Genetic architecture of host specialisation in Yponomeuta
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Introduction and thesis overview

Host plant specialisation and speciation
Species flocks or adaptive radiations, i.e., groups of closely related, usually monophyletic and sympatrically occurring species adapted to different ecological niches, are quite common in nature. A species flock may arise when a species colonizes a new geographical area and diversifies to occupy a variety of ecological niches. Famous examples are the haplochromine cichlids in Lake Victoria (e.g., Meyer 2005; Cristescu 2010) and water snails of the genus *Lavigeria* in Lake Tanganyika (Michel 2000; van Damme & Gautier 2013). For herbivorous insects, many examples can be found of adaptive radiations in which the ecological niche is represented by the host plant (e.g., Farrel 1994; Menken et al. 2010; Turner et al. 2010).

Soon after the publication of Darwin’s *Origin of Species* in 1859, Walsh (1867) proposed that insect speciation could be driven by shifting and adapting to new host plants. Subsequent work on host plant preference and host-specific mating established the possibility of speciation in allopatry (Mayr 1947, 1963) but also the possibility of speciation in sympathy, in the face of gene flow (Maynard Smith 1966; Bush 1969). In the following decades, views of reproductive isolation became polarized, culminating in a major controversy between supporters of seemingly rival theories:

- **Allopatric speciation:** A separation in space is mandatory to provide the necessary level of reproductive isolation that precedes speciation (e.g., Futuyma & Mayer 1980; Coyne & Orr 2004).
- **Sympatric speciation:** Divergent, disruptive or frequency-dependent selection alone can effect adaptive diversification in host use between populations in the face of gene flow (e.g., Doebeli et al. 2005; Mallet 2008).

More recent work has focused on the actual ecological and genetic mechanisms of speciation (Via 2002) and examined in great detail the manner, and to what extent, adaptation to different host plants might reduce gene flow between herbivorous insect populations (Funk 1998; Berlocher & Feder 2002; Drès & Mallet 2002; Fry 2003; Matsubayashi et al. 2009; Thibert-Plante & Gavrilets 2013). Since the end of the 20th century, empirical field studies, laboratory experiments, developments in population genetics theory, and phylogenetic and biogeographic analyses have all been used to provide evidence for what is now often called ecological speciation (Berlocher & Feder 2002; Doebeli et al. 2005; Matsubayashi et al. 2009).

The debate on the question whether ecological speciation is a theoretically plausible evolutionary process centers on two key factors (Doebeli et al. 2005):

- The ecological conditions that are likely to generate disruptive selection on heritable traits underlying adaptation to the ecological niche, and
- The evolution of assortative mating mechanisms in populations experiencing disruptive selection.

Empirical evidence has been found to support the likelihood of both factors occurring (e.g., Smadja et al. 2012). Mathematical models have been developed to describe the population genetics involved in ecological adaptation and shed light on the effect of assortative mating on the fixation of adaptive traits (e.g., Via 2001; Doebeli & Dieckmann 2003; Mizer & Meszéna 2003; Thibert-Plante & Gavrilets 2013). With the rapid growth of large-scale genomic datasets, genetic signatures associated with ecologically driven speciation processes are becoming amenable to analysis. The process of ecological speciation is, however, still poorly understood (Nyman 2010; Nosil & Feder 2013).

Nature provides a wide range of ecological contexts in which insect herbivores and hosts coexist. The exact nature of the adaptations needed to utilize the host, and their relative importance in an ecological context, determine the probability of evolution of assortative mating. This differs between unique cases of speciation: The data presented in this thesis serve to identify the nature of co-adaptation of the host-use traits and the extent of reproductive isolation that has evolved in the course of speciation of European *Yponomeuta* species.

**Yponomeuta** phylogeny and host associations
The nine species of the genus *Yponomeuta* (Lepidoptera: Yponomeutidae) that occur in Western Europe can be seen as an example of speciation facilitated by host shifts. In this thesis, the main actors are three species of small ermine moths, all living in the same ecological habitats in Europe: *Yponomeuta cagnagellus* Hübner (1813), *Y. malinellus* Zeller (1838) and *Y. padellus* Linnaeus (1758).

Characteristic of these species is their host plant specialisation. *Yponomeuta cagnagellus* is strictly monophagous on *Euonymus europaeus* (Celastraceae), *Y. malinellus* is restricted to *Malus* species (Rosaceae) and *Y. padellus* is oligophagous on a small number of host plants within the Rosaceae, including *Crataegus monogyna*, *Prunus spinosa*, and occasionally *Prunus cerasifera*, *Sorbus aucuparia* and *Amelanchier lamarckii*.

In two recent phylogenetic reconstructions of the relationship between *Yponomeuta* species, one molecular (Turner et al. 2010) and the other morphological (Ulenberg et al. 2009), phylogenetic information was linked to host plant use and geographic distribution of the species. Summarized below are the conclusions that were drawn from both studies on the evolution of the host plant associations in the genus.

Within the subfamily Yponomeutinae, the basal genera feed on various plant families, among which *Crassulaceae*, *Rhamnaceae* and *Rosaceae*, which are also exploited by *Yponomeuta* species. The phylogeny shows that an originally specialised host plant range is not an evolutionary dead-end (Kölsch & Pedersen 2008): even if species at the base of the phylogeny seem to be fixed in their specialised host associations, shifts to new hosts occur higher up in the phylogeny (Ulenberg et al. 2009).
Within the genus *Yponomeuta*, basal taxa, occurring mainly in Asia, are restricted to feeding on Celastraceae (Gershenzon & Ulenberg 1998). The host shift(s) in European *Yponomeuta* species—from Celastraceae to other plant families—occurred in recent times, after they dispersed to the West Palearctic from the Far East. The occurrence of these host shifts can be derived from the host associations of the current *Yponomeuta* species (Turner et al. 2010).

Looking more closely at the three species that are the subject of this thesis, both phylogenies show that the common ancestor of *Y. malinellus* and *Y. padellus* shifted from the ancestral Celastraceae to feeding on Rosaceae. Surprisingly, the association of *Y. cagnagellus* with *E. europaeus* originates from a more recent shift back to the Celastraceae from a Rosaceae-feeding ancestor.

To summarize, we are looking at two separate host shift events: one from Celastraceae to Rosaceae, leading to the host associations of *Y. padellus* and *Y. malinellus* (Asian ancestors adapting to European ecosystems that were probably relatively poor in celastraceous species) and the other from these European Rosaceae back to the ancestral Celastraceae, resulting in the host association of *Y. cagnagellus*.

**Sensory ecology and insect physiology**

Invertebrates use a range of sensory inputs to orientate in their small-scaled environment. One of these is the perception of olfactory and gustatory cues. It is well established that host acceptance in herbivorous insects is largely controlled by chemoreception: ‘Insects live in a chemical world’ (Schoonhoven et al. 1998). The detection of chemical compounds in the environment is a key element in insect ecology and evolution (Gardiner et al. 2008; Olsson et al. 2009; Smadja et al. 2012; Briscoe 2013). The physiological basis of the perception of chemical compounds is not very different from that of mammals, and is based on receptor proteins located in the membrane of sensory cells (Sanchez-Gracia et al. 2009). Each receptor protein specifically binds to a particular chemical compound, leading to the firing of the associated neurons and the subsequent secondary processing of this neurological input in the central nervous system, which results in a particular behaviour.

The sensory equipment of invertebrates can discriminate very precisely and sensitively between different chemical compounds. Because of their small size, insects need to be economical in the number of chemical compounds that they can perceive. Lepidopteran larvae in particular often display very distinct food preferences, which are based on a very small set of chemoreceptors (Schoonhoven & van Loon 2002). Chemoreceptor evolution has generally been driven by gene duplications, followed by gain-of-function alleles perceiving the informative compounds being selected for, and loss-of-function alleles being neutral or selected against (Gardiner 2008). Thus herbivores are able to perceive precisely those compounds that enable them to identify suitable host plants, and may not perceive compounds that are not informative (Renwick & Chew 1995; Schoonhoven & van Loon 2002).

Specialised herbivorous insects largely rely on an inherited algorithm in which the olfactory or gustatory input leads to pre-programmed behaviour. Although there is an increasing interest in the role of learning in host acceptance (e.g., Cunningham & West 2008; Salloum et al. 2011) this seems more relevant for insects with a broader diet than for strict host specialists. Fastidious invertebrates will not start feeding or ovipositing unless the sensory input they receive positively signals that they are on a suitable host plant, based on plant compounds that stimulate feeding or oviposition. In addition, many plants contain deterrents that will reduce acceptance for feeding or oviposition (Chapman 2003). Analyses of suites of genes associated with chemoreception have suggested that specialisation is associated with elevated rates of loss-of-function mutations and some evidence of positive selection. The host specialist *Drosophila sechellia*, for example, has lost genes associated with deterrence (bitter receptors), which may have previously (in an ancestor) conferred deterrence to what is now the host of *D. sechellia* (McBride 2007; McBride & Arguello 2007; Dworkin & Jones 2009).

Previous studies in *Yponomeuta* have elucidated a similar mechanism behind host acceptance of the larvae. In each *Yponomeuta* species, the evolutionary shift to a new plant is accompanied by a decrease in sensory response of the larva to specific deterrents present in the new host (van Drongelen 1979; Chapman 2003). The association of the ancestors of *Y. cagnagellus* with Rosaceae has left footprints in the sensory system of its larvae, supporting the idea of a secondary shift back to Celastraceae. *Yponomeuta cagnagellus* can still perceive the plant compound benzaldehyde: this chemosensory trait is typical for the Rosaceae feeders and is not found in the other Celastraceae feeders among the *Yponomeuta* species (Roessingh et al. 2007).

The genes underlying chemosensory differences, and those of gustatory receptors in particular, determine the heritability of diet breadth. The detection of plant compounds and the subsequent acceptance or rejection behaviour is controlled by olfactory (OR) and gustatory (GR) receptor genes. The processes underlying the evolution of these genes are not yet completely elucidated. Gene duplication and subsequent divergence of a copy (neo-functionalization) appears to be a key mechanism in the evolution of chemosensory systems (Ohno 1999; Lynch & Force 2000; Nei et al. 2008; Sanchez-Gracia et al. 2009; Smadja et al. 2012; Briscoe 2013), and it has been suggested that in *Yponomeuta* a duplication of the receptor protein gene sensitive to the sugar alcohol dulcitol provided the basis for the unique shift from ancestral Celastraceae to derived Rosaceae (Menken & Roessingh 1998).

Dulcitol is the major feeding stimulant for larvae of Celastraceae feeders. It functions as an important transport sugar, and is quite specific for this plant family. After duplication of the dulcitol receptor gene, neo-functionalization of the copy could have resulted in a new sensitivity for dulcitol’s stereoisomer sorbitol. Sorbitol is abundantly present in Rosaceae and the major phagostimulant for Rosaceae feeders among the small ermine moths. Interestingly, the small amounts of dulcitol present in some Rosaceae might have allowed the ancestral species to cross the ‘bridge’ between the Celastraceae and the otherwise phytochemically dissimilar Rosaceae (Menken & Roessingh 1998).
Host use, polygenic traits and reproductive isolation in a randomly mating population

There are many mono- or oligophagous herbivorous insect species and probably few truly polyphagous species (Schoonhoven et al. 2005; Menken et al. 2010). Optimal utilization of plants for survival and reproduction apparently requires specific adaptations of the insect.

A new host can only be incorporated into an insect’s diet if adult females accept it for oviposition, larvae accept it for feeding, and the larvae are able to complete their life cycle on it. The term ‘host use’ therefore indicates the suite of traits defining adult and larval host acceptance and larval performance. Shifts to novel hosts seem more probable if there is a common inheritable element underlying adult and larval acceptance of the host, and if this element is somehow linked to larval fitness on the new host. Of course it is also important to consider the similarity of the relevant phytochemistry of the new host to that of the old one (Nyman 2010).

The critical step for completing a host shift is to combine the alleles underlying the traits involved in adaptation to the new host into a co-adapted gene complex. This can take place when a linkage disequilibrium develops in the population between individuals remaining on the old and individuals adapting to the new host. For ecological speciation in the face of gene flow in particular, this disequilibrium needs to become established despite the homogenizing effects of random mating and recombination (Schluter & Nagel 1995; Orr 1998; Hatfield & Schluter 1999; Groman & Pellmyr 2000; Fry 2003). Even if a population does experience persistent disruptive or frequency-dependent selection, adaptive speciation in sexual populations requires the evolution of assortative mating to enable stable inheritance of co-adapted gene complexes (Felsenstein 1981; Via 2009; Debarre 2012).

Assortative mating serves to avoid the production of offspring that would suffer from an unfavorable combination of alleles as the result of the breakdown of the co-adapted gene complex. This implies changes in mate-choice behaviour so that the chances of mating with a partner carrying the same co-adapted alleles are increased (e.g., Berlocher & Feder 2002; Fry 2003; Matsubayashi et al. 2009). One evident mechanism underlying assortative mating in case of host shifts is a tendency for adults to mate on the plant on which they have developed as larva. A well-known example is the *Rhagoletis pomonella* sibling complex, where assortative mating is automatically linked to larval performance through host-fidelity of the adults. The *R. pomonella* complex consists of at least six morphologically very similar, interfertile species of true fruit flies (Berlocher 2000). Courtship and mating occur on the host plant, often on the fruit. Each species is specialised on a different, non-overlapping set of host plants at the larval stage (e.g., *Crataegus* species for *R. pomonella* and *Cornus florida* for the “flowering dogwood fly”; Berlocher 2000; Powell et al. 2013). There is compelling evidence for host-related premating isolation for these flies (Feder et al. 2003, 2010).

Another tephritid fly genus, *Eurosta*, also figures prominently in the sympatric speciation literature (Berlocher & Feder 2002). All *Eurosta* flies make galls on the stems or roots of species of the Asteraceae genus *Solidago*, and display a high degree of host specificity (Foote et al. 1993). Mating in *Eurosta solidaginis* occurs on the host, and its two host races each prefer their own host plants for oviposition and mating (Itami et al. 1998; Craig et al. 2001); larval survival is highest on their own hosts, so trade-offs for survival clearly exist (Craig et al. 1997). Fertile and viable F1s, F2s and backcrosses can be made. Hybrid fitness depends strongly upon individual host genotype; host plant adaptation therefore appears to be more important than intrinsic genetic incompatibility for the reproductive isolation of these flies (Craig & Itami 2011).

In contrast, host fidelity does not appear to be of overriding importance for speciation in the European *Yponomeuta* species. A study by Bakker et al. (2008) did not find evidence for host plant fidelity of adult *Y. padellus* and *Y. cagnagellus* in their choice of a mating place, suggesting that other mechanisms to establish the linkage disequilibrium needed for speciation must have been operating. In nature, *Yponomeuta* species do not hybridize due to the specificity of their sex-pheromones (Löfstedt & van der Pers 1985; Hendrikse 1986, 1988; Löfstedt et al. 1991; Lienard et al. 2010), and moreover the females have been shown to reject heterospecific males for mating during courtship, based on perception of the male pheromones, which work at a short distance (Hendrikse et al. 1984).

An interesting line of research on assortative mating is the direct influence of host-specific compounds in the larval diet on the composition of the male sex pheromone (e.g., Orono 2013). For *Yponomeuta*, this would imply that larvae of *Yponomeuta* sequester secondary compounds from their host plant for the production of the male sex pheromone, and as such advertise their adaptation to that plant at the adult stage. As it is the female that controls the offspring’s diet by her choice of the oviposition site, strict selection of her mate would facilitate co-adaptation of adult host acceptance and larval development. However plausible this line of thought is, very little is currently known about the male pheromones of *Yponomeuta*.

Ruling out high host fidelity, a main driver for the evolution of assortative mating based on sex-pheromones in *Yponomeuta* could be a lower fitness of hybrid offspring. This may provide indirect selection pressure to prevent hybridization. This however, is a chicken-or-egg problem: Do genes underlying mate choice [sex-pheromone production, perception and mating behaviour changes (Lienard et al. 2010)] need to become co-adapted first, before the genes for host plant adaptation can co-adapt? Or did the incipient species pass a phase in which the whole randomly mating population could utilize both hosts simultaneously (Menken & Roessingh 1998), reducing the need for assortative mating; only thereafter did assortative mating based on sex-pheromones develop due to selection pressure from an ecological factor, not related to host plant use per se? This thesis will not deal with the whole scope of assortative mating. It will touch on the occurrence of postzygotic hybrid incompatibility in the absence of assortative mating, and how the nature of inheritance of host use may influence hybrid fitness.

Mating with a partner not carrying the same co-adapted alleles can affect the fitness of the offspring. Offspring may – for instance – inherit the behavior leading to oviposition on one host, whereas the larvae miss the alleles to enable them to develop on it (cf. Nyman 2010). The selection pressure needed to facilitate the evolution of assortative mating is de-
determined by the cost of fitness in the hybrid offspring. This cost will be high in cases in which the number of alleles underlying host use is high, and when genes for adult host acceptance, larval host acceptance and larval survival are not linked to each other genetically; linkage can occur by physical proximity of the genes on the chromosomes, or by a pleiotropic function of the genes.

The challenge lies in distinguishing which of the observed genetic differences in host use occurred after the evolution of reproductive barriers, and which were critical for the evolution of reproductive isolation before the establishment of assortative mating. New technology is allowing us to meet this challenge, providing an opportunity to answer questions that could not be addressed in the past. Rapid developments in DNA sequencing technologies, monitoring gene-expression, eco-genomics and other techniques enabling collection of data on genetic polymorphisms on the individual level are already leading to more detailed insights into molecular evolution. These methods are even suitable for use in model systems such as *Yponomeuta*, which did not allow detailed genetic analysis using the classical methods.

Empirical data on the number, nature and linkage of the alleles underlying host use are still very scant. Matsubayashi et al. (2010) summarize some 25 empirical studies, similar to – and including – the studies in *Yponomeuta*. One of their conclusions is that although genetic studies of ecological speciation are accumulating, further work is needed to understand how genes under selection and genes conferring reproductive isolation become and remain associated with one another. In particular, they point to the possibility that genetic control and opposing dominance for host preference vs. performance may facilitate speciation in the face of gene flow. The work presented here aims to document the genetic architecture of host preference and performance in several species of *Yponomeuta* and may serve as a well-documented example of the factors that have played a role in the speciation process in this genus.

**THESIS OVERVIEW**

In this thesis, data are presented that help understand the genetic architecture of host plant specialisation. The data were acquired from studies of the chemo-ecology and inheritance, in interspecific hybrids between *Yponomeuta* species, of adult host acceptance (Chapter 2, 3, 4 and 5), larval host acceptance and performance on the parental hosts of the hybrids (Chapter 6), and the possibility of genomic (in)compatibility in these hybrids not related to host plant use (Chapter 7).

The work presented in **Chapter 2** documents cues that are important for adult *Y. cagnagellus* in recognising the suitability of a host as a food source for their offspring. It was a study of pre-oviposition behaviour, the behaviour leading up to the moth’s decision to oviposit. It provided information on the nature of the cues used for host plant acceptance and the insect’s perception of these cues.

**Chapter 3** describes a bioassay that was developed to study the effect of isolated plant surface compounds on oviposition behaviour in *Yponomeuta* species. This bioassay was subsequently used to evaluate the oviposition behavior of adult *Y. cagnagellus* in response to host and non-host compounds to test if the predicted chemosensory base for host-specificity indeed could be found.

The sugar alcohol dulcitol is a strong feeding stimulant for larvae of the small ermine moth *Y. cagnagellus*. If dulcitol were also to act as an adult oviposition stimulant, this would simplify the formation of the co-adapted gene complexes needed for a successful host shift and subsequent speciation. The hypothesis that dulcitol acts as an oviposition stimulant for *Y. cagnagellus* was tested in **Chapter 4**.

In **Chapter 5**, the inheritance of oviposition behaviour in the three closely related species *Y. cagnagellus*, *Y. malinellus*, and *Y. padellus* is described. First generation (F1) and second generation hybrids (F2 and Back-crosses) were tested for their acceptance of the parental hosts for oviposition in choice tests, and the degree of dominance and sex-linked inheritance was studied.

Interspecific hybrids (F1) and second generation hybrids (F2 and Backcrosses), between *Y. cagnagellus* and *Y. padellus* were produced to measure larval choice, survival, and pupal weight on one or both of the parental host plants. This design enabled us to look at the fitness consequences of segregation of alleles underlying host acceptance and the ability to develop on the host: in **Chapter 6** we tested if evidence could be found for the existence of multiple genes controlling these traits in a co-adapted gene complex and if disruption of these gene complexes indeed led to reduced fitness. In **Chapter 7**, postzygotic reproductive isolation not related to host plant use was evaluated in hybrids of *Y. padellus* and *Y. cagnagellus*. Traditionally, speciation is thought to be completed by the evolution of hybrid sterility caused by genomic incompatibility. We performed a cytogenetic study of hybrid meiosis in interspecific crosses between *Y. padellus* and *Y. cagnagellus*, documented evidence of irregularities in chromosome pairing and tested their possible consequences for fertility and fecundity. In **Chapter 8** we summarize the results to provide the final conclusion.

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INTRODUCTION AND THESIS OVERVIEW


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