traps: Kagoshima (KG: 31° 21' N, 130° 33' E, collected in October 2009), Amami island (AM: 28° 22' N, 129° 30' E, October 2009), Okinawa island (NH: 26° 13' N, 127° 42' E, October 2009), Iriomote island (IR: 24° 19' N, 123° 49'E, October 2009) and Taipei (TP: 25° 2' N, 121° 38' E, June 2010)(Fig. I-1). Numbers of adult flies and wasps used in the analyses are shown in Tables I-2 and I-3.

Laboratory strains of these flies and wasps were successfully established from Amami, Okinawa and Iriomote islands, with the addition of *D. albomicans* and *D. bipectinata* strains from Taipei. To establish laboratory strains, traps containing banana were placed in the field for a period of 5-7 days, and then brought back to laboratory. When host (i.e., Drosophilid) pupae were formed in the containers, they were placed in Petri dishes and then examined for the emergence of flies and wasps (*Leptopilina* individuals are solitary larvo-pupal parasitoids; i.e., parasitized host larvae grow up to pupae from which new adult wasps emerge). *Drosophila* strains were reared on Drosophila medium. Wasp strains were reared on *D. simulans* as host. Rearing and all consequent experiments were conducted at a constant temperature of 23 ºC under a 15 h light-9 h dark condition. Samples from Kagoshima were too few to successfully establish strains, and the strain of *D. albomicans* from Amami island perished during the course of the experiments. Additional samples of *D. albomicans* from Fukuoka, northern Kyushu were collected in September 2011 (FK: 33° 35' N, 130° 22' E).

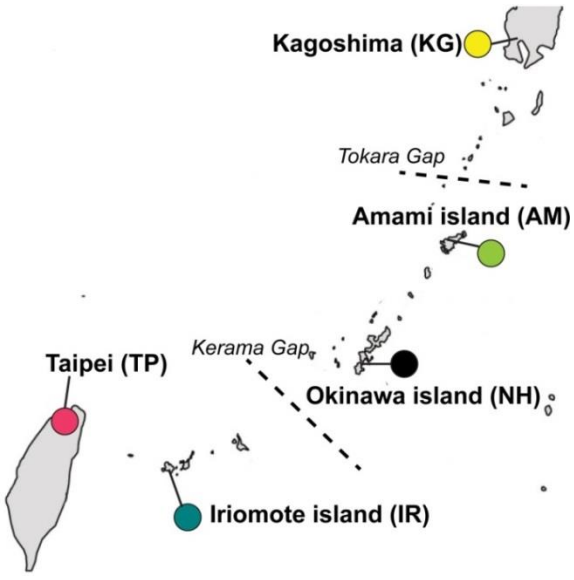


Fig. I-1 Map of Ryukyu archipelago and Taiwan. Circles denote collection sites.

I-2-2. DNA isolation and PCR

Genomic DNA was extracted from each specimen following a modified phenol-chloroform protocol. All amplifications were performed in 23μl reaction volumes containing 1.3 mM $MgCl_2$, 0.042 mM dNTP, 2.6 μM primers, 0.042 U Ampli *Taq* DNA polymerase, and 2.4 μL 10 × PCR buffer. PCR profile consisted of one cycle of denaturation (94°C for 10 min), 35 cycles of denaturation (94°C for 1 min), annealing (50°C for 1 min) and extension (72°C for 1.5min), followed by one cycle of final extension at 72°C for 12min. Amplified products were diluted to 1ng/μL, and used as sequencing templates.

Primers used for amplification of COI and *Gpdh* partial sequences are listed in Table I-1. For all sequence reactions, Big Dye Terminator Cycle Sequencing Kit (ABI) was used. Sequencing was carried out with a 3100 Genetic Analyzer (ABI). *Gpdh* sequences with more than one polymorphic site were re-sequenced utilizing primers that correspond to divergent nucleotide sites. Sequences obtained by different primers were assembled by ProSeq v3.2 (Filatov 2009). *Gpdh* sequences that could not be resolved by direct sequencing using primers, were amplified using PrimeSTAR GXL DNA Polymerase kit following manufacturer`s protocol, blunt-end cloned and sequenced.

I-2-3. Polymorphism and population structure analysis

Partial COI sequences of 611 bp for *D. albomicans*, 612 bp for *D. takahashii* and 574 bp for *D. bipectinata*, and partial *Gpdh* sequences containing 401 bp for *D. albomicans*, 431 bp for *D. takahashii* and 340 bp for *D. bipectinata*, corresponding to part of exon 3,

Table I-1. Primers used for the amplification of COI, and amplification and disambiguation of *Gpdh*

COI	
LCO	5’-GGTCAACAAATCATAAAGATATTGG-3’
HCO	5’-TAAACTTCAGGGTGACCAAAAAATCA-3’
Gpdh	
GNL-mel	5’-GTGGTGCCCCACCAGTTCAT-3’
GNR-mel	5’-GGCTTGAGCTGATTTGTGCA-3’
L4BN	5’-CCATGYGCTGTCTTGATGGG-3’
R4M	5’-ACAGCCGCCTTGGTGTTGTCGCC-3’
bipecti2a	5’-ACGATGTTCTTGAGGGCAA-3’
bipecti2b	5’-ACGATGTTCTTGAGGGCAG-3’
bipecti3a	5’-GCATATTATCACGCGACAT-3’
bipecti3b	5’-GCATATTATCACGCGACAC-3’
bipecti320C	5’-GTTCTTGAGGGCACCACACACCTCG-3’
bipecti320G	5’-GTTCTTGAGGGCACCACACACCTCC-3’
bipecti209C	5’-CAACGAAGTGGCTGAGGGCAAC-3’
bipecti209T	5’-CAACGAAGTGGCTGAGGGCAAT-3’
bipecti209A	5’-CAGCCGATGGTTGTCTCGCARAAA-3’
bipecti209G	5’-CAGCCGATGGTTGTCTCGCARAAG-3’
takaF	5’-TCAAGGGTTTCGACAAGGCCGA-3’
takaR	5’-CAGCTCTCGAAGAAGGTGGACA-3’
albo109A	5’-AGGTGAGTCAATGGACGTTCTTTCA-3’
albo109G	5’-AGGTGAGTCAATGGACGTTCTTTCG-3’
albo128A	5’-CTTTCGTAGTATATTTAGAACACAA-3’
albo128C	5’-CTTTCGTAGTATATTTAGAACACAC-3’
albo162A	5’-CTTTGAAAACCCCTTTGCTAATCTA-3’
albo162C	5’-CTTTGAAAACCCCTTTGCTAATCTC-3’
albo162G	5’-CTTTGAAAACCCCTTTGCTAATCTG-3’
albo162r_C	5’-TATCTGAGCGTGGGAGGAGCGTGTC-3’
albo162r_G	5’-TATCTGAGCGTGGGAGGAGCGTGTG-3’
albo162r_T	5’-TATCTGAGCGTGGGAGGAGCGTGTT-3’

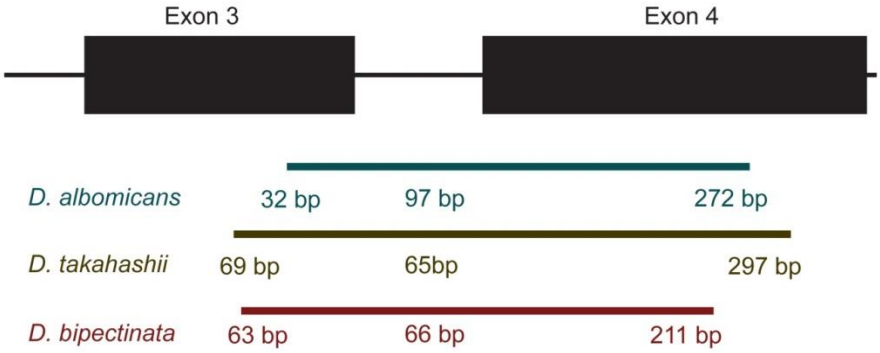


Fig. I-2 Structure of the *Gpdh* gene region and the position of the amplified sequences for *D. albomicans*, *D. takahashii* and *D. bipectinata*

intron and part of exon 4 (Fig. I-2) were used in the analyses.

Haplotype variation within and among populations was assessed using DAMBE5.2 (Xia, 2013). Arlequin 3.0 (Excoffier & Lischer 2010) was used to estimate molecular diversity parameters and pairwise population genetic distances. Significance of F_{ST} values was tested by 1000 permutations. MJ haplotype networks (Bandelt et al. 1999) were constructed using NETWORK 4.6 (fluxus-engineering.com).

I-2-4. Detecting population expansion

To gain more insight into the demographic history of these species, mismatch distribution analyses was employed, based on pairwise differences among individuals in a given population. In populations that have been stationary over a long period of time, these distributions become ragged and erratic, while populations that have passed through a recent demographic expansion have a smooth one-peak mismatch distribution (Harpending 1994). Demographic and spatial expansion models were calculated in Arlequin and fitted with the data (1000 permutations). Tajima`s D statistics (Tajima

1989) and Fu`s Fs (1997) were used to detect deviations from the pattern of polymorphism expected from a neutral evolution model.

I-2-5. Isolation by geographic distance

Genetic distances were expressed through Slatkin`s linearized $F_{ST}$ (Slatkin & Hudson 1991), and the matrices of genetic distances and geographical distances were compared using Mantel`s test (Mantel 1967) with 1000 permutations in Arlequin.

I-2-6. Host acceptance and suitability

To determine host acceptance for parasitoids, second instar drosophilid larvae (up to 80) were placed in a Petri dish (3 cm in diameter) containing a small amount of Drosophila medium. Five wasp females were introduced and left to oviposit for 4 hours. After the removal of wasps, fly larvae were dissected and checked for the presence/absence of wasp eggs. The oviposition rate was calculated as the number of parasitized larvae per total number of larvae.

To estimate host suitability for different wasp species, two-day-old fly larvae were placed in a Petri dish containing a small amount of Drosophila medium and exposed to five wasp females for 24 hours. Thereafter up to 30 larvae were transferred into vials containing Drosophila medium, and additional larvae were dissected to confirm oviposition. Vials with oviposition rates lower than 90% were discarded. The vials were later checked for the emergence of flies and/or wasps.

Host acceptance and suitability data were analyzed using generalized linear models (GLMs) with binomial error and logistic (logit) link function in R statistical

software version 2.13.0 (R Development Core Team, 2009). Significant differences were tested by χ^2 test or Fisher`s exact probability test, followed by Holm`s method for multiple comparisons.

I-3. Results

I-3-1. Nucleotide variation

Sample size, number of haplotypes, number of polymorphic sites per population, haplotype diversity and nucleotide diversity are given in Tables I-2 and I-3. Among the three examined species, *D. albomicans* had the highest number of haplotypes and polymorphic sites, and the highest nucleotide diversity in both COI and *Gpdh* sequences. Forty-eight COI haplotypes were obtained from 101 adult flies. The number of haplotypes within each population ranged from 2 in Kagoshima to 20 in Iriomote island. Of the 58 observed nucleotide substitutions, one was non-synonymous. Average transition:transversion ratio for this species was 13.5. Total nucleotide diversity π was extremely high, 5.85 %. The *Gpdh* partial sequences for 87 samples of *D. albomicans* yielded 7 haplotypes. All substitutions correspond to the intron region. Average transition : transversion ratio was 1.0. Total nucleotide diversity was 2.05%, the highest among the three species.

Thirty-two COI haplotypes were obtained from 84 samples of *D. takahashii*. Number of haplotypes within each population ranged from 5 in Kagoshima and Amami island to 13 in Taipei. One nucleotide substitution resulted in amino acid change. Average transition : transversion ratio was 14.0. Total nucleotide diversity was 2.45%. The *Gpdh* partial sequence of 81 adult flies yielded 2 haplotypes. The single substitution

was located in the exon4 region and was synonymous. Average transition : transversion ratio was 1.0, and the total nucleotide diversity was 0.11%.

The COI partial sequence of 70 individuals of *D. bipectinata* showed 3 different haplotypes, without any non-synonymous mutations. On the other hand, the *Gpdh* sequence of 67 *D. bipectinata* adult flies yielded 10 haplotypes. Number of haplotypes within each population ranged from 4 in Kagoshima to 9 in Okinawa and Iriomote islands. One substitution was located in exon3, and 7 in exon4 region. All substitutions were synonymous. Average transition:transversion ratio was 3.0 for *Gpdh* and 2.0 for COI. Total nucleotide diversity for *Gpdh* was 1.47% and 0.14% for COI.

Table I-2. Sample size (N), no. of haplotypes (H), no. of polymorphic loci (P), haplotype diversity (Hd) and nucleotide diversity (π) for COI.

Species	Population	N	H	P	Hd		π (%)
D. albomicans	KG	2	2	3	1	±0.5000	3
	AM	24	8	16	0.6594	±0.1059	2.424
	NH	24	8	11	0.6957	±0.0954	1.283
	IR	24	20	35	0.9819	±0.0184	6.743
	TP	27	19	27	0.9658	±0.0205	7.100
	Total	*101*					
D. takahashii	KG	17	5	6	0.6912	±0.0753	1.103
	AM	12	5	4	0.803	±0.0777	1.227
	NH	17	8	10	0.8382	±0.0675	2.176
	IR	17	11	13	0.9265	±0.0448	2.853
	TP	21	13	24	0.8905	±0.0604	3.009
	Total	*84*					
D.bipectinata	KG	4	1	0	0	±0.0000	0
	AM	16	1	0	0	±0.0000	0
	NH	20	1	0	0	±0.0000	0
	IR	17	2	1	0.3824	±0.1132	0.382
	TP	13	2	1	0.1538	±0.1261	0.154
	Total	*70*					
L. ryukyuensis	AM	10	1	/	/	/	/
	NH	6	1	/	/	/	/
	IR	21	1	/	/	/	/
	TP	2	1	/	/	/	/
	Total	*39*					

Table I-3 Sample size (N), no. of haplotypes (H), no. of polymorphic loci (P), haplotype diversity (Hd) and nucleotide diversity (π) for *Gpdh*

Species	Population	N	H	P	Hd		π (%)
D. albomicans	KG	2	3	5	0.8333	±0.2224	3.33
	AM	20	3	5	0.5795	±0.0486	2.164
	NH	20	3	4	0.5423	±0.0558	1.892
	IR	24	4	6	0.5842	±0.0374	2.083
	TP	21	6	6	0.77	±0.0382	1.733
	Total	*87*					
D. takahashii	KG	18	2	1	0.0556	±0.0518	0.056
	AM	11	1	0	0	±0.0000	0
	NH	17	2	1	0.3369	±0.0827	0.337
	IR	17	2	1	0.0588	±0.0546	0.059
	TP	18	1	0	0	±0.0000	0
	Total	*81*					
D.bipectinata	KG	4	4	3	0.75	±0.1391	0.929
	AM	16	7	5	0.8528	±0.0280	1.458
	NH	19	9	8	0.8364	±0.0361	1.582
	IR	16	9	7	0.8569	±0.0354	1.718
	TP	12	5	4	0.7464	±0.0525	1.004
	Total	*67*					

The COI sequence of 649 bp was analyzed for 39 individuals of *L. ryukyuensis*. All wasps shared 1 haplotype among all four examined populations.

I-3-2. Genealogical relationship among haplotypes

To gain insight into a more detailed population structure for the three fly species, a Median-joining network was constructed (Figs. I-3-5). *D. albomicans* showed a complex and highly diversified pattern in COI, with a diversity gradient, the southern populations (IR, TP) being much more diverse than the northern ones (KG, AM, NH). The southern and northern population had no shared haplotypes. This pattern was not mirrored in the *Gpdh* network, where the two most common haplotypes were shared by all five populations.

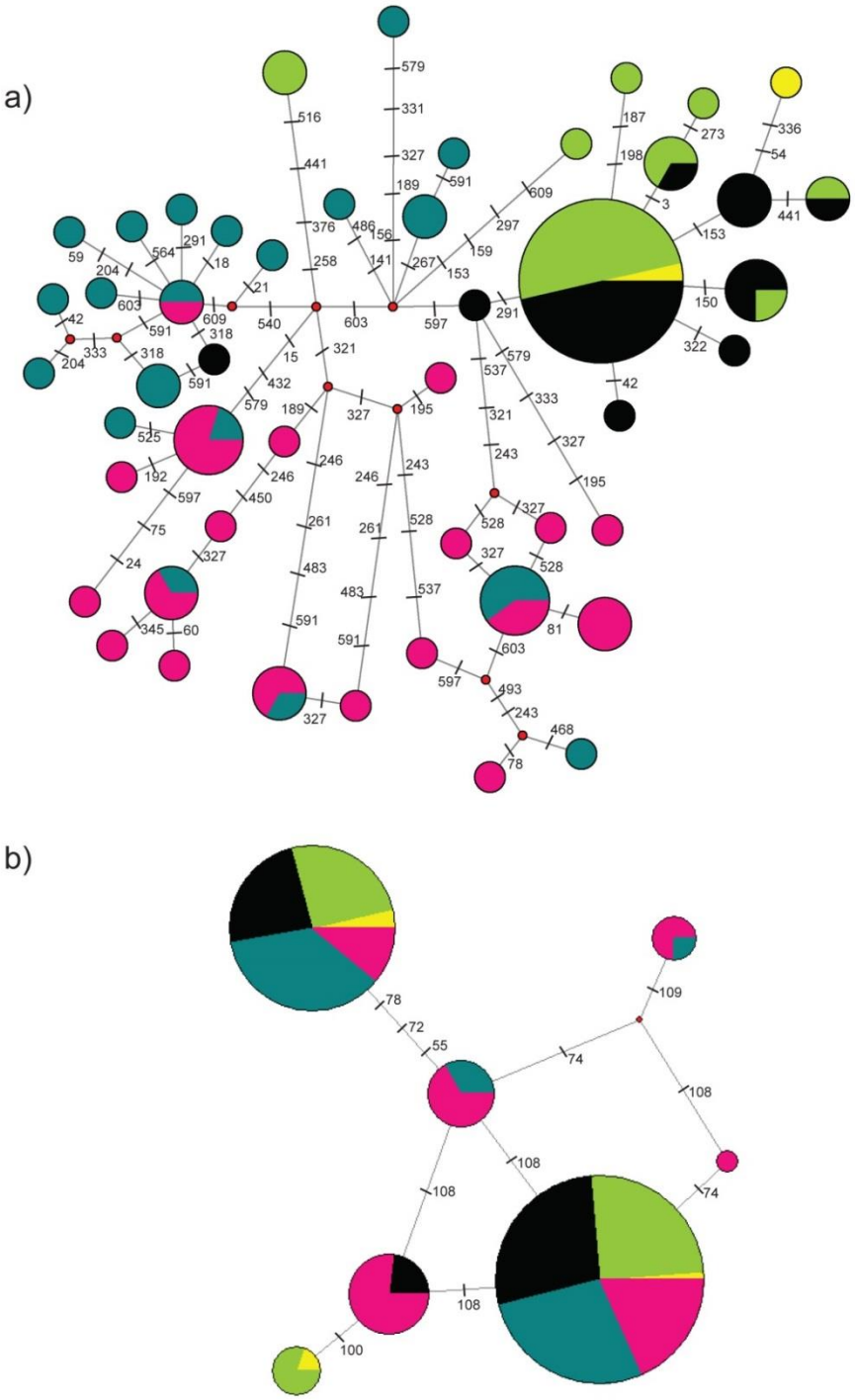


Fig. I-3 MJ network for *D. albomicans*: a) mtDNA haplotype and b) *Gpdh* haplotype lineages. Circles represent haplotypes. Size of the circles corresponds to haplotype frequency. Short intersection lines indicate mutation sites, with numbers representing mutation sites of COI sequences. Colors represent different populations: KG (yellow), AM (green), NH (black), IR (blue) TP (red).

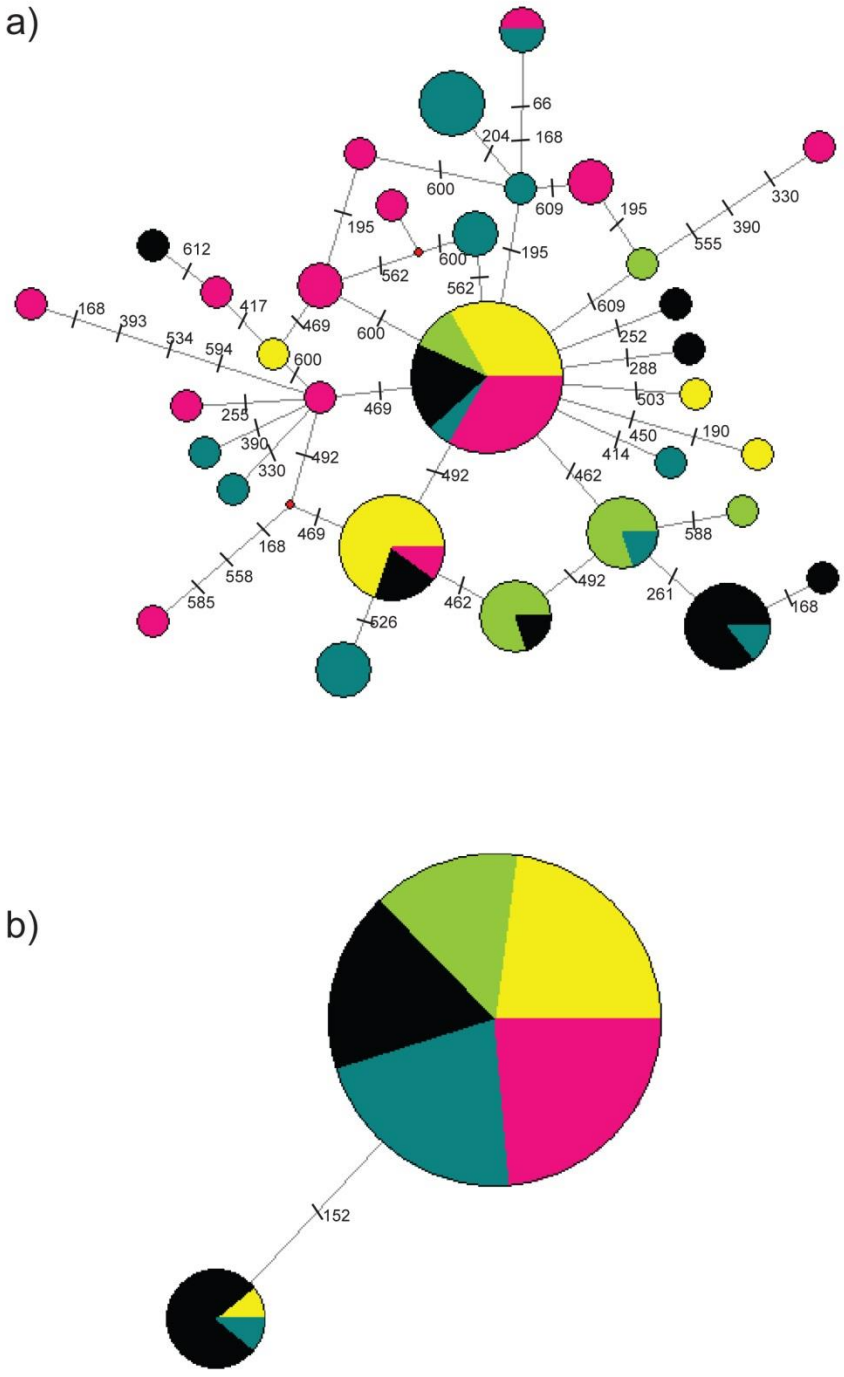


Fig. I-4 MJ network for *D. takahashii*: a) mtDNA haplotype and b) *Gpdh* haplotype lineages. Circles represent haplotypes. Size of the circles corresponds to haplotype frequency. Short intersection lines indicate mutation sites, with numbers representing mutation sites of COI sequences. Colors represent different populations: KG (yellow), AM (green), NH (black), IR (blue) TP (red).

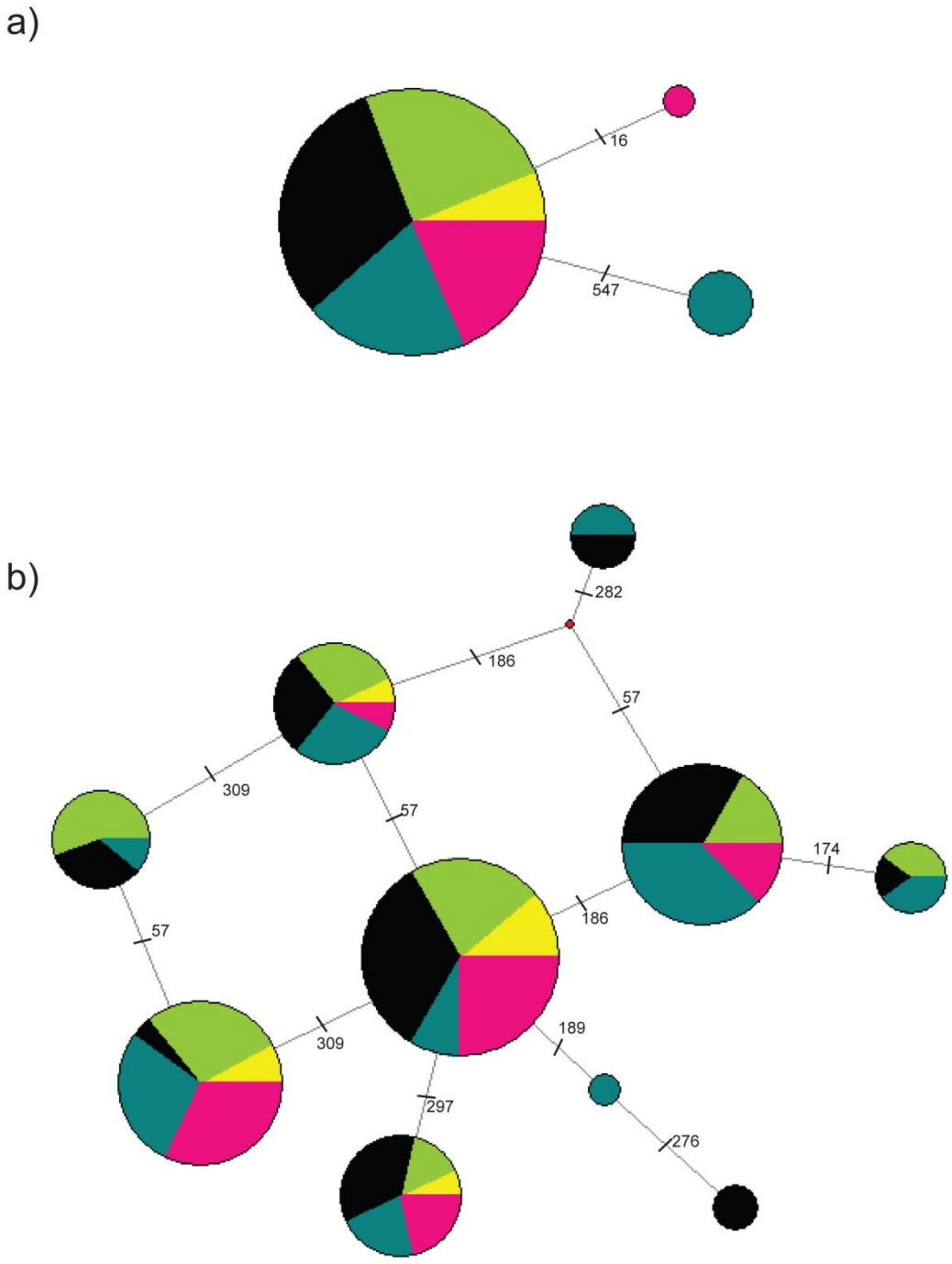


Fig. I-5 MJ network for *D. bipectinata*: a) mtDNA haplotype and b) *Gpdh* haplotype lineages. Circles represent haplotypes. Size of the circles corresponds to haplotype frequency. Short intersection lines indicate mutation sites, with numbers representing mutation sites of COI sequences. Colors represent different populations: KG (yellow), AM (green), NH (black), IR (blue) TP (red).

Drosophila takahashii showed a star-like pattern, with one core haplotype shared by all five populations. Haplotype diversity was highest in the two southern populations (IR and TP). Only two haplotypes were observed in *Gpdh*.

Drosophila bipectinata showed a reverse pattern where haplotype diversity was much lower for COI compared to *Gpdh*. The *Gpdh* network showed a near star-like pattern, with major haplotypes distributed in all sampled populations.

Table I-3. Pairwise F_{ST} values for COI of *D. albomicans*

D. albomicans	Kagoshima	Amami	Naha	Iriomote	Taipei
Kagoshima	0.00000				
Amami	0.03631	0.00000			
Naha	0.18644	0.00607	0.00000		
Iriomote	0.21245*	0.31994***	0.36711***	0.00000	
Taipei	0.24714*	0.36389***	0.40650***	0.09353**	0.00000

* P<0.05 **P<0.01 ***P<0.001

Table I-4. Pairwise F_{ST} values for COI of *D. takahashii*

D. takahashii	Kagoshima	Amami	Naha	Iriomote	Taipei
Kagoshima	0.00000				
Amami	0.30982***	0.00000			
Naha	0.18718***	0.09282*	0.00000		
Iriomote	0.09838**	0.19839***	0.12545**	0.00000	
Taipei	0.07325**	0.22195***	0.13937***	0.03514*	0.00000

* P<0.05 **P<0.01 ***P<0.001

Table I-5. Pairwise F_{ST} values for COI of *D. bipectinata*

D. bipectinata	Kagoshima	Amami	Naha	Iriomote	Taipei
Kagoshima	0.00000				
Amami	0.00000	0.00000			
Naha	0.00000	0.00000	0.00000		
Iriomote	0.01723	0.18025	0.20781*	0.00000	
Taipei	-0.1343	0.01655	0.03465	0.12860	0.00000

* P<0.05 **P<0.01 ***P<0.001

Table I-6. Pairwise F_{ST} values for *Gpdh* of *D. albomicans*

D. albomicans	Kagoshima	Amami	Naha	Iriomote	Taipei
Kagoshima	0.00000				
Amami	-0.07230	0.00000			
Naha	-0.01428	-0.01856	0.00000		
Iriomote	-0.07233	-0.00326	0.00172	0.00000	
Taipei	0.11075	0.06071*	0.05352*	0.10832***	0.00000

* P<0.05 **P<0.01 ***P<0.001

Table I-7. Pairwise F_{ST} values for *Gpdh* of *D. takahashii*

D. takahashii	Kagoshima	Amami	Naha	Iriomote	Taipei
Kagoshima	0.00000				
Amami	-0.01460	0.00000			
Naha	0.12014*	0.14416*	0.00000		
Iriomote	-0.02939	-0.01335	0.11346*	0.00000	
Taipei	0.00000	0.00000	0.18743**	0.00171	0.00000

* P<0.05 **P<0.01 ***P<0.001

Table I-8. Pairwise F_{ST} values for *Gpdh* of *D. bipectinata*

D. bipectinata	Kagoshima	Amami	Naha	Iriomote	Taipei
Kagoshima	0.00000				
Amami	-0.01740	0.00000			
Naha	0.00971	0.03518	0.00000		
Iriomote	0.03720	0.01466	-0.00387	0.00000	
Taipei	-0.05642	0.01921	0.0553*	0.05079*	0.00000

* P<0.05 **P<0.01 ***P<0.001

I-3-3. Population structure and population history

The net interpopulation distance for COI for *D. albomicans* varied from 0.01% between AM and NH to 3.26% between KG and TP. There was significant differentiation between the northern (KG, AM, NH) and southern (IR, TP) populations (F_{ST}=0.34438, P<0.000). The variation between the northern and southern populations represented 32.3% of the total variation within the species (AMOVA). No similar geographical

pattern was observed in *Gpdh*, or in any of the other two species, *D. takahashii* and *D. bipectinata*. The largest net interpopulation difference for *D. takahashi* COI was 0.56% between AM and IR. F_{ST} values for all 3 species are listed in Tables I-3-8.

Table I-9. Polymorphism analyses, neutrality test and mismatch analysis. SSD (sum of squared deviations between the observed and the expected mismatch); τ (scaled time elapsed since the demographic event); *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001

		COI	*Gpdh*
D. albomicans	No. of specimens	101	87
	No. of polymorphic sites	58	7
	Nucleotide diversity π	0.009576±0.00511	0.005103±0.003185
	Neutrality test		
	Tajima`s D	-1.52756*	1.46274
	Fu`s Fs	-25.21435***	2.06776
	Mismatch analysis		
	SSD (*P*-value)	0.012 02 (0.18)	0.10466 (0.1)
	τ(95%CI)	7.80 (3.94-10.35)	5.04 (0.00-6.78)
D. takahashii	No. of specimens	84	81
	No. of polymorphic sites	30	1
	Nucleotide diversity π	0.004000 ± 0.002423	0.000245±0.000453
	Neutrality test		
	Tajima`s D	-1.83817**	-0.42144
	Fu`s Fs	-26.74479***	-0.22013
	Mismatch analysis		
	SSD (*P*-value)	0.00687 (0.55)	0.00008 (0.3)
	τ(95%CI)	1.1 (0.36-1.96)	3.00 (0.29-3.00)
D. bipectinata	No. of specimens	70	67
	No. of polymorphic sites	2	8
	Nucleotide diversity π	0.000240±0.000396	0.004332±0.002923
	Neutrality test		
	Tajima`s D	-1.10330	0.01688
	Fu`s Fs	-1.89220	-1.6938
	Mismatch analysis		
	SSD (*P*-value)	0.00044 (0.3)	0.01123 (0.00)
	τ(95%CI)	3.00 (0.58-3)	1.61 (1.35-1.89)

A unimodal mismatch distribution was obtained for COI and *Gpdh* of *D. takahashii* and *D. bipectinata* (Fig. I-6). *D. albomicans* had a bimodal COI distribution

with the first peak corresponding to the expansion event of the northern populations, and the second to the expansion event of the southern populations (Fig. I-7). There was no significant deviation between the observed and expected distributions (Table I-9), with an exception of *Gpdh* of *D. albomicans*.

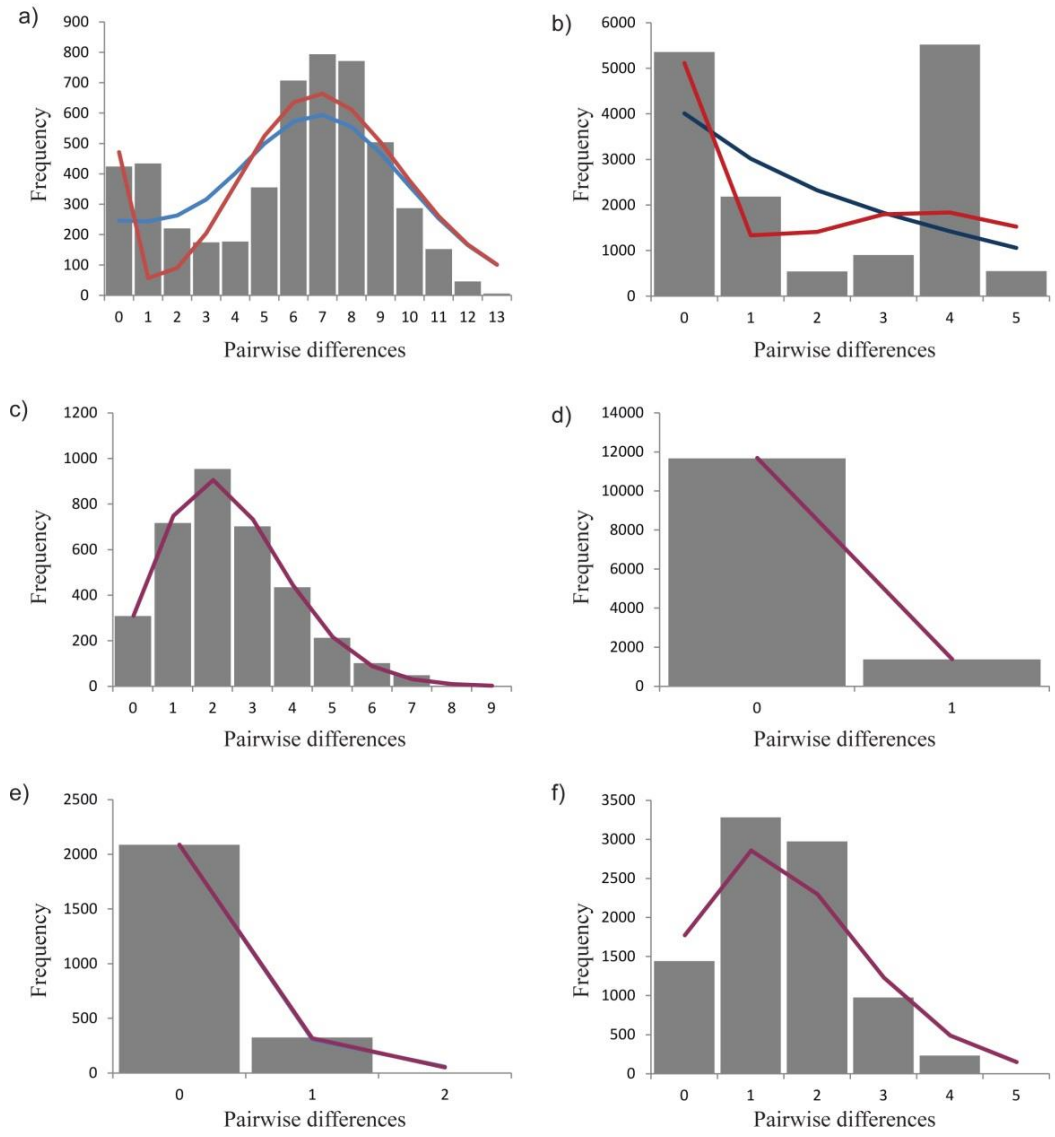


Fig. I-6 Pairwise mismatch distribution for *D. albomicans* COI (a); *D. albomicans Gpdh* (b); *D. takahashii* COI (c); *D. takahashii Gpdh* (d); *D. bipectinata* COI (e); *D. bipectinata Gpdh* (f). Solid histograms represent observed differences, the blue line the expected distribution compatible with a sudden-expansion model, the red line the distribution compatible with the spatial expansion model, and the purple line the distribution compatible with both models.

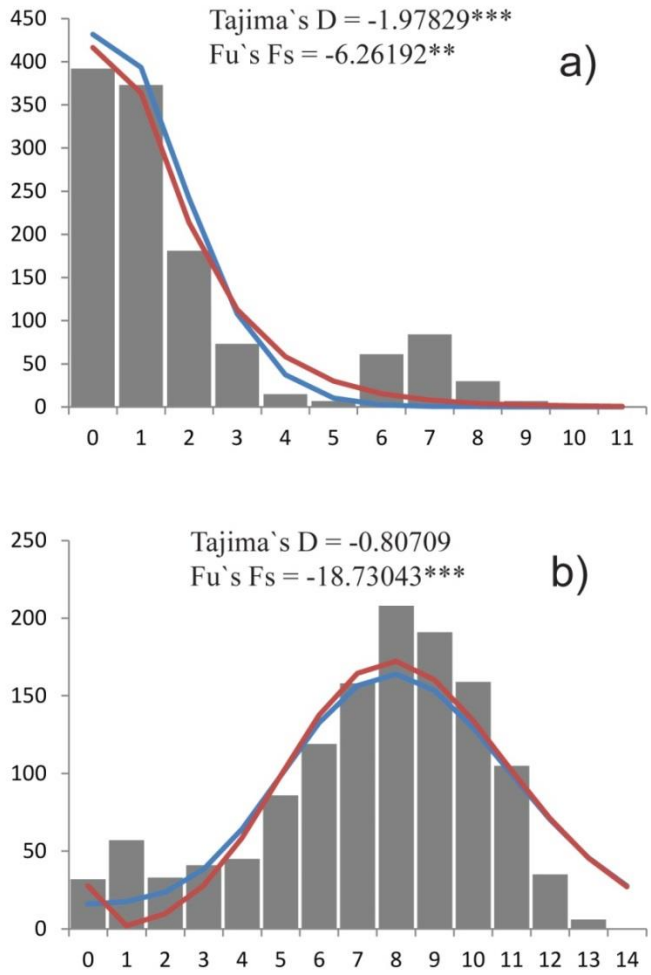


Fig I-7. Pairwise mismatch distribution for a) *D. albomicans* COI northern populations KG, AM and NH and b) *D. albomicans* COI southern populations IR and TP. Solid histograms represent observed differences, the blue line the expected distribution compatible with a sudden-expansion model and the red line the distribution compatible with the spatial expansion model.

Estimates of the parameter τ were used to determine the time elapsed since the expansion events based on mutation rate (μ) of 5.88×10^{-7} mutations per sequence per generation (Su et al. 1999), at 10 generations per year. The population expansion event of *D. takahashii* was estimated to have occurred during the last glacial period of the upper Pleistocene, c. 94 ka (31 – 167 ka). The first expansion event of *D. albomicans*

was estimated at c. 724 ka (497 – 866 ka), corresponding to the interglacial period during the middle Pleistocene, and the second c. 122 ka (15 – 242 ka), corresponding to the Eemian interglacial in the upper Pleistocene. Expansion time was not estimated for *D. bipectinata* due to the low resolution of haplotype differences.

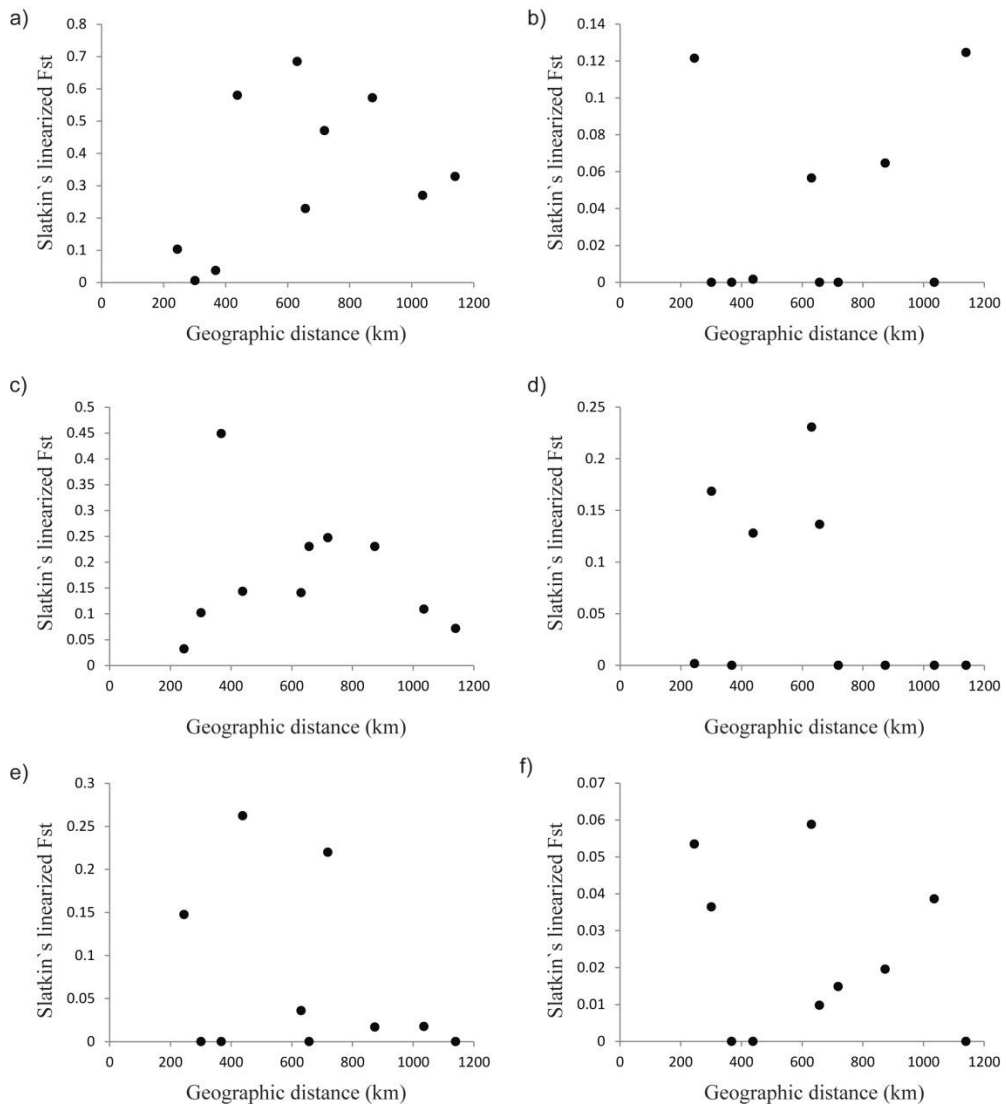


Fig. I-8 Correlation analyses of inter-population genetic distance and geographic distance between pair-wise populations. *D. albomicans* COI (a); *D. albomicans Gpdh* (b); *D. takahashii* COI (c); *D. takahashii Gpdh* (d); *D. bipectinata* COI (e); *D. bipectinata Gpdh* (f).

The significant negative Tajima`s D values (Tajima 1989) and highly significant negative values of Fu`s Fs for COI indicate excess of recent mutations (Fu 1997) and support the occurrence of population range expansion for *D. albomicans* and *D. takahashii* (Table I-9).

The relationship between genetic divergence and geographic distance for populations of all 3 species is shown in Fig. I-8. No significant relationship between genetic divergence and distance was observed for any of the species for neither of the two sequences (Mantel test, 1000 permutations).

I-3-4. Host acceptance and suitability

The host acceptance of *L. ryukyuensis* was highest for *D. albomicans*, with an oviposition rate of over 89% in all strains except the AM strain (Fig. I-9). Neither host nor wasp strains showed any significant inter-population differences in host acceptance or suitability in this interaction.

Leptopilina ryukyuensis host acceptance was lowest for *D. takahashii*, ranging from 3.2% for a combination of the IR wasp strain and the NH fly strain, to 43.6% for a combination of the AM wasp strain and the AM fly strain. The acceptance of *D. takahashii* was significantly higher in the AM wasp strain compared to the NH and IR strains (AM-NH: $P<0.05$, AM-IR: $P<0.01$). Similarly, the AM flies were significantly more readily accepted as hosts compared to the IR flies (AM-IR: $P<0.05$)

The acceptance for *D. bipectinata* was moderately high, with an oviposition rate of over 50% in all cases except for the IR wasp strain ovipositing in the NH fly strain (34.1%). The AM wasp strain had the highest host acceptance rate, with 95%

oviposition in the IR fly strain and 93.33% in the AM fly strain. The IR wasp strain had a significantly lower host acceptance for *D. bipectinata* compared to the AM and NH strains (P<0.001).

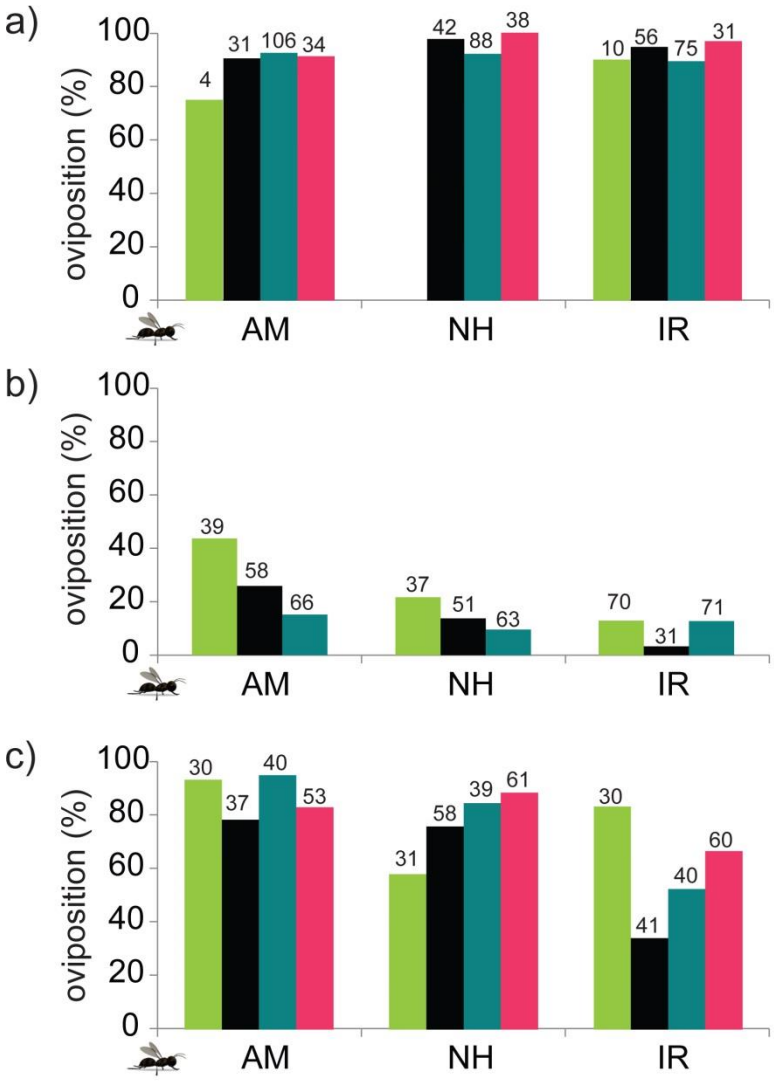


Fig. I-9 Host acceptance AM, NH and IR strains of *L. ryukyuensis* for *D. albomicans* (a), *D. takahashii* (b) and *D. bipectinata* (c). Numbers above each bar indicate the number of hosts tested. Colors represent different fly populations: AM (green), NH (black), IR (blue) TP (red).

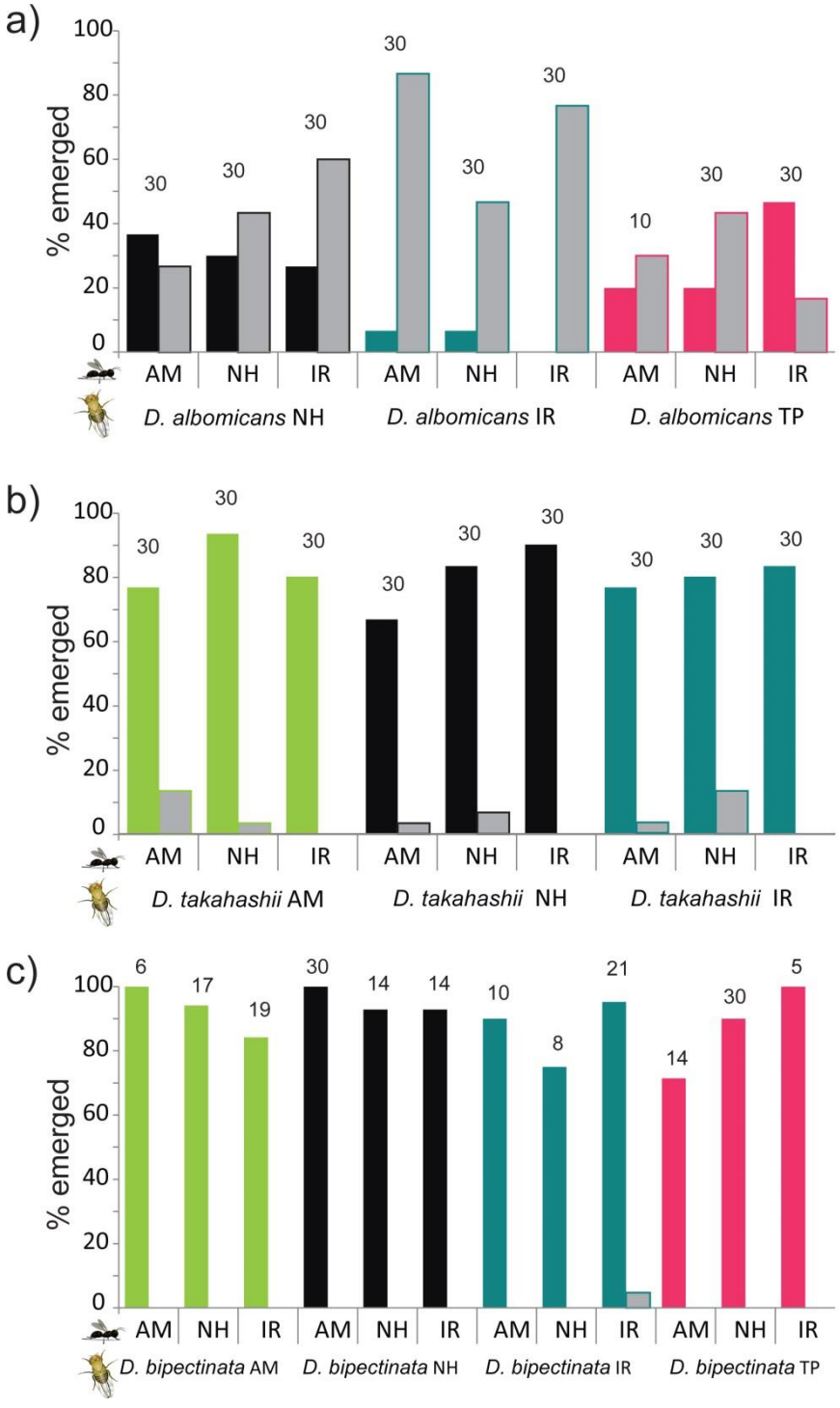


Fig. I-10 Emergence of flies (in color) and wasps (gray) in host suitability experiments. *D. albomicans* (a), *D. takahashii* (b) and *D. bipectinata* (c) measured for three *L. ryukyuensis* wasp strains (AM, NH and IR). Numbers above each bar indicate the number of host larvae tested. Colors represent different fly populations: AM (green), NH (black), IR (blue) TP (red).

Drosophila albomicans was the most suitable host for *L. ryukyuensis*, with wasp emergence ranging from 16.7% for the IR wasps in TP flies, to 86.7% for AM wasps in IR flies (Fig. 1-10). The IR fly population was significantly more suited as a host compared to the NH and TP populations ($P<0.001$).

Drosophila takahashii was not a good host for this wasp, with wasp emergence lower than 13.3% in all tested combinations. No wasps emerged from the IR strain of this fly. *D. bipectinata* was the least suitable host for *L. ryukyuensis*, with a single wasp emerging from the IR fly strain parasitized by IR wasps.

I-4. Discussion

In the Ryukyu archipelago and Taiwan, the observed COI and *Gpdh* haplotype patterns differed greatly among the three *Drosophila* species, while *Leptopilina ryukyuensis* did not show any nucleotide diversity in COI. *D. albomicans* had the highest diversity among the three fly species for both COI and *Gpdh.* Moreover, the diversity of COI was extremely high, with 48 haplotypes recovered from 101 adult flies. This finding is in accordance with the results of Chen et al. (1994) and Wang et al. (1994), who report a remarkable polymorphism in *D. albomicans* populations uncovered by RFLP, with a total of 29 haplotypes recovered from 29 isofemale lines (Chen et al. 1994), and 34 nucleomorphs detected from 82 isofemale lines (Wang et al. 1994). Wang et al. (1994) suggest that a mechanism which maintains mtDNA diversity exists in this fly. They further suggested that the intra-populational polymorphism numerically conceals the extent of differentiation between populations. In this study, however, some populations were highly differentiated with the highest net interpopulation distance of 7.1%

observed between IR and TP. A great divergence was further observed between the northern (KG, AM, NH) and southern (IR, TP) populations of *D. albomicans*. These two areas did not share a single haplotype, indicating a restricted gene flow between these two population clusters. No significant relationship was found between genetic divergence and geographic distance in *D. albomicans*. However, the over 250 km break in the island chain between Okinawa island in the north and Miyako island in the south, representing the Kerama gap, may represent a strong barrier for gene flow in this particular species. This pattern is further supported by the two expansion demographic events, estimated at c.724 ka for the southern and 122 ka for the northern populations. According to the Kizaki and Oshiro`s hypothesis modified by Hikida and Ota (Ota 1998), the Kerama gap was already wide in middle Pleistocene, which corresponds to the first expansion of the southern populations, and may explain why their haplotypes did not spread further north. The northern areas may have been colonized directly from the south or via another route shortly before the second expansion event. The Tokara gap does not seem to be a barrier for this species, as the northern populations spread up to Kagoshima (KG). The additional samples from the northern Kyushu island (FK, Appendix I-1) show, however, that the *D. albomicans* from this population did not originate from southern Kyushu, but probably has its origins in the Taiwanese populations, in agreement with Ohsako et al. (1994).

Drosophila takahashii showed a star-like haplotype network, with a more recent estimated expansion time of c. 94ka. The lowest nuclear *Gpdh* diversity among the three fly species further testifies to the recent expansion of this species.

Drosophila bipectinata had the lowest diversity in COI, also much lower than the diversity found in the autosomal *Gpdh* of this species. This extremely low

mitochondrial diversity and a reverse autosomal – mitochondrial diversity pattern may result from a selective sweep, potentially due to a *Wolbachia* infection (Kopp & Barmina 2005). Ravikumar et al. (2011) found that *D. bipectinata* from India was infected by *Wolbachia* supergroup A, subgroup Mel, whereas *D. albomicans* and *D. takahashii* were *Wolbachia* free. This may explain the differences in mitochondrial diversity of these three species, but *Wolbachia* infection has yet to be looked into for the three species in the Ryukyus.

According to the estimated expansion time and the diversity seen in the autosomal locus, *D. albomicans* is the oldest species in this area, while *D. takahashii* colonized the region most recently. For all three species in this study, the area around Kagoshima represents the northern limit of natural distribution, and the gradient of diversity of COI in *D. albomicans* and *D. takahashii* most likely reflects the colonization history from south to north, with founder and bottlenecks effects in the northern areas, colonized most recently.

What is the reason behind the different phylogeographical patterns in these species, and why does *D. albomicans* show population differentiation and the other two species do not? Additionally, why does the Kerama gap represent a barrier for the dispersal of this species, and not for *D. takahashii* and *D. bipectinata*? It is possible that the dispersal abilities, feeding and breeding preferences, and behavior of these species are different. A similar case was observed in the populations of three sympatric cactophilic *Drosophila* from Sonoran Desert, where *D. patchea* showed differentiation between continental and peninsular populations, with the Sea of Cortez being an effective dispersal barrier to this species, which was not the case for the other two species *D. mettleri* and *D. nigrospiracula*, despite these three species having similar

niches and overlapping distributions (Hurtado et al. 2004). Further studies of the ecology of the three fly species from the Ryukyu archipelago and Taiwan might shed more light on this issue.

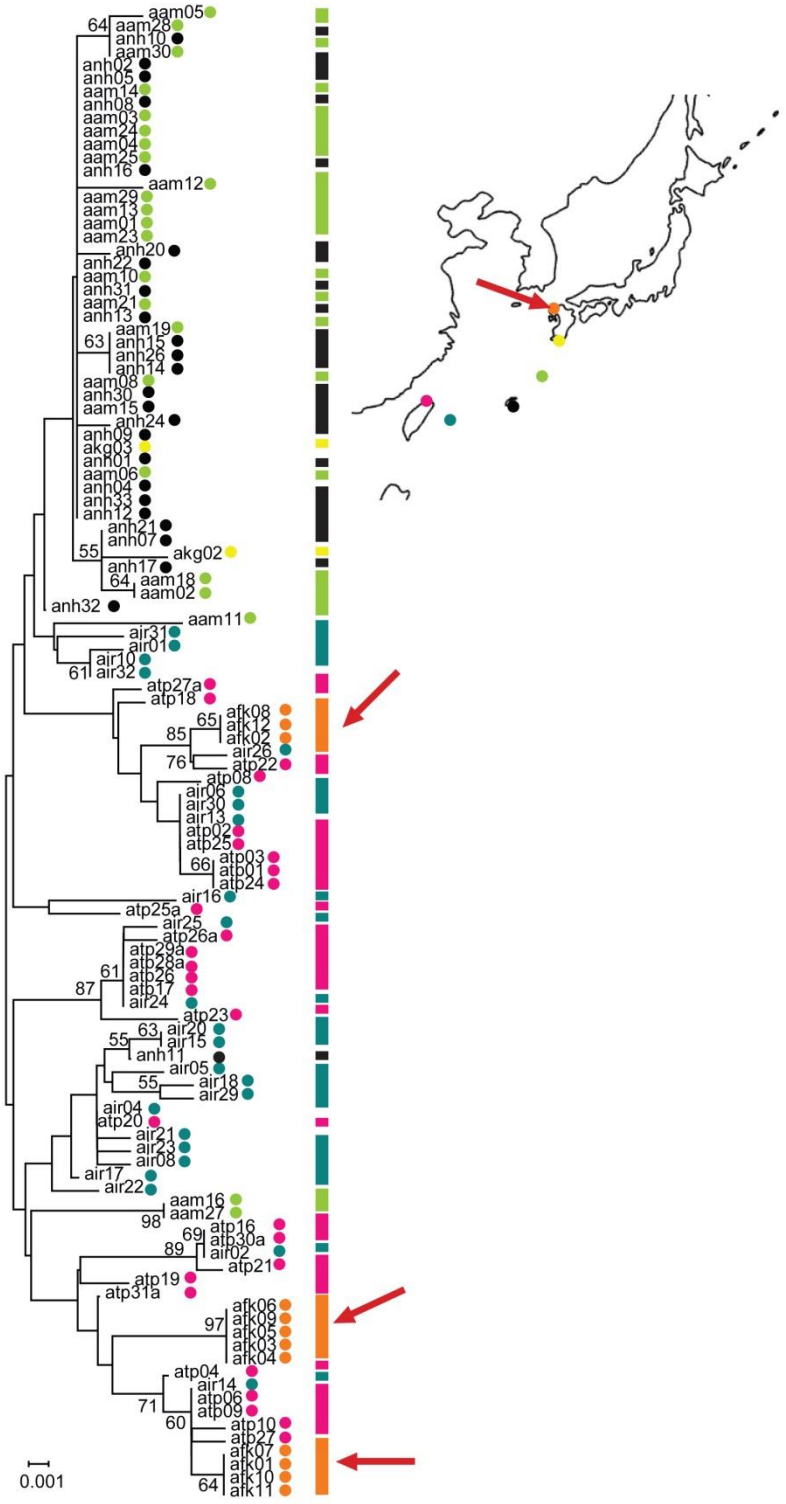
Leptopilina ryukyuensis wasp, which shows no COI diversity, may have colonized the archipelago most recently. This is also supported by the low diversity in ribosomal inter-transcribed spacer sequences of ITS1 and ITS2 observed in this species (Novković et al. 2011).

Host suitability experiments revealed that *D. albomicans* was the most suitable host for *L. ryukyuensis*, while *D. takahashii* and *D. bipectinata* were resistant to this wasp. *L. ryukyuensis* rarely oviposited in the less-suitable host species *D. takahashii*, but readily oviposited in *D. bipectinata* showing a disparity between the virulence and host preference. The evolution of host preference is expected to lag behind the evolution of virulence, and wasps should gradually lose preference for hosts which newly acquired resistance. Accordingly, in the Ryukyu archipelago and Taiwan *D. bipectinata* may have recently acquired resistance to *L. ryukyuensis*, and *L. ryukyuensis* still has to lose its preference for this former host.

Slight differentiation between wasp populations in host acceptance behavior points to coevolutionary interactions in the respective *Drosophila – L. ryukyuensis* communities. The northern AM wasp strain is more likely to accept less or non-suitable hosts, i.e. *D. takahashii* and *D. bipectinata*. The southern IR wasp strain, on the other hand, is least likely to accept these two fly species as hosts. Host preference of IR wasps seems to be better adapted and matched with their virulence. If we assume that this adaptation to readily oviposit in the least resistant host, and oviposit less in the resistant ones, corresponds to the longest coevolutionary host-parasitoid–interactions in

respective populations, these data support a northward expansion of this wasp. It is plausible that *L. ryukyuensis* expanded northwards following its host populations.

Host acceptance and suitability experiments also revealed some differences in fly populations of *D. albomicans* and *D. takahashii*. The *D. takahashii* AM strain was more readily accepted as a host compared to the IR strain. These two localities also had the largest COI net inter-population distance in the phylogeographic analysis. Further, the IR strain of *D. albomicans* was the least resistant and, therefore, more suited as a host for *L. ryukyuensis* than NH or TP strains. Populations of this fly were also highly differentiated as shown by COI haplotypes. While differences in wasp traits did not relate to any observed phylogeographic patterns, more studies encompassing the adjacent geographic regions are needed to establish if the observed fly trait differences are results of phylogeographic events, or due to different coevolutionary selective pressures on different fly populations, resulting from differences in respective host-parasitoid community structures. Further inclusion of additional populations from all over the distribution range of these species may reveal how these patterns formed, and shed more light to their significance in the overall coevolution of these species.



Appendix I-1. Neighbor-joining tree based on COI partial sequences of *D. albomicans* (611bp). Numbers at nodes denote bootstrap values (>50%) for the NJ method. Colors represent different populations: KG (yellow), AM (green), NH (black), IR (blue) TP (red). Arrows indicate the position of haplotypes collected in Fukuoka (orange).

Chapter II

Geographical variation of host resistance and parasitoid virulence in *D. bipectinata* and *L. victoriae*

II-1. Introduction

The coevolution in *Drosophila* - parasitoid interactions has been one of the major models for testing the hypothesis of the geographic mosaic theory. The majority of the available studies focus on *Drosophila melanogaster* and its major parasitoids *L. boulardi* and *A. tabida*. European populations of *Drosophila melanogaster* show a considerable difference in their resistance to *Asobara tabida*, measured through their encapsulation ability, with highest values in central-southern Europe and lower values elsewhere. Resistance is low in northern Europe where *A. tabida* attacks another species, *D. subobscura*, in the Iberian peninsula where this parasitoid is absent, but also in southeastern Europe where *D. melanogaster* is readily attacked by *A. tabida* (Kraaijeveld & Godfray 1999). The northern populations of *A. tabida* have a lower ability to resist encapsulation compared to the south European populations (Kraaijeveld & Godfray 1999). These differences are explained by differences in host community structures. In northern Europe, the major host of *A. tabida* is *D. subobscura*, in which no encapsulation ability has been recorded, while in the south this species is rare, and the main host is the better defended *D. melanogaster* (Kraaijeveld & Godfray 1999). There is further a correlation of virulence with host acceptance behavior, with *A. tabida* females of low virulence rejecting *D. melanogaster* larvae (Kraaijeveld et al. 1995).

Drosophila melanogaster further shows a patchwork distribution of defense when subjected to a test strain of *Leptopilina boulardi* (Kraaijeveld & Godfray 1999). The geographic distribution of the encapsulation pattern under sympatric conditions for this host-parasitoid pair matches the geographic distribution of the virulence phenotype in *L. boulardi* populations, measured by one reference host strain, with high encapsulation and low virulence ability in tropical Africa (Dupas et al. 2003). These differences have also been partially linked to the composition of host communities, with lower virulence towards *D. melanogaster* in more host-diverse communities (Dupas et al. 2003). Geographic variation has been further found in the host-selection behavior of *L. clavipes*, where the southern populations of this parasitoid accept all hosts offered, while the northern populations show lower affinity towards *D. melanogaster*, as, again, a likely adaptation to differences in local host communities (Pannebakker et al. 2008). The species-rich communities of various drosofilid flies and their parasitoids in other geographic regions are yet to be explored in terms of the geographic variation of resistance and virulence traits and local adaptation.

Along with field studies, mathematical models that address the host-parasitoid coevolution have been developed over the years. In a model where one parasitoid species attacks several host species, coevolutionary alternation with or without escalation can arise (Nuismer & Thompson 2006). Natural selection favors escalation of defenses in the most commonly attacked host. It also favors parasitoids that preferentially attack less-defended species. If defenses are costly, selection will further favor decreased defense in the host species that are no longer a major target. Over time, the relative preference of parasitoids will shift, and the relative defenses in the hosts change. In other words, the degree to which a parasitoid population is specialized to a

particular host species will vary in time. Escalation will occur if the preference of the parasitoid evolves faster than the loss of defenses in the hosts that are currently rarely attacked (Nuismer & Thompson 2006; Thompson 2009). Accordingly, some currently unattacked hosts show high levels of defense, as evidence of past defense that has not yet been completely lost, and some currently attacked local host populations show low levels of defense, indicating that the parasitoid has newly shifted towards this species, or that this species was a former host that lost its defenses during the time parasitoid focused its attack on another species (Thompson 2005).

The aim of this study was to capture the geographic mosaic of coevolution using a matrix of populations of a parasitoid *Leptopilina victoriae* and a host *Drosophila bipectinata*. Both of these species are distributed over a wide geographic area and, unlike their more famous counterparts in Europe, the geographic variation of these species has not been studied before. Their wide distribution and dispersion over many Asian islands make them ideal for the study of coevolutionary interactions. Considering that *D. bipectinata* has a resistance mechanism that differs from the encapsulation – melanization mechanism encountered in *D. melanogaster* (personal observation), resistance and virulence of *D. bipectinata* and *L. victoriae* are measured not through encapsulation rates, but through host suitability experiments. Finally, the implications of the observed variation are addressed, and the results are discussed in the light of the hypothesis, predictions and mathematical models of the theory of geographic mosaic of coevolution.

II-2. Material and Methods

II-2-1. Study species

Drosophila bipectinata, is one of the four species in the *D. bipectinata* species complex, inside the *ananassae* subgroup, which belongs to the large *melanogaster* species group. *D. bipectinata* is the most widely distributed in the complex, spanning both Oriental and Australian biogeographic zones, ranging from India, Thailand, Borneo, Philipines and Japan, across New Guinea to Samoa in the Pacific Ocean (Fig. II-1). *D. bipectinata* is widely studied for speciation, reproductive isolation and evolutionary history (Barmina & Kopp 2005; Kopp & Frank 2005; Matsuda, Tomimura & Tobari, 2005). Three subspecies are described within this species: *D. bipectinata bipectinata* from Southeast Asia and Okinawa, *D. bipectinata szentivanii* from Papua New Guinea (Mather &

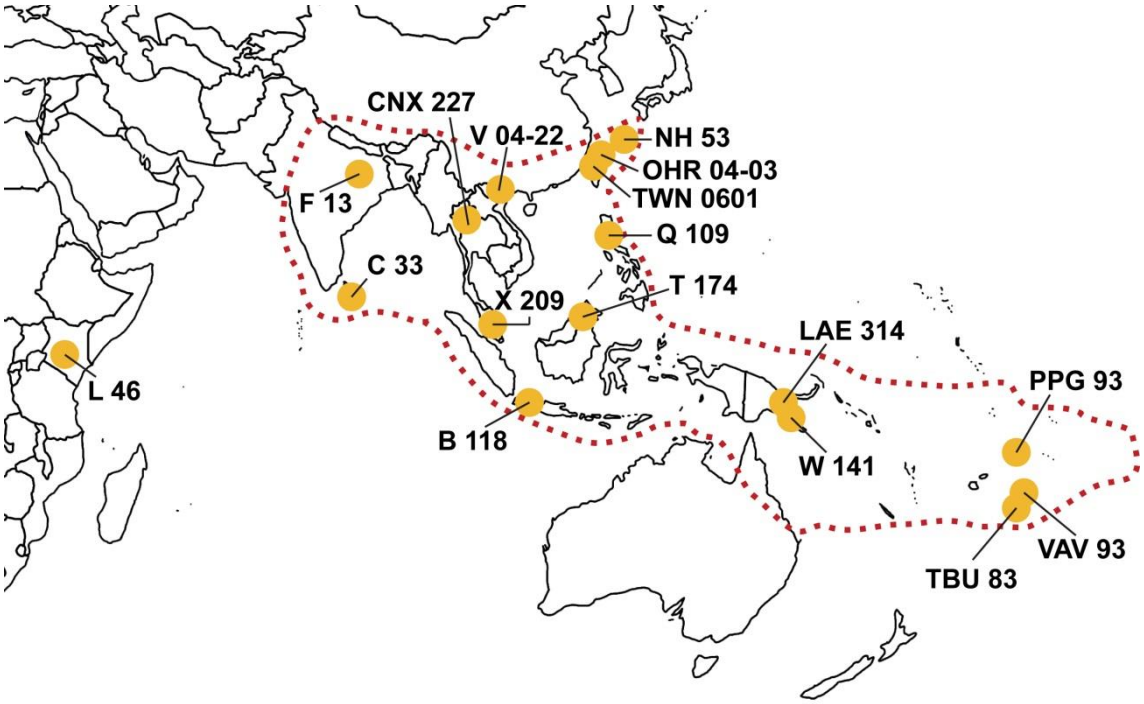


Fig. II-1 Reported distribution of *D. bipectinata* based on Kopp & Barmina 2005; yellow circles denote the 17 strains used in host suitability experiments.

Dobzhansky, 1962) and *D. bipectinata pacificae* in the Pacific islands (Matsuda, Tomimura & Tobari, 2005).

Seventeen isofemale lines of *D. bipectinata* were used in the experiments. All lines were obtained from the Kyorin University. They cover all of the *D. bipectinata* distribution range, and one strain comes from Africa, which is currently not recognized as the natural distribution of this species. Strain names, locality and collection dates are given in Table II-1.

Table II- 1. Location and year of collection for the study strains of *D. bipectinata*

Strain	Country	Locality	Collection date
D. bipectinata bipectinata			
L 46	Kenya	Nairobi	1979
C 33	Sri Lanka	Kandy	1979
F 13	India	Varanasi	1979
CNX 227	Thailand	Chiang Mai	1981
X209	Malaysia	Kuala Lumpur	1979
VT04-22	Vietnam	Hanoi	2004
BG118	Indonesia	Bogor	2008
T174	Malaysia	Kota Kinabalu	1979
Q 109	Philippines	College	1979
TWN 0601	Taiwan	Taipei	2006
OHR 04-03	Japan	Ohara, Iriomote-jima	2004
NH 53	Japan	Naha, Okinawa	2008
D. bipectinata szentivanii			
W141	Papua New Guinea	Wau	1979
LAE 314	Papua New Guinea	Lae	1981
D. bipectinata pacificae			
PPG 93	Samoa	Pago Pago	1981
VAV 93	Tonga	Vav`u	1981
TBU 83	Tonga	Tongatapu	1981

Leptopilina victoriae used in this study belongs to the *L. heterotoma* group. *L. victoriae* is a species with a wide distribution, ranging from Ivory Coast, Kenya, Madagascar, Seychelles, over the islands in the Indian Ocean, to Thailand, Indonesia (Bogor), Malaysia (Kota-Kinabalu) and up to Iriomote-jima in southen Japan (Allemand et al. 2002; Nordlander 1980; Novković et al. 2011). The host recorded under natural conditions is *D. malerkotliana*, and in the case of Iriomote-jima *D. bipectinata*. The two strains used in the experiments originated from Kota Kinabalu, Malaysia (KK: 5° 59' N, 116° 4' E) and Bogor, Indonesia (BG: 6° 35' S, 106° 47' E) (Fig. II-2).

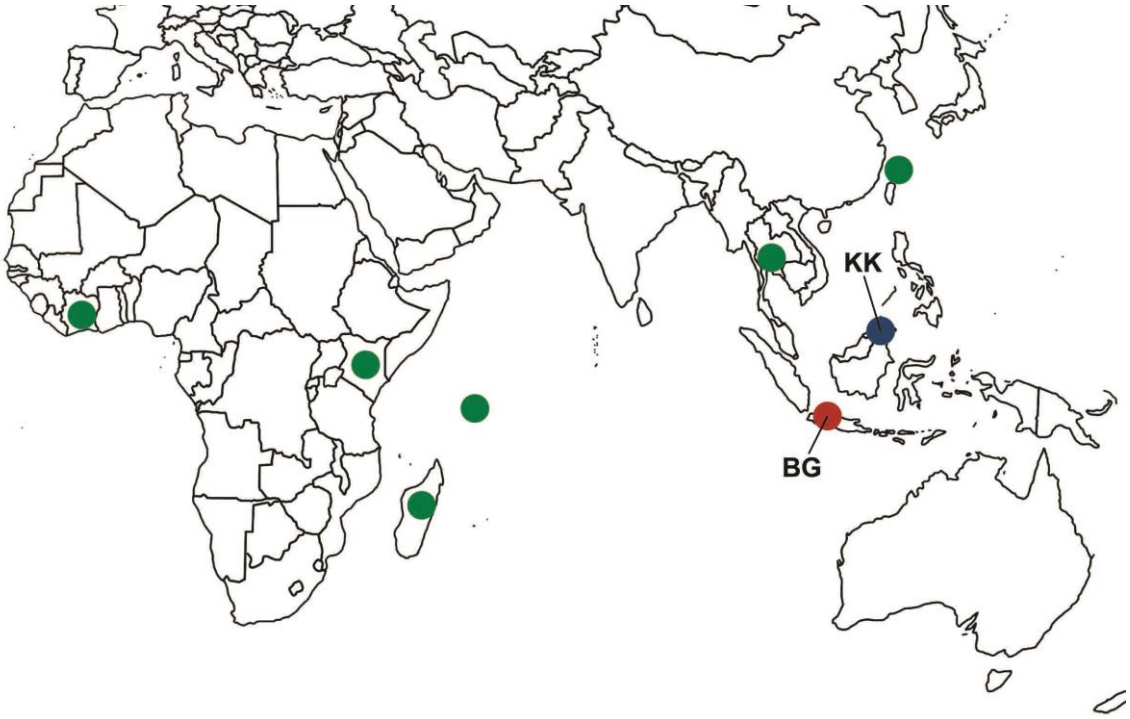


Fig. II- 2. Reported collection sites of *L. victoriae*, and the origin of two study strains (KK and BG).

Wasps for the KK and BG strains were collected in 2008 by M. Kondo and MT Kimura respectively. Wasp strains were reared in laboratory using *D. simulans* as host.

Rearing and all consequent experiments were conducted at a constant temperature of 23 ºC under a 15 h light-9 h dark condition.

II-2-2. Host suitability

To determine host suitability for different wasp species, two-day-old fly larvae were first placed in a Petri dish containing a small amount of rearing medium and exposed to several (2-3) wasp females. Wasps were monitored for oviposition under a stereoscopic microscope. Characteristic oviposition behavior such as full extension of the ovipositor after contact with the host (van Lenteren 1976; van Lenteren et al. 1998), and longer insertions of the ovipositor into larvae (>10s) (Vet & Bakker 1985; Visser 1995; Dubuffet et al. 2006) were taken as successful oviposition indicators. When oviposition was confirmed, parasitized fly larvae were transferred into vials containing Drosophila medium. Some of the larvae were dissected from each strain, to confirm the presence of parasitoid eggs. The vials were later checked for the emergence of flies and/or wasps. Over 100 parasitized larvae were obtained for each fly strain. After 30 days, all the pupae in vials were counted and dissected, and the number of dead pupae/flies/wasps was recorded.

Control experiments were carried out for all strains to account for strain mortality. Fifty *D. bipectinata* larvae were placed under the same conditions as in the host suitability experiments, without being subjected to parasitism, and transferred into vials. Each control experiment was carried out in triplicate.

II-3. Results

In host suitability experiments, the KK strain of *L. victoriae* emerged from all *D. bipectinata* strains, except the strain L 46 from Nairobi (Fig. II-3). Nairobi strain had an emergence of 76.4% In addition to the Nairobi strain, the Bogor (BG 118) strain showed a high resistance to the KK strain of *L. victoriae*, with a fly emergence of 68%. KK was most successful in parasitizing the three strains of *D. bipectinata pacificae*, with a wasp emergence of 62.4 – 71.3 %. No flies emerged in the Tonga TBU 83 strain. Parasitism

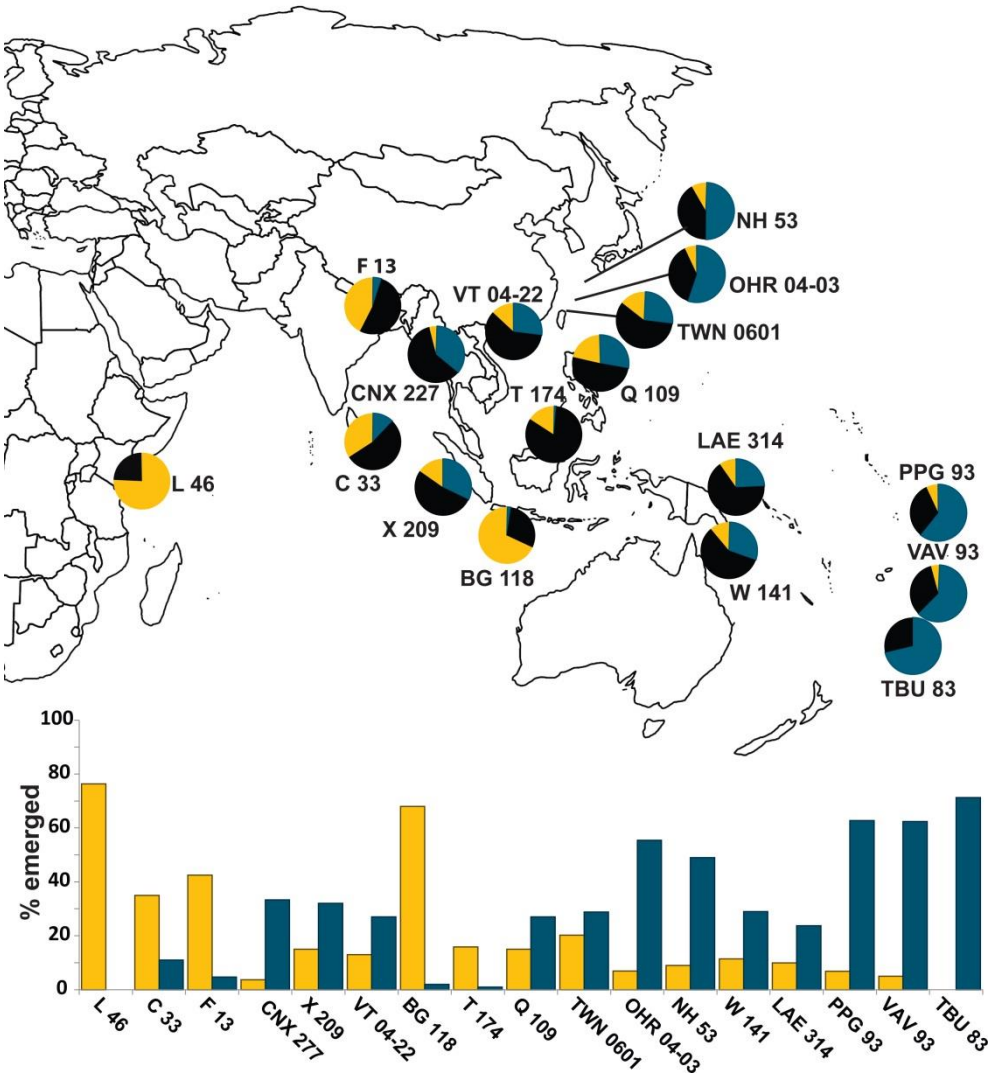


Fig. II-3 Emergence of flies (yellow), KK wasps (blue) and dead individuals (black) in host suitability experiments.

success varied in the *D. bipectinata bipectinata* subspecies with the two highest parasitism rates in Okinawa strains NH 53 (49%) and OHR 04-03 (55.4%).

Wasps of the BG strain emerged from 10 of the 17 wasp strains (Fig. 4). In all *D. bipectinata* strains, wasps emerged at a lower frequency when parasitized by the BG, than when parasitized by the KK strain. All three strains of *D. bipectinata pacificae* were successfully parasitized, with wasp emergence from 44 to 48.5%. BG wasps emerged with very low frequencies from the two strains of *D. bipectinata szentivani* (1

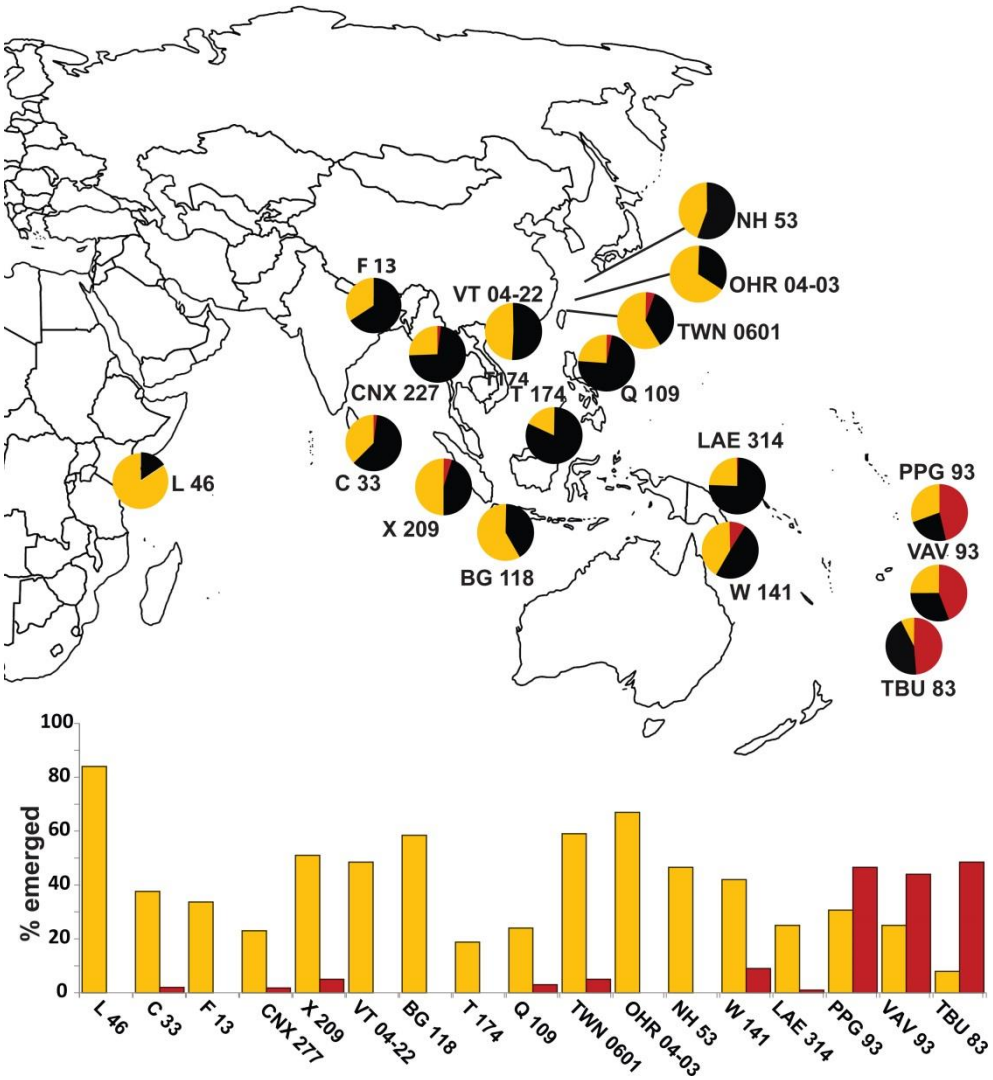


Fig II-4. Emergence of flies (yellow), BG wasps (red) and dead individuals (black) in host suitability experiments.

and 9%), and five of the strains belonging to *D. bipectinata bipectinata* (1.8 to 5%). The two Okinawa strains were completely resistant to this *L. victoriae* strain.

Both KK and BG strains encountered high resistance in their sympatric strains T 174 and BG 118, respectively. The strains T 174 and LAE 314 showed high mortality even when unparasitized, with over 74.7 and 62% mortality, respectively. Over 60% of LAE 314 and 55% of T174 did not reach the pupal stage in host suitability experiments (Appendix II-3). Four other strains, C33, F13, CNX 227 and Q 109, had mortality rates over 50%. Observed high mortality in some strains may result from the fixation of harmful mutations when establishing the isofemale lines. Wasp induced mortality ranged from 0 up to 37.6% (Appendix III-2). In the pooled dataset for all strains combined, flies exposed to KK and BG had similar mortality rates (48.4 and 50.4%). However, flies more often died in the larval stage when exposed to BG, with a 41.2% of total parasitized larvae, compared to 35.4% when exposed to KK. When parasitized by KK, on the other hand, flies died more in the pupal stage (11.9%) than when parasitized by BG (7.6%).

II-4. Discussion

A considerable geographic variation was found in host resistance towards *L. victoriae*. The resistance to the KK strain of *L. victoriae* was overall lower in all *D. bipectinata* strains compared to the resistance to the BG strain. Resistance to KK was highest in the strains from India, Indonesia and Africa, and lowest in the pacific island strains.

Most of the variation was found in the *D. bipectinata bipectinata* subspecies. High host resistance in Africa, India and Indonesia, may be facilitated and selected for

by high parasitoid pressures, due to *L. victoriae* and/or other larval parasitoid species. These tropical areas are known to have a rich parasitoid community, with parasitism rates as high as 80 % (Kimura & Suwito 2012). Mounting a strong defense can topple the possible cost of resistance, in cases where trade-off occurs.

An interesting geographic region is the south of Japan. The strains NH 53 from Okinawa and OHR 04-03 from Iriomote island are completely resistant to the weaker BG strain of *L. victoriae*, but very much susceptible to the KK strain. Novković et al. (2012) observed that *D. bipectinata* remains unparasitized for most of the season in Iriomote island, and that the major parasitoid species in this island cannot successfully parasitize *D. bipectinata* in laboratory experiments. We suspect that *L. victoriae* is not usually present this far north, judging from the only event when *L. victoriae* was recorded, in winter 2003 (Novković et al. 2012). The populations of *D. bipectinata bipectinata* in southern Japan may have found an enemy free space, and escaped the selective pressure for high resistance.

All three strains of *D. bipectinata pacificae* were susceptible to both the KK and BG strains of *L. victoriae*. One possibility is that, in the pacific islands, flies escaped from the parasitoid and evolved inside the enemy-free space, similarly to populations from southern Japan, losing resistance due to its possible cost. Another possibility is that these flies have fixed low resistance during colonization through a bottleneck/founders event. Further sampling in this geographic area is necessary to establish the presence/absence of parasitoids.

In terms of the geographic mosaic theory of coevolution, pacific islands and southern Japan would represent a coevolutionary cold spot. Java and Borneo where the wasp strains originated from, on the other hand, represent an evolutionary hot-spot, with

flies actively selected for resistance against the parasitoid. In these areas, where *D. bipectinata* is highly resistant, evolutionary alternation may be taking place, with *L. victoriae* more readily attacking *D. malerkotliana*, *D. parabipectinata* and *D. pseudoananassae*, the sister species belonging to the *D. bipectinata* species complex (Novković, unpublished data).

A considerable difference was also found in virulence between the two *L. victoriae* strains, BG from Java and KK from Borneo. *L. victoriae* KK was able to emerge from all but one strains of *D. bipectinata*, whereas *L. victoriae* BG, on the other hand, was much less virulent. Models show that in fluctuating unpredictable environments, and due to the fact that not all hosts encounter parasitoids, hosts tend to be generalist, but wasps tend to be partially specialized as they cannot survive without successful parasitization, thus causing an asymmetry in host-parasitoid coevolution (Lapchin 2002). The BG and KK strains of *L. victoriae* may be locally adapted to cope with different host species or populations differing in their levels of resistance. Rapid and repeated shifts in the patterns of specialization can be expected in parasitoid populations that interacts with multiple host species (Nuismer & Thompson 2006). The variation in *L. victoriae* virulence may further indicate an ongoing arms race, leading to an increased selection for virulence.

The geographical variation in *D. bipectinata* and *L. victoriae* resistance-virulence patterns shows that there are different coevolutionary interactions taking place in different geographic regions/communities. Some study areas qualify as hot spots and some as cold spots, and resistance and virulence traits are continually remixed through gene flow, satisfying the tripartite hypothesis of the geographic mosaic theory of coevolution and its predictions. Obtaining further information about the parasitoid and

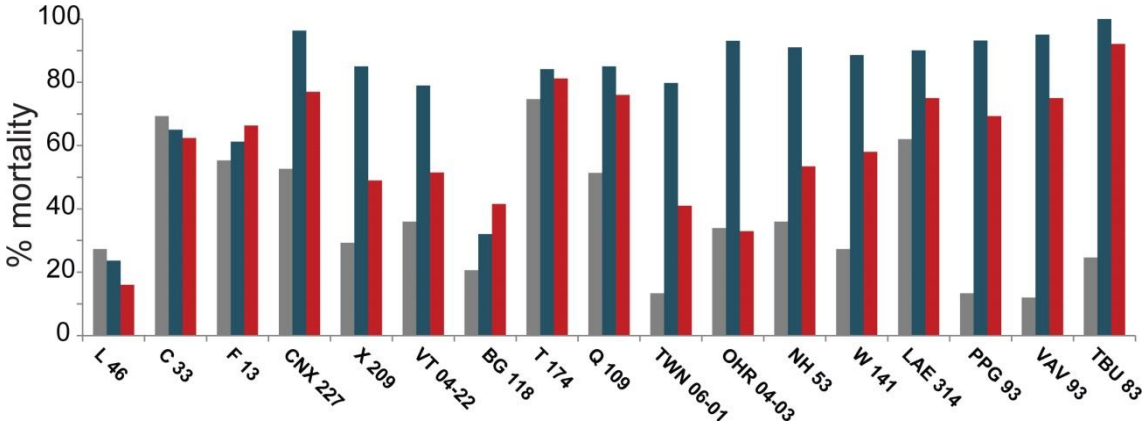
host species building these communities across the whole region would further clarify the coevolutionary mosaic involving *L. victoriae* and *D. bipectinata*.

Appendix II-1. Dissection data for host suitability; N= total number of larvae parasitized; P= number of pupae recovered; F=no. of flies emerged; W= no. of wasps emerged; DF= flies developed but died inside puparium; DW: wasps developed but died inside puparium; DP: dead pupae; %DL: percentage of larvae that did not reach pupal stage; M: mortality of the strain without parasitism

***Leptopilina victoriae* KK**									
Strain	**N**	**P**	**F**	**W**	**DF**	**DW**	**DP**	**%DL**	**M**
L 46	110	92	84	0	2	0	6	16.36	27.33
C 33	100	68	35	11	0	0	23	32	69.33
F 13	116	57	45	5	1	0	8	50.86	55.33
CNX 227	108	55	4	36	1	1	16	49.07	52.67
X 209	100	61	15	32	4	4	6	39	29.33
VT 04-22	100	57	21	27	3	1	4	43	36
BG 118	100	70	68	3	0	0	4	30	20.67
T 174	101	41	16	1	1	1	22	59.41	74.67
Q 109	100	62	15	27	1	4	15	38	51.33
TWN 0601	104	70	21	30	2	5	18	32.69	13.33
OHR 04-03	101	71	7	56	0	0	8	29.7	34
NH 53	100	63	9	49	0	0	5	37	36
W 141	114	61	13	33	1	2	13	46.49	27.33
LAE 314	101	39	10	24	0	1	4	61.39	62
PPG 93	102	95	7	64	0	0	24	6.86	13.33
VAV 93	101	90	5	63	1	0	21	10.89	12
TBU 83	101	85	0	72	1	1	12	15.84	24.67
total	1759	1137	375	533	18	20	209	35.4	48.4

Appendix II-2. Dissection data for host suitability; N= total number of larvae parasitized; P= number of pupae recovered; F=no. of flies emerged; W= no. of wasps emerged; DF= flies developed but died inside puparium; DW: wasps developed but died inside puparium; DP: dead pupae; %DL: percentage of larvae that did not reach pupal stage; M: mortality of the strain without parasitism

***Leptopilina victoriae* BG**									
Strain	**N**	**P**	**F**	**W**	**DF**	**DW**	**DP**	**%DL**	**M**
L 46	100	87	84	0	0	0	3	13	27.33
C 33	101	47	38	2	0	1	6	53.47	69.33
F 13	101	40	34	0	1	0	5	60.4	55.33
CNX 227	113	44	26	2	1	0	15	61.06	52.67
X 209	100	57	51	5	0	0	1	43	29.33
VT 04-22	101	50	49	0	0	0	1	50.5	36
BG 118	101	62	59	0	1	0	2	38.61	20.67
T 174	101	44	19	0	0	0	25	56.44	74.67
Q 109	100	45	24	3	4	0	8	55	51.33
TWN 0601	100	70	59	5	2	0	7	30	13.33
OHR 04-03	100	68	67	0	1	0	0	32	34
NH 53	101	54	47	0	1	0	6	46.53	36
W 141	100	59	42	9	0	1	7	41	27.33
LAE 314	104	34	26	1	0	0	7	67.31	62
PPG 93	101	91	31	47	2	2	9	9.90	13.33
VAV 93	100	90	25	44	2	1	18	10	12
TBU 83	101	73	8	49	2	3	11	27.72	24.67
total	1725	1015	689	167	17	8	131	41.2	50.4



Appendix II-3. Mortality of *D. bipectinata* strains: without parasitism (gray), when parasitized by KK (blue), when parasitized by BG (red).

Chapter III

Genetic aspects of host resistance and parasitoid virulence in *D. bipectinata* and *L. victoriae*

III-1. Introduction

Genetics of resistance and virulence in *Drosophila*-parasitoid interactions has been studied for more than two decades. However, the genetic basis of the fly cellular effector response, the mechanisms mediating the nonspecific recognition process, and the factors underlying target specificity have not been thoroughly investigated (Carton & Nappi 2001). Similarly, there is yet a lot to be revealed about the underlying mechanisms of parasitoid virulence.

Most of the studies of resistance available to date focused on the resistance of *D. melanogaster* against its two parasitoids *L. boulardi* and *A. tabida*. Once inside the host, large eukariotic parasites, such as parasitoid eggs, provoke series of immune responses mediated largely by circulating blood cells – hemocytes. Among the first detectable steps of the encapsulation response are the proliferation, release and differentiation of host hemocytes (Carton et al. 2008). Hemocytes form multilayer capsules around the foreign organisms, engaging in a type of communal phagocytosis (Carton & Nappi 2001, Dubuffet et al. 2009). In *D. melanogaster*, spherical plasmatocytes and large flattened lamellocytes are the main cells involved in cellular encapsulation. Lamellocytes are rarely observed in unparasitized, but proliferate greatly in parasitized larvae, where they increase in numbers via morphological transformation

of plasmatocytes. Finally, crystal cells are involved in the melanogenesis, leading to a fully formed melanotic capsule. Although hemocytes are also responsible for recognition and recruiting (Carton & Nappi 2001), presently no immune-inducing component has been recognized, and the mechanisms leading to the recognition of the invader are largely unknown (Dubuffet et al. 2009). Thus, the resistance of *D. melanogaster* can be said to contain two components, a nonspecific component, mirrored in the number of hemocytes, and a specific component that would correspond to a recognition factor (Dupas &Carton 1999). As a confirmation, lines selected for resistance against *L. boulardi* also increased resistance to *A. tabida*, while the opposite was not the case, leading to a conclusion that improved resistance had a nonspecific component effective against both wasps, and a specific one required for encapsulation of *L. boulardi* (Fellowes et al. 1999).

It is important to note that many *Drosophila* species do not follow this resistance mechanism. The death of the parasitoid is not associated with encapsulation is some *Drosophila* species, and the specific hemocytes that play a key role in capsule formation of *D. melanogaster* are not found in all of *Drosophila* species (Nappi 1970, Eslin & Doury 2006).

Carton et al. (1992) found that the resistance of *D. melanogaster* to *L. boulardi* is governed by a single major segregating locus with two alleles, named Rlb^{+} and Rlb^{-} with the resistant allele dominant to the susceptible one. Similarly, Benassi et al. (1998) found that resistance to *A. tabida* is due to a single major segregating locus with two alleles and complete dominance of the resistant allele, Rat^{+} over Rat^{-}. Thereafter, both of these genes for resistance against *L. boulardi* and *A. tabida* have been precisely localized on the second chromosome, 35 centimorgans apart. In other words, these

genes represent two separate genetic systems, and are not clustered, like the resistance genes to pathogens are clustered in plants (Poirie et al. 2000). *Rat* locus has not been cloned yet (Dubuffet et al. 2009), while there are two possible candidates for *Rlb*, the *mae/edl* gene, encoding for a signaling protein, and CG15086, a gene of unknown function (Hita et al. 1999). mae/edl is involved in proliferation of hemocytes and formation of lamelocytes. Conversely, some studies involving selection found evidence for additive genetic variation in resistance, with the most extreme example of the survival of *D. melanogaster* from *L. boulardi* attack increasing from 0.5 to 50% in about six generations (Fellowes et al. 1998).

Parasitoids counteract host resistance mechanisms by injection of immunosupresive factors, notably venom proteins and virus-like particles during oviposition (Colinet et al. 2012). The aphid parasitoid *Aphidus ervi* showed substantial additive genetic variation in virulence (Henter 1995). Selection experiments in *Drosophila* parasitoids, however, failed to find strong support for additive genetic variation (Kraaijeveld & Godfray 1999). In the *Drosophila* parasitoid *L. boulardi*, immunosuppression has been linked to a single gene, one for each host species. Two non-linked genes for specific virulence of *L. boulardi* against *D. melanogaster* and *D. yakuba* were found (Dupas & Carton 1999; Dupas et al 1998). They were named Immune Suppression ISm and ISy. ISm and ISy are semidominant and recessive, respectively. Semidominance is explained by a threshold model where presence or absence of immune suppression depends on whether parasitoid injects more or less immunosuppressive material (Dupas & Carton 1999). Immune suppressive ability was found to be dominant in *L. heterotoma* (Walker 1959). In the case of these parasitoids, success is more related to the genotype of their mothers than to their own genotype,

where hybrid eggs have the same success of that of the maternal line (Dupas et al. 1998, Dupas & Carton 1999). Natural selection for virulence against one host species does not influence the evolution of virulence against another, and parasitoids are thought to evolve toward narrow host specialization (Dupas et al. 2003). These genes additionally evolve at different costs. Parasitoid invests more in suppression of *D. yakuba* than *D. melanogaster* (Dupas & Boscaro 1999). Virulence genes are thought to evolve in response to the spectrum of host species present in the given locality (Dupas et al. 2003), with less specific virulence in more host-diverse communities. In conclusion, the results available to date support a gene-for-gene model of interaction for *Drosophila* resistance and parasitoid virulence, while selection experiments indicate an additive effect in host resistance.

The aim of this chapter is to offer an insight into the genetic mechanisms of resistance and virulence of *D. bipectinata* and *L. victoriae*, adding a genetic dimension to the geographic mosaic of coevolution encountered in these species. *D. bipectinata* shows some major differences from *D. melanogaster* in the encapsulation response, mainly the absence of melanization, and the late stage of the encapsulation – encapsulating parasitoids at the stage of larvae and not eggs (Novković, unpublished data). Similarly, *L. victoriae* is phylogenetically distant from the well-studied *L. boulardi*. Five *D. bipectinata* isofemale lines from different geographic localities with different resistance properties are crossed to investigate genetic mechanism of host resistance. KK and BG strains of *L. victoriae* are crossed to shed light on the genetics of parasitoid virulence. Possible models of inheritance are discussed, and the effects of egg factors evaluated for these species.

III-2. Material and Methods

III-2.1. Fly Crosses

Five of the 17 isofemale lines of *D. bipectinata* studied in Chapter II were selected for the cross experiments (Fig. III-1). Two of these strains, L 46 and BG 118 (R) showed high resistance towards both wasp strains, and were crossed with three susceptible strains (S), to check for host resistance levels. Among the three susceptible strains, TBU 83 was susceptible to both wasp strains and was used to test resistance towards both KK and BG wasp strains, while NH 53 and OHR 04-03 were susceptible only to the KK strain of *L. victoriae* and were used only in experiments with this strain.

Drosophila have an XY sex-determination system. Crossing-over occurs only in females. Accordingly, parental and F_1 individuals were used in the experiments. To obtain F_1 larvae, 10 virgin females and 10 virgin males were crossed in a vial containing Drosophila medium. Second instar larvae were collected after three days, and

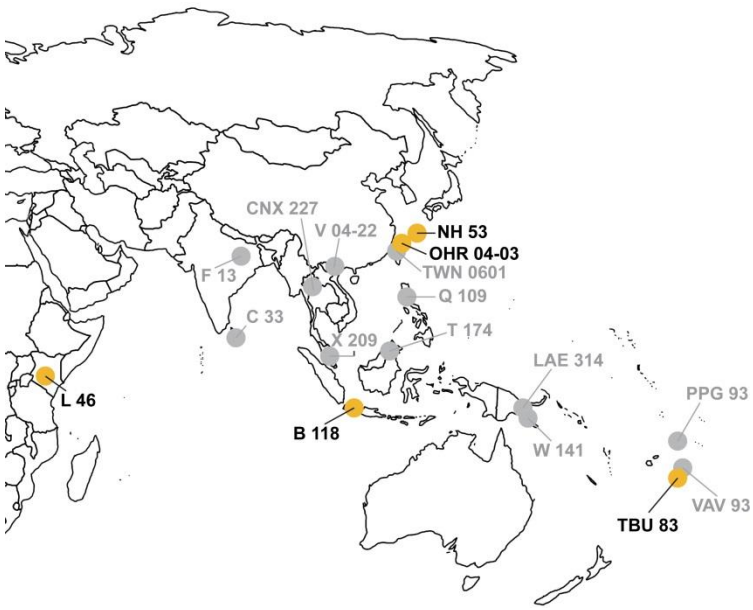


Fig. III-1. Geographic origins of five *D. bipectinata* strains used in fly cross experiments

transferred into a petri dish. Five wasp females, aged 4-11 days were introduced into the petri dish and left to oviposit for 24h. After the removal of wasps, 30 parasitized larvae were collected and transferred into a new vial containing Drosophila medium. 10 additional larvae were dissected to confirm the presence of wasp eggs. In all instances, parasitism rate was above 90%. The vials were regularly monitored for emergence of flies and/or wasps. The number of emerging flies and wasps was recorded, and after 30 days all pupae were dissected and dead flies, wasps and pupae were counted. Every cross experiment was carried out in triplicate. Control experiments were carried out to account for any reduced viability due to the cross itself.

For statistical tests, rates of fly emergence were normalized with arcsine function. The mode of inheritance was tested by contrast analyses of variance (ANOVA) using the method of De Belle & Sokolowski (1987). Following contrasts were considered:

1) resistant (R) versus susceptible (S) parent strain – to test the genetic differentiation between parental strains.
2) F_1 versus F_1 of reciprocal crosses – to check for deviation from an autosomal model of inheritance.
3) R × 2 versus F_1+F_1 - to investigate dominance, assuming that in complete dominance $S<R=F_1$
4) S+R versus F_1+F_1 – to test the semidominant hypothesis ($S<F_1<R$).
5) S × 2 versus F_1+F_1 – to test the recessive hypothesis ($S=F_1<R$).

III-2.2. Wasp Crosses

Similarly to fly crosses, five *D. bipectinata* isofemale lines were chosen for the wasp cross experiments (Fig. III-3): the most resistant strain L46, and four strains that showed

differential susceptibility to the KK and BG wasp strains: VT 04-22, X 209, NH 53 and OHR 04-03.

Wasp virulence can be potentially attributed to two factors, 1) wasp female factors injected with the eggs and venom into the fly larve, and 2) wasp egg/larval factors. Additionally, hymenopterans have an XO sex determination system, with haploid males and diploid females. To account for all of these factors, a more complicated crossing scheme (Fig. III-4) was used. Second instar larvae of five strains of *D. bipectinata* were parasitized for 24h in petri dishes, by five wasp females of the C (control, wasp females crossed with the males of the same wasp strain), V (virgin female wasps of the parental line), P (wasp females that were crossed with males of a different wasp strain), F_1V (virgin females of the F_1 generation), $F_1\times$KK (F1 female wasps crossed with KK males) and $F_1\times$BG (female wasps crossed with BG males).

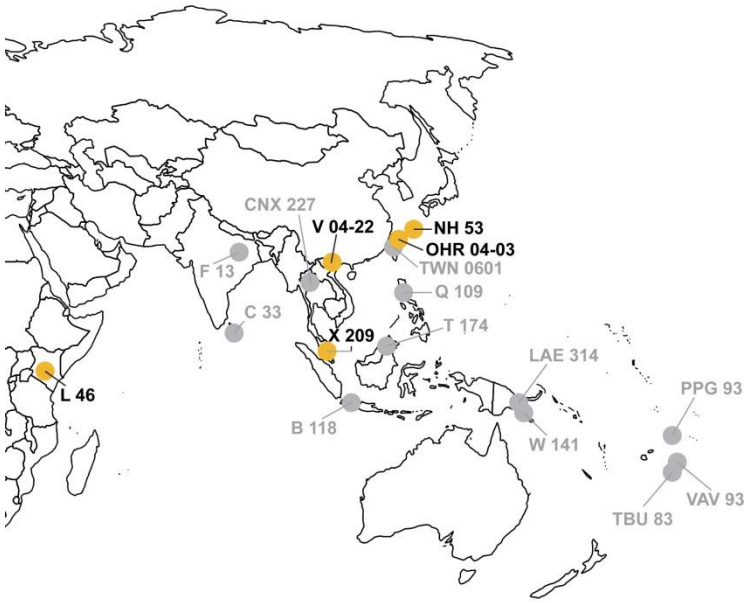


Fig. III-3 Geographic position of five *D. bipectinata* strains used in wasp cross experiments

Thirty parasitized larvae were prepared as described above, and transferred into a new vial containing Drosophila medium, and 10 additional larvae were dissected to confirm the presence of wasp eggs. The vials were regularly monitored for emergence of flies and/or wasps. The number of emerging flies and wasps was recorded, and after 30 days all pupae were dissected and dead flies, wasps and pupae were counted. Each cross experiment was carried out in triplicate.

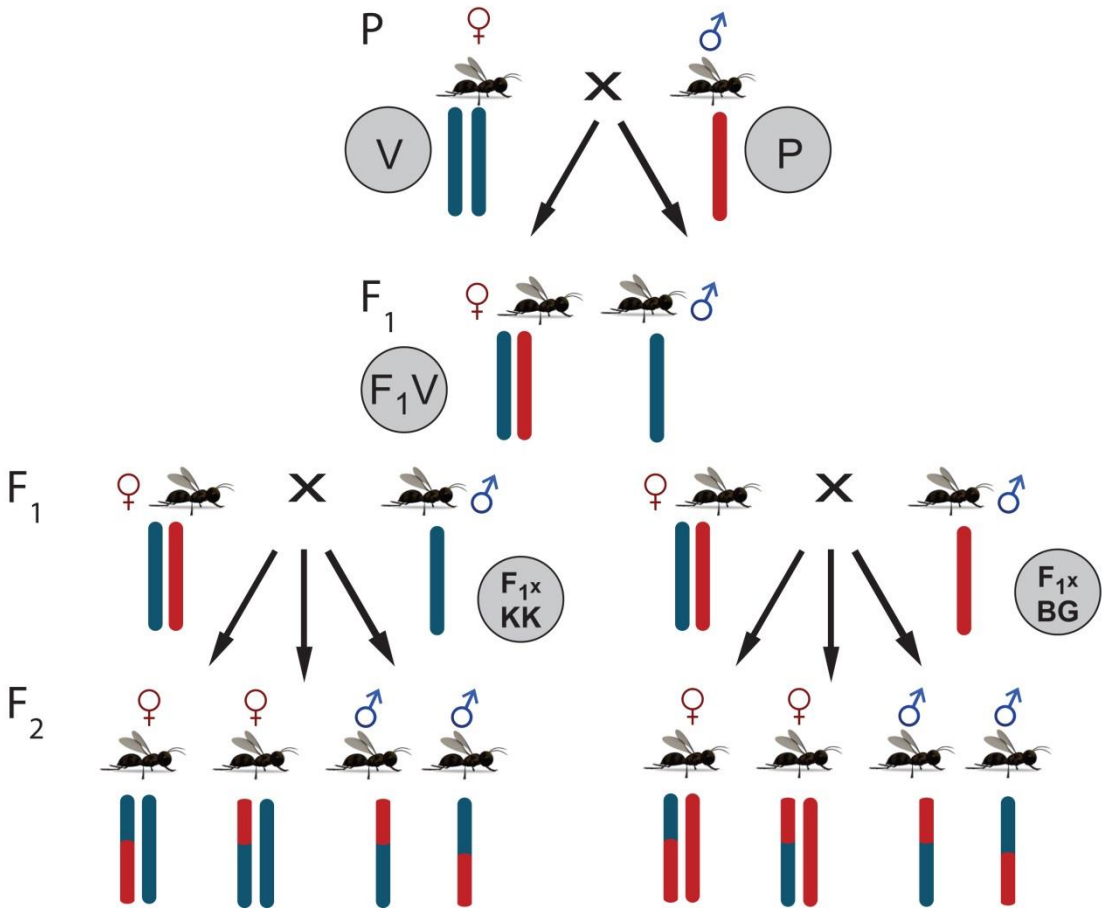


Fig. III-4 Cross scheme for *L. victoriae*.

For statistical tests, rates of wasp emergence were normalized with arcsine function, and the mode of inheritance and the effect of egg factors were tested by contrast analyses of variance (ANOVA):

1. KK versus BG - test of genetic differentiation among parental strains.
2. F_1V versus F_1V of reciprocal crosses – test of deviation from a chromosomal mode of inheritance.
3. KK V$\times$2 versus F_1V+ F_1V – test of the hypothesis of dominance.
4. KKV+BGV versus F_1V+ F_1V – test of the semidominant hypothesis.
5. BGV$\times$2 versus F_1V+ F_1V – test of the recessive hypothesis.
6. C versus P versus V for KK– test of the influence of egg factors for the KK strain.
7. C versus P versus V for BG– test of the influence of egg factors for the BG strain.
8. F_1V versus $F_1\times$KK versus $F_1\times$BG – test of the influence of egg factors in the F_1.

In 6-8. virgins were compared with females mated with virulent KK and avirulent BG wasp males. This allowed for testing for the influence of egg factors, as the genotype of the mother was the same, but the genotype of the eggs differed.

III-3. Results

III-3.1. Genetic aspects of *Drosophila bipectinata* resistance

Significant difference was observed between the parental strains in all crosses (Tables III-1-2). Fly emergence was highest in the BG 118 strain parasitized by BG wasps, and

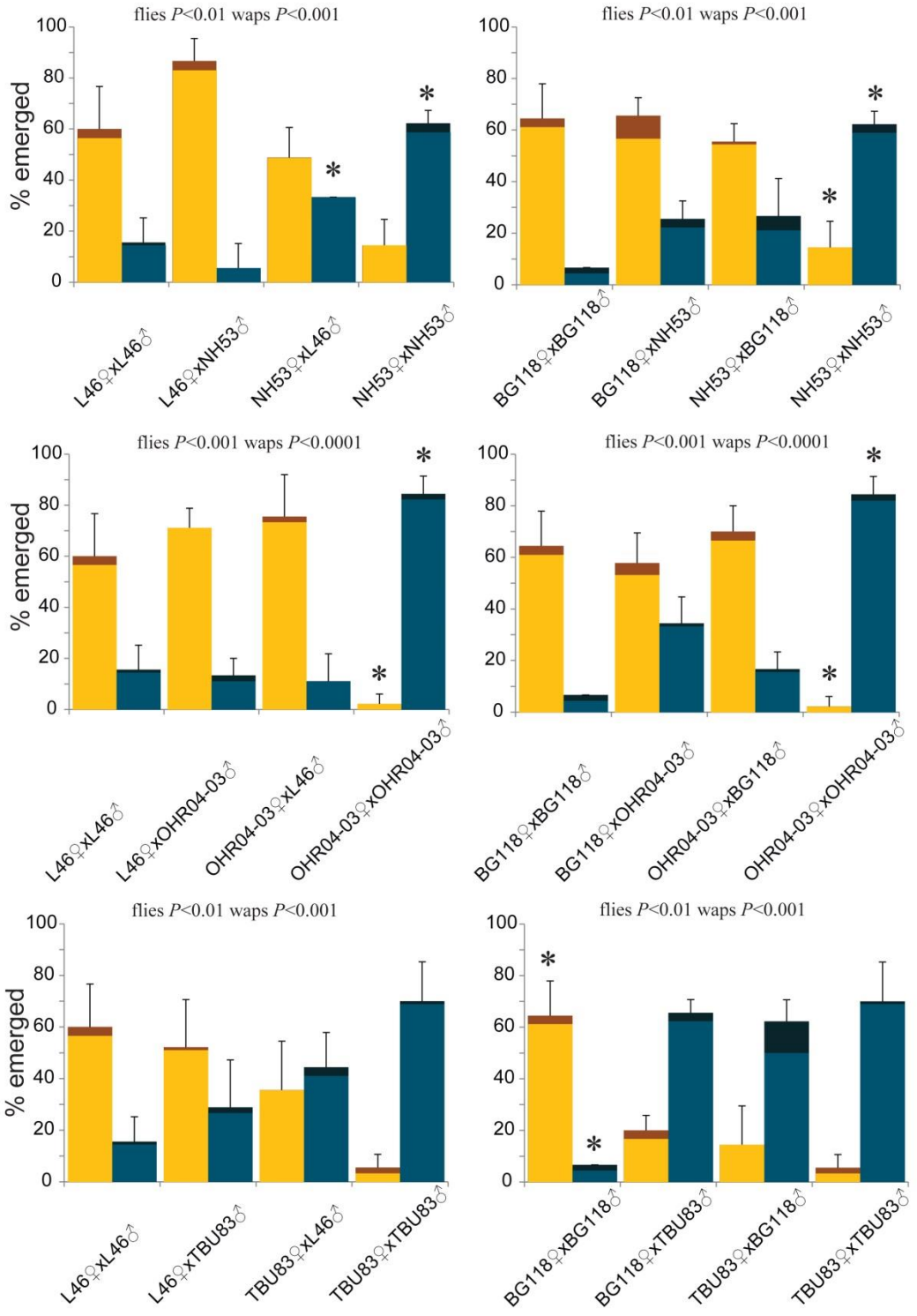


Fig. III-5 Outcomes of parasitism by the KK wasp strain on parental and F_1 individuals. Flies that emerged (yellow), dead flies that remained inside the puparium (brown), wasps that emerged (red), dead wasps that remained inside the puparium (dark red). Average + SD shown. Significant differences by Tukey`s test, after ANOVA shown by asterisk .

lowest in the OHR 04-03 strain parasitized by KK wasps. Deviation from the chromosomal mode of inheritance was significant only in the crosses of L 46 × NH 53, with fly emergence of 83.3 and 48.9% in L 46♀ × NH 53♂ and NH 53♀ × L 46♂, respectively (wasp emergence was 5.6 vs. 33.3%), indicating a possible effect of transient or permanent extra-chromosomal factors.

Fly emergence did not significantly differ from that of the more resistant parental line in F_1 of all crosses except the crosses of BG 118 × TBU 83 subjected to KK wasp parasitism, ranging from 35.6 to 83.3% (Figs III-5-6). In the BG 118 × TBU 83 crosses subjected to KK, fly emergence was 16.7 and 14.4% in BG 118♀ × TBU 83♂ and TBU 83♀ × BG 118♂, respectively. These values were significantly lower than the 61.1% of the more resistant parental BG 118 line.

Deviation from dominance was non-significant, supporting the dominance hypothesis, for all crosses except the crosses involving TBU 83 subjected to KK wasp parasitism. For the L 46 × TBU 83 crosses the semidominant model could not be

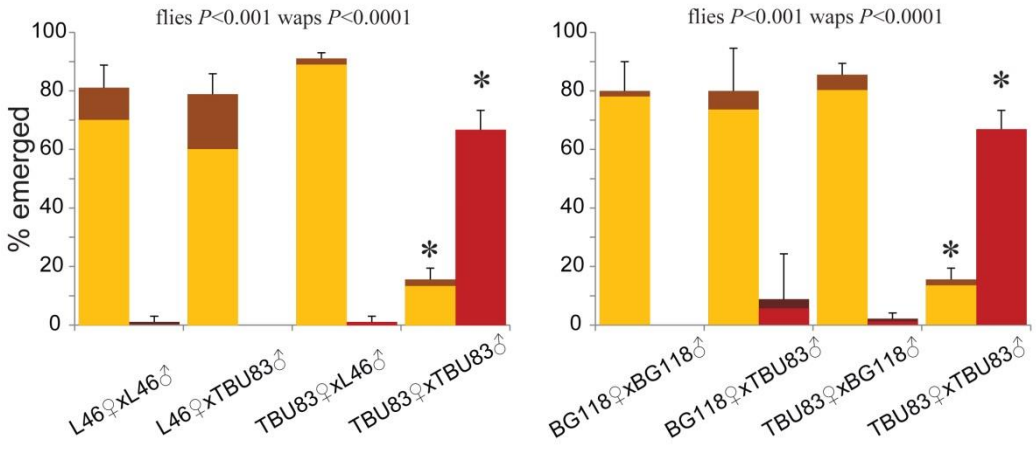


Fig. III-6 Outcomes of parasitism by the BG wasp strain on parental and F_1 individuals. Flies that emerged (yellow), dead flies that remained inside the puparium (brown), wasps that emerged (red), dead wasps that remained inside the puparium (dark red). Average + SD shown. Significant differences by Tukey`s test, after ANOVA shown by asterisk .

Table III-1. Contrast ANOVA of fly emergence from crosses between resistant (L 46, BG 188) and susceptible (NH 53, OHR 04-03, TBU 83) strains of *D. bipectinata* parasitized by the *L. victoriae* KK line. * $P<0.05$ ** $P<0.01$ *** $P<0.001$.

Source	df	SS	MS	*F*	*P*
L 46 × NH 53					
Model (between crosses)	3	0.961	0.320	14.558	**
1. Genetic differentiation between parental strains	1	0.352	0.352	13.574	*
2. Deviation from a chromosomal mode of inheritance	1	0.227	0.227	12.565	*
3. Deviation from the dominant model	1	0.079	0.079	1.510	NS
4. Deviation from the semidomint model	1	0.764	0.764	40.878	**
5. Deviation from the recessive model	1	2.154	2.154	36.559	**
Error (within crosses)	11	1.137	0.103		
L 46 × OHR 04-03					
Model (between crosses)	3	1.794	0.598	29.668	***
1. Genetic differentiation between parental strains	1	0.882	0.882	37.557	**
2. Deviation from a chromosomal mode of inheritance	1	0.001	0.001	0.084	NS
3. Deviation from the dominant model	1	0.168	0.168	2.270	NS
4. Deviation from the semidomint model	1	1.820	1.820	34.2547	**
5. Deviation from the recessive model	1	5.236	5.236	73.8419	**
Error (within crosses)	11	1.955	0.178		
L 46 × TBU 83					
Model (between crosses)	3	0.925	0.308	10.502	**
1. Genetic differentiation between parental strains	1	0.747	0.747	35.376	**
2. Deviation from a chromosomal mode of inheritance	1	0.041	0.041	1.093	NS
3. Deviation from the dominant model	1	0.116	0.116	1.592	NS
4. Deviation from the semidomint model	1	0.273	0.273	5.671	NS
5. Deviation from the recessive model	1	1.924	1.924	31.703	**
Error (within crosses)	11	1.160	0.105		
BG 118 × NH 53					
Model (between crosses)	3	0.551	0.184	13.829	**
1. Genetic differentiation between parental strains	1	0.422	0.422	19.853	*
2. Deviation from a chromosomal mode of inheritance	1	0.001	0.001	0.140	NS
3. Deviation from the dominant model	1	0.020	0.020	0.515	NS
4. Deviation from the semidomint model	1	0.257	0.257	23.013	**
5. Deviation from the recessive model	1	1.337	1.337	20.521	*
Error (within crosses)	11	0.658	0.060		
BG 118 × OHR 04-03					
Model (between crosses)	3	1.489	0.496	28.808	***
1. Genetic differentiation between parental strains	1	0.991	0.991	52.700	**
2. Deviation from a chromosomal mode of inheritance	1	0.028	0.028	1.802	NS
3. Deviation from the dominant model	1	0.001	0.001	0.012	NS
4. Deviation from the semidomint model	1	0.939	0.939	14.356	*
5. Deviation from the recessive model	1	3.860	3.860	51.230	**
Error (within crosses)	11	1.627	0.148		
BG 118 × TBU 83					
Model (between crosses)	3	0.935	0.312	10.288	**
1. Genetic differentiation between parental strains	1	0.847	0.847	51.571	**
2. Deviation from a chromosomal mode of inheritance	1	0.015	0.015	0.350	NS
3. Deviation from the dominant model	1	1.694	1.694	20.513	*
4. Deviation from the semidomint model	1	0.145	0.145	2.548	NS
5. Deviation from the recessive model	1	0.291	0.291	3.280	NS
Error (within crosses)	11	1.178	0.107		

rejected, while for the BG 118 × TBU 83 crosses, deviation from both semidominant and recessive models was non-significant (wasp emergence followed the recessive model, F= 35.606; P=0.208).

Some increase in wasp emergence was observed in all crosses subjected to KK wasps, with the exception of the L46 × OHR 04-03 crosses. In the BG 118 × TBU 83 crosses, wasp emergence was not significantly different from that of the less resistant parental TBU 83 line. In all control crosses, fly emergence ranged from 83.3 to 92.2%, and differed little from that of the original parental lines (85.5 - 96.7%), hence all the crosses were considered viable.

In all crosses subjected to KK wasps, the progeny of L 46 showed higher resistance to wasps, than the progeny of BG 118 when crossed to the same susceptible strains. Similarly, among the susceptible strains, the progeny of the most susceptible strain TBU83 showed the least resistance. Parasitism by the less resistant BG wasp strain resulted in much higher fly emergence compared to the parasitism by KK wasps.

Table III-2. Contrast ANOVA of fly emergence from crosses between resistant (L 46, BG 188) and susceptible (TBU 83) strains of *D. bipectinata* parasitized by the *L. victoria* BG line. * P<0.05 ** P<0.01 *** P<0.01.

Source	df	SS	MS	*F*	*P*
L 46 × TBU 83					
Model (between crosses)	3	1.198	0.399	17.190	***
1. Genetic differentiation between parental strains	1	0.599	0.599	27.682	**
2. Deviation from a chromosomal mode of inheritance	1	0.169	0.169	6.806	NS
3. Deviation from the dominant model	1	0.024	0.024	0.236	NS
4. Deviation from the semidomint model	1	0.861	0.861	25.280	**
5. Deviation from the recessive model	1	2.896	2.896	70.376	**
Error (within crosses)	11	1.384	0.126		
BG 118 × TBU 83	df	SS	MS	*F*	*P*
Model (between crosses)	3	1.144	0.381	26.048	***
1. Genetic differentiation between parental strains	1	0.780	0.780	54.381	**
2. Deviation from a chromosomal mode of inheritance	1	0.009	0.009	0.618	NS
3. Deviation from the dominant model	1	0.002	0.002	0.036	NS
4. Deviation from the semidomint model	1	0.710	0.710	47.546	**
5. Deviation from the recessive model	1	2.978	2.978	189.993	***
Error (within crosses)	11	1.261	0.115		

III-3.2. Genetic aspects of *Leptopilina victoriae* virulence

Significant difference was observed between the parental wasp strains in all crosses (Table III-3). Wasp emergence was highest in the OHR 04-03 strain parasitized by KK wasps (82.2%), and very low or nonexistent in all 5 fly strains parasitized by BG wasps (< 6%). No deviation from the chromosomal mode of inheritance was observed.

Wasp emergence in the F_1 generation ranged from 34.4 - 70% when ovipositing in the NH 53 fly strain, 35.6 - 47.8% in the OHR 04-03 fly strain, 31.1 - 61.1% in the X 209 fly strain, 11.1 - 28.9% in the VT 04-22sly strain and 0 - 8.9% in the L 46 fly strain (Fig. III-7). In all F_1 crosses wasp emergence was significantly higher than that of the BG parent, rejecting the recessive hypothesis, except when wasps oviposited in the L 46 strain which generally had a very low wasp emergence (<15%). Deviation from the dominant model was not significant when ovipositing in the NH 53, OHR 04-03 and VT 04-22 strains. Deviation from the semidominant model was not significant in the OHR 04-03, X 209, and the L 46 strains.

The influence of egg factors on the emergence of F_1 wasps was statistically significant only in the VT 04-22 and L 46 strains. However, fly emergence was higher in all fly strains when parasitized by KK parent females that mated with BG males, compared to KK virgin females or KK control. Similarly, the influence of egg factors was statistically significant only in the X 209 strain for the F_2 generation. Nevertheless, in many cases fly emergence was increased when F_1 females were crossed with BG males. Additionally, wasp emergence was lower in $BG_{♀} \times KK_{♂}$ F_1 females when these were crossed to BG males compared to the $BG_{♀} \times KK_{♂}$ virgins.

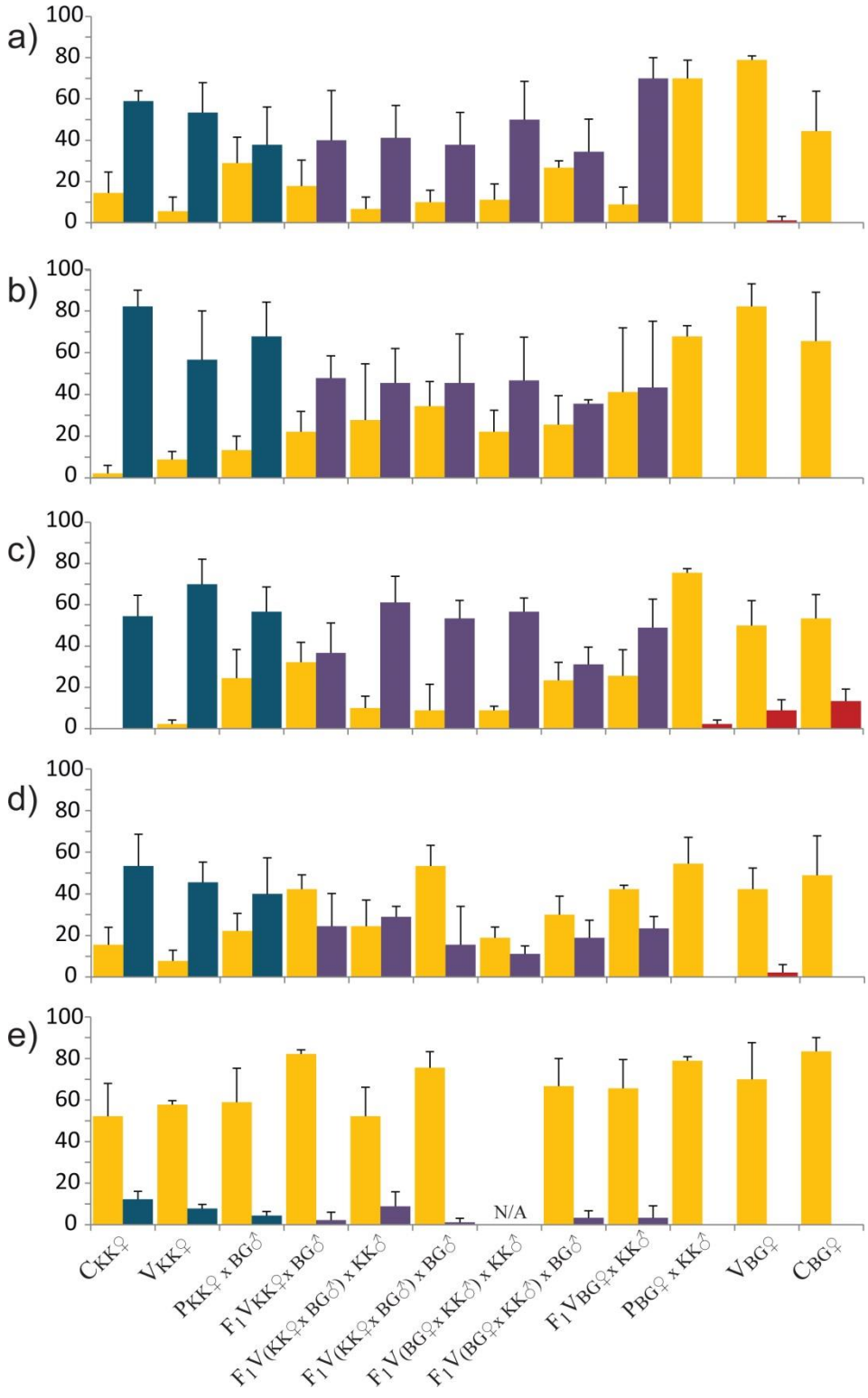


Fig. III-7 Outcomes of parasitism in KK × BG crosses, in NH 53 (a), OHR 04-03 (b), X 209 (c), VT 04-22 (d) and L46 (e) fly strains. Flies that emerged (yellow), wasps that emerged: KK parents (blue), BG parents (red) and F_1 generation parasitism (purple). Average + SD shown.

Table III-3. Contrast ANOVA of wasp emergence from crosses between the virulent KK and the avirulent BG strains of *L. victoriae*. * $P<0.05$ ** $P<0.01$ *** $P<0.001$.

Source	df	SS	MS	*F*	*P*
NH 53					
Model (between crosses)	11	4.004	0.364	16.024	***
1. Genetic differentiation between parental strains	1	0.865	0.865	51.887	**
2. Deviation from a chromosomal mode of inheritance	1	0.150	0.150	3.978	NS
3. Deviation from the dominant model	1	0.002	0.002	0.017	NS
4. Deviation from the semidomint model	1	0.939	0.939	17.802	*
5. Deviation from the recessive model	1	3.606	3.606	53.101	**
6. Influence of egg factors in the parental generation (KK)	2	0.078	0.039	1.882	NS
7. Influence of egg factors in the parental generation (BG)	2	0.007	0.004	1.000	NS
8.Influence of egg factors in the F_1 generation	5	0.284	0.057	1.716	NS
Error (within crosses)	24	0.545	0.022		
OHR 04-03					
Model (between crosses)	11	5.030	0.457	14.163	***
1. Genetic differentiation between parental strains	1	1.095	1.095	37.799	**
2. Deviation from a chromosomal mode of inheritance	1	0.008	0.008	0.110	NS
3. Deviation from the dominant model	1	0.099	0.099	0.478	NS
4. Deviation from the semidomint model	1	0.536	0.536	4.481	NS
5. Deviation from the recessive model	1	3.164	3.164	34.880	**
6. Influence of egg factors in the parental generation (KK)	2	0.124	0.062	1.792	NS
7. Influence of egg factors in the parental generation (BG)	2	0	0	/	/
8.Influence of egg factors in the F_1 generation	5	0.033	0.007	0.140	NS
Error (within crosses)	24	0.775	0.032		
X 209					
Model (between crosses)	11	2.478	0.225	17.378	***
1. Genetic differentiation between parental strains	1	0.744	0.744	52.331	**
2. Deviation from a chromosomal mode of inheritance	1	0.025	0.025	1.111	NS
3. Deviation from the dominant model	1	0.498	0.498	10.759	*
4. Deviation from the semidomint model	1	0.024	0.024	0.822	NS
5. Deviation from the recessive model	1	1.038	1.038	36.976	**
6. Influence of egg factors in the parental generation (KK)	2	0.049	0.024	1.668	NS
7. Influence of egg factors in the parental generation (BG)	2	0.095	0.047	4.836	NS
8.Influence of egg factors in the F_1 generation	5	0.222	0.044	3.247	*
Error (within crosses)	24	0.311	0.013		
VT 04-22					
Model (between crosses)	11	4.004	0.364	16.024	***
1. Genetic differentiation between parental strains	1	0.865	0.865	51.887	**
2. Deviation from a chromosomal mode of inheritance	1	<0.001	<0.001	0.002	NS
3. Deviation from the dominant model	1	0.002	0.002	0.017	NS
4. Deviation from the semidomint model	1	0.939	0.939	17.802	*
5. Deviation from the recessive model	1	3.606	3.606	53.101	**
6. Influence of egg factors in the parental generation (KK)	2	0.078	0.039	1.882	NS
7. Influence of egg factors in the parental generation (BG)	2	0.214	0.107	6.549	*
8.Influence of egg factors in the F_1 generation	5	0.116	0.023	1.066	NS
Error (within crosses)	24	0.545	0.022		

Table III-3. continued Contrast ANOVA of wasp emergence from crosses between the virulent KK and the avirulent BG strains of *L. victoriae*. * *P*<0.05 ** *P*<0.01 *** *P*<0.001.

L 46					
Model (between crosses)	10	0.486	0.0486	4.967	***
1. Genetic differentiation between parental strains	1	0.119	0.119	194.046	***
2. Deviation from a chromosomal mode of inheritance	1	<0.001	<0.001	0.021	NS
3. Deviation from the dominant model	1	0.204	0.204	11.931	*
4. Deviation from the semidomint model	1	0.011	0.011	0.746	NS
5. Deviation from the recessive model	1	0.057	0.057	3.874	NS
6. Influence of egg factors in the parental generation (KK)	2	0.032	0.016	7.322	*
7. Influence of egg factors in the parental generation (BG)	2	0	0	/	/
8.Influence of egg factors in the F_1 generation	4	0.096	0.024	1.194	NS
Error (within crosses)	22	0.215	0.010		

III-4. Discussion

Fly emergence of F_1 individuals did not significantly differ from that of the more resistant parental line used in the cross, and the dominant model for resistance was supported for all crosses except the BG 118×TBU 83 cross subjected to KK parasitism. This cross showed a recessive-like pattern, with the F_1 progeny emergence similar to that of the less resistant parental TBU 83 line. These results do not necessarily refute the single-gene-two-alleles resistance model of Carton et al. (1992) and Benassi et al. (1998). Instead, they may indicate a possible threshold effect, where a single dominant resistant allele can be toppled by a nonspecific resistance factor such as a lowered hemocyte count, differences in hemocyte types, or variations in hemocyte structure. The fact that the progeny of L 46 always showed higher resistance to wasps than the progeny of BG 118 when crossed to same susceptible fly strains, indicates that these strains may differ in that particular nonspecific component, which slightly modifies their resistance. A similar pattern can be observed in the susceptible strains, in which the progeny of the

most susceptible strain TBU83 showed the least resistance, possibly due to the same nonspecific factor.

In localities where parasitoids are not present, the cost of maintaining a high hemocyte load, for example, may select against it. This nonspecific component probably accounts for the additive effect observed in selection experiments (Bouletreau 1986, Kraaijeveld 1994, Hughes and Sokolowski 1996, Kraaijeveld and Godfray 1997, Fellowes et al. 1998). While the evolution of the recognition factor that potentially represents the resistant allele, may quickly become fixed in populations under parasitoid attack, and lost quite slowly through mutation when populations seize to be attacked, the nonspecific resistance component should show much more variation among populations, and faster evolutionary dynamics, leading to potential rapid local adaptation, and further modifying the coevolutionary mosaic, leading to more subtle differences, as observed in Chapter II.

A possible effect of transient or permanent extra-chromosomal factors influencing both fly and wasp eclosion was discovered in the L 46 × NH 53 cross, indicating a possible maternal effect. Such extra-chromosomal factors may also lead to the formation of geographic mosaic in resistance, but such a case has not been reported before, and warrants further analyses.

The encapsulation mechanism of *D. bipectinata* differs from the well-known response of *D. melanogaster*. Further studies of the cellular and molecular mechanisms will shed light on the role of nonspecific and specific resistance components in this interaction. It would be interesting to check if these genes for resistance are present in the other three species of the *D. bipectinata* complex. Locating and cloning the specific-component resistance gene, and comparing it to that of *D. melanogaster*, would

further tell a story about the conservative properties of the immune response in *Drosophila*.

In wasp crosses, *L. victoriae* emergence showed a more complicated pattern than in fly crosses, with both the dominant and the semidominant models supported, depending on the fly strains the wasps oviposited in. In other *Leptopilina* wasps the virulence property was found to be dominant in *L. heterotoma* (Walker 1959), semidominant in *L. boulardi* when ovipositing in *D. melanogaster* and recessive in *L. boulardi* when ovipositing in *D. yakuba* (Dupas & Carton 1999). Dupas and Carton (1999) proposed a threshold model where the presence or absence of immune suppression in *L. boulardi* depends on whether the parasitoid injects more or less immune suppressive material than a threshold value, characteristic for the host species. Subsequently, virulence in *L. boulardi* has been linked to LbGAP, a RacGAP protein present in the wasp`s venom (Labrosse et al. 2005). A much higher level of both mRNA and LbGAP protein was found in the venom-producing tissues of the virulent parasitoid, compared to the avirulent one, suggesting that a quantitative difference rather than a qualitative one is responsible for the observed differences between the virulent and avirulent strain, indicating, again, a threshold effect of this molecule on parasitoid virulence (Colinet et al. 2010). In the case of *L. victoriae*, the same threshold model can be applied to explain the observed patterns: heterozygous *L. victoriae* wasps inject more than the threshold to suppress host immune reaction in more than half of the infestations in NH 53 and OHR 04-03 strains, but inject enough to suppress host immune reaction in only about half of the infestations in the X 209 and VT 04-02 strains. Most likely this threshold is a function of the host hemocyte numbers or hemocyte properties, and therefore, in this study, the same amount of immunosupresive particles had a different

effect in different host strains.

The influence of egg factors was significant in only some of the fly strains the wasps oviposited in. Similarly to *L. boulardi* (Dupas & Carton 1999), the success of *L. victoriae* appears to be more related to the genotype of the mother than to the wasp`s own genotype. This finding agrees with the crucial role of immune suppressive components that are injected with the egg and venom by the mother into the host larvae during oviposition. However, a higher emergence of flies in the F_1 generation, when virulent KK parent females were crossed to avirulent BG males, and the boost of fly emergence in the F_2 generation when F_1 females were crossed back to BG males, indicate a week influence of egg factors modifying slightly the overall infestation outcome.

Virus like particles (VLPs) responsible for lamellocyte lysis have been found in *L. victoriae* (Morales et al. 2005). A further study of these and other protein components of the venom will shed additional light on the exact mechanism of virulence in these wasps.

Finally, I propose an interaction model (Fig. III-8) in which the resistant strain of *D. bipectinata*, carrying the resistance allele Rlv^+ (resistance to *L. victoriae*), comes out as a winner from the interaction with both virulent and avirulent wasps. The virulent wasp in this model carrying the immune suppressive allele Isb^+ (Immune suppression of *D. bipectinata*) can only parasitize susceptible fly strains, homozygous for Rlv^-. The success of the avirulent *L. victoriae* strain, homozygous for Isb^-, in the susceptible strain of *D. bipectinata* differs depending on the nonspecific resistance component.

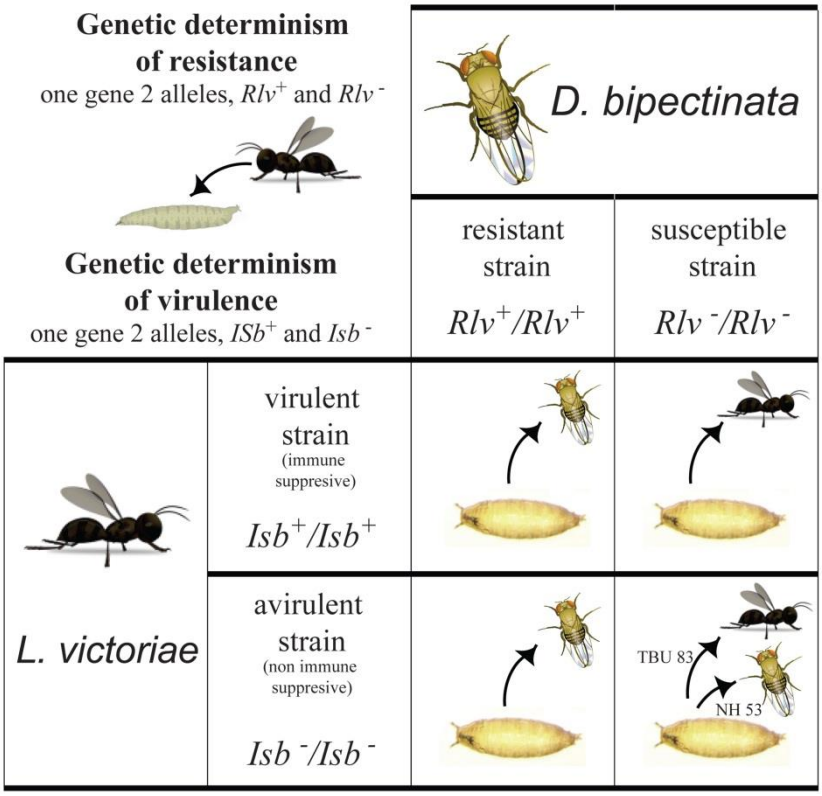


Fig III-8 Interaction outcomes in the *D. bipectinata – L. victoriae* system, when resistance of the host and virulence of the parasitoid are considered as mainly due to one major gene. Flies carrying the resistant allele *Rlv* $^{+}$ are resistant to both virulent and avirulent parasitoid strains. Virulent wasp strains carrying the *Isb*$^{+}$ allele can parasitise all susceptible fly strains, while avirulent wasp strains differ in their parasitism success depending on host nonspecific resistance levels.

In the only other *Drosophila* parasitoid model, that combines the genetic interactions of both the fly and the parasitoid wasp to date, that of *D. melanogaster* and *L. boulardi*, the virulent wasp strain always wins in all interactions (Dupas et al. 2003). This model argues that there is low genetic variability of parasitoid virulence compared to host resistance (Dupas et al. 2003), arising from the differences in the strength of the reciprocal selection. In this case, arms race is won by the parasitoid. Again, asymmetric selective forces push parasitoids to develop higher levels of virulence, as all parasitoids need a host to survive, but hosts will develops specific resistance only if that parasitoid

is a major mortality factor (Lapchin 2002). In the model of *D. bipectinata* and *L. victoriae*, however, parasitoid does not have the final word. It seems that selection on *D. bipectinata* in some regions of its distribution has been strong enough to select for a strong immune response, and this especially seems to be the case in tropical areas such as East Africa, Indonesia and Malaysia. *L. victoriae* may have lost the arms race with this particular host species, and resorted instead to host alternation attacking less defended species of the same species complex (Kimura & Suwito 2012). Unlike fly resistance, the cost of which has yet to be found, the cost of virulence was observed in actual experiments (Dupas & Boscaro 1999), and may be what is preventing high virulence from being fixed in wasp populations.

In this chapter, not inbreed isogenic lines but isofemale lines were used. These isofemale lines have been reared in laboratory conditions for several years up to several decades, and can be considered genetically homogenous. However, this interaction model should preferably be verified by using inbreed homozygous lines of both *D. bipectinata* and *L. victoriae*. Additionally, the significance of the nonspecific additive components should be confirmed by selection experiments.

General Discussion

Asian *Drosophila*-parasitoid interactions were studied for the first time in the light of the geographic mosaic theory of coevolution. The diverse *Drosophila*-parasitoid communities across this region proved to be excellent test subjects for coevolutionary studies, and the results of this thesis shed new lights on how these interactions coevolve over broad landscapes, and provide a basis for further comprehensive research in this area.

Here I review the tripartite hypothesis of the geographic mosaic theory of coevolution, focusing on the results of this thesis.

1) Interspecific interactions are subjected to a selection mosaic that favors different evolutionary trajectories in different populations. Local differences in host resistance were observed in *D. bipectinata*, where a mosaic of different resistance levels was observed across 17 populations distributed from Africa, across Asia to the Pacific islands. This difference was not supported by phylogeographic patterns, implicating local coevolution as the main differentiation factor. Considerable difference was also found in parasitoid virulence between the two strains of *L. victoriae*, KK and BG, despite the fact that these strains originated from relatively close geographic areas – Borneo island, Malaysia and Java island, Indonesia. Finally, minor geographic differences were observed in resistance levels of *D. albomicans*, host acceptability of *D. takahashii* and the host acceptance of *L. ryukyuensis*. The selection mosaic differentially affecting all these traits is most likely resulting from the different composition of the host-parasitoid communities between given localities (also in Kraiijeveld & Godfray 1999, Dupas et al. 2003).

2) There are coevolutionary hot spots in which reciprocal selection is actually occurring, while in cold spots it is not. In terms of the geographic mosaic theory of coevolution, pacific islands and southern Japan would represent a coevolutionary cold spot for *D. bipectinata*, as the parasitoid *L. victoriae* may not be present to exert a selection pressure on a resistance response in these potential host populations. Java and Borneo, on the other hand, where the experimental *L. victoriae* wasp strains originated from, represent an evolutionary hot-spot, with flies actively selected for resistance against this parasitoid. More research is necessary in order to access the status of the Ryukyu archipelago and Taiwan in terms of hot and cold spots for the interaction of the three *Drosophila* species with *L. ryukyuensis*.

3) There is a continual geographic remixing of the range of coevolving traits, that results from the selection mosaic, coevolutionary hot spots, gene flow, random genetic drift and local extinction of populations. A study on the evolutionary history of the *D. bipectinata* complex shows little genetic diversity within this species, and no indications of interrupted or decreased gene flow (Kopp & Barmina 2005). Furthermore, gene flow may occur among *D. bipectinata* and its three sister species, as the female hybrids of these species are fertile when crossed in laboratory and backcrossed to either parent. Resistance and virulence traits are potentially further remixed by the expansion and contraction of the areas of distribution of these species, or extinction and colonization in/of specific areas.

The three ecological predictions arising from this theory give a direction for further research of the coevolutionary interactions in this complex.

1) Populations will differ in the traits shaped by an interaction. The populations of *D. bipectinata* and *L. victoriae* differ in both host resistance and

parasitoid virulence levels. These differences were further shown to have a strong genetic basis, with an additive nonspecific host resistance component fine-tuning the outcome of the interaction. Even populations of *D. albomicans*, *D. takahashii* and *L. ryukyuensis* studied in a small-scale setting showed differences in host acceptance and host suitability traits, possibly due to the differences in their local-specific interactions.

2) Traits of interacting species will be well matched in some communities while mismatched in others, leading in some cases to local maladaptation. In the case of antagonistic reactions, maladaptation of the host can result from the adaptation of the parasitoid and vice versa. In at least some populations, such as Thailand and parts of Malaysia, flies may potentially suffer great mortality by *L. victoriae*, but the virulence of this parasitoid in these areas is yet to be determined. The KK and BG strains may be maladapted to parasitize *L. bipectinata* in sympatric communities, but are well adapted to parasitize other related host-species. The match and mismatch of traits need to be further studied by analyzing additional *L. victoriae* strains from other areas in which these two species exist in sympatry. In host-parasitoid relations, the maladaptation represents a smaller issue for the host, compared to the parasitoid, as not all hosts are parasitized, but all parasitoids need a host to survive. *D. albomicans*, for example, may seem maladapted in the Iriomote island, having no resistance towards *L. ryukyuensis*, unless we consider that the parasitism in these populations varies among seasons, and/or never gets high enough to exert a strong selective pressure for resistance in this fly.

3) *There will be few species-level coevolved traits.* Neither host resistance nor parasitoid virulence are fixed for either of the interacting species in the *D. bipectinata – L. victoriae* model. Further studies are necessary to establish the same for *D. albomicans, D. takahashii* and *L. ryukyuensis*.

In terms of coevolutionary alternation, host shifts and arms race may explain some of the patterns observed across the *Drosophila*-parasitoid populations. Previous results regarding the *D. melanogaster – L. boulardi* system showed that if the wasp is virulent enough, it overrides fly resistance. This is explained as a result of the asymmetry in selective pressures, with wasp actively selected for high levels of virulence, and hosts losing the arms race against the parasitoid (Dupas et al. 2003). *D. bipectinata* however exhibited high resistance to both *L. victoriae* and *L. ryukyuensis*, at least in some geographic areas. This indicates that *D. bipectinata* was under a strong selective pressure to develop high resistance levels in spite of the potential costs.

Leptopilina victoriae, on the other hand, instead of continuing with a possible arms race with the well-defended *D. bipectinata*, may have shifted to less defended hosts in Indonesia and Malaysia. There may be a cycle of alternation between these host species, where they alternatively gain and lose defenses, with the wasp switching to the least defended host. It would be interesting to tackle parasitoid populations from a broader geographic area to further analyze the interactions between *L. victoriae* and the *D. bipectinata* species complex.

Drosophila takahashii was not readily oviposited in by *L. ryukyuensis*, indicating either a presence of avoidance mechanisms in the host such as a ticker larval cuticle, or low host acceptability on the part of the parasitoid. It is possible that *D. takahashii* was a former host of *L. ryukyuensis*, and that after developing resistance this wasp was selected for abandoning acceptance of this *Drosophila* species as a host. If, however, there is an avoiding mechanism in this fly, one other parasitoid has found a way to overcome it, as *Asobara japonica* specializes on *D. takahashii* in the Ryukyu archipelago (Novković et al. 2012).

Although *D. albomicans* showed the highest genetic diversity, and was possibly the historically longest present species among the four analyzed species in the area, this species had the lowest resistance for *L. ryukyuensis*. *D. albomicans* may represent a new host for this parasitoid, and thus may have not yet developed resistance mechanisms. This is potentially supported by the low mitochondrial and nuclear (Novković et. al 2011) diversity of *L. ryukyuensis*, which may have colonized the archipelago most recently. It is also possible that *D. albomicans* is not attacked enough for this wasp to exert a strong selective pressure on the immune response of this host species.

Studies available up to date gave all the credit for the interaction outcome in *Drosophila* – parasitoid interactions to one of the resistance components disregarding the other. Some supported the importance of the specific resistance component, i.e. the gene-for-gene interaction model (e.g. Dupas et al. 2003), while others were of the opinion that this kind of interaction is unimportant in natural populations, where a more additive variation plays the key role (e.g. Godfray 2000). Based on the cross experiments in this study, I propose a gene-for-gene model, in which the outcome is fine-tuned and modified by the additive nonspecific component with a threshold effect. Cross results indicate that a single dominant resistant allele may be toppled by wasp virulence, depending on a nonspecific resistance factor, most likely hemocyte related.

While the gene-for-gene interaction paints a very simple picture for the outcome of these *Drosophila*-parasitoid interactions, the nonspecific component can harbor much more variation among populations, with a faster evolutionary dynamics, further modifying and diversifying the coevolutionary mosaic, leading to field cases similar to those observed in *D. bipectinata* populations.

Models based on the relatively nonresistant host *D. melanogaster* may not be applicable to the majority of cases found in natural populations, due to the recent spread of *D. melanogaster* (Stephan & Li 2007), and the much longer coevolutionary interaction history of native fly species and parasitoids over broad geographic areas. Moreover, parasitoid pressures in some areas, especially tropics, seem to be high enough to select for high resistance in the hosts, the cost of which is yet to be evaluated. The specific encapsulation response of *D. bipectinata* raises many more questions about the differences in the mechanisms in which resistance to parasitoids evolves in different species and across phylogenetic lineages. Further physiological, molecular, and genetic research of the *Drosophila* – parasitoid models studied in this thesis will potentially answer these and many other questions related to the coevolutionary dynamics of these species.

Summary

Chapter I

Phylogeography provides a crucial context for differentiating between the effect of gene flow, hybridization, random genetic drift and natural selection in creating the geographic mosaic of coevolution. This chapter had two main aims, to determine the phylogeography of three *Drosophila* species, *D. albomicans*, *D. takahashii* and *D. bipectinata* and the parasitoid *Leptopilina ryukyuensis* in the Ryukyu archipelago and Taiwan, and to explore the differentiation in host resistance and parasitoid virulence in these potential host-parasitoid interactions among different islands. Mitochondrial COI and autosomal *Gpdh* partial sequences were used to establish geographic patterns, and host-parasitoid differentiation was tested via host acceptance and host suitability experiments. COI and *Gpdh* haplotype patterns differed greatly among the three *Drosophila* species. *D. albomicans* had the highest diversity, with 48 COI haplotypes recovered from 101 adult flies. A great divergence was observed between the northern (KG, AM, NH) and the southern (IR, TP) populations of this species, without a single shared haplotype. The Kerama gap, a break of over 250 km in the island chain between Okinawa island in the north and Miyako island in the south, may represent a strong barrier for gene flow in this particular species. This is further supported by two separate demographic expansion events, estimated at c.724 ka for the southern and 122 ka for the northern populations. *D. takahashii* showed a star-like haplotype network, with a more recent estimated expansion time of c. 94 ka, and the lowest nuclear *Gpdh* diversity among the three fly species. *Drosophila bipectinata* had the lowest diversity in COI,

much lower than the diversity found in the autosomal *Gpdh*, potentially resulting from a selective sweep due to a *Wolbachia* infection. *L. ryukyuensis* did not show any COI nucleotide diversity. Host acceptance experiments revealed differences between the strains of *L. ryukyuensis* and *D. takahashii*, while host suitability ecperiments revealed some differences in the resistance of *D. albomicans* strains. Further widening of the study area for these interacting species will establish the significance of the coevolutionary mosaic as the key process in the formation of these patterns.

Chapter II

The aim of this Chapter was to capture the geographic mosaic of coevolution using a matrix of populations of a host *Drosophila bipectinata* and a parasitoid *Leptopilina victoriae*. The wide distribution of these two species and their dispersion over many Asian islands make them ideal for the study of coevolutionary interactions. Resistance and virulence of 17 isofemale lines of *D. bipectinata* and two strains of *L. victoriae* were measured through host suitability experiments. A considerable geographic variation was found in host resistance towards *L. victoriae*, and parasitoid virulence towards *D. bipectinata*. The resistance to the KK strain of *L. victoriae* was overall lower compared to the resistance to the BG wasp strain. Resistance to KK was highest in the strains from Africa, India and Indonesia and lowest in the pacific island strains. High host resistance in Africa, India and Indonesia may be facilitated and selected for by high parasitoid pressures. Two Japanese strains, NH 53 from Okinawa and OHR 04-03 from Iriomote island, were completely resistant to the weaker BG strain of *L. victoriae*, but very much susceptible to the KK strain. In southern Japan, as well in the pacific islands,

flies may have escaped from the parasitoid and evolved inside the enemy-free space, losing resistance due to its possible cost. In terms of the geographic mosaic theory of coevolution, pacific islands and southern Japan would represent a coevolutionary cold spot. Java and Borneo where the wasp strains originated from, on the other hand, would represent evolutionary hot-spots, with flies actively selected for resistance against this parasitoid. In these areas, where *D. bipectinata* is highly resistant to *L. victoriae,* evolutionary alternation may be taking place, with *L. victoriae* more readily attacking the sister species belonging to the *D. bipectinata* species complex. The BG and KK strains of *L. victoriae* may be locally adapted to cope with different host species or populations differing in their levels of resistance. This difference in virulence may also indicate an ongoing arms race, driven by an increased selection for virulence. The geographical variations in *D. bipectinata* resistance and *L. victoriae* virulence patterns show that there are different coevolutionary interactions taking place in different geographic regions/communities, satisfying the tripartite hypothesis of the geographic mosaic theory of coevolution. Obtaining further information about the parasitoid and host species building these communities across the whole region would further clarify the coevolutionary mosaic involving *L. victoriae* and *D. bipectinata*.

Chapter III

The aim of this chapter was to offer an insight into the genetic mechanisms of host resistance and parasitoid virulence in *D. bipectinata* and *L. victoriae,* adding a genetic dimension to the geographic mosaic of coevolution encountered in these species. Five *D. bipectinata* isofemale lines from different geographic localities with different resistance

properties were crossed to investigate the genetic mechanism of host resistance. Further, the KK and BG strains of *L. victoriae* were crossed to shed light on the genetics of parasitoid virulence. In fly crosses the dominant model for resistance was supported for all but one cross. These results indicate a possible threshold effect, in which a single dominant resistant allele could be toppled by a nonspecific resistance factor. The fact that the progeny of the stronger resistant strain always showed higher resistance among the two resistant strains, and that the progeny of the most susceptible strain always showed the least resistance of the three susceptible strains, indicate further that these strains may differ in a nonspecific component, which slightly modifies their resistance. A possible effect of transient or permanent extra-chromosomal factors influencing both fly and wasp emergence was discovered in the L 46×NH 53 cross. In wasp crosses, both the dominant and the semidominant models were supported, depending on the fly strains the wasps oviposited in. In the case of *L. victoriae* a threshold model can be applied to explain the observed patterns with heterozygous *L. victoriae* wasps inject more than the threshold to suppress host immune reaction in some but not other fly strains. Results indicate a possible week effect of egg factors modifying the overall infestation outcome. An interaction model is proposed in which the resistant strain of *D. bipectinata* comes out as a winner from the interactions with both virulent and avirulent *L. victoriae*. The virulent wasp in this model can only parasitize susceptible fly strains. Finally, the success of the avirulent *L. victoriae* strain in susceptible strains of *D. bipectinata* differs depending on the nonspecific resistance component. Results indicate that the parasitism of *D. bipectinata* in some regions of its distribution has been strong enough to select for a strong immune response, and this especially seems to be the case in tropical areas such as East Africa, Indonesia and Malaysia. *L. victoriae* may have lost

the arms race with this particular host species, and resorted instead to host alternation attacking less defended species of the same species complex.

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