Colour Pattern Evolution and Development in *Vanessa* Butterflies

by

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Abstract

The evolution and development of eyespot and non-eyespot colour pattern elements was studied in *Vanessa* butterflies using a phylogenetic approach. A Bayesian phylogeny of the genus *Vanessa* was reconstructed from 7750 DNA base pairs from 10 genes. Twenty-four non-eyespot and forty-four eyespot color pattern elements from the Nymphalid ground plan were defined and studied and their evolutionary history was traced on the *Vanessa* phylogeny. Ancestral character states were predicted and the direction of evolutionary changes was inferred for all characters. Five serially arranged eyespots were predicted for the ancestral *Vanessa* on all wing surfaces. Homologous eyespot and non-eyespot characters on the surfaces of the forewing were more similar than those on the surfaces of the hindwing. Homologous eyespot characters on the dorsal surfaces of fore and hindwings show more similarities than the ventral surfaces, in contrast to what was found for non-eyespot characters. Independent Contrast analysis was also used to study correlations between eyespot characters. Independent Contrast analysis revealed significant correlations between eyespots 2 and 5 and eyespots 3 and 4 on all wing surfaces. This consistency among highly variable eyespot characters suggested a structural hypothesis: the existence of a Far-Posterior (F-P) compartment boundary and organizer could be responsible for the observed correlations. This hypothesis was tested in several ways. First, examination of wing patterns across species from all families of butterflies revealed correspondence between wing cells 1 and 4 and between cells 2 and 3. Second, evaluation of spontaneous mitotic clones in butterflies and moths reveals a peak abundance of clonal boundaries along the vein dividing wing cells 2 and 3. Finally,

experimentally generated FLP/FRT mitotic wing clones produced in *Drosophila*, reveal a clonal boundary posterior to the L5 wing vein, which is homologous to the vein dividing wing cells 3 and 4 in butterflies. Collectively, this suggests the existence of an additional compartment boundary associated with an organizer in wing cell 3 responsible for patterning the posterior portion of insect wings. A model is proposed that predicts that the wing developmental compartment boundaries produce unique combinations of gene expression for each wing sector, permitting eyespot individuation.

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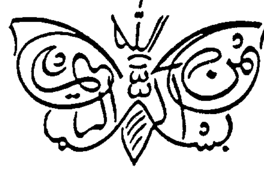

"Men from the land of Persia will attain scientific knowledge even if it is as far as the Pleiades (a constellation of stars)."

– a quote from the Prophet Mohammed (PBUH)

To my parents
Without whom none of my success would be possible

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Chapter 1 General Introduction

General Background

Order Lepidoptera Linnaeus 1758

Lepidoptera is the third largest order of insects with 175,000 known species and includes butterflies and moths (Mallet 2015). Butterflies as defined in the traditional sense include about 17,500 described species, that belong to two superfamilies: the skippers (Hesperioidea) with approximately 3750 described species, and true butterflies (Papilionoidea) with approximately 13,750 described species (Robbins and Opler 1997). However, higher-level phylogenetic studies place skippers within the true butterflies superfamily (see Fig. 1-1) (Regier et al. 2013). The six major families of butterflies are as follows: skippers (Hesperiidae); swallowtails (Papilionidae); whites, marbles and sulphurs (Pieridae); metalmarks (Riodinidae); gossamer wings (Lycaenidae); and brush-footed butterflies (Nymphalidae), with the last five families traditionally being assigned to superfamily Papilionoidea (Mallet 2015).

Family Nymphalidae Rafinesque 1815

With approximately 6,452 described species (Shields 1989; Harvey 1991), Nymphalidae is perhaps the largest and best studied family of butterflies. Based on a traditional morphology-based classification by Harvey (1991), the Nymphalidae contains 13 subfamilies but recent molecular studies have changed the composition of several of the subfamilies and decreased the number of subfamilies to 11 (Wahlberg and Peña 2015).

Genus *Vanessa* Fabricius 1807

The butterfly genus *Vanessa* Fabricius, 1807 (Nymphalidae, subfamily Nymphalinae, tribe Nymphalini) includes colourful butterflies distributed around the world, with the exception of Antarctica, and contains 22 described species to date (See *Vanessa* plate in chapter 2) (Braby 2000; Mecenero et al. 2013). They live in every biome except for deserts (Mecenero et al. 2013). Some species are geographically widespread, while six *Vanessa* species are endemics and live in restricted habitats as small as a few thousand square kilometres (Field 1971; Wahlberg and Rubinoff 2011) (see Table 1-1 and Figure 1-2). *Vanessa cardui* and *V. atalanta* are among the most widely distributed butterflies in the world (Emmel 1991) and the painted lady, *Vanessa cardui*, has been considered to be the only butterfly meriting being called cosmopolitan (Scudder 1876). Table 1-1 and Figure 1-2 summarize the distribution of *Vanessa* around the world. Field (1971) divided the genus into 3 genera (*Vanessa* Fabricius, 1807, *Cynthia* Fabricius, 1807 and *Bassaris* Hübner, 1821) based on the study of both male and female genitalia, tarsi, and habitus (morphology and colouration) (Field 1971). However, a molecular phylogenetic study of the genus using 9 genes of nuclear and mitochondrial origin, supported a monophyletic status of the genus *Vanessa* (Wahlberg and Rubinoff 2011). Wahlberg and Rubinoff (2011) also transferred two *Antanartia* species into *Vanessa*, *V. hippomene* and *V. dimorphica,* increased the number of species in *Vanessa* from 20 to 22.

Life cycle

Butterflies are among the holometabolous flying insects that have complete metamorphosis, and their life cycle is composed of four distinct stages: egg, larvae, pupa

and adult. The length of the life cycle in *Vanessa* is dependent on the temperature of the environment. At room temperature (20° C), it has a short life cycle that starts with barrel-shaped eggs that have a varying number of glassy vertical ribs, dependent on the species, (Braby 2000; Mecenero et al. 2013) that take 3 to 5 days to hatch. Caterpillars are black to green and yellowish-beige colour with branched spines along their body (Mecenero et al. 2013). They eat continuously for about 4 weeks and molt 4 times before pupation at the fifth instar larvae stage. Caterpillars of different *Vanessa* species feed on nettle, false nettle, pellitory, thistle, mallow, sunflower, pearly everlasting, pussytoes, cudweed, knapweed, groundsel, burdock and other plants (Braby 2000; Wagner 2005; Glassberg 2011; Allard 2013). An adult will emerge about 7 to 10 days after the chrysalis is formed by pupation. Adults feed on nectar, rotting fruit, and tree sap, and can mate within a week of emerging as an adult; *Vanessa* adults only live about 2-6 weeks. Figure 1-3 depicts the life cycle of *Vanessa cardui* graphically.

Overwintering (Hibernation and Migration)

Overwintering in butterflies is achieved by hibernation and migration during the cold and dry time of the year, and in different stages of the life cycle; egg, larvae, caterpillar and adult. In *Vanessa* butterflies, *V. atalanta* overwinters as an adult (Mikkola 2003a, 2003b; Hall et al. 2014) and occasionally as a pupae (Cech and Tudor 2005). It is shown that this butterfly migrates long distances from the Mediterranean to northern Europe, and returns back south using high-elevation northerly winds (Mikkola 2003a, 2003b). Following warm winters, there have been some reports of fresh *V. cardui* in Ontario, suggesting the possibility of overwintering as pupae (Hall et al. 2014). There are

reports of southward migrations for *V. cardui* (Scott 1986), and cold tolerant *V. virginiensis* in the fall (Cech and Tudor 2005; Hall et al. 2014). Stefanescu et al. (2012) studied the migration patterns and parasitism in *V. cardui* in Spain and Morocco, and found that population of parasites increased after establishment of several generations of *Vanessa* population in the same region in both countries. Therefore, they proposed that parasitoids may be a selective force driving the migration of *Vanessa* populations to new parasitoid-free regions (Stefanescu et al. 2012).

Genetics (number of chromosomes and sex determination)

More than 50% of the butterflies examined in terms of the haploid number of chromosomes, shows n=29, 30, or 31, with n=31 being the most common (White 1973, 1978; Saura et al. 2013). There is an apparent trend showing that the lower chromosome number is favoured as opposed to the higher chromosome number (White 1973, 1978; Saura et al. 2013). The situation in moths is also similar to that in butterflies (Robinson 1971; Saura et al. 2013). The number of chromosomes in most Trichoptera, which is the sister order to Lepidoptera, is also n=30 (Suomalainen 1969; Saura et al. 2013). Studies on the number of chromosomes in *V. atalanta* (Federley 1938), *V. cardui* (Lorkovic 1941), *V. indica* (Maeki and Makino 1953; Maeki 1953a, 1953b) and *V. virginiensis* (Maeki and Remington 1960) show that all of these species have chromosome numbers of n=31. Given the fact that three outgroup species used in *Vanessa* phylogeny, *Polygonia c-album* (Kernewitz 1914, 1915; Beliajeff 1930; Federley 1938; Maeki and Makino 1953; Maeki 1953a, 1953b), *Nymphalis polychloros* (Lorkovic 1941; Maeki and Makino 1953; Maeki 1953a, 1953b) and *Araschnia levana* (Lorkovic 1941; Maeki and

Makino 1953; Maeki 1953a, 1953b), also have n=31 chromosomes, it is probable that the whole genus has n=31 chromosomes. Sex in butterflies is determined by the ZW sex-determination system, in which the Z chromosome is larger and contains more genes. Males are ZZ and females are ZW (Traut et al. 2007). During development female butterflies sometimes lose chromosome W in some cells, and become hemizygous for Z. This phenomenon creates mosaic or bilateral gynandromorphs depending on the timing of the phenomenon (Packard 1875; Gerould 1925; Jahner et al. 2015).

Communication in butterflies

Intraspecific communication in Lepidopterans is similar to other insects and occurs through several modalities including chemicals (i.e. pheromones) (Schneider 1975), wing colour patterns (Blest 1957; Stevens 2005), as well as sound and ultrasound (Conner 1999; Yack and Fullard 2000). Based on the enormous diversity in their colour patterns, visual communication may be considered an important mode of communication in this group of insects. Butterflies are known for their colourful wing patterns (Nijhout 1991). These patterns are often so constant and reliable as to be used as field marks and characters for butterfly species identification (Nijhout 1991). It is shown that these patterns have a significant contribution to the butterfly survival (Blest 1957; Blum 2002). This occurs through communication with the opposite sex and mate attraction (Robertson and Monteiro 2005), and sending warning or misleading signals to predators (Kodandaramaiah 2011; Kodandaramaiah et al. 2013).

Colour vision

Adult butterflies have two compound eyes made of ommatidia. Each ommatidium is composed of a microscopic lens and photoreceptor cells coated by pigment cells (Land and Nilsson 2012). The visual pigments in ommatidia are made of a chromophore (Smith and Goldsmith 1990) and an opsin protein (Briscoe 2008). The ability to distinguish between different wavelengths of light regardless of the intensity is called true color vision (Menzel 1979; Goldsmith 1991). Studies have revealed that several moth and butterfly species including the hummingbird hawkmoth *Macroglossum stellatarum* (Kelber and Henique 1999), orchard butterfly *Papilio aegeus* (Kelber and Pfaff 1999), Asian swallowtail butterfly *Papilio xuthus* (Kinoshita et al. 1999), the common blue butterfly *Polyommatus icarus* (Sison-Mangus et al. 2008), and monarch butterfly *Danaus plexippus* (Blackiston et al. 2011) have true colour vision. Blackiston et al. (2011), based on their findings on the monarch butterfly, speculate that true colour vision might be common across Lepidoptera (Blackiston et al. 2011). A broad review of spectral sensitivity data shows that insects have pigment cells with the highest sensitivity of wavelength from 312 (*Proxylocopa sp.* (carpenter bee)) (Peitsch et al. 1992) to 620 nanometers (nm) (*Polygonia c-album* (nymphalid butterfly)) (Eguchi et al. 1982; Briscoe and Chittka 2001). In particular, all of the butterflies have three rhodopsins sensitive to ultraviolet, blue and green light, respectively (Briscoe and Chittka 2001). Also, some butterflies developed one or two other kinds of rhodopsins derived from a green-sensitive rhodopsin to be able to absorb and detect red light (Briscoe and Chittka 2001; Briscoe et al. 2003). Flowers have ultraviolet patterns that can guide butterflies. A study by Bernard (1979) of the eyes of 17 species of butterflies showed that 9 of the species possess a rhodopsin that is highly sensitive at 610 nanometers, which is in the yellow, orange and

red end of the light spectrum (Bernard 1979). Given these findings, it was suggested that the yellow, orange and red colour pattern on butterfly wings not only can be important for warning vertebrate predators, but can also be important for communication among butterflies (Bernard 1979). A study on visual pigment absorption spectrum of *V. cardui* using microspectrophotometry showed that it has a rhodopsin with maximum absorption at 530 nm (Bernard 1983). Another intense study with similar methodology showed that *V. cardui* also has two other rhodopsins (at much lower abundance than the first one) with maximum absorption at 470 and 360 nm (Briscoe et al. 2003). The study suggested that colour vision in *Vanessa* is trichromatic colour vision and simpler than the tetrachromatic colour vision in some other butterflies (Briscoe et al. 2003).

Wing venation system

The primary tracheal system of the wing (which is a component of the respiratory system of insects) is formed during the larval stages and turns into a secondary tracheal system during pupation when it finally produces wing veins (Nijhout 1991). Wing venation for *Vanessa* species is given in Figure 1-4. Veins are labeled based on previous studies (Miller and Brown 1989; Martin and Reed 2010). Veins are used to define sectors (also known in the field of Entomology as wing "cells") within the butterfly wing and many colour patterns are bounded on one or more sides by wing veins.

Wing scales

Lepidoptera is a Greek term that means "Scale wing". When looked at using microscopy, a butterfly wing can be seen to be made of a two layers of cells (thick

transparent or brown membrane), with scales on either side that overlap partially (Nijhout 1991; NISE 2015). Scales are flat structures made of cuticle that emerges from specialized cells (basal socket cells) on the wing during pupation (Nijhout 1991). Two types of scales are characterized in terms of size and role, including small ground scales and large cover scales. Ground scales and cover scales arrange alternately and typically produce background and main pattern elements respectively, by synthesizing the colour pigments during late pupation (Nijhout 1991). There are two types of colour shown by organisms including pigmentary and structural colours (Fox 1976). Differential absorption of light by pigments produces pigmentary colours, while physical interaction of light with biological nanostructures with varying reflective indices produce structural colours (Fox 1976; Prum et al. 2006). Scale cells synthesize the pigments during pupation. Colour pattern elements are produced first and then the remaining areas (which make up the ground colour of the wing) produce black or brown melanin pigments (Koch 2000; Koch et al. 2000). A study of 12 representative species of butterflies known for their structural colours demonstrated that coherent scattering or change of direction of photons without any modification is a method by which scales produce iridescent colour (Prum et al. 2006). *Vanessa* butterflies generally lack the blue, green, and violet iridescence associated with structural colouration, but there has been no systematic study to investigate this. When present, structural colour in butterflies seems to play a role in sexual communication (Robertson and Monteiro 2005) and thermoregulation (Bosi et al. 2008).

Functional significance of colour pattern

Colour patterns in most animals contribute to offensive and defensive mechanisms for survival, as well as courtship and mate selection and thermoregulation. Animals may use colour patterns to minimize the contrast between their body and environment (camouflage), to be able to avoid detection by their predators or prey successfully (Poulton 1890; Vignieri et al. 2010). The animals that are under attack by predators use colour patterns in multiple ways to hide from predators (camouflage), to falsely represent themselves with intimidating patterns (mimicry of predators), and to send (true or false) signals about toxicity or unprofitability as a prey item (aposematism) (Poulton 1890; Stuart-Fox and Moussalli 2009). In ectothermic land animals, dark colouration patterns may aid in efficient basking and thermoregulation (Geen and Johnston 2014). It is shown that *Pieris* butterflies use their wing melanization pattern to reflect solar radiation to their body for thermoregulation (Kingsolver 1985). Also, in some animals, two sexes may differ in their ornamentation by colour patterns, and this may play an important role in sexual selection (Kottler 1980). However there are examples that a character is not dimorphic but it is under sexual selection. In *Bicyclus anynana* butterflies, the UV reflecting central white pupils of dorsal eyespots of the wings are the most important feature in males under selection by females, but female butterflies also have evolved that character probably due to developmental constraints (Robertson and Monteiro 2005).

Studies have demonstrated that colour patterning in butterflies, is used to avoid vertebrate (Kodandaramaiah et al. 2009; Mukherjee and Kodandaramaiah 2015) and invertebrate predators (Tietz 1972). It is also used as a sexual signal (Breuker and Brakefield 2002; Robertson and Monteiro 2005; Costanzo and Monteiro 2007; Martin et

al. 2011), as well to facilitate behavioural thermoregulation (Kingsolver 1985, 1985b, 1987, 1988; Kingsolver and Wiernasz 1991). Seasonal dimorphism in butterfly colour patterns can also be understood in the context of camouflage or thermoregulation in seasonally changing environments (Kingsolver and Wiernasz 1991; Kingsolver 1995). Sexual dimorphism in terms of wing colouration pattern is present in a number of butterflies such as *Bicyclus* and *Junonia* (Oliver and Monteiro 2011). Male and female *Vanessa* are different in terms of overall body size (female is bigger), and shape of the abdomen (abdomen is curved because of egg mass and rounded at the posterior part in female), but they do not seem to have sexual dimorphism in colour pattern. There are reports showing that *V. virginiensis* will have brighter or paler colours in the early and late seasons, respectively (Opler and Krizek 1984).

Colour pattern elements

Colour pattern elements in Lepidoptera are organized in a series of repetitive pattern elements called the nymphalid ground plan (NGP) (Schwanwitsch 1924; Süffert 1927; Nijhout 1991; Otaki 2012). It is made of three symmetry systems; border, central and basal symmetry systems from wing margin to wing hinge respectively (Nijhout 2001). Otaki revised the NGP and proposed that both the central and border symmetry system each included one “core element” and two “paracore elements” on either side of the core element. He also considered the wing root band a symmetry system independent from the basal symmetry system. In addition, he unified marginal and submarginal bands as one “marginal band system” (Otaki 2012). Eyespots are part of the border symmetry system that occurs in between Media I and Externa III (see Fig. 3-1). Eyespots can differ

in terms of number, location, shape, size, colour, number of elements within and between the wing surfaces (Monteiro 2015; Abbasi and Marcus 2015b). Table 1-2 shows all of the NGP elements by different authors. *Vanessa braziliensis* is used as a representative for the genus *Vanessa,* to demonstrate the NGP elements (diagrams in chapters 2, 3 and 4).

Due to differences in their structure and origin, I have decided to divide the colour patterns of the NGP into two categories of non-eyespot and eyespot patterns. The non-eyespot patterns in order from the wing margin to the wing hinge are: the Externae I, II, and III; Media I, Discalis I, Media II, Discalis II, and the Basal Symmetry System (See Chapters 2, 3 and 4 for diagrams of NGP) (Nijhout 2001; Martin and Reed 2010). Eyespot patterns include eyespots and their components. Gene expression studies have demonstrated the association of genes *aristaless 2* (Martin and Reed 2010), *wingless* (Carroll et al. 1994) and *WntA* (a paralogue of *wingless*) (Martin et al. 2012), with the formation of non-eyespot colour patterns. Also QTL mapping of the mimicry locus H in swallow tail butterfly revealed the role of transcription factor *invected* in female specific colour pattern (Clark et al. 2008). There are at least 12 genes including *Distal-less*, *engrailed*, *Notch*, *spalt*, *Antennapedia, cubitus interruptus*, *patched*, *hedgehog*, *wingless*, *SMAD1*, *Ecdysone receptor* and *decapentaplegic* that are expressed in the eyespot position on the developing wing (Brakefield et al. 1996; Beldade and Brakefield 2002; Monteiro et al. 2006; Saenko et al. 2011; Oliver et al. 2012; Shirai et al. 2012), but only the first 5 of these genes are found in the eyespots of most nymphalid butterflies examined (Oliver et al. 2012; Shirai et al. 2012). Functional studies on the above genes are required to confirm their role on eyespot and non-eyespot pattern formation. Chapters 2 (Abbasi and Marcus 2015a) and 3 (Abbasi and Marcus 2015b) will specifically discuss

the evolution and development of non-eyespot and eyespot patterns in *Vanessa* respectively.

Evolutionary history of a trait

The study of evolution can be challenging because it usually occurs too slowly to observe directly in controlled experiments, and so the evolutionary history of organisms must often be inferred rather than measured directly (Lenski and Travisano 1994; Ronquist 2004). Knowing the ancestral state of a particular character is important for understanding the evolution and adaptation of that character. The fossil record can provide this information, but often there is no fossil record or the nature of the character being studied would not allow it to be fossilized (Ronquist 2004). Fortunately evolution leaves traces in extant organisms. Inferring the ancestral state of a particular character using the information from the current state of that character and a phylogeny is a popular and alternative method to predict the ancestral state (Harvey and Pagel 1991; Ronquist 2004). In this method, the current state of characters in a group of organisms are mapped on the phylogenetic tree of that group and the ancestral states in each node are inferred (Schultz et al. 1996; Schluter et al. 1997; Cunningham et al. 1998; Pagel 1999; Ronquist 2004).

There are number of methods for inferring the ancestral state of the character including maximum parsimony (Maddison et al. 1984; Maddison and Maddison 1992), maximum likelihood (Edwards 1972; Schluter et al. 1997; Pagel 1999) and Bayesian inference (Schultz and Churchill 1999; Ronquist 2004). The maximum parsimony method takes the minimum number of evolutionary changes or steps into account in

inferring the ancestral character state (Maddison et al. 1984). The limitation of this method is that it ignores uncertainties in mapping, meaning that it provides only one solution to the ancestral state problem by a criterion of a minimum number of steps. Ancestral state reconstruction by parsimony generally assumes equal probabilities of all character state transitions, so that the probability of a character state gain is equal to the probability of a character state loss. Also the rate of evolution (represented by branch length) in character states is not considered by parsimony reconstructions. Finally, parsimony-based ancestral character state reconstruction assumes that the phylogenetic tree being used is correct and does not take the uncertainties about the reconstructed phylogenetic tree into account (Reviewed in (Cunningham et al. 1998; Ronquist 2004). Despite these problems, most of ancestral state reconstruction studies rely on parsimony reconstruction method (Cunningham et al. 1998; Ronquist 2004).

The maximum likelihood is an alternative method that takes some evolutionary models for the characters into account to provide the highest possibility of observing current state of the characters from the inferred ancestral states (Edwards 1972; Schluter et al. 1997; Pagel 1999). Bayesian reconstruction method is another method that accounts for the phylogenetic inference uncertainty and character mapping uncertainties at the same time. Unlike the likelihood method, the relative rates of changes i.e. from 0 to 1 do not have to be fixed and can change in the reconstruction (Ronquist 2004). Similar to most of the reconstruction studies (Reviewed in (Cunningham et al. 1998; Ronquist 2004), I present the parsimony reconstruction in this dissertation.

Compartment boundaries

Each wing sector in butterfly wings can be similar to or different from neighbouring sectors but they were derived from similar cells during development. Two of the fundamental questions that developmental biologists ask are (1) how the progeny of a cell acquire different fates and develop into different tissues adjacent to each other and (2) what forces or mechanisms keep those populations of cells separated. Two types of boundaries have been characterized that contribute in these processes: lineage boundaries (Garcia-Bellido 1968; Garcia-Bellido et al. 1973) and non-lineage boundaries (Mann and Morata 2000). Dahmann et al. (2011) provides a useful scheme for understanding the development of boundaries in developing tissues that form barriers between gene expression domains. Non-lineage boundaries are non-cell autonomous meaning that cell populations require continuous signals from other cells to maintain their fate (Vincent 1998; Dahmann et al. 2011).

At lineage boundaries, which are also called compartment boundaries and are the focus of this study, the fate of the cells is inherited, require no constant input signals, therefore these boundaries are cell autonomous (Vincent 1998; Dahmann et al. 2011). As early developmental processes that trigger cascade of events, these boundaries are very important and any deficiency would affect all of the subsequent patterning and depending on the place may kill the organism or produce anomaly (Dahmann et al. 2011). It is known that compartment boundaries play important roles in pattern formation in holometabolous insect wings and vertebrates (Dahmann and Basler 1999). The important function of compartment boundaries in maintaining the position of organizer cells in the developing tissues is well studied and illustrated in the *Drosophila* wing development (Dahmann and Basler 1999). The wing, similar to all appendages, originated from an

imaginal disk that is, in turn, formed by invagination of the embryonic epidermis (Dahmann and Basler 1999). Two different boundaries have been discovered in the wing disk of *Drosophila* as Anterior/Posterior (A/P) (Garcia-Bellido et al. 1973) and Dorsal/Ventral (D/V) (Garcia-Bellido 1968) compartment boundaries. Chronologically, A/P boundary in *Drosophila* occurs before D/V boundary during embryogenesis (Vincent 1998). The present study hypothesizes existence of an additional A/P compartment boundary posterior to the A/P boundary that has been described previously, reports several lines of evidence for the additional boundary, and predicts its possible role in the patterning of the insect wings.

Homeosis

Homeosis is a phenomenon in which part of the organism grows in the place of another part due to mutation and missexpression of developmentally important genes (homeotic genes) that regulate positional information (Garcia-Bellido 1975, 1977; Lewis 1978; Hombria and Lovegrove 2003). This phenomenon may be seen in insects on the integument that covers their body. The integument may convert between body segments, between tissues derived from different imaginal disks or within an imaginal disk (Sibatani 1980). As an example, *apterous (ap)* is a gene that is only expressed on the dorsal surface of the insect wing. If a cell on the dorsal surface of the wing loses the expression of apterous protein due to mutation, the descendants of that cell will also inherit the loss and that lineage of cells will show the morphology of a ventral wing surface (Blair et al. 1994). In a second example, *Ultrabithorax (Ubx)* is a gene product that is only expressed in the hindwing and in thoracic segment 3. Loss of function

mutations transform the tissue so that it is similar to the equivalent position on the forewing, which is attached to thoracic segment 2 (Lewis 1978; Weatherbee et al. 1999). The mosaic tissues of homeotic and gynandromorph cell lineages (also called clones) provide an excellent tool to study the position of compartment boundaries. These naturally and genetically marked cells are similar to normal cell in that they will not cross compartment boundaries and due to their distinct morphology, can be used to show the location of the compartment boundary. I will use data from clones found in naturally occurring Lepidoptera homeotic mutants, as well as from experimentally-induced clones in *Drosophila melanogaster*, to show the placement of the second hypothetical A/P compartment boundary.

Objectives and rationale of the dissertation

The scientific question that I address in this dissertation is how the high diversity of colour patterns found in the butterfly genus *Vanessa* arose and changed over time. I first divide the colour pattern elements based on their genetic architecture and possible function into non-eyespot and eyespot colour pattern characters. Next, I predict the ancestral states in each set of characters for all wing surfaces in these butterflies. I then discuss the direction of changes in each character, and discuss the possible adaptive advantages of different pattern element character states. Finally, I hypothesize how these colourful patterns on the wing surfaces adjacent to each other can individuate to take on distinct individual phenotypes. I present a model for eyespot individuation that based on the previously published observations and the data collected during the early parts of my PhD studies. Finally, I provide a number of lines of evidence including comparative

morphological and experimental genetic approaches in butterflies and fruit flies to test the predictions of the hypothesis. The evidence I have collected is consistent with the model that I have proposed.

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Table 1-1. The localities of *Vanessa* specimens used in 4 phylogenetic studies (Otaki et al. 2006, Wahlberg et al. 2005; Wahlberg and Rubinoff 2011, Abbasi and Marcus 2015a), plus a more general description of the global distribution of each species (Savela 2015).

Species \ References	(Otaki et al. 2006)	(Wahlberg et al. 2005; Wahlberg and Rubinoff 2011)	(Abbasi and Marcus 2015a)	(Savela 2015)
Vanessa abyssinica (C. & R. Felder, 1867)		Tanzania	Kenya	Abyssinia, Kenya, Tanzania, Ethiopia, Uganda, Rwanda, Congo
Vanessa altissima (Rosenberg & Talbot, 1914)		Peru	Peru	Ecuador, Peru
Vanessa annabella (Field, 1971)		Wyoming	California	California
Vanessa atalanta (Linnaeus, 1758)	Slovakia	USA, Switzerland, Sweden, Finland, Spain	Canada, USA, Poland, Romania	Azores Islands (Portugal), Canary Islands (Spain), Hawaii, USA, Guatemala, Haiti, Iran, Pakistan, New Zealand, Europe, North America
Vanessa braziliensis (Moore, 1883)	Peru	Brazil, Ecuador	Argentina, Peru	Brazil, Argentina, Bolivia
Vanessa buana (Fruhstorfer, 1898)	Sulawesi (Indonesia)	Sulawesi (Indonesia)	Sulawesi (Indonesia)	Sulawesi (Indonesia)
Vanessa cardui (Linnaeus, 1758)	Japan	Spain, USA, Russia, Tanzania, Greece	Poland, Romania, Vietnam, France, Canada, USA, South Korea	Hawaii (USA), Iran, Asia, Africa, Europe, North America, Australia
Vanessa carye (Hübner, 1812)		Chile	Chile, Argentina	Colombia, Ecuador, Peru, Bolivia, Paraguay, Uruguay, Argentina, Patagonia (Argentina), Galapagos (Ecuador), Easter Island (Chile), Juan Fernandez (Chile), Tuoamotu Archipelago
Vanessa dejeanii (Godart, 1824)	Java (Indonesia)	Bali (Indonesia)	Java, Bali (Indonesia)	Java, Bali, Lombok (Indonesia), Philippines
Vanessa dilecta (Hanafusa, 1992)	Timor (Indonesia)		Timor (Indonesia)	Timor (Indonesia)
Vanessa dimorphica (Howarth, 1966)		South Africa	Kenya	
Vanessa gonerilla (Fabricius, 1775)	New Zealand	New Zealand	New Zealand	New Zealand, Chatham Islands (New Zealand)
Vanessa hippomene (Hübner, 1823)		South Africa	Congo, Tanzania, Uganda	
Vanessa indica (Herbst, 1794)	Japan	Russia, Japan, China	Vietnam, China	India, Myanmar
Vanessa itea (Fabricius, 1775)	Australia	New Zealand, Australia	New Zealand, Australia	New Zealand, Australia
Vanessa kershawi (McCoy, 1868)		Australia	Australia	Australia
Vanessa myrinna (Doubleday, 1849)	Peru	Brazil	Peru	Brazil, Ecuador, Peru, Colombia
Vanessa samani (Hagen, 1895)	Sumatra (Indonesia)	Sumatra (Indonesia)	Sumatra (Indonesia)	Sumatra (Indonesia)
Vanessa tameamea (Eschscholtz, 1821)	Hawaii (USA)	Hawaii (USA)	Hawaii (USA)	Hawaii (USA)
Vanessa terpsichore (Philippi, 1859)		Chile		Chile
Vanessa virginiensis (Drury, 1773)		USA, Dominican Republic	USA, Canada, Canary Islands	USA, Canada, Guatemala, Cuba, Galapagos, Canary Islands (Spain), Hawaii (USA)
Vanessa vulcania (Godart, 1819)		Canary Islands (Spain)	Canary Islands (Spain)	Canary Islands (Spain)

Table 1-2. Nymphalid ground plan nomenclature reproduced from Nijhout (1991) with new data from Otaki (2012). Key to acronyms: BSS, Basal Symmetry System, CSS, Central Symmetry System, BoSS, Border Symmetry System, MBS, Marginal Band System.

(Schwanwitsch 1924)	(Süffert 1927)	(Nijhout 1978, 1985b, 1991)		(Otaki 2012)	
Basalis (B)	Wurzelbinde (W)	Wing root band		Wing root band (WRB)	
Discalis II (DII)	Hohlbinde (H)	Basal symmetry system	BSS	Basal symmetry system (BSS)	BSS
Media II (MII); Granulata II (GII)	Innenbinde des centralen; Symmetriesystems (Cp)	Proximal band of central symmetry system		Paracore element (Proximal band of central symmetry system (pBC))	
Discalis I (DI)	Discoidalfleck (D)	Discal spot	CSS	Core element (Discal spot (DS))	CSS
Media I (MI); Granulata I (GI)	Aussenbinde des centralen; Symmetriesystems (Cd)	Distal band of central symmetry system		Paracore element (Distal band of central symmetry system (dBC), [Element f])	
(Not recognized)	Innere Binde des ocellaren Symmetriesystems (Op)	(Not named)		Paracore element (Proximal parafocal element (pPFE) [Element g])	
Circulus (C); Ocellata (OC)	Ocellenreihe (O)	Border ocelli	BoSS	Core element (Border ocelli (eyespot) [Element h])	BoSS
Externa III (EIII)	Aussere Binde des ocellaren Symmetriesystems (Od)	Parafocal element		Paracore element (Distal parafocal element (dPFE) [Element i])	
Externa II (EII)	Randbinde 2 (R_2)	Submarginal band	MBS	Submarginal band (SMB) [Element j]	MBS
Externa I (EI)	Randbinde 1 (R_1)	Marginal band		Marginal band (MB)	
Venosa (V)	(Not recognized)	Venous stripe		(Not recognized)	
Intervenosa (I)	(Not recognized)	Intervenous stripe		(Not recognized)	

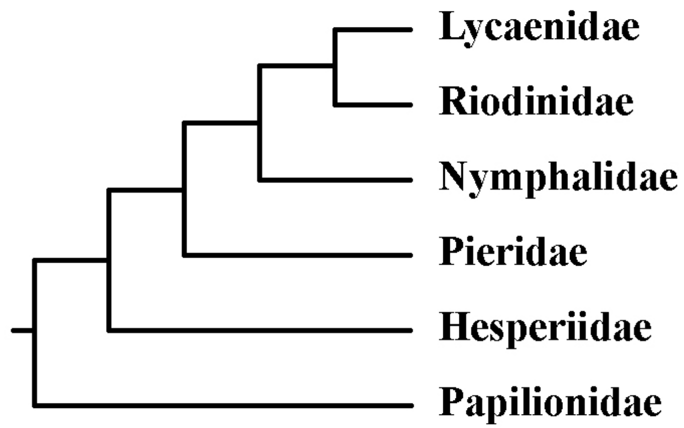

Figure 1-1. Phylogeny of the major butterfly families, reproduced from Regier et al. (2013) and tree of life project website (Brower 2015).

Figure 1-2. Approximate distribution of the species of *Vanessa* around the world.

Figure 1-3. Life cycle of *Vanessa* showing the four distinct stages: adult, egg, larvae and pupa (Photographs by Jeffrey Marcus).

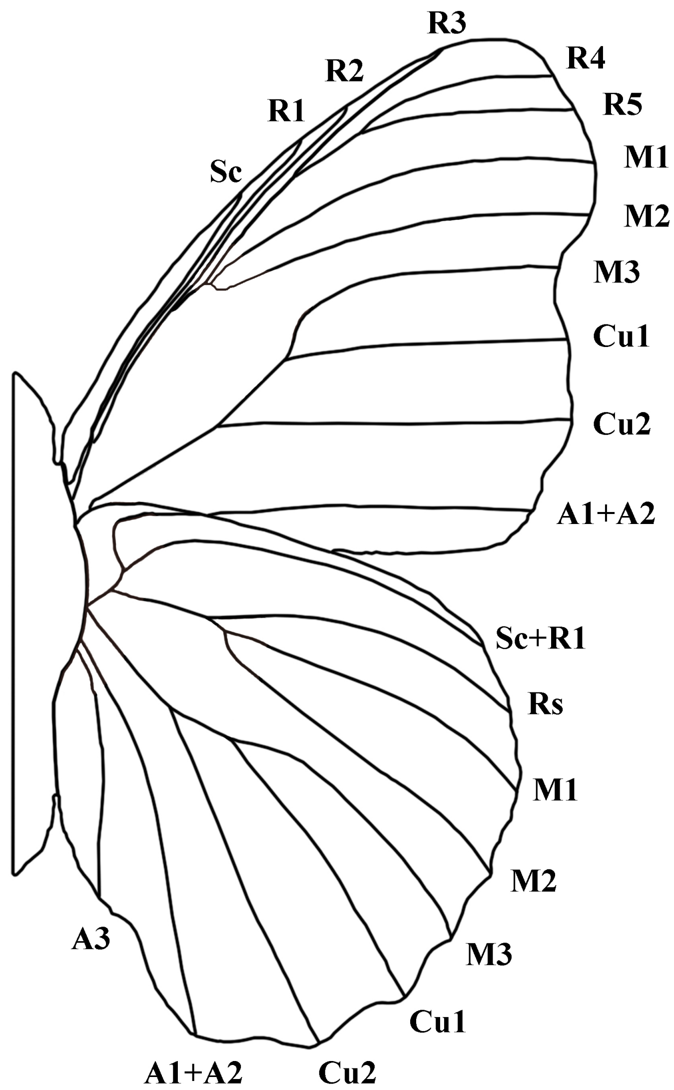

Figure 1-4. Wing venation system in adult *Vanessa* butterflies, adapted from references (Miller and Brown 1989; Martin and Reed 2010).

Chapter 2 Colour pattern evolution in *Vanessa* butterflies (Nymphalidae: Nymphalini): Non-eyespot characters[1]

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[1] This chapter has been previously published as:
Abbasi, R. and J. M. Marcus. 2015. Color pattern evolution in *Vanessa* butterflies (Nymphalidae: Nymphalini): Non-eyespot characters. Evolution & Development **17** (1): 63-81. © John Wiley & Sons, 2015. The chapter is published here under the terms of the Copyright Transfer Agreement between the author and John Wiley & Sons Publishers.
RA conceived the research, collected the data, and wrote the manuscript. JMM supervised the project, scored the morphological characters independently, double-checked the analyses, and made extensive comments and revisions to the manuscript.

Summary

A phylogenetic approach was used to study colour pattern evolution in *Vanessa* butterflies. Twenty-four colour pattern elements from the Nymphalid ground plan were identified on the dorsal and ventral surfaces of the fore- and hind wings. Eyespot characters were excluded and will be examined elsewhere. The evolution of each character was traced over a Bayesian phylogeny of *Vanessa* reconstructed from 7750 DNA base pairs from 10 genes. Generally, the correspondence between character states on the same surface of the two wings is stronger on the ventral side compared to the dorsal side. The evolution of character states on both sides of a wing correspond with each other in most extant species, but the correspondence between dorsal and ventral character states is much stronger in the forewing than in the hindwing. The dorsal hindwing of many species of *Vanessa* is covered with an extended Basal Symmetry System and the Discalis I pattern element is highly variable between species, making this wing surface dissimilar to the other wing surfaces. The Basal Symmetry System and Discalis I may be contribute to behavioral thermoregulation in *Vanessa.* Overall, interspecific directional character state evolution of non-eyespot colour patterns is relatively rare in *Vanessa,* with a majority of colour pattern elements showing non-variable, non-directional, or ambiguous character state evolution. The ease with which the development of colour patterns can be modified, including character state reversals, has likely made important contributions to the production of colour pattern diversity in *Vanessa* and other butterfly groups.

Keywords: Colour pattern evolution, *Vanessa*, Nymphalidae, Non-eyespot character evolution, butterfly wing

Abstract word count: 246 words

Introduction

Wing colour patterns in butterflies are used for visual communication, serving in courtship, camouflage, and aposematism (Nijhout 1991). As such, colour patterns serve a variety of important behavioural and ecological functions and are subject to the effects of both natural and sexual selection (Hiyama et al. 2012). Butterflies provide an attractive model system for evolutionary developmental studies because of the 2-dimensional nature of their wings, which are only two cells thick (Marcus 2005). Monteiro and Prudic (2010) classified studies on butterfly colour pattern evolution into proximate studies of developmental mechanism and ultimate studies of the functional significance of colour pattern change. Numerous studies have focused on both the mechanistic details of colour pattern genetics and development (Reed and Nagy 2005; Evans and Marcus 2006; Monteiro and Prudic 2010; Martin et al. 2012), and the functional significance of the patterns in the natural environment (Kingsolver 1988; Mallet and Barton 1989; Kodandaramaiah 2009; Kodandaramaiah et al. 2013). A phylogenetic framework is essential for understanding the direction and pattern of evolution over time for morphological characters such as colour patterns (Harvey and Pagel 1991; Marcus and McCune 1999; Monteiro and Prudic 2010).

The Nymphalid butterfly genus *Vanessa* has a nearly global distribution. It includes 22 described species (Wahlberg and Rubinoff 2011) that display impressive wing colour pattern diversity (Vane-Wright and Hughes 2007), including a majority of the elements of the nymphalid ground plan (see below and Fig. 2-1). The combination of impressive phenotypic diversity with a manageable number of species makes *Vanessa* an

especially good model system for the study of butterfly colour pattern evolution. The natural history of the genus is well-studied, and the ranges, phenologies, larval hosts, and migration patterns of most species are known (Williams 1930; Opler and Krizek 1984; Myers 1985; Blitzer and Christoffel 2014). *Vanessa* includes two of the most widely distributed butterfly species, the painted lady (*V. cardui*) and red admiral (*V. atalanta*) (Emmel 1991), allowing butterfly researchers all over the world ready access to the genus (and very often to the same species). *Vanessa cardui* is among the easiest butterflies to rear and there is an artificial diet available, making it a useful and convenient butterfly model for laboratory studies (Ellis and Bowers 1998).

Otaki and Yamamoto (2004a, 2004b) studied the colour patterns in *Vanessa* that are produced by the administration of sodium tungstate, a chemical substance that may mimic the effects of a putative cold-shock hormone and inhibits the activity of protein tyrosine phosphatases (PTPases). Sodium tungstate injected pupae of *V. indica* and *V. cardui* produces phenotypes that resemble the normal colour patterns found in the number of other *Vanessa* species (Otaki and Yamamoto 2004a, 2004b). Otaki and Yamamoto (2004b) suggested that cold shock-associated phenotypes might have played a role in the evolution of *Vanessa* wing patterns and speciation in high altitude populations that experience large fluctuations in temperature. In a separate study, Otaki (2008) proposed a physiological side-effect model to explain the existence of interspecific variation in non-functional colour patterns (with no currently identified roles in mating, mimicry, aposematism, or camouflage) seen in *Vanessa* and other butterflies. He suggested that the existence of diverse but apparently non-functional patterns might be a by-product of the effects of the putative cold shock hormone in butterflies (Otaki 2008).

There is also a long history of taxonomic study of *Vanessa* butterflies (reviewed in Field, 1971). Field (1971) divided the 16 species then-known from the group into 3 genera: *Vanessa* (5 species, type species *V. atalanta*), *Cynthia* (9 species, type species *C. cardui*) and *Bassaris* (2 species, type species, *B. itea*) based on the structure of genitalia, tarsi, and habitus. An early molecular phylogenetic study with incomplete taxon sampling appeared to show that *Bassaris* and *Cynthia* were monophyletic, but was equivocal about the monophyly of *Vanessa* as defined by Field (1971) and did not sample enough outgroup species to verify the overall monophyly of the group (Otaki et al. 2006). More recent molecular studies support the monophyletic status of the genus *Vanessa* in the broad sense, and identified additional *Vanessa* species that had previously been assigned to other genera based on morphology (Wahlberg and Rubinoff 2011).

Otaki et al. (2006) combined the study of *Vanessa* colour patterns with phylogenetic methods. They focused on 2 clades: the *indica* group (*V. indica, V. samani, V. dejeanii, V. dilecta* and *V. buana*) and the *atalanta* group (*V. atalanta, V. tameamea*) based on results of a phylogenetic analysis (Otaki et al. 2006). They studied the relative area of orange (RAO) values on the forewings that were defined as the percentage of orange area (ground colour of the wing) relative to the entire wing surface. They assigned three character states based on RAO values: large or orange type (e.g. *V. samani*), intermediate (e.g. *V. buana*), and small or black type (e.g. *V. dejeanii*). Otaki et al. (2006) did not find a clear relationship between colour pattern and phylogeny, suggesting that RAO may be subject to homoplasy, and that similarities in RAO values may be due to convergence. However, the RAO value is a problematic character because it only encompasses a small fraction of the complexity observed in the colour patterns in

Vanessa butterflies. RAO values only describe the ratio of background to the area covered by pattern elements on a portion of the wing. A higher relative amount of background colour, or orange area, only implies smaller colour pattern elements, and does not describe the complexity or nature of the pattern itself. Approaches to the study of colour pattern evolution that refer directly to individual colour pattern elements may be more practical than RAO for tracing the evolutionary history of colour patterns in *Vanessa*.

The objective of this study is to reconstruct the ancestral states and trace the direction of changes in wing pattern evolution in *Vanessa* butterflies using a phylogenetic approach. The nymphalid ground plan provides a framework for scoring morphological characters on the dorsal and ventral surfaces of both wings. The nymphalid ground plan is the set of pattern elements composed of basal, central and border symmetry systems that make up butterfly wing patterns (Fig. 2-1) (Schwanwitsch 1924; Nijhout 1991; Otaki 2012b). We have chosen to focus only on non-eyespot patterns here because these patterns are similar to each other in terms of their developmental biology and appear to be associated with domains of expression of the secreted ligand *wingless* (Martin and Reed 2010). Butterfly eyespots appear to be evolutionarily derived from components of the submarginal bands (Fig. 2-1) (Nijhout 1994, 2001; Oliver et al. 2014) and there appear to be signaling processes occurring between the incipient eyspots and other elements of the border symmetry system that determine the relative placement of these patterns (Otaki 2012a, 2012b). However, the structure of the eyespots themselves develops via a more complex mechanism that seems to be *wingless*-independent (Evans and Marcus 2006; Martin and Reed 2010) and will be considered elsewhere in separate

publications (Abbasi and Marcus 2015b, ms).

First, we will identify which *Vanessa* colour pattern characters are variable, and which show no change across species. For those characters that show interspecific variation, we postulate a null hypothesis that there are no directional trends in colour pattern evolution, and that interspecific variation in colour pattern elements in *Vanessa* is characterized primarily by random changes. The alternative hypothesis is that there are directional trends in the evolution of colour pattern elements across *Vanessa* phylogeny associated with diversification of the genus.

Material and Methods

Specimens were acquired for 38 species of butterflies: 22 ingroup taxa from *Vanessa* and 16 outgroup taxa within Nymphalini including *Aglais, Antanartia, Araschnia, Hypanartia, Kaniska, Mynes, Nymphalis, Polygonia,* and *Symbrenthia*. Outgroup taxa were selected with reference to Wahlberg et al. (2005). Many sequences from the 38 exemplar species were available from GenBank (NCBI) as part of previously published studies (Wahlberg et al. 2005; Otaki et al. 2006; Wahlberg and Rubinoff 2011). We obtained additional DNA sequences ourselves to confirm specimen identifications based on morphology and to gather sequence data missing from previously published phylogenetic studies (Supplemental Table S2-1). New sequences were deposited in GenBank (accession numbers KJ648948-KJ649143 and KM225792- KM225794).

Legs were removed from each specimen and DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's animal tissue extraction protocol with minor modifications (Borchers and

Marcus 2014). Briefly, legs were removed from each specimen and pulverized with a ceramic mortar and pestle. DNA concentration was measured by a Nanodrop 2000 spectrophotometer (Nanodrop, Wilmington, Delaware, USA), before samples were stored at -20 C for future use. PCR amplification was carried out using previously published primers and established PCR protocols (Table 2-1). PCR reactions were conducted in 25 μl volumes using Quick-Load Taq 2X (New England Biolabs, Ipswich, MA, USA) and primer concentrations of 1 μM. Template concentrations varied from 5 ng to 50 ng of genomic DNA per PCR reaction.

PCR reactions were performed in MyCycler and S1000 Thermal Cyclers (BioRad, Hercules, California, USA). PCR products were evaluated using agarose gel electrophoresis, stained with ethidium bromide, and evaluated under UV light to detect successful amplifications. Correctly sized PCR products were prepared for sequencing using fluorescent-tagged Sanger dideoxy sequencing as previously described (Borchers and Marcus 2014) and ABI Big Dye V3.1 Dye Termination sequencing chemistry (Applied Biosystems, Carlsbad, California, USA). PCR products were sequenced in both directions using the same primers used to generate the PCR products. Sequencing reactions were analyzed on an ABI 3730xl automated sequencer (Applied Biosystems) located at the University of Calgary Core DNA Services laboratory (Alberta, Canada). Sequencher 4.6 (Sequencher 2005) was used to assemble contigs, edit chromatograms and prepare sequences for alignment. Sequences for each gene were aligned separately using ClustalW2.1 (Thompson et al. 1994) as implemented for the EMBL-EBI online server (McWilliam et al. 2013) under the default settings. Polymorphic sites were double-checked by eye in Sequencher 4.6 to ensure data quality. Before initiating more complex

phylogenetic analyses with the complete dataset, we confirmed the species identification of our specimens based on morphology by comparing the barcode fragment of the *cytochrome oxidase subunit I* (*COI*) gene (Hebert et al. 2003) from our specimens with each other and with previously sequenced exemplars of *Vanessa* and outgroup species from GenBank (Otaki et al. 2006; Wahlberg and Rubinoff 2011). Sequences were aligned with ClustalW (Thompson et al. 1994) as described above, then we generated a neighbour-joining tree with PAUP* 4.0610 (Swofford 2002) and ensured that each of our samples belonged to a monophyletic group with other specimens from the same species.

For each gene, taxon names and the order of the samples in each alignment were standardized using MEGA5 (Tamura et al. 2011) and the alignments were concatenated manually to create the data matrix composed of 38 taxa and 10 genes, for a total of 7750 base pairs. The whole concatenated dataset for 10 genes, as well as the dataset for each gene separately was analyzed under the Bayesian information criterion (BIC) (Schwarz 1978) and the Akaike information criterion (AICc) (Akaike 1974) using MEGA5 (Tamura et al. 2011). The following models were recovered as the best model under the chosen criterion for each gene and whole dataset: GTR+G+I for *COI*, HKY+G for *GAPDH*, K2+G+I for *EF1-a*, K2+I for *ArgKin* and *Wg*, T92+G for *CAD, IDH* and *MDH*, T92+G+I for *RpS5,* T93+G+I for *ND5,* and GTR+G+I for the whole dataset combined. MrBayes v3.2.1 (Ronquist and Huelsenbeck 2003) was used for Bayesian phylogenetic inference. The combined dataset was divided into 10 partitions corresponding to the 10 genes. Commands were written for the selected models and their parameters for each gene in the Nexus file and run in MrBayes v3.2.1 for 10,000,000 generations. Sampling was set for every 1000th generation and printfreq=1000 seed=5748 swapseed=5748

temp=0.3. The combined dataset was also run as a whole without partitioning to the 10 genes under a gained model of GTR+G+I. All the Bayesian analyses were run with a default setting of 2 independent runs and 4 chains. The run was stopped after 10,000,000 generations where the average standard deviation of split frequency of the 2 runs was less than 0.05 and close to zero (showing that 2 runs converged on the same optimal tree) (Lemey et al. 2009). Tracer v1.6 (Rambaut et al. 2014) was used with its default setting to assure the stationarity and convergence of 2 independent runs by producing plots of log probability of the data versus the number of generations as well as convergence diagnostics (Sharanowski et al. 2010). In 10,000,000 generations, both independent runs were determined to be at stationary phase with effective sample size values (ESS) of 5175.0264 and 5143.0522 respectively (Rambaut et al. 2014).

Colour pattern character states were evaluated from specimens in our laboratory's research collection. For those species with small numbers of specimens, we supplemented our direct observations with previously published photos (Layberry et al. 1998; Vane-Wright and Hughes 2007; Wahlberg and Rubinoff 2011; Dec 2012; Fric et al. 2012; Warren et al. 2012). Species were examined to assess the probable polymorphism among regional populations, sexes and seasonal forms. We examined our *Vanessa* samples for intraspecific variation in non-eyespot characters and found that the amount of variation is negligible compared to interspecific variation. Specimens with colour pattern phenotypes evaluated for this study are listed in supplemental Table S2-2. The dorsal and ventral wing surfaces of each species are shown in Figs. 2-2 and 2-3. Morphological characters were defined based on the Nymphalid ground plan (Table 2-2). States are assigned for the variation of each character and then scored for each species in

both the ingroup and outgroup (Table S2-3). Abbreviations for characters were made based on the position and name of the compartments in which they occur. Mesquite version 2.75 (Maddison and Maddison 2011) was used for mapping the phenotypic characters on the Bayesian phylogenetic tree for ancestral state reconstruction using a parsimony reconstruction method. Reconstructions and mappings were carried out for all of the taxa including the outgroups, but due to limited space, the outgroups were removed from the figures and only the *Vanessa* is shown.

Results

The phylogeny inferred based on a separate Bayesian model for each gene and the phylogeny based on a single Bayesian model for all genes produced identical tree topologies. There were some minor differences between the trees in terms of posterior probabilities, all of which were less than two percent. Therefore only the tree without partitioning and including all base positions is shown here (Fig. 2-4). The phylogeny was generally well supported with high Bayesian posterior probabilities and was broadly congruent at the species level with the relationships reported by Wahlberg and Rubinoff (2011a).

Most of the intraspecific variation in colour pattern that we observed in *Vanessa* was in the eyespot phenotypes, which we address elsewhere (Abbasi and Marcus 2015b, ms). While both wild-caught and laboratory induced aberrations in non-eyespot patterns in *Vanessa* are known (Köhler and Feldotto 1937; Otaki 2003; Otaki 2007; Otaki 2008), these phenotypes are quite rare in wild-caught individuals. While specimens varied somewhat in the hue of their colour patterns, we observed very little intraspecific

variation in the size and shape of non-eyespot characters, the basis for our character state definitions, within the series of *Vanessa* specimens that we studied. Character states for each colour pattern character was mapped individually onto the phylogeny and the evolutionary history of each character was reconstructed using parsimony. For clarity, the results of the character state reconstruction and optimization will be discussed in spatial order from the wing margin to the wing hinge: The Externae I, II, and III; Discalis I and II and the Basal Symmetry System.

Externa I

On both surfaces of both wings, a dotted Externa I is the only character state observed (Table 2-3). The reconstruction of the ancestral state on all four wing surfaces showed that the ancestral *Vanessa* had a dotted line in Externa I (Table 2-4). There are no character state changes observed in Extranae I within *Vanessa* (data not shown).

Externa II

On both surfaces of the forewing, a continuous Externa II is the most frequent character state. In the forewing, 21 out of 22 species show the same pattern as continuous form of Externa II on two surfaces of the wing. On the hind wing, 14 out of 22 species show the same pattern as either a continuous or dotted line in Externa II on both surfaces of the wing. Correspondence between characters states on the dorsal and ventral surfaces of the forewing was stronger than on the hindwing (Table 2-3). Also the ancestral *Vanessa* probably had continuous lines making up Externae II on all wing surfaces (Table 2-4). All extant species of *Vanessa* show the ancestral form of Externae II on both

surfaces of the forewings except for *V. altissima* that show dotted Externa II on its ventral forewing (data not shown). On the dorsal and ventral hindwing more variation from the ancestral state is seen. Less than half of the species show the derived dotted line character state in Externae II (Fig. 2-5A and 2-5B).

Externa III

On both surfaces of the forewing, a continuous Externa III is the predominant character state. On the forewing, all of the 22 *Vanessa* shows the same pattern: a continuous line on both surfaces of the wing. On the hind wing, 11 out of 22 show the same pattern as either a continuous or dotted line in Externa III on the two surfaces of the wing. Due to the lack of variation on the forewing, correspondence among character states on the forewing was greater than on the hindwing. Correspondence between character states on the ventral surfaces of the wings was stronger than on the dorsal surfaces (Table 2-3). Also the ancestral *Vanessa* probably had continuous lines making up Externae III on all wing surfaces (Table 2-4). All extant species of *Vanessa* show the ancestral form of Externae III on both surfaces of the forewings. On the dorsal and ventral hindwing more variation from the ancestral state is seen. Less than half of the species show the derived dotted line character state in Externae III (Fig. 2-6A). On the ventral hindwing, only *V. hippomene* and *V. tameamea* show the derived dotted line character state in Externae III (Fig. 2-6B).

Discalis I

Discalis I on the dorsal and ventral surfaces of the forewing and on the ventral

surface of the hindwing show the united form of Discalis I with no variation among *Vanessa* species (trees not shown). On the dorsal hindwing, all members of the *cardui* and *carye* groups plus *V. dilecta* from *atalanta* group show the united form while the remaining species in the genus do not have a recognizable Discalis I (Fig. 2-7B). Since there is no variation in Discalis I on the surfaces the forewing, there is greater correspondence between character states for this pattern element on the forewing than on the hindwing. Correspondence between Discalis I character states on the ventral surface of the forewing and hindwing was stronger than on the dorsal surface (Table 2-3). The reconstruction of Discalis I on both surfaces of both wings indicates that the probable ancestral phenotype in *Vanessa* was the united form of Discalis I with the exception of the dorsal hindwing on which the ancestral *Vanessa* species had either the absent or united character state form of Discalis I (Table 2-4, Fig. 2-7B). On the dorsal hindwing, there are two obvious gains of the united character state: one in *V. dilecta* in the *atalanta* group and the other in the entire *cardui* lineage. There is also a third possible gain of the united form in the *carye* lineage.

Discalis II

Discalis II on the dorsal and ventral surfaces of the forewing shows a mostly segmented state (number of black coloured blocks). Also, on the forewing, 17 out of 22 *Vanessa* species show the same character state on both wing surfaces (Fig. 2-8, Table 2-3). On the dorsal surface of the forewing, the *carye*, *itea* and *hippomene* groups plus *V. viginiensis* from *cardui* group and *V. abyssinica, V. dejeanii, V. dilecta* and *V. buana* show the ancestral character state of united Discalis II (Fig. 2-8A). The reconstruction of

Discalis II on the dorsal forewing suggests the probable ancestral *Vanessa* had the united character state. All of the species in the *cardui* and *atalanta* groups except for *V. virginiensis* from the *cardui* group and *V. abyssinica, V. dejeanii, V. dilecta,* and *V. buana* from the *atalanta* group show the derived segmented state. The *carye*, *itea* and *hippomene* groups show the ancestral united character state for Discalis II on the dorsal forewing. Extant species are equally divided with respect to the two character states (Table 2-4, Fig. 2-8A).

In the ventral surface of the forewing, *itea* and *hippomene* group plus *V. annabella* from *carye* group and *V. abyssinica* from *atalanta* group show the united form and the rest of the genus shows the derived segmented character state (Fig. 2-8B). The reconstruction of Discalis II on the ventral forewing also confirms the ancestor of *Vanessa* had the united character state even though this character evolved over time in the *cardui* and *atalanta* groups (except for *V. abyssinica*) and in *V. carye* to the segmented form (Table 2-4, Fig. 2-8B). There is a clear trend towards repeated evolution of the derived segmented form of Discalis II in the ventral forewing.

On the dorsal hindwing, Discalis II is mostly absent from the whole genus except for *V. dilecta* from *atalanta* group and *V. altissima, V. brazilliensis, V. kershawi* and *V. myrinna* from *cardui* group (Fig. 2-7A). The reconstruction of Discalis II in the dorsal hindwing indicates that the ancestral *Vanessa* probably did not have a distinguishable Discalis II (Table 2-4). This character state remained unchanged in all *Vanessa* except for *V. dilecta* from *atalanta* group and *V. altissima, V. braziliensis, V. kershawi* and *V. myrinna* from *cardui* group (Fig. 2-7A). These species show a gain of the united form of Discalis II.

Finally no segmentation of Discalis II is seen on the ventral surface of the hindwing in any species of *Vanessa* (tree not shown). Correspondence between characters states on the dorsal and ventral surfaces of the forewing was stronger than on the hindwing. Correspondence between character states on the ventral surface of the forewing and hindwing were stronger than on the dorsal surface (Table 2-3). The reconstruction of Discalis II on the ventral hindwing indicates that the ancestral *Vanessa* species had united form of Discalis II and this phenotype was conserved during *Vanessa* diversification (Table 2-4).

Basal Symmetry System

Tracing the Basal Symmetry System in both the dorsal and ventral forewing shows that it has a greater tendency to meet Discalis II in the dorsal forewing than in the ventral forewing in *Vanessa*. Species in which the Basal Symmetry System were attached to Discalis II on the dorsal forewing include *V. abyssinica, V. atalanta, V. buana, V. dilecta, V. dejeanii, V. dimorphica, V. hippomene, V. gonerilla* and *V. itea* (Fig. 2-9A). In the ventral forewing those include *V. abyssinica, V. dimorphica, V. hippomene, V. gonerilla* and *V. itea* (Fig. 2-9B). The Basal Symmetry System in the dorsal hindwing shows an equal tendency to meet the Discalis II or pass through Discalis II and reach to the eyespots. The whole *cardui* and *carye* group plus *V. samani* and *V. itea* show the Basal Symmetry System extended to Discalis II. The whole *atalanta* group except for *V. samani* and *hippomene* group plus *V. gonorilla* show the Basal Symmetry System extended to the eyespots (Fig. 2-10A). The Basal Symmetry System in the ventral hindwing shows equal tendency to join or remain separate from Discalis II. All members

of the *cardui* and *hippomene* groups plus *V. abyssinica,* and *V. atalanta* show the Basal Symmetry System separate from Discalis II. The whole *atalanta* group, except for *V. atalanta* plus the *carye* and *itea* groups show the Basal Symmetry System extended to Discalis II (Fig. 2-10B). Correspondence between character states on the dorsal and ventral surface of the forewing was stronger than on the hindwing for Discalis II. Correspondence between character states on the ventral surfaces of the forewing and hindwing were stronger than on the dorsal surfaces (Table 2-3).

The reconstruction of the Basal Symmetry System on the dorsal surface of the forewing indicates that the Basal Symmetry System was probably attached to Discalis II in the ancestral *Vanessa* species (Table 2-4, Fig. 2-9A). Thirteen out of 22 extant species have the Basal Symmetry System separate from Discalis II (Fig. 2-9A). Character state changes to the derived condition occur in three or four lineages in the genus and there are one or two character state reversals. During the evolution of the *cardui* group, the Basal Symmetry System separated from Discalis II on the dorsal surface of the forewing. In most of the species in the *atalanta* group it remained attached to Discalis II, except for *V. tameamea, V. samani, V. vulcania* and *V. indica.* Also the *carye* group shows the Basal Symmetry System separate from Discalis II, while the *hippomene* and *itea* group show the Basal Symmetry System attached to the Discalis II.

The reconstruction of the Basal Symmetry System on the ventral surface of the forewing suggests that ancestral *Vanessa* had a Basal Symmetry System that was separate from Discalis II (Table 2-4). There are apparent changes to the attached character state in the *itea* and *hippomene* lineage groups and in *V. abyssinica* (Fig. 2-9B). It is not clear from reconstruction whether the ancestors of *atalanta* and *cardui* lineage had the Basal

Symmetry System separate from or attached to Discalis II. The dorsal and ventral forewing shows a high level of consistency as 18 out of 22 species have the same character states on both surfaces. It seems ventral surface of the forewings in both *cardui* and *atalanta* groups retained the ancestral state of Basal Symmetry System, separate from Discalis II, with the exception of *V. abyssinica* which experienced a change to derived character attached to Discalis II on the ventral surface.

The reconstruction of the Basal Symmetry System on the dorsal surface of the hindwing shows that the ancestral *Vanessa* likely had the Basal Symmetry System attached to Discalis II (Table 2-4). Currently, 11 out of 22 species have the Basal Symmetry System separate from Discalis II and 11 out of 22 species have it attached to Discalis II (Fig. 2-10A). This finding shows no directional changes in favour of a specific character state in the lineage. The *cardui* group kept the ancestral state or regained it, but the *atalanta* group gained the Basal Symmetry System extended to ocelli, except for *V. samani,* which reverted to the ancestral character state. The *carye* group shows the Basal Symmetry System attached to Discalis II. The entire *hippomene* group and *V. gonorilla* in the *itea* group shows the Basal Symmetry System extended to ocelli, while *V. itea* shows the ancestral character state.

Finally, the reconstruction of the Basal Symmetry System on the ventral surface of the hindwing shows that the probable ancestral *Vanessa* likely had the Basal Symmetry System either separate from or attached to Discalis II (Table 2-4). Today, 11 out of 22 species have the Basal Symmetry System attached to Discalis II (Fig. 2-10B). There is a gain of Basal Symmetry System attached to Discalis II in the entire *atalanta* group with the exception of *V. abyssinica* and *V. atalanta.* The rest of the *cardui* group

remained unchanged and thus show the Basal Symmetry System separate from Discalis II.

Discussion

Topology of the tree

The phylogeny of *Vanessa* is generally well-supported and is largely congruent at the species level with the relationships reported by Wahlberg and Rubinoff (2011a) (Fig. 2-4). Both our phylogeny and that of Wahlberg and Rubinoff (2011a) support the monophyly of *Bassaris* and *Vanessa* as defined by Field (1971) (the *V. itea* group and the *V. atalanta* group in Fig. 2-4, respectively), but *Cynthia* is paraphyletic and split into distantly related clades (the *V. carye* group and the *V. cardui* group). Otaki et al. (2006) also supported a monophyletic *Bassaris*, were equivocal about the monophyly of *Vanessa,* and found *Cynthia* to be monophyletic (but species in the *V. carye* group were not included in their analysis). Our tree clearly shows monophyly of *Vanessa* in the broad sense. At the generic level, there are rearrangments in the placement of outgroups compared to Wahlberg and Rubinoff (2011a), but there is only one major change in the placement of species within *Vanessa*. Wahlberg and Rubinoff (2011a) place *V. myrinna* within the *cardui* group while our results show that it is the basal taxon in the *cardui* group. The placement of *V. dilecta* does not show any change even though we have added sequence data for eight genes missing from the Wahlberg and Rubinoff (2011a) data set. Based on the estimation by Wahlberg and Rubinoff (2011a) the probable ancestral *Vanessa* occurred in the mid Oligocene approximately 25 to 30 million years

ago.

Overall, there was very little intraspecific variation in the colour pattern elements studied here. Many species of *Vanessa* are known to migrate, in some cases across large distances (Williams 1930; Opler and Krizek 1984; Myers 1985; Blitzer and Christoffel 2014). Over time, in the absence of other evolutionary processes, migration will tend to homogenize the populations (Gemmell et al. 2014), and may contribute to the consistency of colour pattern phenotypes we have observed among wild-caught specimens from most *Vanessa* species.

Evaluating the directionality of colour pattern evolution

We anticipated several patterns of colour pattern evolution might be apparent in *Vanessa* butterflies. First, based on the obvious colour pattern diversity in the genus (Fig. 2-2), we predicted that many non-eyespot colour pattern elements will be variable. For variable pattern elements, we proposed a null hypothesis that predicts that character state changes will be random or nondirectional. In this case, we predict that there will be lineages that show both the evolution of novel character states as well as reversals to the original state of the character. Of the 24 characters evaluated in this study, 13 of them are variable (parsimony informative and autapomorphic) and 11 are completely uniform within *Vanessa*. Only 3 of the variable characters showed clearly nondirectional changes including FDB, HDB and FDDII (Refer to Table 2-2 for abbreviations). The alternative hypothesis predicts that characters will show character state changes that are nonrandom or directional. This hypothesis predicts that changes from the original character state are irreversible. The reconstructions show that only 3 of the 24 characters show directional

changes including HDDI, FVB and HVEIII and only HDDI shows strong directional change (Fig. 2-7). One character, FVEII, shows no variation except for an apomorphy in *Vanessa altissima* (Data not shown), which is insufficient to assess the directionality of character state change. The 6 remaining variable characters (HDEII, HVEII, HDEIII, HDDII, FVDII and HVB) include ambiguous character state reconstructions and therefore the presence of character state reversals cannot be detected or ruled out with certainty. Clearly, both directional and nondirectional character state changes have contributed to colour pattern variation in *Vanessa.* However, it is remarkable that in spite of the obvious colour pattern diversity within *Vanessa*, only a minority of the non-eyespot colour pattern elements present on the wings contribute to that diversity.

Functional significance of wing colour patterns

While many butterfly species have very similar colour patterns on their dorsal and ventral wing surfaces (Cech and Tudor 2005), studies of the Satyrine butterfly *Bicyclus anynana* have shown that the degree of immutable genetic coupling between the dorsal and ventral wing pattern elements is actually very limited (see Allen (2008), for a review). This suggests that it is fairly easy to decouple the phenotypes of the dorsal and ventral wing surfaces which have different functional roles in butterflies (Fraser 1871; Oliver et al. 2009). The dorsal wing surfaces frequently play roles in thermoregulation (Kingsolver 1985a, 1988) and sexual displays (Kemp 2007; Rutowski et al. 2010; Kamalanathan and Mohanraj 2012). The ventral surfaces of butterfly wings often include colour patterns associated with camouflage (Kamalanathan and Mohanraj 2012) or disruptive colouration (Platt and Brower 1968) to avoid detection by predators. Due to

these distinct selection pressures, colour patterns on dorsal and ventral wing surfaces of a species can evolve in opposite directions (Kodandaramaiah 2009; Oliver et al. 2009). Both dorsal and ventral wing surfaces sometimes include patterns that may distract or intimidate predators (Lyytinen et al. 2004; Kodandaramaiah et al. 2013) or that advertise distastefulness (Prudic and Oliver 2008; Rutowski et al. 2010). The non-eyespot components of butterfly colour patterns contribute substantially to each of these functional roles.

The results of the character state reconstruction and optimization among *Vanessa* species show that the overall correspondence between character states on the two surfaces of the forewing was higher than on the two surfaces of the hindwing (Table 2-3). Correspondence between character states on the ventral surfaces of the forewing and hindwing is stronger than correspondence between the wings on the dorsal surface (Table 2-3). Both of these patterns of correspondence are caused by the divergent phenotype on the dorsal hindwing in some *Vanessa* species, on which the Basal Symmetry System has been extended distally to cover much of the wing surface (Fig. 2-10), and in many cases the darkly pigmented Discalis I pattern element assumes a united character state (Fig. 2-7). Basking behaviour is common in a number of *Vanessa* butterfly species (Cech and Tudor 2005), so we suspect that the large and often darkly pigmented basal symmetry system and Discalis I pattern elements found on the dorsal hindwing of many species may contribute to behavioural thermoregulation, but this needs to be confirmed experimentally. This or other colour pattern elements on the dorsal wing surfaces of *Vanessa* may also contribute to sexual displays, though most species of *Vanessa* are not sexually dimorphic with respect to colour pattern (except male and female *V. tameamea*

which have subapical bands of different colours on the dorsal forewing (Otaki 2008)).

The ventral hindwings of *Vanessa* are generally exposed when its wings are folded and the animal is at rest. The ventral forewings are often largely obscured in this posture (Cech and Tudor 2005), so colour patterns with roles in camouflage, predator avoidance, or intimidation are often more prominent on the ventral hindwing (Lyytinen et al. 2003; Kamalanathan and Mohanraj 2012; Wilts et al. 2013). Non-mimetic species of *Limenitis* butterflies typically obscure their outline through disruptive colouration created by a prominent white band (Platt and Brower 1968), and the intricate colour patterns featuring contrasting colours on the ventral hindwings of many species of *Vanessa* butterflies may serve a similar function.

The genetics of non-eyespot colour patterns

Several genes have been implicated in the formation and diversification of non-eyespot colour patterns in butterflies. Clark et al. (2008) studied the locus H, an autosomal locus defining colour variation and mimicry among female *Papilio dardanus* butterflies. Using amplified fragment length polymorphism (AFLP) analysis and linkage mapping, the transcription factor *invected* was identified as a candidate gene that may be responsible for the development of mimicry-associated colour patterns (Clark et al. 2008). Martin and Reed (2010) described a novel gene, *aristaless 2,* which is expressed in a pattern that suggests that it may play a role in the determination of Discalis II. They examined the development of the Basal Symmetry System, Discalis I and II, and Externa I in a broad survey of the Lepidoptera. They also expanded on earlier results (Carroll et al. 1994) which suggested that *wingless* gene expression may also have roles in the

determination of these colour pattern elements (Martin and Reed 2010). Genetic variation in *WntA,* a paralogue of *wingless,* has been associated with the production of different mimetic colour pattern phenotypes in *Heliconius* butterflies (Martin et al. 2012). The association between the *wingless* morphogen and the induction of wing colour patterns may extend beyond the Lepidoptera. Spots on the wings of the fly *Drosophila guttifera* are also driven by *wingless* expression (Werner et al. 2010). Like all proposed mechanisms based on associations between phenotypes and gene expression patterns, confirmation of links between *invected, aristaless 2, wingless, and WntA* will require functional studies of the gene products in butterfly developing wing tissues (Marcus 2005). Fortunately, technologies for doing these functional studies in the Lepidoptera are developing rapidly (Dhungel et al. 2013; Beaudette et al. 2014). The role of these gene products in the developing wings of *Vanessa* butterflies will be a subject of future research.

Conclusion

The non-eyepot colour patterns have evolved extensively during the radiation of the genus *Vanessa* from the inferred set of ancestral character states. Non-eyespot pattern elements cover the bulk of the wing surfaces and thus would be expected to contribute substantially to the overall colour pattern variation in the genus (Fig. 2-2). However, many of these patterns (11 of 24) are invariable across *Vanessa.* Among pattern elements that do vary, 3 show non-directional character state evolution, 3 show directional character state evolution (1 with strong directional evolution) among species, and 7 are difficult to categorize due to ambiguities with respect to the ancestral state reconstruction

within *Vanessa.* Even in the case of colour pattern traits with possible roles in behavioral thermoregulation on the dorsal hindwings of *Vanessa,* our character state reconstructions show that one character (Discalis I) has evolved directionally towards a united character state, with no apparent character state reversals. Another character that may have a similar role (Basal symmetry system) shows enlargement, followed by reduction in surface area in some *Vanessa* lineages. Overall, strong directional evolution of character states in non-eyespot colour pattern elements appears to be very rare in *Vanessa.* Computational models for butterfly eyespot development suggest that very small changes in the concentration of some expressed gene products can easily cause character state reversals in these colour pattern elements (Evans and Marcus 2006). Our results in *Vanessa* suggest that we might expect to see very similar phenomena influencing the size, placement, and connectedness during the development of non-eyespot patterns, facilitating non-directional evolution and character state reversals in these patterns as well. The ease with which the development of colour patterns can be modified has likely been an important contributor to the production of butterfly colour pattern diversity (Nijhout 1991).

Supporting Information

Table S2-1: Genbank Accession numbers for DNA sequences used for phylogenetic analysis. See appendix 1.

Table S2-2: Specimens consulted for the study of colour pattern evolution and used for DNA extractions. See appendix 2.

Table S2-3: Scored values for the non-eyespot colour pattern characters found on the four wing surfaces of *Vanessa* butterflies and outgroups. See appendix 3.

Tables can also be downloaded from this link as well:
http://onlinelibrary.wiley.com/doi/10.1111/ede.12109/suppinfo

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Table 2-4. Genes, primer sets, PCR conditions, and reference for source of each primer set. Standard degenerate nucleotide symbols are used except for I, which refers to the unconventional nucleotide inosine, which is capable of binding to all 4 conventional nucleotides.

Gene	Primers	Primer sequences (5′ to 3′)	PCR protocol conditions	Reference
cytochrome oxidase subunit I (COI)	HCO2198 /LCO1490	HCO2198 TAA ACT TCA GGG TGA CCA AAA AAT CA LCO1490 GGT CAA CAA ATC ATA AAG ATA TTG G	95°C for 5 min 35 cycles of: [94°C for 1 min 46°C for 1 min 72°C for 1.5 min] 72°C for 5 min	(Folmer et al. 1994)
NADH dehydro-genase subunit 5 (ND5)	ND5(F)/ ND5(R)	ND5(F) GTT CAT TCA TCT ACT TTA GTA ACT GCT G ND5(R) AAG GAA TAC CAC ATA AAG CTA AAT TTG	95°C for 5 min 35 cycles of: [95°C for 1 min 56°C for 1 min 72°C for 1 min] 72°C for 10 min	(Otaki, Kimura, and Yamamoto 2006)
elongation factor 1α (EF-1α)	1: ef44 (f)-240/ ef51r (r)-650 2: ef51.9(f)-798/ efrcM4(r)-1351 3: HybStarsky/ HybMonica (R) 4: HybAlF/ HybEFrcM4	ef44 (f)-240 GCY GAR CGY GAR CGT GGT ATY AC ef51r (r)-650 CAT GTT GTC GCC GTG CCA AC ef51.9(f)-798 CAR GAC GTA TAC AAA ATC GG efrcM4(r)-1351 ACA GCV ACK GTY TGY CTC ATR TC HybStarsky TAA TAC GAC TCA CTA TAG GGC ACA TYA ACA TTG TCG TSA TYG G HybMonicaR ATT AAC CCT CAC TAA AGC ATR TTG TCK CCG TGC CAr CC HybAlF TAA TAC GAC TCA CTA TAG GGG AGG AAA TYA ARA ARG AAG HybEFrcM4 ATT AAC CCT CAC TAA AGA CAG CVA CKG TYT GYC TCA TRT C	Primer pairs 1 and 2: 95°C for 5 min 40 cycles of: [95°C for 1 min 50°C for 1 min 72°C for 1.5 min] 72°C for 10 min Primer pairs 3 and 4: 95°C for 5 min 40 cycles of: [94°C for 30 sec 50°C for 30 sec 72°C for 1.5 min] 72°C for 10 min	(Monteiro and Pierce 2001), (Wahlberg and Wheat 2008)
ribosomal protein S5 (RpS5)	HybrpS5deg (F)/ HybrpS5deg (R)	HybrpS5degF TAA TAC GAC TCA CTA TAG GGA TGG CNG ARG ARA AYT GGA AYG A HybrpS5degR ATT AAC CCT CAC TAA AGC GGT TRG AYT TRG CAA CAC G	95°C for 5 min 40 cycles of: [94°C for 30 sec 55°C for 30 sec 72°C for 1.5 min] 72°C for 10 min	(Wahlberg and Wheat 2008)
glyceraldehy de-3-phosphate dehydro-genase (GAPDH)	HybFrigga/ HybBurre	HybFrigga TAA TAC GAC TCA CTA TAG GGA ARG CTG GRG CTG AAT ATG T HybBurre ATT AAC CCT CAC TAA AGG WTT GAA TGT ACT TGA TRA GRT C	95°C for 5 min 40 cycles of: [94°C for 30 sec 55°C for 30 sec 72°C for 1.5 min] 72°C for 10 min	(Wahlberg and Wheat 2008)
arginine kinase (ArgKin)	Arginine (F) and (R)	ArginineF TAA TAC GAC TCA CTA TAG GGT NAC YGA RKC CCA RTA YAA G ArginineR ATT AAC CCT CAC TAA AGT TGA TSA GYT CRG CGA TG	95°C for 5 min 40 cycles of: [94°C for 30 sec 55°C for 30 sec 72°C for 1.5 min] 72°C for 10 min	(Wahlberg and Wheat 2008)
carbamoyl-phosphate synthase domain	1: CAD743n (F) CADmid (R) 2: CADmid (F)	CAD743nF TAA TAC GAC TCA CTA TAG GGG GNG TNA CNA CNG CNT GYT TYG ARC C	95°C for 5 min 40 cycles of: [94°C for 30 sec 55°C for 30 sec	(Wahlberg and Wheat 2008)

protein (CAD)	CAD1028 (R)	CAD1028R ATT AAC CCT CAC TAA AGT TRT TNG GNA RYT GNC CNC CCA T CADmidF TAA TAC GAC TCA CTA TAG GGK GGA TTY TCN GAY AAA CAA ATN GC CADmidR ATT AAC CCT CAC TAA AGC ATT CWG CKG CWA CTG TAT C	72°C for 1.5 min] 72°C for 10 min	
isocitrate dehydro-genase (IDH)	IDHdeg27 (F)/IDHdeg (R)	IDHdeg27F TAA TAC GAC TCA CTA TAG GGG GWG AYG ARA TGA CNA GRA THA THT GG IDHdegR ATT AAC CCT CAC TAA AGT TYT TRC AIG CCC ANA CRA ANC CNC C	95°C for 5 min 40 cycles of: [94°C for 30 sec 55°C for 30 sec 72°C for 1.5 min] 72°C for 10 min	(Wahlberg and Wheat 2008)
cytosolic malate dehydro-genase (MDH)	1: HybMDH (F) /MDHmid (R) 2: MDHmid (F) /HybMDH (R)	HybMDHF TAA TAC GAC TCA CTA TAG GGG AYA TNG CNC CNA TGA TGG GNG T MDHmidR ATT AAC CCT CAC TAA AGA AYT GNG TRG ATG ART GRT TNC C MDHmidF TAA TAC GAC TCA CTA TAG GGG CNC CNT CWA TNC CNA AAG A HybMDHR ATT AAC CCT CAC TAA AGA GNC CYT CNA CDA TYT TCC AYT T	95°C for 5 min 40 cycles of: [94°C for 30 sec 55°C for 30 sec 72°C for 1.5 min] 72°C for 10 min	(Wahlberg and Wheat 2008)
wingless (wg)	1: Lepwg1 (F)/ Lepwg2 (R) 2: wg(F)/wg(R)	lepwg1 GAR TGY AAR TGY CAY GGY ATG TCT GG lepwg2 ACT NCG CRC ACC ART GGA ATG TRC A wg(F) GGN TTC AGA TTC AGT AGR GAR TTY G wg(R) ACT ICG CAR CAC CAR TGG AAT GTR CA	94°C for 5 min 40 cycles of: [94°C for 1 min 46°C for 1 min 72°C for 2 min] 72°C for 10 min	(Brower and DeSalle 1998; Kronforst 2005)

Table 2-5. Morphological characters, their abbreviations, and character states in fore- and hindwings.

Wing	Surface	Characters	Abbrev-iation	States and their codes
Forewing	**Dorsal**	Basal Symmetry System	**FDB**	0=Absent, 1=Separate from Dll, 2=Attached to Dll, 3=Extended to ocelli
		Discalis II	**FDDII**	0=Absent, 1= Segmented, 2= United
		Discalis I	**FDDI**	0=Absent, 1= Segmented, 2= United
		Externa III	**FDEIII**	0=Absent, 1= Dotted line, 2= Continuous line
		Externa II	**FDEII**	0=Absent, 1= Dotted line, 2= Continuous line
		Externa I	**FDEI**	0=Absent, 1= Dotted line, 2= Continuous line
	Ventral	Basal Symmetry System	**FVB**	0=Absent, 1=Separate from Dll, 2=Attached to Dll, 3=Extended to ocelli
		Discalis II	**FVDII**	0=Absent, 1= Segmented, 2= United
		Discalis I	**FVDI**	0=Absent, 1= Segmented, 2= United
		Externa III	**FVEIII**	0=Absent, 1= Dotted line, 2= Continuous line
		Externa II	**FVEII**	0=Absent, 1= Dotted line, 2= Continuous line
		Externa I	**FVEI**	0=Absent, 1= Dotted line, 2= Continuous line
Hindwing	**Dorsal**	Basal Symmetry System	**HDB**	0=Absent, 1=Separate from Dll, 2=Attached to Dll, 3=Extended to ocelli
		Discalis II	**HDDII**	0=Absent, 1= Segmented, 2= United
		Discalis I	**HDDI**	0=Absent, 1= Segmented, 2= United
		Externa III	**HDEIII**	0=Absent, 1= Dotted line, 2= Continuous line
		Externa II	**HDEII**	0=Absent, 1= Dotted line, 2= Continuous line
		Externa I	**HDEI**	0=Absent, 1= Dotted line, 2= Continuous line
	Ventral	Basal Symmetry System	**HVB**	0=Absent, 1=Separate from Dll, 2=Attached to Dll, 3=Extended to ocelli
		Discalis II	**HVDII**	0=Absent, 1= Segmented, 2= United
		Discalis I	**HVDI**	0=Absent, 1= Segmented, 2= United
		Externa III	**HVEIII**	0=Absent, 1= Dotted line, 2= Continuous line
		Externa II	**HVEII**	0=Absent, 1= Dotted line, 2= Continuous line
		Externa I	**HVEI**	0=Absent, 1= Dotted line, 2= Continuous line

Table 2-6. Correspondence of characters between wings and surfaces. Numbers indicate the number of *Vanessa* species in which the character states between the two wing surfaces match exactly.

	FVBSS	HDBSS	FVDII	HDDII	FVDI	HDDI	FVEIII	HDEIII	FVEII	HDEII	FVEI	HDEI
FDBSS	**18**	**0**										
HVBSS	**10**	**4**										
FDDII			**17**	**1**								
HVDII			**6**	**5**								
FDDI					**22**	**10**						
HVDI					**22**	**10**						
FDEIII							**22**	**11**				
HVEIII							**20**	**11**				
FDEII									**21**	**15**		
HVEII									**16**	**14**		
FDEI											**22**	**22**
HVEI											**22**	**22**

Table 2-7. Ancestral characters predicted by reconstructions for each wing surface.

	Forewing Dorsal	Forewing Ventral	Hindwing Dorsal	Hindwing Ventral
Basal Symmetry System	Attached to Dll	Separate from Dll	Attached to Dll	Either separate from DII or attached to DII
Discalis II	United	United	Absent	United
Discalis I	United	United	Either absent or united	United
Externa III	Continuous line	Continuous line	Continuous line	Continuous line
Externa II	Continuous line	Continuous line	Continuous line	Continuous line
Externa I	Dotted line	Dotted line	Dotted line	Dotted line

B: Basal symmetry system
DII: Discalis II
DI: Discalis I
Oc: Ocelli
EIII: Externa III
EII: Externa II
EI: Externa I

Sc: Subcosta
R: Radius
M: Media
Cu: Cubitus
A: Anal vein

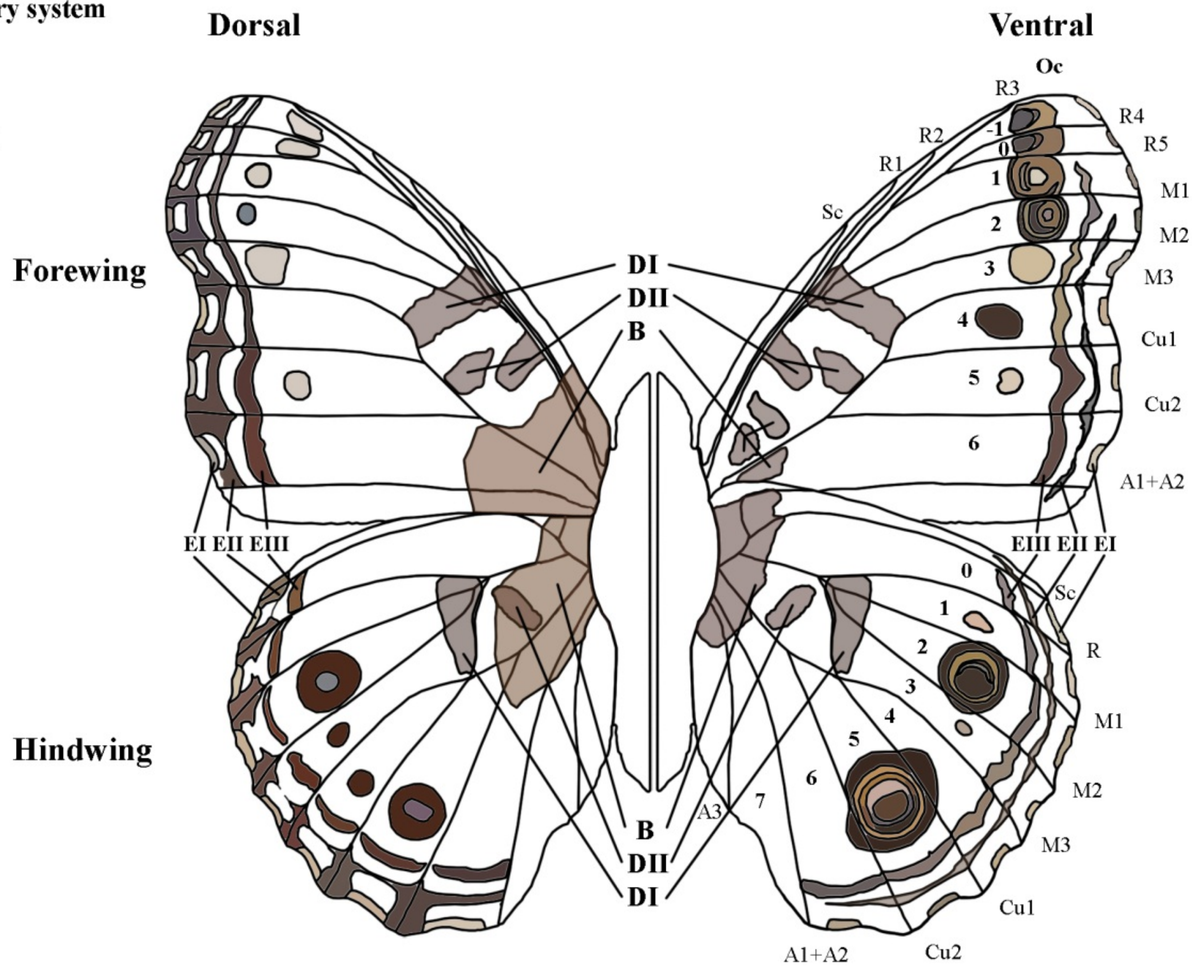


Figure 2-1. The Nymphalid Ground Plan as applied to the dorsal ventral wing surfaces of *Vanessa braziliensis* and labeled using the nomenclature of Schwanwitsch (Schwanwitsch 1924). The major ground plan elements are: ‘‘Basalis’’ (B), ‘‘Discalis II’’ (DII), ‘‘Discalis I’’ (DI), the two bands MII and MI, that form the large central symmetry system, generally centered on DI; the ‘‘border ocelli’’ (Oc), ‘‘Externa patterns’’ (E) are the parafocal, submarginal, and marginal elements (EIII, EII, and EI, respectively) that border the wing (After Martin and Reed 2010; Nijhout 2001).

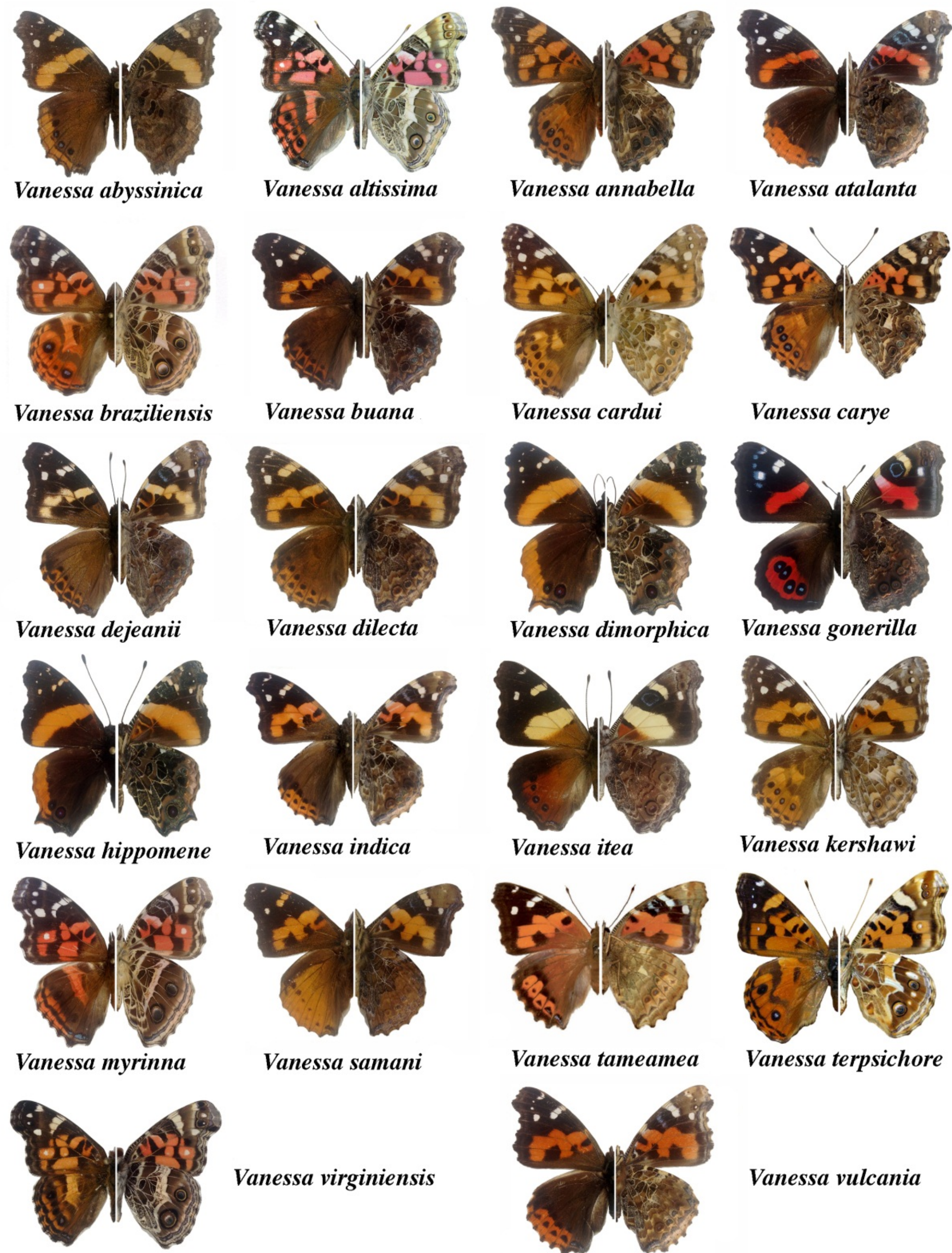


Figure 2-2. Plate of *Vanessa* species showing wing dorsal (left) and ventral (right) surface. There is little sexual dimorphism in *Vanessa. Vanessa terpsichore* was modified from Fric et al. (Fric et al. 2012) and *Vanessa tameamea* was modified from Warren et al. (Warren et al. 2012). Photos of *Vanessa altissima* are courtesy of Loran Gibson.

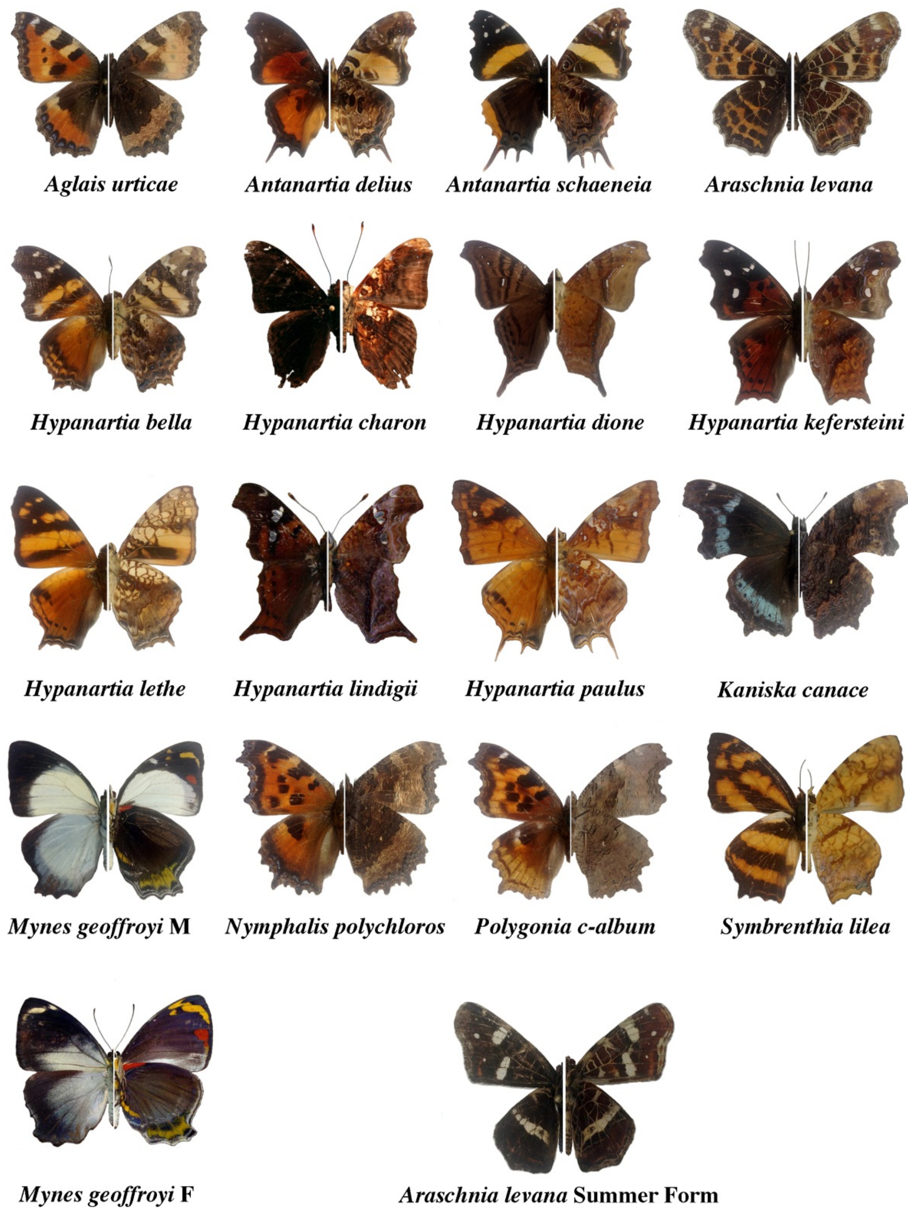


Figure 2-3. Plate of outgroup species showing wing dorsal (left) and ventral (right) surface. Unless otherwise specified, outgroup species are monomorphic. M and F indicate male and female butterflies respectively. *Hypanartia charon* was modified from Wahlberg and Rubinoff (2011a). *Hypanartia lindigii* and *Mynes geoffroyi* female were modified from Fric et al. (2012).

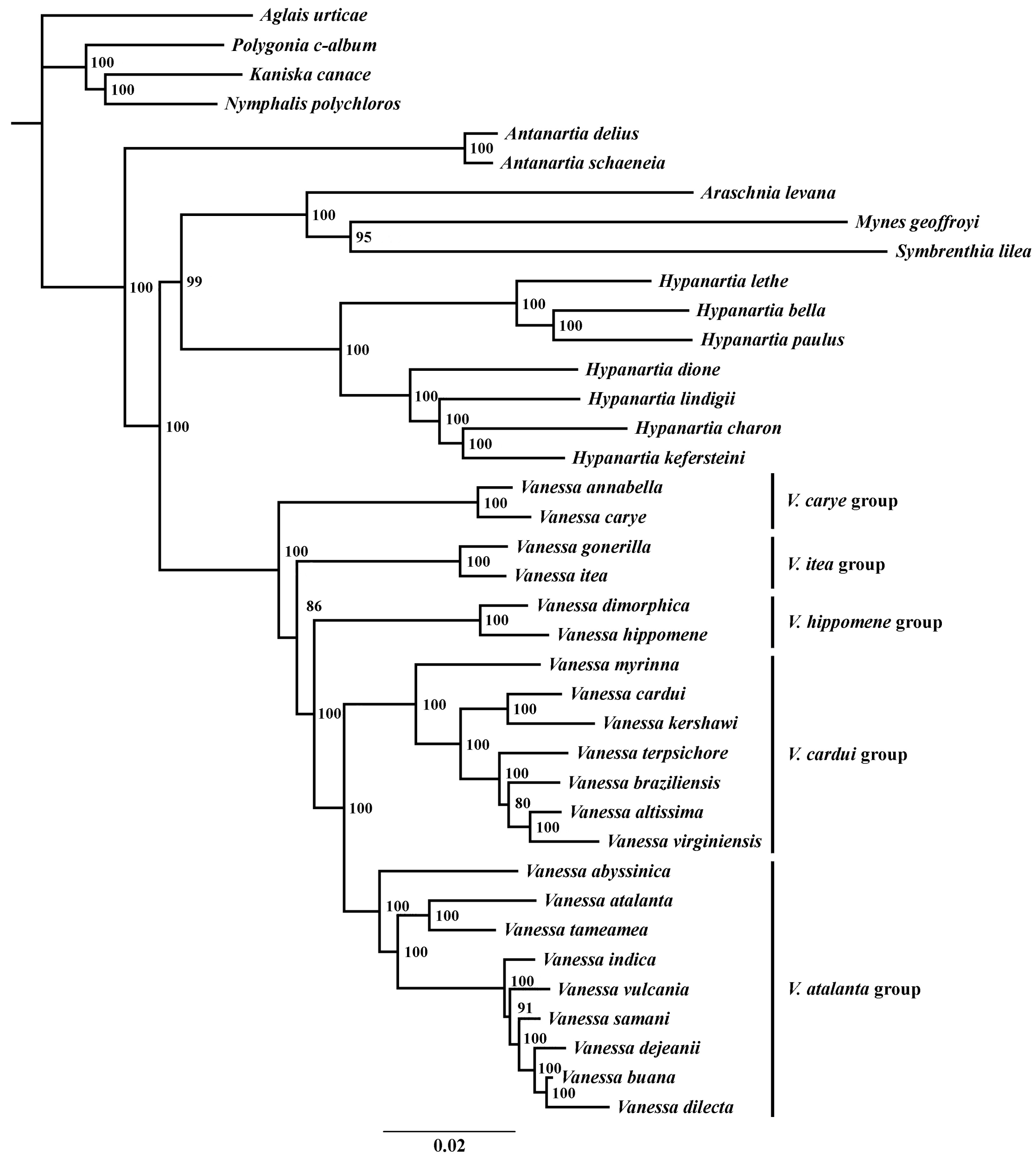


Figure 2-4. Topology and branch lengths from a Bayesian analysis of all DNA sequence data. Numbers adjacent to nodes indicate the posterior probability. The *V. indica* group defined by Otaki et al. (2006) refers to *V. indica, V. vulcania, V. samani, V. dejeanii, V. buana and V. dilecta.*

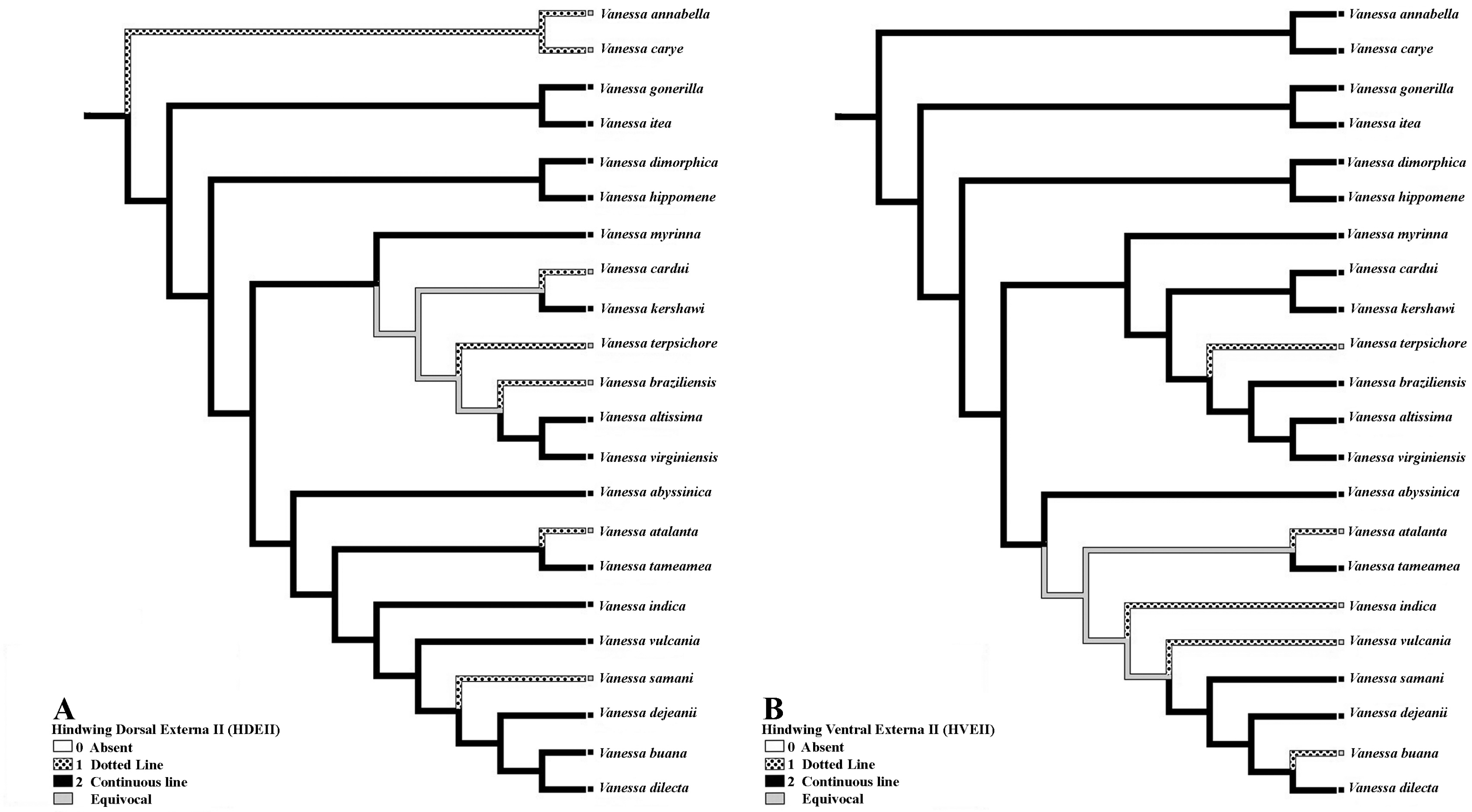
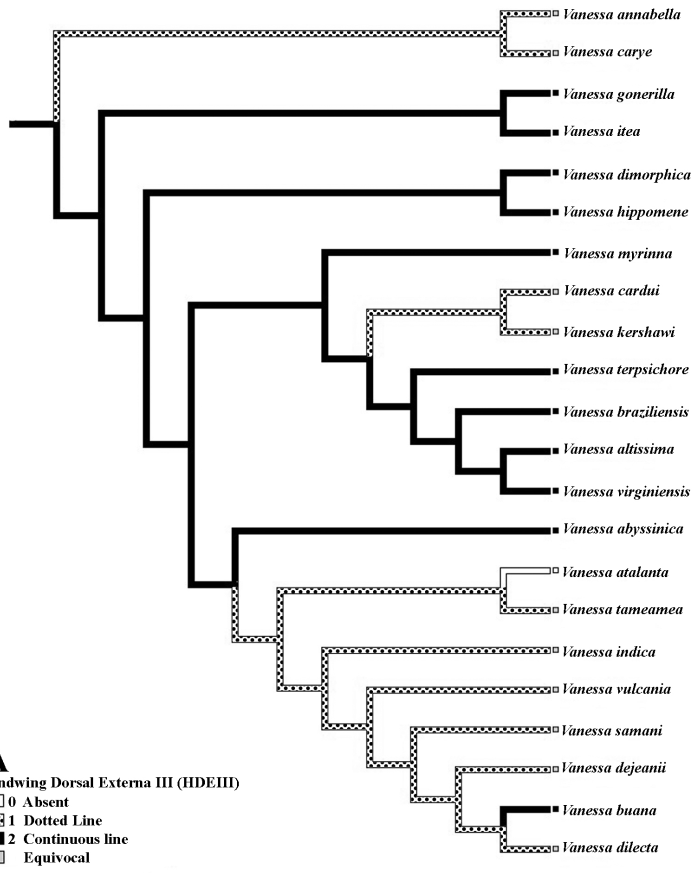


Figure 2-5. Ancestral state reconstruction of the Externa II on the dorsal (A) and ventral (B) surface of the hindwing in *Vanessa*.



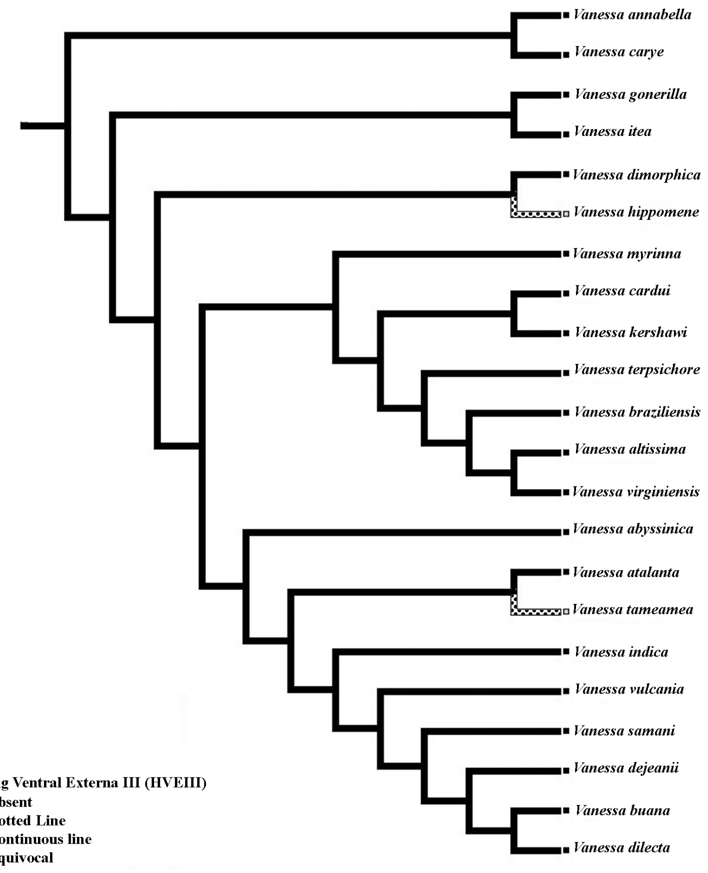


Figure 2-6. Ancestral state reconstruction of the Externa III on the dorsal (A) and ventral (B) surface of the hindwing in *Vanessa*.

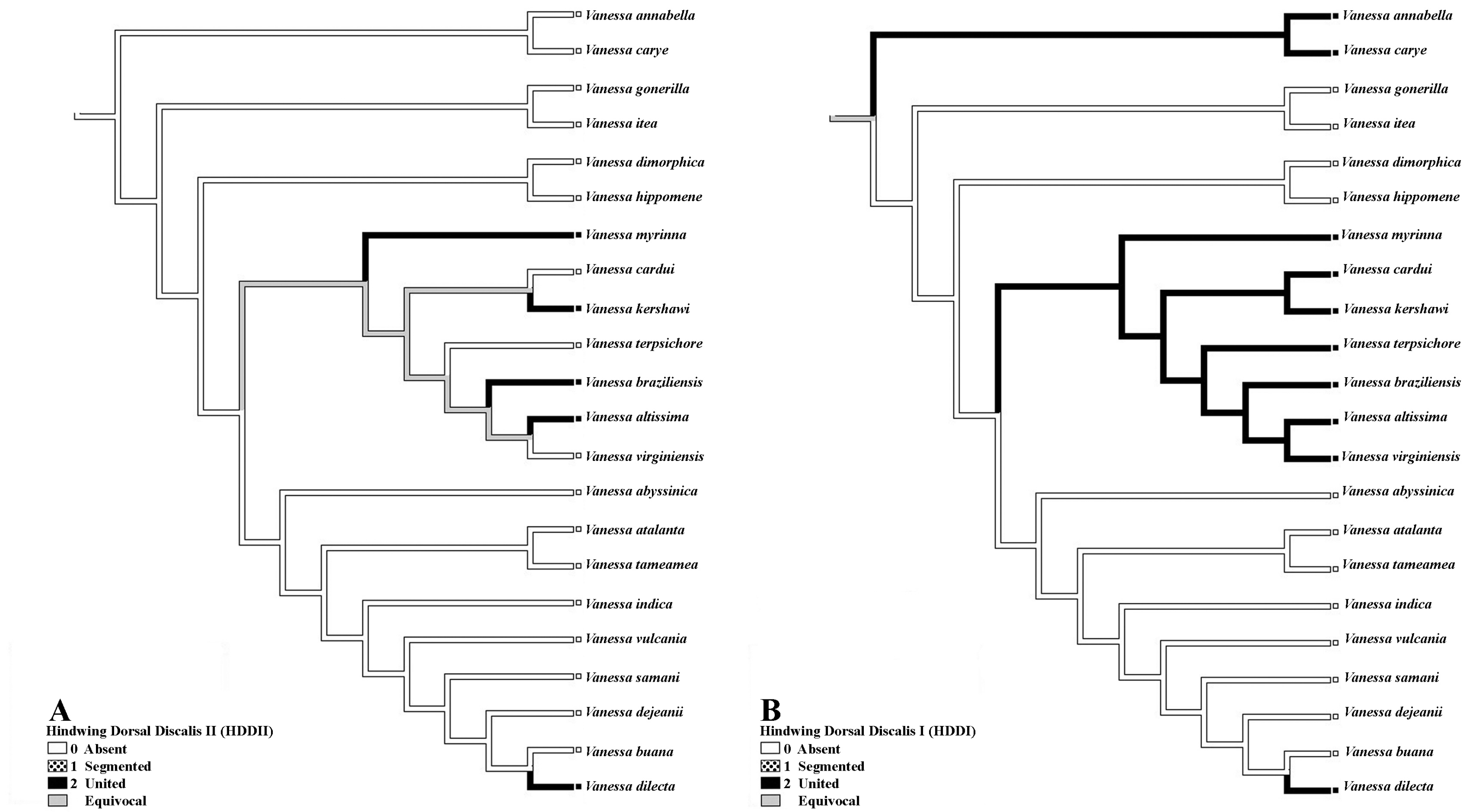


Figure 2-7. Ancestral state reconstruction of the Discalis II (A) and I (B) on the dorsal surface of the hindwing in *Vanessa*.

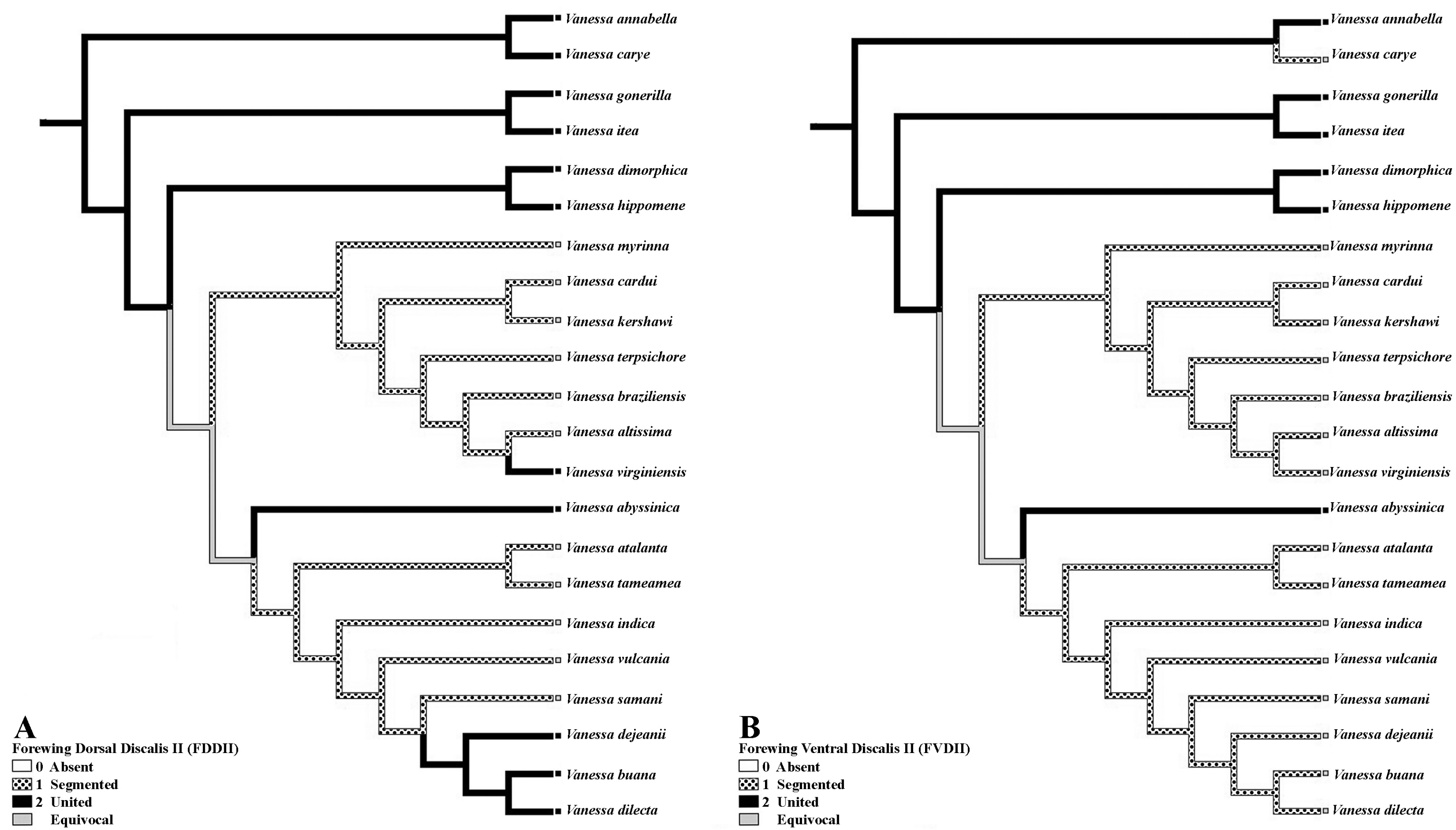


Figure 2-8. Ancestral state reconstruction of the Discalis II on the dorsal (A) and ventral (B) surface of the forewing in *Vanessa*.

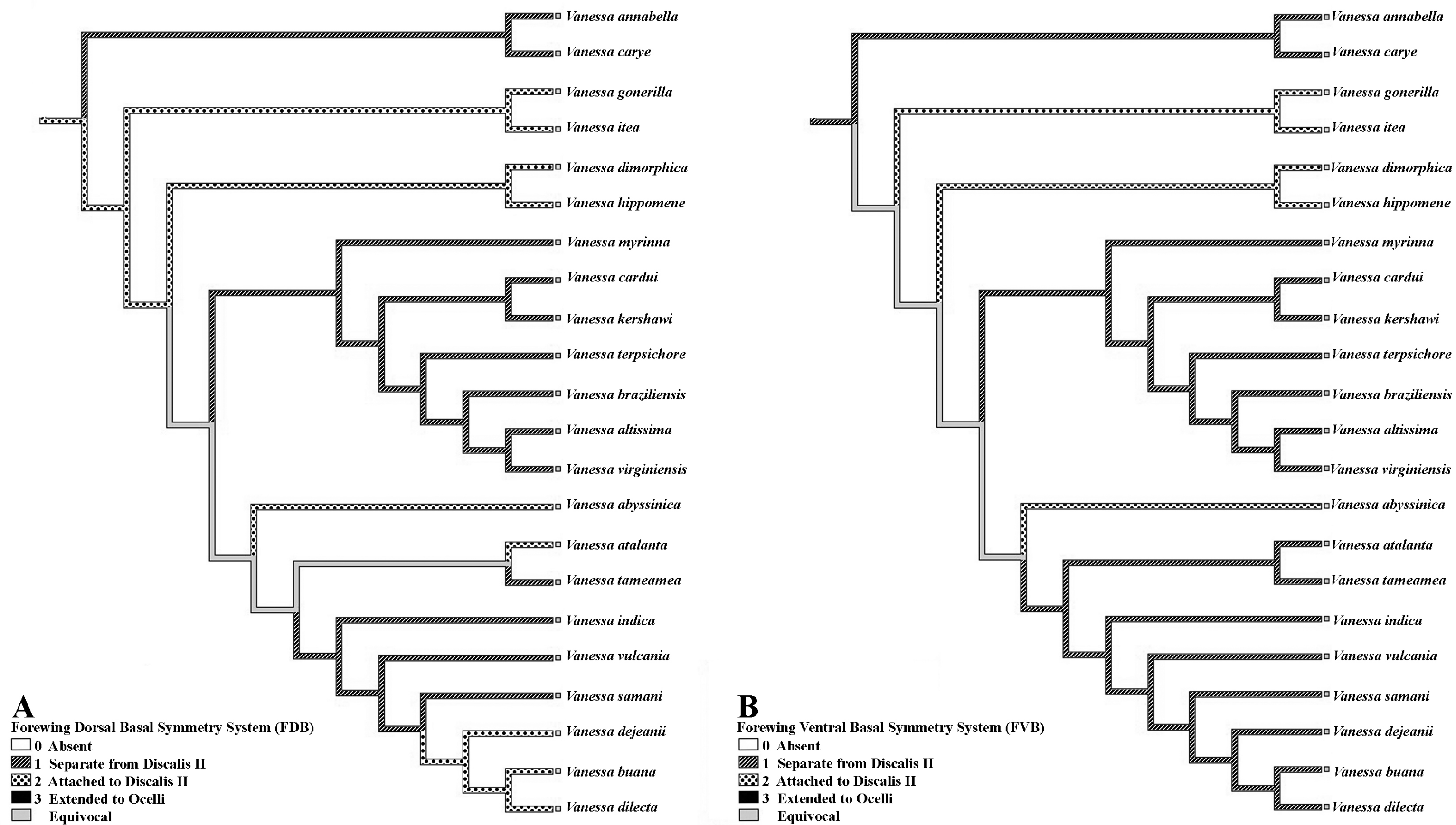


Figure 2-9. Ancestral state reconstruction of the Basal Symmetry System on the dorsal (A) and ventral (B) surface of the forewing in *Vanessa*.

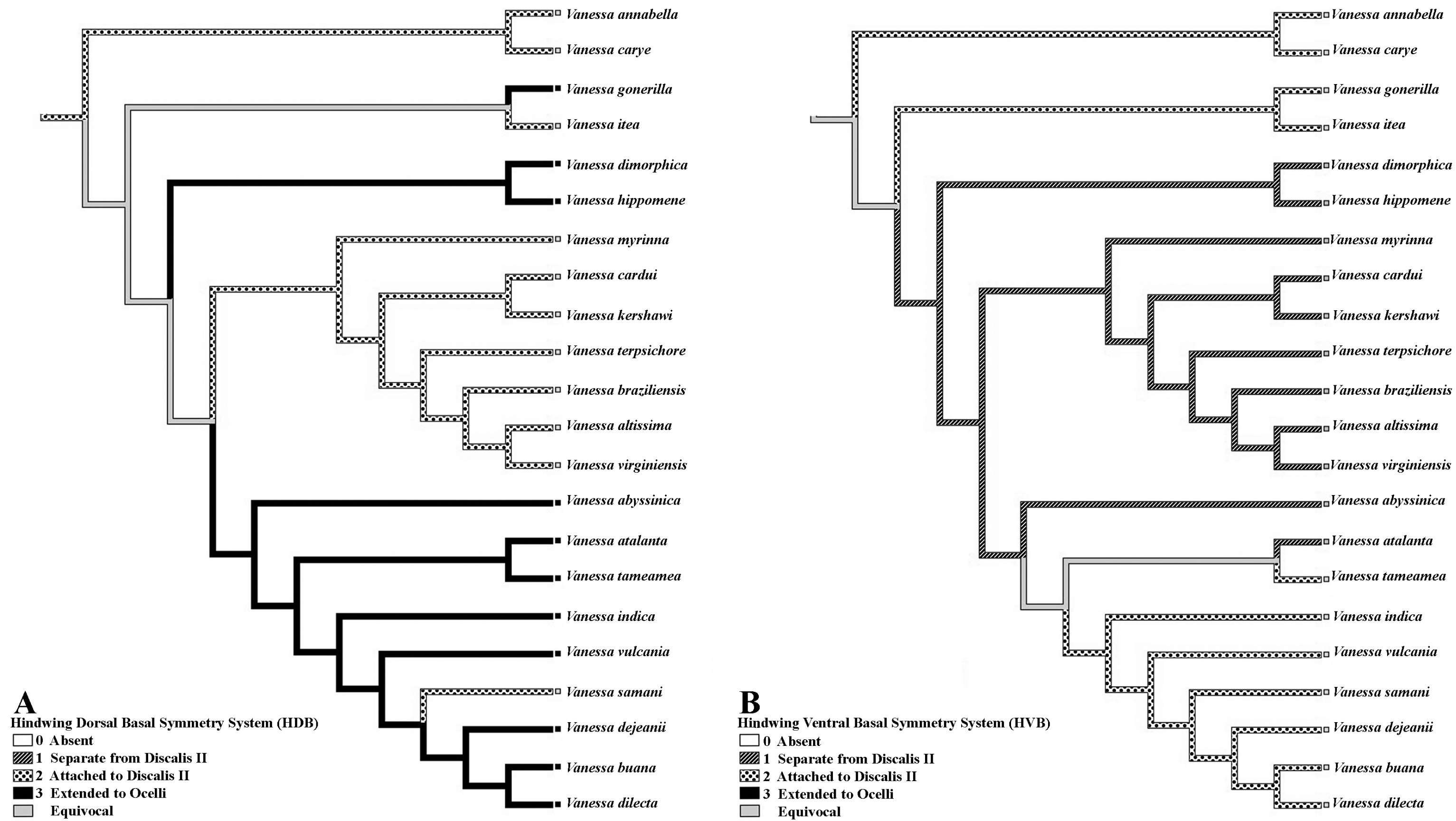


Figure 2-10. Ancestral state reconstruction of the Basal Symmetry System on the dorsal (A) and ventral (B) surface of the hindwing in *Vanessa*.

Chapter 3 Colour pattern homology and evolution in *Vanessa* butterflies (Nymphalidae: Nymphalini): Eyespot characters[2]

[2] This chapter has been previously published as:
Abbasi, R. and J. M. Marcus. 2015. Color pattern evolution in *Vanessa* butterflies (Nymphalidae: Nymphalini): Eyespot characters. Journal of Evolutionary Biology doi: 10.1111/jeb.12716 © John Wiley & Sons, 2015. The chapter is published here under the terms of the Copyright Transfer Agreement between the author and John Wiley & Sons Publishers. RA conceived the research, collected the data, and wrote the manuscript. JMM supervised the project, scored the morphological characters independently, double-checked the analyses, and made extensive comments and revisions to the manuscript, particularly on the sections related to homology.

Summary

Ocelli are serially repeated colour patterns on the wings of many butterflies. Eyespots are elaborate ocelli that function in predator avoidance and deterrence as well as in mate choice. A phylogenetic approach was used to study ocelli and eyespot evolution in *Vanessa* butterflies, a genus exhibiting diverse phenotypes among these serial homologs. Forty-four morphological characters based on eyespot number, arrangement, shape, and the number of elements in each eyespot were defined and scored. Ocelli from eight wing cells on the dorsal and ventral surfaces of the forewing and hindwing were evaluated. The evolution of these characters was traced over a phylogeny of *Vanessa* based on 7750 DNA base pairs from 10 genes. Our reconstruction predicts that the ancestral *Vanessa* had 5 serially arranged ocelli on all four wing surfaces. The ancestral state on the dorsal forewing and ventral hindwing was ocelli arranged in two heterogeneous groups. On the dorsal hindwing, the ancestral state was either homogenous or ocelli arranged in two heterogeneous groups. On the ventral forewing, we determined that the ancestral state was organized into three heterogeneous groups. In *Vanessa,* almost all ocelli are individuated and capable of independent evolution relative to other colour patterns except for the ocelli in cells -1 and 0 on the dorsal and ventral forewings, which appear to be constrained to evolve in parallel. The genus *Vanessa* is a good model system for the study of serial homology and the interaction of selective forces with developmental architecture to produce diversity in butterfly colour patterns.

Keywords: Serial homology, colour pattern evolution, *Vanessa*, Nymphalidae, eyespot character evolution, butterfly wing

Abstract word count: 250

Introduction

It has been stated that “Homology is the central concept for all biology” (Wake 1994) and homology is particularly important in the realm of evolutionary biology (Hall 2000). Modern use of the concept of homology originates from Owen (1843) who defined it as “The same organ in different animals under every variety of form and function”. Two distinct criteria have been proposed as the fundamental basis for homology. The phylogenetic criterion for homology, which is the more commonly used criterion, defines structures as “the same” when they are inherited from a common ancestor (Lankester 1870; Mayr 1942; Patterson 1988). The ontogenetic criterion for homology defines structures as “the same” when they are derived from the same embryonic primordium or otherwise share fundamental developmental features (Wilson 1891, 1892; Spemann 1915; Riedl 1978). The field of evolutionary developmental biology is fundamentally engaged in understanding the interplay of these two fundamental bases for defining homology and creating biological structures (Van Valen 1982; Roth 1988; Müller and Wagner 1996).

Although many structures, such as vertebrate forelimbs, are clearly homologous to each other based on both phylogenetic and ontogenetic criteria for homology (Owen 1848; Duboule 1992), the homology of many other structures is far more ambiguous. This is due to the fact that many structures are clearly descended from one structure in an ancestral taxon, but may share fundamental developmental features with possibly multiple other structures in the same ancestral taxon (Roth 1984). For example, in some plants, phylloclades are leaf-like organs that develop in physical positions where branches normally form. They are both specialized for photosynthesis

(like leaves) and produce bracts and flowering structures from their axils (like branches), thus sharing developmental similarities with both tissue types (Sattler 1984, 1988).

Serial homologs are structures that are repeated several times in the same individual organism (Owen 1848). The vertebrate forelimb and hindlimb are classic examples of serially homologous structures that clearly share structural and developmental similarities, but that also develop from completely distinct embryonic primordia that are not derived from a single structure in an ancestral species (Owen 1848; Duboule 1992). This has led some authors to conclude that serial homology is qualitatively distinct from phylogenetic homology (Lankester 1870). Serially homologous characters (both morphological and molecular) are often avoided for phylogenetic reconstruction because they can introduce ambiguity into the resulting trees (Patterson 1988; Hillis 1994). At the same time, the deployment of similar developmental programs repeatedly within single organisms provides rich opportunities for generating evolutionary novelties (Hanken 1985; Averof and Patel 1997). By mapping features of serially homologous structures onto phylogenetic trees (created from independent data sets to avoid problems of circularity (deQueiroz 1996)), it becomes possible to understand the morphological commonalities among serial homologs as they evolve. It also becomes possible to understand which features of the serially homologous structures vary independently through evolutionary time, and which features are interdependent (Marcus and McCune 1999).

Many serially homologous structures found in animals (e.g. limbs, vertebrae, teeth) are hundreds of millions of years old and may not be as evolutionarily labile as structures of more recent origin (Oliver et al. 2014). Butterfly colour patterns in general and eyespot patterns in particular also exhibit serial homology, but are both highly labile (Nijhout 1991) and much more

recent in origin (Oliver et al. 2014). The entire superfamily (Papilionoidea) that includes the butterflies is less than 100 million years old (Regier et al. 2013). This, plus the fact that many butterfly colour patterns are serially repeated four times in each individual (on both the dorsal and ventral surfaces of the forewings and the hindwings) (Nijhout 1991) make butterflies an ideal system in which to explore the phenomenon of serial homology.

Butterfly colour patterns are used for camouflage, predator deterrence, thermoregulation, and mate choice (Monteiro and Prudic 2010). Colour patterns in Nymphalid butterflies can be understood in the context of the Nymphalid Ground Plan. The Nymphalid Ground Plan is a set of pattern elements organized into basal, central and border symmetry systems that make up the wing patterns of butterflies (Nijhout 1991) (Fig. 3-1). The border symmetry system includes ocelli, which are defined as concentric rings of colourful scales around an organizing center. Typically, there is one ocellus in each wing cell, a wing cell being defined as a region of wing membrane (made up of thousands of cytological cells) bounded on all sides by wing veins or the wing margin (Evans and Marcus 2006). Wing cells have traditionally been identified using the adjacent wing veins as reference points (Comstock 1918; Oliver et al. 2012), but for ease of reference, we follow earlier authors in numbering the wing cells sequentially from anterior to posterior (Fig. 3-1) (Monteiro et al. 2003; Monteiro et al. 2007; Kodandaramaiah 2009). When ocelli become very elaborate, they are often referred to as eyespots (Nijhout 1991).

Ocelli are arrayed on the wing in two distinct arrangements: serial and individual. The serial arrangement is defined as a character state in which ocelli occur in 6 consecutive wing cells while the individual arrangement is characterized by the interruption that occurs by deletion of ocelli in a subset of wing cells (Kodandaramaiah 2009). The serial arrangement of the border

symmetry system is the basis for the symmetry hypothesis for the origin of ocelli (Nijhout 1994, 2001; Oliver et al. 2014). This hypothesis suggests that over evolutionary time, the colourful submarginal bands that occur near the wing margin (Fig. 3-1) became constricted in the wing tissues adjacent to the wing veins, producing a series of serially identical homologous ocelli. Over evolutionary time, some ocelli were then deleted while others became individuated and developed distinct phenotypes (Nijhout 1994, 2001; Monteiro 2008; Held 2012). The time series of Distal-less protein expression associated with ocelli development in the late 5th instar larval wing imaginal discs may reflect this evolutionary history, in that the domains of Distal-less expression in all wing cells are uniform early in wing development, but later diverge in wing cells with different ocellus/eyespot phenotypes (Evans and Marcus 2006; Nijhout 2010). An alternative to the symmetry hypothesis, the co-option hypothesis, suggests that the serial arrangement of ocelli is a derived character produced by co-opting the developmental pathway responsible for making the original ancestral ocellus (Monteiro 2008; Oliver et al. 2014). In this proposed scenario, the developmental pathway is redeployed repeatedly in different wing cells to produce a series of ocelli which might have either phenotypically distinct or uniform phenotypes (Monteiro 2008; Oliver et al. 2014). These two hypotheses make fundamentally different predictions about the nature of serial homology. The serial hypothesis predicts that ocelli were initially uniform when they first appeared and became individuated later in evolutionary time. In contrast, the co-option hypothesis predicts that ocelli may have been individuated as soon as the ocellus developmental pathway was iteratively re-deployed in different wing cells (or on different wing surfaces) due to the genetic regulation necessary to produce this iterative pattern (McMillan et al. 2002; Monteiro et al. 2003; Monteiro 2008).

Comparing butterfly wing colour pattern elements among species is a traditional approach for understanding both colour pattern development and evolution (Schwanwitsch 1924; Süffert 1927; Nijhout 1991) that has been enhanced by character state mapping on phylogenetic trees (Maddison and Maddison 2011). Mapping colour pattern elements over a phylogenetic tree can be used to trace the evolutionary history of the characters by predicting the ancestral state of the pattern elements, and direction of evolutionary change among groups of butterflies (Otaki et al. 2006; Kodandaramaiah 2009; Oliver et al. 2014; Abbasi and Marcus 2015). This approach has been employed to reconstruct the evolutionary history of ocelli arrangements (serial vs. individual) on the wing surfaces of butterflies (Kodandaramaiah 2009; Oliver et al. 2014).

Evolution of butterfly eyespot arrangements in *Junonia* was examined by Kodandaramaiah (2009) using a phylogenetic approach. He traced the evolution of eyespot arrangements on the dorsal surface of the hindwing over a phylogenetic tree of the genus *Junonia*. His data predicted that the 'serial arrangement' or 'no eyespots' were the ancestral character state on the dorsal hindwing in the tribe Junoniini (Kodandaramaiah 2009).

Using a similar approach on a much broader phylogenetic scale, Oliver et al. (2014) carried out a character state reconstruction at the family level to estimate the ancestral form of ocelli and their evolution in the butterfly family Nymphalidae. Using 394 species of Nymphalid butterflies and 29 outgroup species, they scored seven wing cells on each wing surface for which ocelli or eyespots can occur for a total of 28 wing cells. Considering all estimates for each wing cell, they predicted that the most likely ocelli pattern in the ancestor of all Nymphalids living approximately 85–90 million years ago had ocelli restricted to the ventral hindwing. There were either four or five original ocelli on the ventral surface of the hindwing. They predicted that

either the four original ocelli occurred in wing cells 1, 2, 4, and 5 (an individual arrangement according to our definition, nomenclature of the wing cells is given in Fig. 3-1) or that the five original ocelli occurred on wing cells 1 to 5 (a serial arrangement according to our definition) (Oliver et al. 2014). Their study also provided some evidence for the co-option hypothesis for the origin of homologous ocelli (Oliver et al. 2014).

When mapping the character states of serial homologs like ocelli or eyespots onto phylogenetic trees, care must be taken to evaluate the interdependence of the character states being mapped (deQueiroz 1996). Phenotypes exhibited by the ocelli within a single wing cell on the dorsal and ventral surfaces of a wing might be expected to exhibit some interdependence because they may both be under similar selection pressures (Kodandaramaiah 2011) or both express some components of a shared developmental program (Evans and Marcus 2006; Marcus and Evans 2008). The same would apply to adjacent ocelli on a single wing surface, or ocelli in homologous wing cells on the forewing and the hindwing.

At the same time, ocelli on opposite sides of the same wing cell, in adjacent wing cells on a wing surface, or in homologous wing cells on the forewing and the hindwing are often dramatically different from each other in Nymphalid butterflies (Brunetti et al. 2001; Cech and Tudor 2005). In extreme cases, such as in *Morpho* butterflies, the ocelli may be highly elaborate eyespots on one wing surface and completely absent on the other (Neild 2008). Further, there are examples of point mutations in species that normally lack ocelli in certain wing cells that produce mutant phenotypes with fully formed eyespots in those wing cells (Brakefield and French 1993; Monteiro et al. 2007), suggesting that the competency to produce elaborate eyespots in particular wing cells may be retained, even when not normally expressed (Evans and

Marcus 2006; Marcus and Evans 2008). Thus, it is not clear whether it is more relevant biologically to score ocelli phenotypes in a binary fashion (presence/absence) or using a graduated score on the basis of the number of colour components in each ocellus. Collectively, this makes it very difficult to predict *a priori* which features of ocelli are likely to be interdependent prior to mapping them onto a phylogeny, so by scoring them in several different ways, it may be possible to identify the most informative ways of examining phenotypic evolution in butterfly ocelli.

We chose to focus on genus *Vanessa* that contains 22 species that have a particularly diverse array of ocelli and eyespot patterns on their wings (Cech and Tudor 2005; Otaki et al. 2006; Vane-Wright and Hughes 2007). Here we take a phylogenetic approach to the study of ocelli and eyespot colour pattern evolution similar to that of Kodandaramaiah (2009), Oliver et al. (2014), and Abbasi and Marcus (2015). We examine the quantity, quality (serial vs. individual), overall state (homogenous vs. heterogeneous), and number of elements or rings of the ocelli on all wing surfaces in the genus *Vanessa* to predict the ancestral form for each of these characters and to examine the degree of individuation exhibited by each ocellus. This may contribute to evaluating patterns of ocelli evolution and individuation in Nymphalid butterflies.

Materials and Methods

Specimens of 38 species of butterflies were acquired for this study: 22 *Vanessa* species and 16 outgroup species within tribe Nymphalini including *Aglais, Antanartia, Araschnia, Hypanartia, Kaniska, Mynes, Nymphalis, Polygonia,* and *Symbrenthia*. Outgroups were selected with reference to Wahlberg et al. (2005) . Sequences for 10 genes from 38 species (Wahlberg et

al. 2005; Otaki et al. 2006; Wahlberg and Rubinoff 2011) were analyzed by Bayesian phylogenetic inference using MrBayes v3.2.1 (Ronquist and Huelsenbeck 2003) as described in Abbasi and Marcus (2015) (Fig. 3-2).

Ocellus and eyespot character states were evaluated from specimens in our laboratory research collection, supplemented by examination of previously published photographs (Layberry et al. 1998; Vane-Wright and Hughes 2007; Wahlberg and Rubinoff 2011; Dec 2012; Fric et al. 2012; Warren et al. 2012). Multiple specimens from each species were examined to assess variation among regional populations, sexes, and seasonal forms. A complete list of specimens examined for this study is listed in Abbasi and Marcus (2015). Each specimen was photographed to document the diversity of eyespots in *Vanessa* (Fig. 3-3) and in the outgroup taxa (Fig. 3-4).

The presence or absence of an ocellus in each wing cell, the number of components or rings in each ocellus/eyespot, and the overall homogeneity or heterogeneity of ocelli on each wing surface were recorded (Table 3-1). Eight wing cells on the dorsal and ventral surfaces of the forewing and hindwing were examined for a total of 32 wing cells. Three different character states were defined for ocelli quality on each wing surface: absent, serial, and individual. We also scored the number of different colour components found in the ocellus of each wing cell (Table S3-1).

The term absent refers to a complete absence of ocelli on a wing surface. The character state serial ocelli refers to a group of ocelli that occur in adjacent wing cells on a wing surface with no intervening empty wing cells. This definition of serial arrangement generalizes the serial arrangement character state as defined by Kodandaramaiah (2009) to all wing surfaces. We

elected to do this due to the variation in the maximum number of ocelli found on different wing surfaces and in different species. If one were to define a distinct number of ocelli (i.e. six) as a requirement for designation as a serial arrangement, this would force the categorization of most of the arrangements on different wing surfaces and in different species as individual arrangements, even when the wing surface features an uninterrupted series of eyespots. We changed the definition to generalize the term (compared to the usage in Kodandaramaiah (2009)) to fully encompass the serial arrangement of ocelli observed in this study and to possibly make it more applicable for future studies. The maximum number of serial ocelli observed on a wing surface in *Vanessa* is 6. The term individual ocelli refers to the presence of one or more wing cells without ocelli in between two or more wing cells containing eyespots.

Homogenous eyespots refer to a state in which all ocelli on a wing surface have a similar morphology. Heterogeneous ocelli refers to a state in which all ocelli on a wing surface are not similar and may be categorized into several groups, which are internally consistent, but different from one another. Up to six heterogeneous groups of ocelli can be found on a single *Vanessa* wing surface. Homogeneity or heterogeneity of the ocelli was evaluated independently by each author on the basis of general appearance, relative size, and colour(s) of the ocelli on a given wing surface. Any specimens where co-authors assigned conflicting character states were re-evaluated and subsequently assigned a consensus state. Abbreviations for the character states we examined are included in Table 3-1. The characters were scored for both ingroup and outgroup species from digital photographs and by direct observation of spread specimens under an Olympus SZ-61 stereomicroscope.

Mesquite version 2.75 (Maddison and Maddison 2011) was used to map characters on the Bayesian phylogenetic tree for ancestral state reconstruction. In *Vanessa,* there are no ocelli on either surface of the hindwing in wing cell -1, or on either surface of the forewing in wing cell 7. Thus, these four constant characters were excluded from our analyses. Therefore, a total of 44 characters out of a total of 48 were mapped individually onto phylogenetic trees using a maximum parsimony reconstruction approach. While they were included in all determinations of ancestral character states for genus *Vanessa,* reconstructions of the characters for the outgroups were removed from the final figures due to limited space.

Results

Correspondence between characters

Correspondences between characters evaluated in this study are summarized in Table 3-2. The ocelli on the ventral hindwing are the most evolutionarily labile, and the most likely to diverge from the phenotypes on the other wing surfaces. Thus, the correspondence of character states of ocelli on opposite sides of the forewing and the correspondence of character states between equivalent ocelli on the dorsal surfaces of the forewing and hindwing are somewhat greater than comparisons involving the ventral hindwing. However, paired 2-tailed t-tests comparing the number of *Vanessa* species with identical character states for each ocellus between two wing surfaces show that these patterns of correspondence are not statistically significant (Forewing dorsal and ventral surfaces compared with correspondence between hindwing dorsal and ventral surfaces: $t_{12} = 0.440$, $P = 0.668$; Forewing dorsal and hindwing

dorsal surfaces compared with forewing ventral and hindwing ventral surfaces $t_{12} = 0.103$, $P = 0.920$)

Ocelli quality (individual vs. serial)

Results of the ancestral state reconstruction show that the ancestral *Vanessa* had serial ocelli on both surfaces of both wings (Fig. 3-5 and 3-6). There are, however, a number of species within the genus with derived individual arrangements. The cardui group (Fig. 3-2) includes several species with an individual arrangement of ocelli on the forewing and hindwing surfaces. *Vanessa terpsichore*, *V. altissima,* and *V. virginiensis* possess the individual arrangement on the dorsal forewing, whereas *V. terpsichore* and *V. altissima* show the individual arrangement on the ventral forewing (Fig. 3-5). *Vanessa terpsichore* also shows the individual arrangement on the dorsal hindwing, whereas *V. myrinna, V. braziliensis, V. altissima,* and *V. virginiensis* show it on the ventral hindwing (Fig. 3-6). The atalanta group (Fig. 3-2) includes three species with an individual arrangement of ocelli on the forewing and hindwing surfaces. *Vanessa tameamea* shows the individual arrangement on the dorsal forewing, whereas *V. buana* and *V. dilecta* exhibit an individual arrangement of ocelli on the ventral forewing (Fig. 3-5).

Ocelli quantity

Results of the ancestral state reconstruction show that the ancestral *Vanessa* had 5 ocelli on both surfaces of the forewing and hindwing (Fig. 3-7 and 3-8).

Overall state of ocelli (homogeneous vs. heterogeneous)

Examination of the ocelli in terms of overall similarity using defined character states shows that the ancestral *Vanessa* had eyespots in the heterogeneous form with two distinct groups on the dorsal forewing and ventral hindwing. It also had heterogeneous ocelli with three distinct groups on the ventral forewing. On the dorsal hindwing, the ancestral *Vanessa* had either the homogenous or heterogeneous character state with two distinct groups (Fig. 3-9 and 3-10).

Number of elements in ocelli -1 to 7

The ancestral states for the number of elements in each ocellus on each wing surface are summarized in Table 3-3. Position 7 on the forewing (Fig. 3-1) does not have a completely enclosed wing cell (the veins do not surround this region of wing membrane completely) and thus, no ocelli were seen in this position in *Vanessa* or the outgroup species. Position 1 on the hindwing (Fig. 3-1) does not have a complete wing cell and thus no ocellus was seen in this position in the ingroup or outgroups. The number of colour elements in the remaining ocelli in the ancestral *Vanessa* was determined to be between zero and five elements depending on which ocellus was being considered (Table 3-3).

Reconstructing the colour pattern elements of the ocelli on the dorsal forewing reveals that the ancestral *Vanessa* likely had simple ocelli consisting of only one colour in wing cells -1 to 3 (Table 3-3). In contrast, the ventral forewing of the ancestral *Vanessa* likely had more diverse ocelli consisting of one colour in wing cells -1 and 0, four colours in wing cells 1 and 2, and two colours in wing cell 3 (Table 3-3). The ocelli in wing cells -1 and 0 on the dorsal and ventral forewing are also the most consistently similar to each other in terms of the number of pattern elements of any ocelli located on the same wing surface. These two ocelli almost always

showed identical phenotypes within a species (22/22 *Vanessa* species for the dorsal surface and 20/22 species for the ventral surface) and appear to have changed character state in parallel in nearly all cases (only two exceptions on the ventral forewing).

Our reconstruction of ocelli on the dorsal hindwing suggests that the ancestral *Vanessa* had simple ocelli composed of only one colour in wing cells 1 and 2. The number of colours in eyespots 3, 4, and 5 was not resolved and thus remains ambiguous. Eyespot 3 was likely composed of one, two, or three colours; eyespot 4 was likely made up of two or three colours and eyespot 5 likely had two or four colours (Table 3-3). On the ventral hindwing, eyespot 1 likely consisted of three colours, whereas eyespot 2 was likely composed of four colours. The number of colours making up eyespot 3 was not resolved and thus its state remains ambiguous between two, three or four colours. Ocelli 4 and 5 were reconstructed as containing three and five colour components, respectively (Table 3-3).

Overall, most of the diversity in the colour pattern elements in the predicted *Vanessa* ancestor is seen in the ocelli that occur in wing cells 1 to 5. The dorsal forewing had the least diversity among its ocelli, whereas the ventral hindwing had the most. The dorsal hindwing and ventral forewing showed intermediate levels of diversity.

Examining the reconstructed trees for the number of eyespot elements reveals that for a particular eyespot, some extant species show different numbers of eyespot components than the predicted ancestor. To summarize these results, we defined a "net direction of evolutionary change". The net direction of evolutionary change in each eyespot was calculated by subtracting the number of species that show more eyespot elements than the predicted ancestor from the number of species that show fewer eyespot elements than the predicted ancestor. We did not

consider species that show the predicted ancestral character state in the calculations. In cases where wing cells lacked an eyespot, the number of colour pattern elements was considered to be zero. The results of these calculations are presented in Table 3-4. Overall, the results show that 15 of the 32 ocelli examined in 22 *Vanessa* species show a tendency to become more complex by increasing the number of colour elements over evolutionary time. This is especially prevalent among ocelli that occur on the dorsal (7 out of 7 ocelli) and ventral forewing (4 out of 7 ocelli) (Table 3-4). In contrast, only 7 of the 32 eyespots tend to become less complex by decreasing the number of colour elements over time. This is most apparent on the ventral forewing (3 of 7 ocelli) and ventral hindwing (3 of 7 ocelli) (Table 3-4).

Discussion

Correspondence between characters

Overall, our observations show that homologous ocelli on the two surfaces of the forewing may have a tendency (not significant) to be more similar than homologous ocelli on the two surfaces of the hindwing. This finding parallels what was reported in Abbasi and Marcus (2015) for non-eyespot colour pattern elements. This is also consistent with the different functions that wing pattern elements, including ocelli, have on the dorsal and ventral surfaces of the wing (Robertson and Monteiro 2005; Stevens 2005). Our observations show that homologous eyespots on the dorsal side of the forewing and hindwing are somewhat more similar to each other (again, not significant) than those on the ventral side. This is consistent with the proposed limited coupling between dorsal and ventral wing pattern elements (see (Allen 2008) for a review). The pattern is opposite to what we reported previously for non-eyespot characters

(Abbasi and Marcus 2015): the correspondence between non-eyespot characters is stronger on the ventral surface of the wing than on the dorsal surface. This contrast emphasizes the complexity and flexibility of the developmental architecture responsible for producing colour patterns on butterfly wings, various components of which can evolve in different directions at the same time (Kodandaramaiah 2009).

Evolution of ocelli quality and quantity

Our reconstruction indicates that the predicted ancestor of *Vanessa* had ocelli in a serial arrangement in all wing cells and was competent to produce ocelli on both the dorsal and ventral surfaces of the forewing and hindwing. One possible mechanism for the evolution of an individual arrangement of ocelli from the ancestral serial arrangement is the deletion of an eyespot in a wing cell. Such an eyespot deletion can be a result of mutations in genes controlling eyespot placement (Monteiro et al. 2003; Monteiro et al. 2007; Monteiro 2008) or by regulatory mutations in genes responsible for eyespot development (Evans and Marcus 2006; Marcus and Evans 2008).

The cardui group includes several species with an individual arrangement of ocelli on all 4 wing surfaces. According to the estimated times of divergence for *Vanessa* by Wahlberg and Rubinoff (2011), the individual arrangement of eyespots arose within in the late Miocene epoch, approximately 10 million years ago. Compared with the estimated age of *Vanessa* (approximately 25 to 30 million years), this character appears to be recent among *Vanessa* species. The atalanta group also includes 3 species with an individual arrangement of ocelli on

the forewing and hindwing surfaces. Overall, the individual arrangement phenotype appears to have evolved in parallel several times in the genus.

The reconstruction of the number of ocelli indicates that the probable ancestral *Vanessa* had 5 ocelli on the dorsal and ventral forewing and hindwing. The number of ocelli on both sides of the forewing shows a tendency to increase over time, especially among the cardui group. Members of the atalanta group retain the ancestral state more often than members of the cardui group. In contrast with the forewing, the number of ocelli on both sides of the hindwing tends to decrease over time, especially among the cardui group. The atalanta group shows much less change from the ancestral state compared to the cardui group. Our observations suggest that the evolutionary changes that altered the number of ocelli on the wing surfaces of *Vanessa* are reversible, which is consistent with the findings of Kodandaramaiah (2009) in *Junonia* butterflies and with simulation studies of eyespot development (Evans and Marcus 2006; Marcus and Evans 2008).

The structure, character, composition, and dynamics of a developmental system may impose limitations on phenotypic variability that produce developmental constraints to evolution (Maynard Smith et al. 1985). Butterfly ocelli are serially homologous structures that share similar developmental processes during their formation (Beldade and Brakefield 2002), but that can diverge from one another under natural and sexual selection (Allen 2008). Both serial and individual arrangements of ocelli occur in *Vanessa* and the serial arrangement seems to be ancestral in this genus. However, the origination of the serial arrangement of ocelli in butterflies (Oliver et al. 2014) is far older than the genus *Vanessa* (which is approximately 25 to 30 million

years (Wahlberg and Rubinoff 2011)), and so we cannot determine whether this is the ancestral state for all Nymphalid butterflies with our data.

In modern butterflies, substantially similar pathways are used to create serially homologous ocelli on the wings (Brunetti et al. 2001). Based on the observations of a shift between serial and individual arrangements of ocelli in *Junonia,* Kodandaramaiah (2009) proposed that the genetic mechanism for eyespot formation remains intact, but is regulated during development to turn ocelli on and off. Evans and Marcus (2006) proposed specific gene products that may be responsible for the presence or absence of ocelli in particular wing cells. Kodandaramaiah (2009) concluded that most of the changes in eyespot phenotypes are based on selective forces and that there is little developmental constraint affecting the process. Artificial selection of the two ocelli on the dorsal surface of the forewing of the butterfly *Bicyclus anynana* showed that these two ocelli can evolve in opposite directions (Beldade et al. 2002). However, the ease with which different phenotypes can be decoupled depends on the specific eyespots and the direction of selection (Beldade et al. 2002), suggesting that particular groups of ocelli may have a tendency to co-vary based on the underlying developmental architecture of the wing.

In *Vanessa*, ocelli on all wing surfaces appear to be individuated and capable of some phenotypic evolution independent from all other ocelli, with the possible exception of the ocelli in wing cells -1 and 0 on the dorsal and ventral forewing. The number of elements in these ocelli is identical within nearly all species and changes in character state appeared to have occurred parallel in nearly all cases. At the same time, some pairs of ocelli (2+5 and 3+4 on the ventral hindwing, 3+4 on the dorsal forewing) identified in previous work focused on other Nymphalid butterfly genera (*Junonia, Bicyclus*)(Monteiro et al. 2003; Monteiro et al. 2007; Kodandaramaiah

2009), also appear to co-vary and evolve in parallel across species in *Vanessa.* These pairs of eyespots do show a degree of independent phenotypic evolution from one another and vary independently somewhat more than ocelli -1 and 0, but do consistently show a high degree of similarity in terms of the number of colour pattern elements. When *Vanessa* species lose eyespots, they tend to lose ocelli 3 and 4, and they tend to lose them simultaneously. These patterns in interspecific variation in butterfly ocelli could be related to the developmental architecture of the wing (Garcia-Bellido et al. 1973), or they may represent the phenotypic consequences of patterns of selection that are broadly applicable to Nymphalid butterflies.

Evolution of number of elements in ocelli -1 to 7

The number, shape, and colour of the rings around the organizing center of an eyespot define the size, overall shape, and diversity of the eyespots on a wing surface. Our observations and analysis of the ocelli of butterflies of the genus *Vanessa* demonstrate that these butterflies display extensive variation in eyespot shape, size, and colour. The size of an eyespot is defined by the strength of the signal originating from each eyespot-organizing center and the sensitivity of the surrounding responsive tissue to that signal (Monteiro et al. 1994). *Vanessa* butterflies also vary in the shape of their ocelli (i.e. circular, semicircular, elliptical, triangular, quadrangular), even within single butterfly specimens. Spacing of the scales that produce ocelli in the anterior-posterior axis of a wing surface or changes in wing shape are responsible for shaping ocelli (Monteiro et al. 1997b; Monteiro et al. 1997c). *Vanessa* butterflies display a variety of different scale colours within their ocelli (i.e. white, blue, orange, red, brown, green). The composition of the coloured rings is defined by the property of the response by the wing tissue to the signals that

are emitted by the organizing centre (Monteiro et al. 1997a). Reconstruction of the number of eyespot elements showed that the evolutionary changes are bidirectional, producing both more and less complex ocelli in the genus *Vanessa*. This is also consistent with the findings of artificial selection and immunohistochemistry experiments, which showed that there is a high amount of variability in eyespot developmental pathways with respect to eyespot shape, size and colour (Monteiro et al. 1997a; Monteiro et al. 1997b; Monteiro et al. 1997c; Brunetti et al. 2001; Beldade et al. 2002).

Functional significance of ocelli in butterflies

The border symmetry system in Nymphalid butterflies includes a number of ocelli that vary in shape, size, and colour depending on their position on the wing surface and the functional role that they play in any given species. There are three main hypotheses to describe the role of ocelli: the intimidation hypothesis, the deflection hypothesis, and the sexual signaling hypothesis. The intimidation hypothesis suggests that large and conspicuous ocelli may intimidate visual predators from attacking a butterfly and thus, increase their chances of survival (Blest 1957). The deflection hypothesis suggests that smaller ocelli on the wing margin may deflect attacks of visual predators away from the body of a butterfly and thereby increase his or her chances of survival (Kodandaramaiah 2011). The sexual signaling hypothesis suggests that eyespot size (Breuker and Brakefield 2002), composition (Robertson and Monteiro 2005), and number (Westerman et al. 2012) may contribute to mate choice among conspecifics. A number of studies have tested these hypotheses and provide evidence to support each of them in different species (Robbins 1980; Wourms and Wasserman 1985; Stevens 2005; Olofsson et al. 2010;

Kodandaramaiah 2011; Kodandaramaiah et al. 2013; Mukherjee and Kodandaramaiah 2015; Prudic et al. 2015).

In *Vanessa,* the ancestral eyespot arrangement was a serial arrangement, which is most often associated with a visual predator deflection strategy (Kodandaramaiah 2011; Kodandaramaiah et al. 2013). As the genus diversified, examples of individual eyespot arrangements evolved on all 4 wing surfaces in at least some *Vanessa* species (Figs. 3-4 and 3-5). Both surfaces of the forewing and the ventral hindwing show the individual eyespot arrangement evolving repeatedly within different lineages within the genus. Of these 3 wing surfaces, the dorsal forewings are most prominent when the wings are open and the ventral hindwings are most prominent when the wings are closed, potentially visible to both visual predators and conspecifics.

It would be very interesting to see if visual predators that feed on butterflies, such as birds (Ota et al. 2014), praying mantises (Prudic et al. 2015), lizards (Vleiger and Brakefield 2007), and frogs (Stefanescu and Paramo 2010) change their behaviour when interacting with *Vanessa* with serial or individual eyespots on different wing surfaces. This would determine whether the presence of eyespots changes predator behavior and frequency of attack, and to identify which wing surface(s) are important in eliciting different behaviours. *Vanessa* is an ideal genus to test predator interactions with different wing surfaces because it includes species that have serial and individual eyespot arrangements on all 4 wing surfaces. This would be a useful supplement to behavioural tests of visual predators using paper models of butterflies rather than actual specimens (Mukherjee and Kodandaramaiah 2015).

Although it has not yet been demonstrated in *Vanessa,* in some butterflies, ocelli (especially on the dorsal wing surface) may also play a role in courtship by acting as a sexual signal (Robertson and Monteiro 2005; Westerman et al. 2012). Many *Vanessa* engage in open-winged basking behaviour, were the dorsal wing surfaces are exposed for extended periods of time (Braby 2000; Cech and Tudor 2005; Mecenero et al. 2013), so the eyespots on these wing surfaces are perhaps the most likely to be employed for communication with conspecifics. The interactions between these different forms of natural and sexual selection are thought to act upon the series of homologous ocelli found on each wing surface to produce the diversity eyespot colour patterns on the wings of butterflies (Monteiro and Prudic 2010). Butterfly ocelli thereby represent an excellent system to study the effects of these evolutionary forces on a serially repeated phenotype whose developmental basis is better understood than most other organismal phenotypes with known fitness consequences (Marcus 2005).

Supporting Information

Table S3-1: Number of colour components found in the ocellus of each wing cell on the four wing surfaces of *Vanessa* butterflies and outgroups. See appendix 4.

Table can also be downloaded from this link as well:
http://onlinelibrary.wiley.com/doi/10.1111/jeb.12716/suppinfo

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Table 3-2. Morphological characters of the forewing and hindwing. Serial ocelli refers to consecutive ocelli in adjacent wing cells with no intervening empty wing cells. Individual ocelli refer to the character state in which there is a cell without an eyespot between two cells containing eyespots.

Wing	Position	Characters	Abbreviation	States and Their Codes
Forewing	Dorsal	Ocelli Quality	**FDOcQl**	0=Absent, 1=Serial, 2=Individual
		Ocelli Quantity	**FDOcQt**	0, 1, 2, 3, 4, 5, 6, 7, 8
		Overall State of Ocelli on the Wing Surface	**FDOSOc**	0=No Ocelli, 1=Homogenous, 2=Heterogeneous 2 groups, 3=Heterogeneous 3 groups, 4=Heterogeneous 4 groups, 5=Heterogeneous 5 groups, 6=Heterogeneous 6 groups
		No. of Elements in Ocellus -1	**FDNEOc-1**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 0	**FDNEOc0**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 1	**FDNEOc1**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 2	**FDNEOc2**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 3	**FDNEOc3**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 4	**FDNEOc4**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 5	**FDNEOc5**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 6	**FDNEOc6**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 7	**FDNEOc7**	0, 1, 2, 3, 4, 5
	Ventral	Ocelli Quality	**FVOcQl**	0=Absent, 1=Serial, 2=Individual
		Ocelli Quantity	**FVOcQt**	0, 1, 2, 3, 4, 5, 6, 7, 8
		Overall State of Ocelli on the Wing Surface	**FVOSOc**	0=No Ocelli, 1=Homogenous, 2=Heterogeneous 2 groups, 3=Heterogeneous 3 groups, 4=Heterogeneous 4 groups, 5=Heterogeneous 5 groups, 6=Heterogeneous 6 groups
		No. of Elements in Ocellus -1	**FVNEOc-1**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 0	**FVNEOc0**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 1	**FVNEOc1**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 2	**FVNEOc2**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 3	**FVNEOc3**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 4	**FVNEOc4**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 5	**FVNEOc5**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 6	**FVNEOc6**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 7	**FVNEOc7**	0, 1, 2, 3, 4, 5
Hindwing	Dorsal	Ocelli Quality	**HDOcQl**	0=Absent, 1=Serial, 2=Individual
		Ocelli Quantity	**HDOcQt**	0, 1, 2, 3, 4, 5, 6, 7, 8
		Overall State of Ocelli on the Wing Surface	**HDOSOc**	0=No Ocelli, 1=Homogenous, 2=Heterogeneous 2 groups, 3=Heterogeneous 3 groups, 4=Heterogeneous 4 groups, 5=Heterogeneous 5 groups, 6=Heterogeneous 6 groups
		No. of Elements in Ocellus -1	**HDNEOc-1**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 0	**HDNEOc0**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 1	**HDNEOc1**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 2	**HDNEOc2**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 3	**HDNEOc3**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 4	**HDNEOc4**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 5	**HDNEOc5**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 6	**HDNEOc6**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 7	**HDNEOc7**	0, 1, 2, 3, 4, 5
	Ventral	Ocelli Quality	**HVOcQl**	0=Absent, 1=Serial, 2=Individual
		Ocelli Quantity	**HVOcQt**	0, 1, 2, 3, 4, 5, 6, 7, 8
		Overall State of Ocelli on the Wing Surface	**HVOSOc**	0=No Ocelli, 1=Homogenous, 2=Heterogeneous 2 groups, 3=Heterogeneous 3 groups, 4=Heterogeneous 4 groups, 5=Heterogeneous 5 groups, 6=Heterogeneous 6 groups
		No. of Elements in Ocellus -1	**HVNEOc-1**	0, 1, 2, 3, 4, 5

No. of Elements in Ocellus 0	**HVNEOc0**	0, 1, 2, 3, 4, 5
No. of Elements in Ocellus 1	**HVNEOc1**	0, 1, 2, 3, 4, 5
No. of Elements in Ocellus 2	**HVNEOc2**	0, 1, 2, 3, 4, 5
No. of Elements in Ocellus 3	**HVNEOc3**	0, 1, 2, 3, 4, 5
No. of Elements in Ocellus 4	**HVNEOc4**	0, 1, 2, 3, 4, 5
No. of Elements in Ocellus 5	**HVNEOc5**	0, 1, 2, 3, 4, 5
No. of Elements in Ocellus 6	**HVNEOc6**	0, 1, 2, 3, 4, 5
No. of Elements in Ocellus 7	**HVNEOc7**	0, 1, 2, 3, 4, 5

Table 3-3. Correspondence of characters between wings and wing surfaces. Numbers indicate the number of *Vanessa* species in which the character states between the two wing surfaces are exact matches.

	Forewing Dorsal vs. Forewing Ventral	Forewing Dorsal vs. Hindwing Dorsal	Forewing Ventral vs. Hindwing Ventral	Hindwing Dorsal vs. Hindwing Ventral
Ocelli Quality	18	19	16	17
Ocelli Quantity	14	10	10	12
Overall State of Ocelli on the Wing Surface	4	9	3	12
No. of Elements in Ocellus -1	6	Incomplete wing cell	Incomplete wing cell	Incomplete wing cell
No. of Elements in Ocellus 0	6	0	0	22
No. of Elements in Ocellus 1	0	12	6	2
No. of Elements in Ocellus 2	0	14	10	0
No. of Elements in Ocellus 3	11	13	3	3
No. of Elements in Ocellus 4	18	4	1	1
No. of Elements in Ocellus 5	16	0	0	0
No. of Elements in Ocellus 6	22	20	22	20
No. of Elements in Ocellus 7	Incomplete wing cell	Incomplete wing cell	Incomplete wing cell	21

Table 3-4. Ancestral characters on each wing surface predicted by reconstructions.

	Forewing Dorsal	Forewing Ventral	Hindwing Dorsal	Hindwing Ventral
Ocelli Quality	Serial	Serial	Serial	Serial
Ocelli Quantity	5	5	5	5
Overall State of Ocelli on the Wing Surface	Heterogeneous 2 groups	Heterogeneous 3 groups	Homogenous or Heterogeneous 2 groups	Heterogeneous 2 groups
No. of Elements in Ocellus -1	1	1	Incomplete wing cell	Incomplete wing cell
No. of Elements in Ocellus 0	1	1	0	0
No. of Elements in Ocellus 1	1	4	1	3
No. of Elements in Ocellus 2	1	4	1	4
No. of Elements in Ocellus 3	1	2	1, 2 or 3	2, 3 or 4
No. of Elements in Ocellus 4	0	0	2 or 3	3
No. of Elements in Ocellus 5	0	0	2 or 4	5
No. of Elements in Ocellus 6	0	0	0	0
No. of Elements in Ocellus 7	Incomplete wing cell	Incomplete wing cell	0	0

Table 3-5. Net direction of evolution in the number of elements in *Vanessa* ocelli based on character state reconstructions (Calculated by subtracting the number of species with more ocellus components than the predicted ancestor from the number of species that show fewer ocellus components than the predicted ancestor).

	Trend towards more ocellus components		Trend towards fewer ocellus components		Ocelli absent in this position		Undetectable due to ambiguous ancestral state	
	Dorsal	**Ventral**	**Dorsal**	**Ventral**	**Dorsal**	**Ventral**	**Dorsal**	**Ventral**
Forewing	FDNEOc-1 FDNEOc0 FDNEOc1 FDNEOc2 FDNEOc3 FDNEOc4 FDNEOc5	FVNEOc-1 FVNEOc0 FVNEOc4 FVNEOc5		FVNEOc1 FVNEOc2 FVNEOc3	FDNEOc6	FVNEOc6		
Hindwing	HDNEOc2	HVNEOc2 HVNEOc6 HVNEOc7	HDNEOc1	HVNEOc1 HVNEOc4 HVNEOc5	HDNEOc0 HDNEOc6 HDNEOc7	HVNEOc0	HDNEOc3 HDNEOc4 HDNEOc5	HVNEOc3

B: Basal symmetry system
DII: Discalis II
MII: Media II
DI: Discalis I
MI: Media I
Oc: Ocelli
EIII: Externa III
EII: Externa II
EI: Externa I

Sc: Subcosta
R: Radius
M: Media
Cu: Cubitus
A: Anal vein

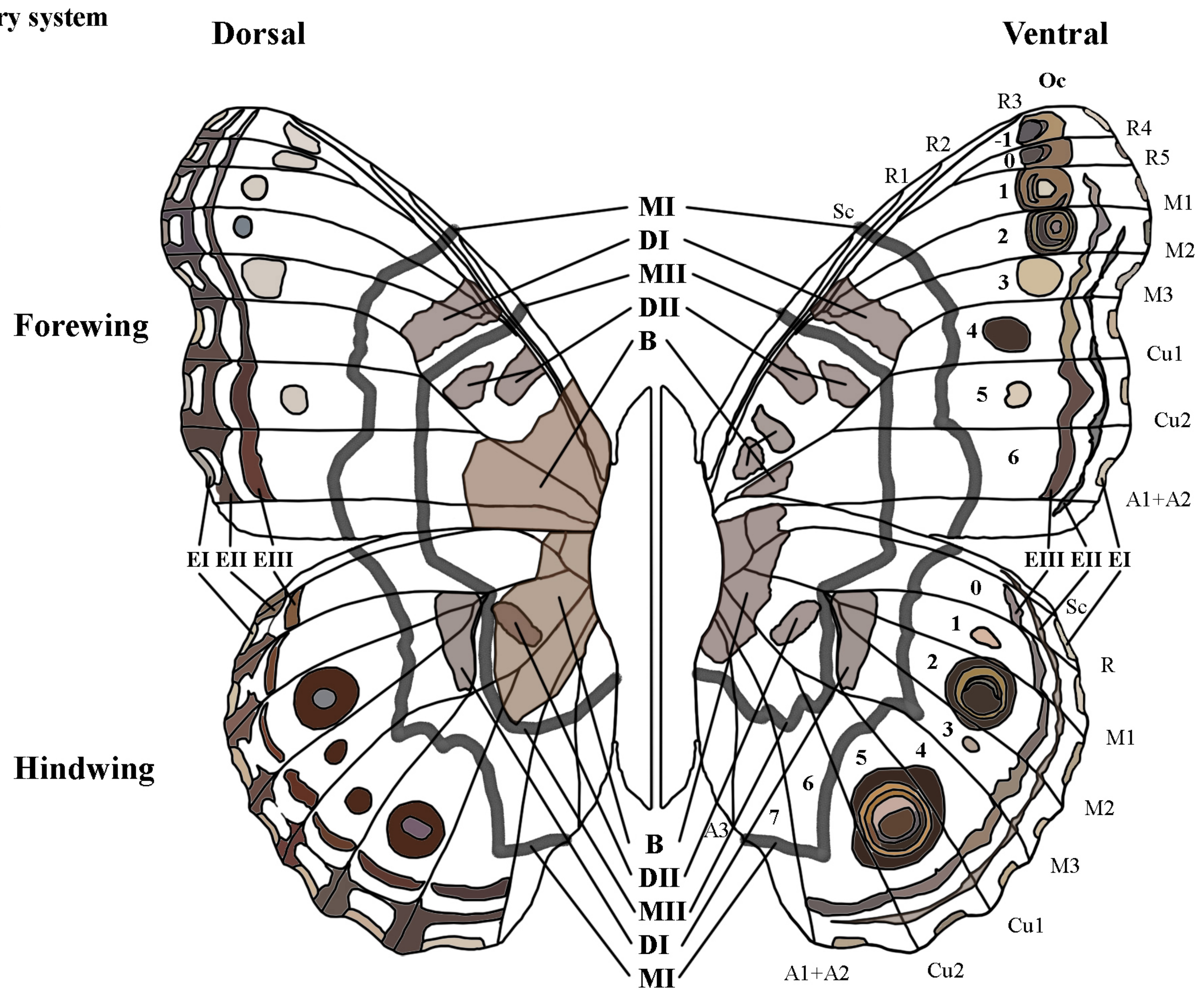

Figure 3-1. The Nymphalid Ground Plan as found in the Lepidoptera. The color pattern phenotypes shown are based on those found on the ventral wing surfaces of *Vanessa braziliensis* and labeled using the nomenclature of Schwanwitsch (Schwanwitsch 1924). The major ground plan elements are as follows: 'Basalis' (B); 'Discalis II' (DII); 'Discalis I' (DI); the two Media bands MII and MI, which form the large central symmetry system generally centered on DI; 'Border ocelli' (Oc); and 'Externa patterns' (E), which are the parafocal, submarginal, and marginal elements (EIII, EII, and EI, respectively) that occur at the wing margin (After (Nijhout 2001; Martin and Reed 2010)).

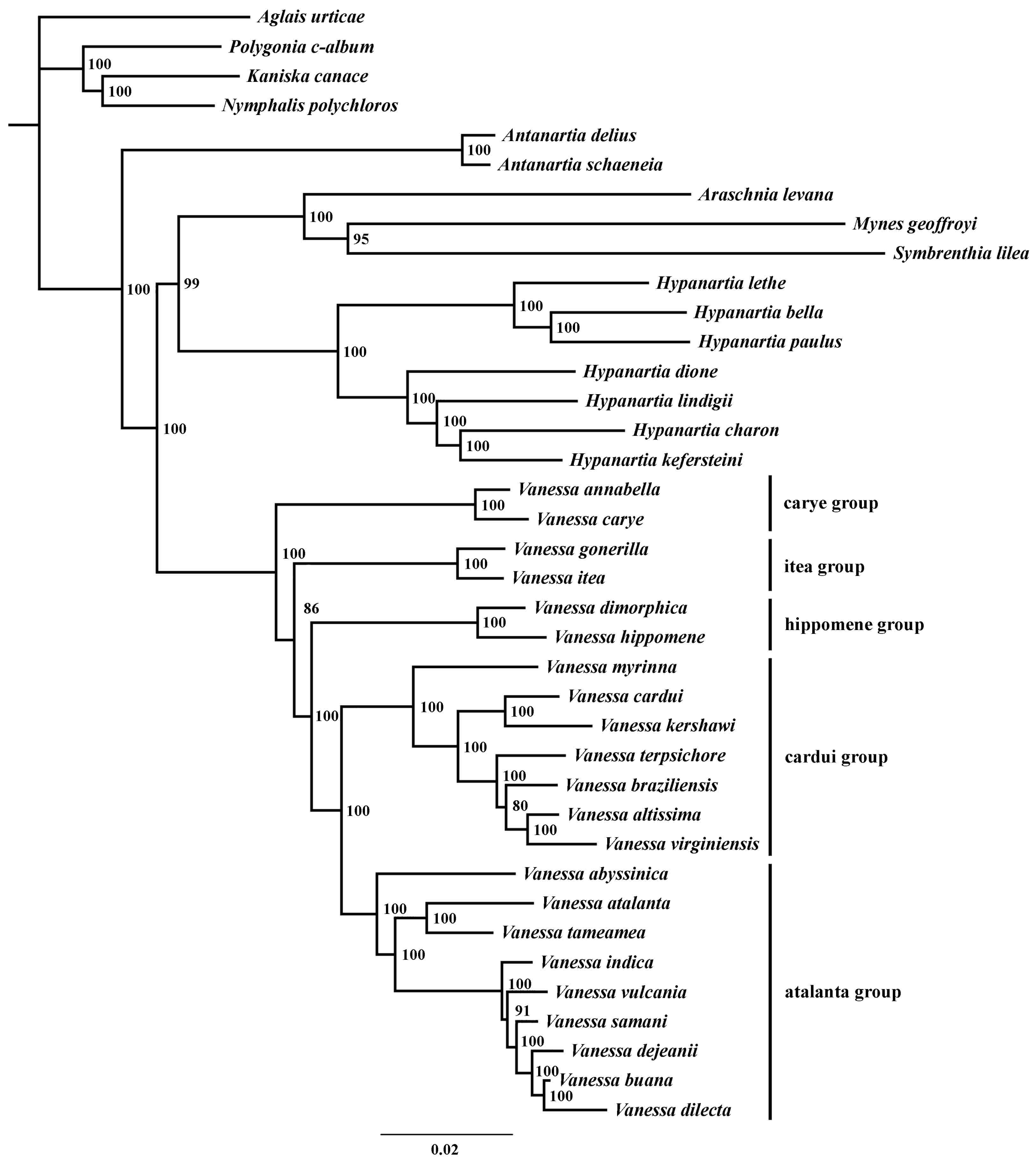

Figure 3-2. Topology and branch lengths from a Bayesian analysis of all DNA sequence data. Numbers adjacent to nodes denote posterior probabilities.

Figure 3-3. Eyespots of the 22 *Vanessa* species showing dorsal (left) and ventral (right) surfaces of the forewing (left) and hindwing (right).

Figure 3-4. Eyespots of the 16 outgroup species showing dorsal (left) and ventral (right) surfaces of the forewing (left) and hindwing (right). M and F indicate male and female butterflies, respectively.

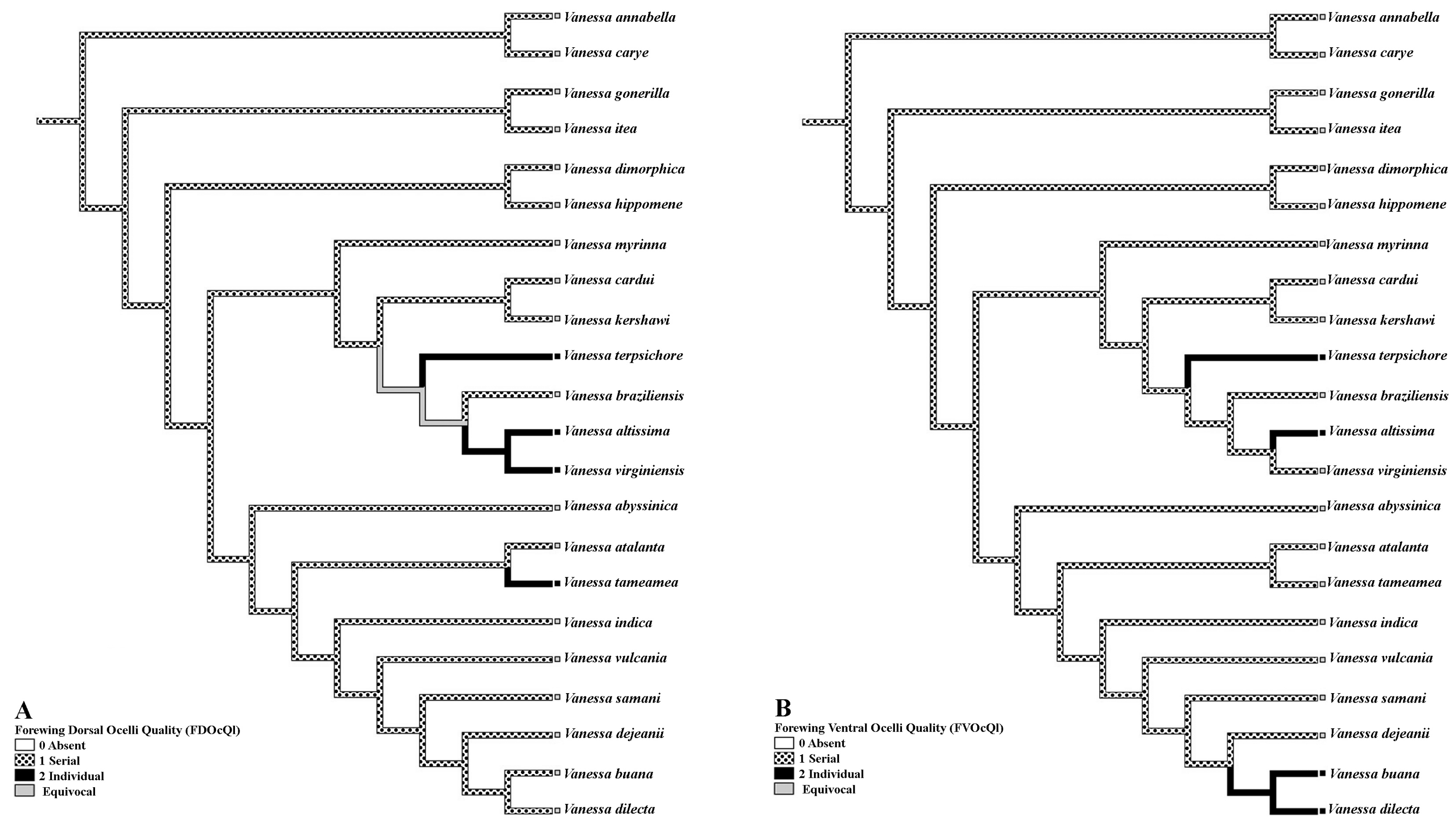

Figure 3-5. Maximum parsimony reconstruction of ocelli quality (serial vs. individual) on the dorsal (A) and ventral (B) surfaces of the forewing in *Vanessa*.

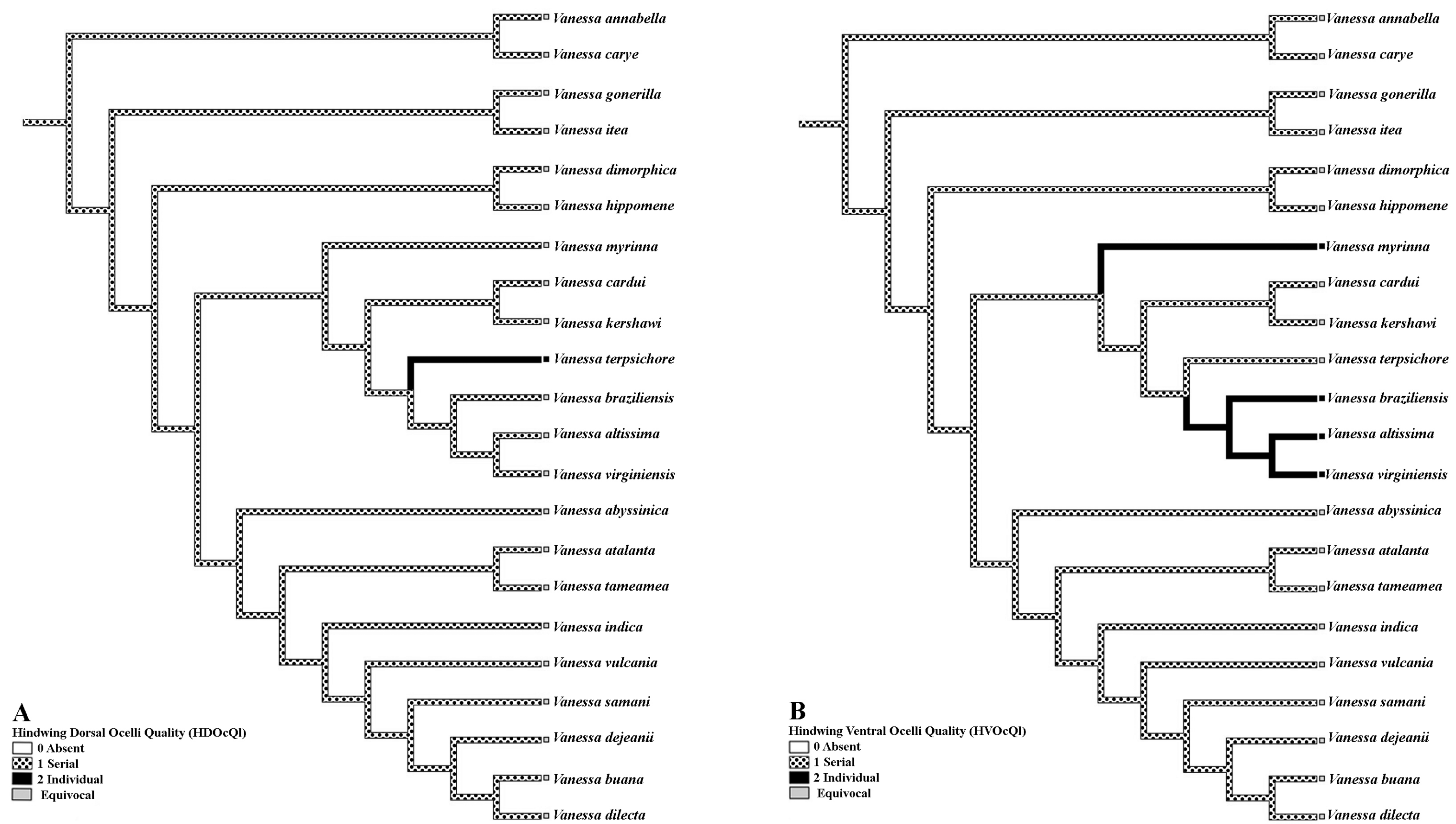

Figure 3-6. Maximum parsimony reconstruction of ocelli quality (serial vs. individual) on the dorsal (A) and ventral (B) surfaces of the hindwing in *Vanessa*.

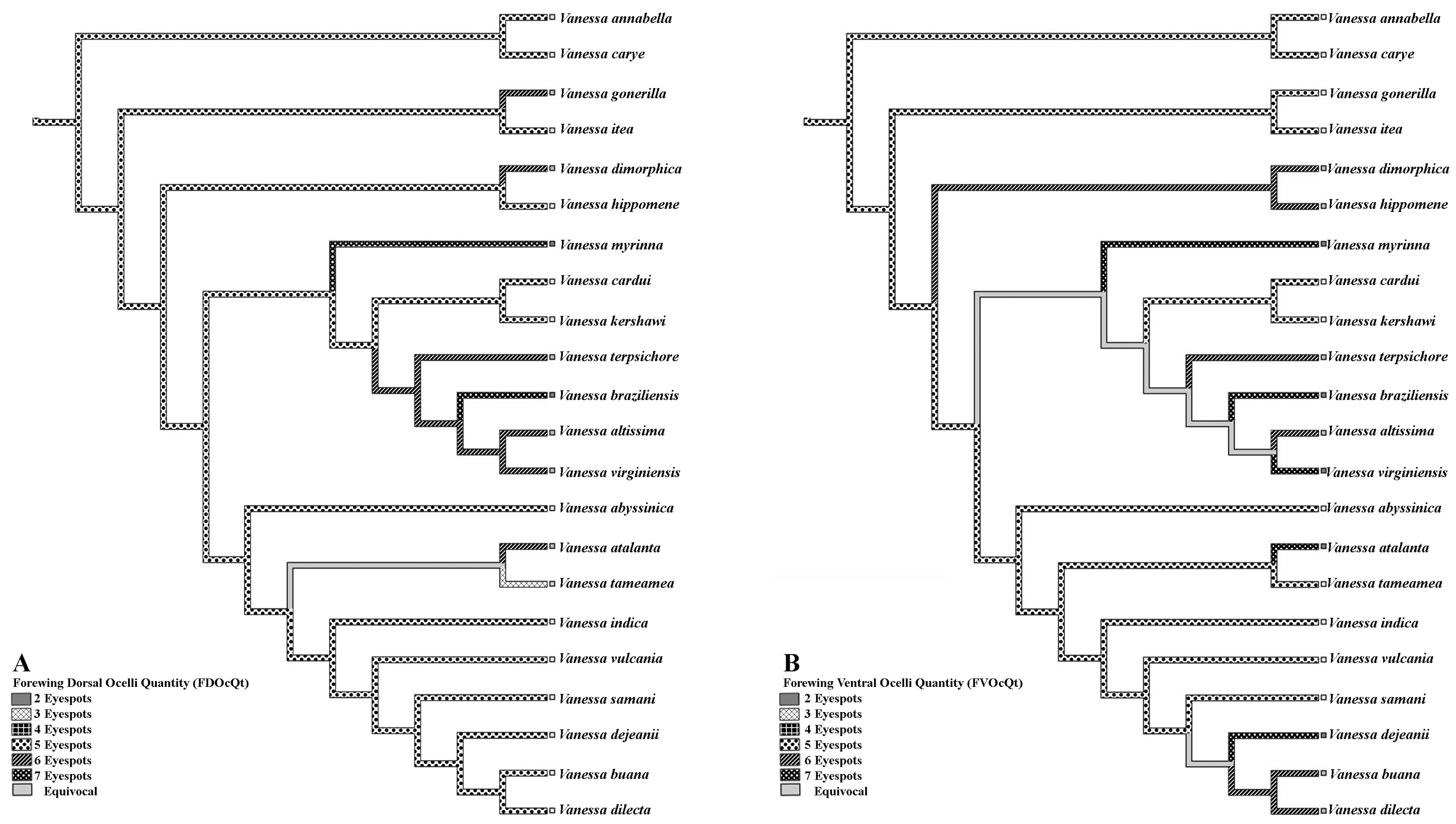

Figure 3-7. Maximum parsimony reconstruction of ocelli number on the dorsal (A) and ventral (B) surfaces of the forewing in *Vanessa*.

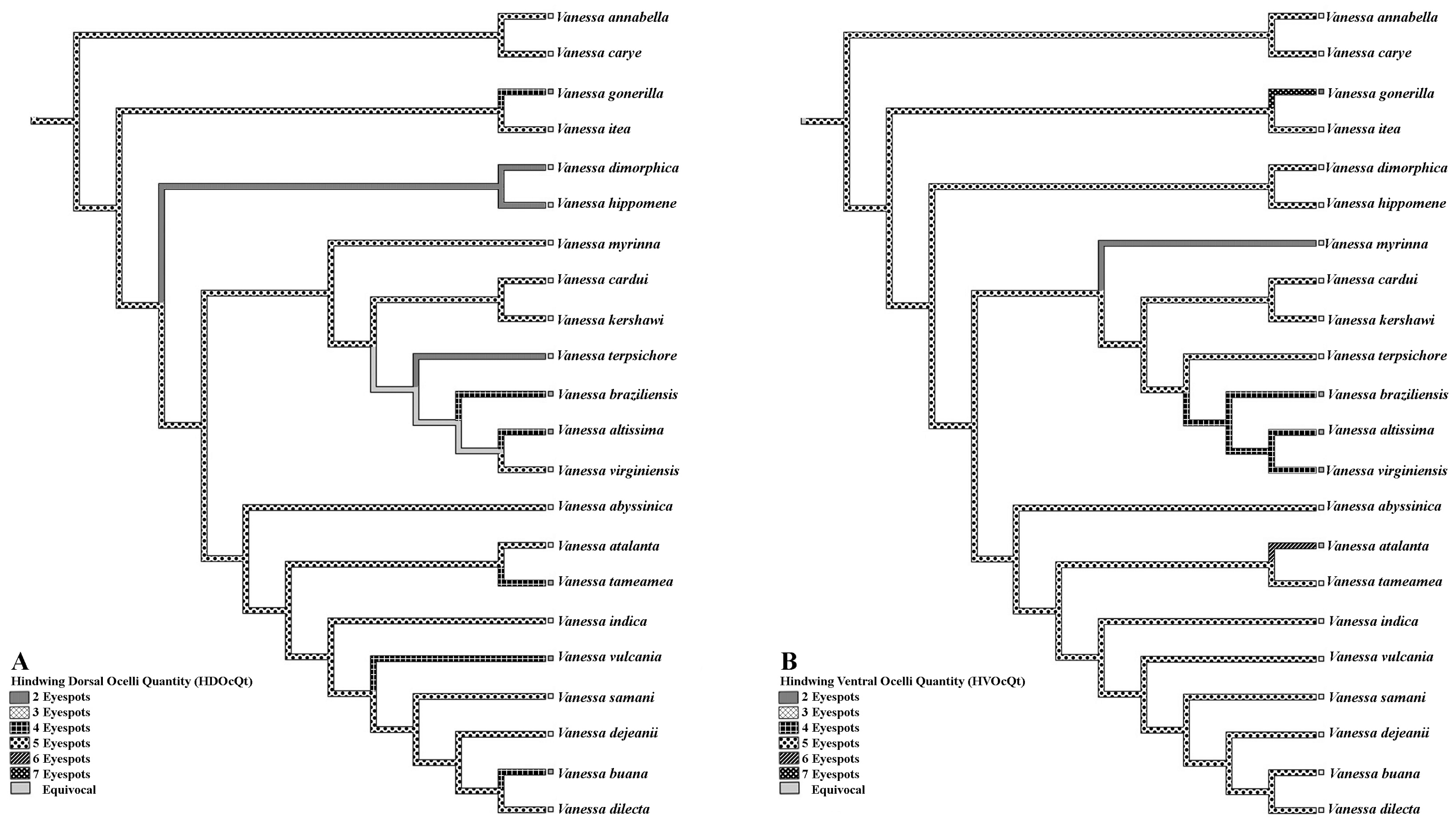

Figure 3-8. Maximum parsimony reconstruction of ocelli number on the dorsal (A) and ventral (B) surfaces of the hindwing in *Vanessa*.

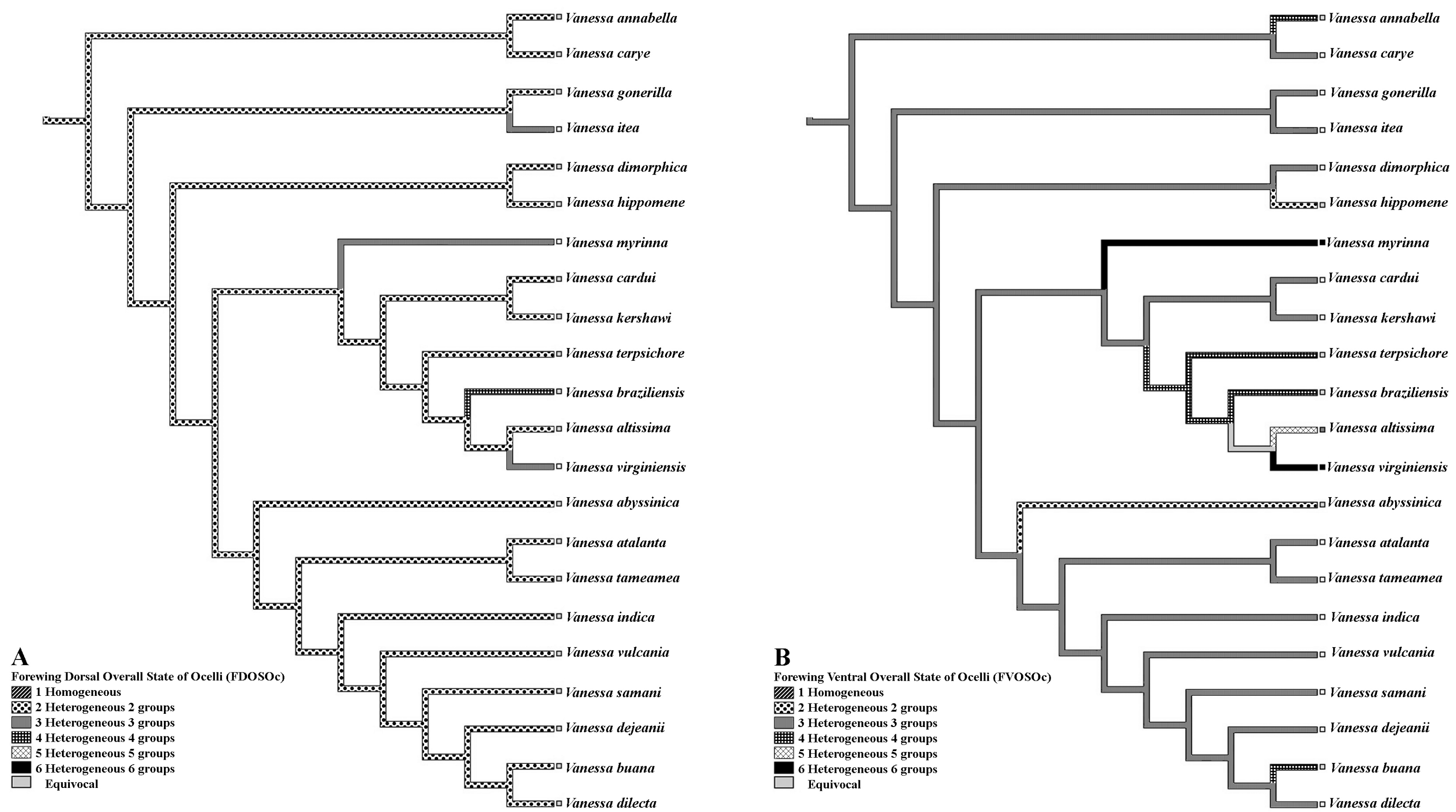

Figure 3-9. Maximum parsimony reconstruction of overall state of the ocelli (homogenous vs. heterogenous) on the dorsal (A) and ventral (B) surfaces of the forewing in *Vanessa*.

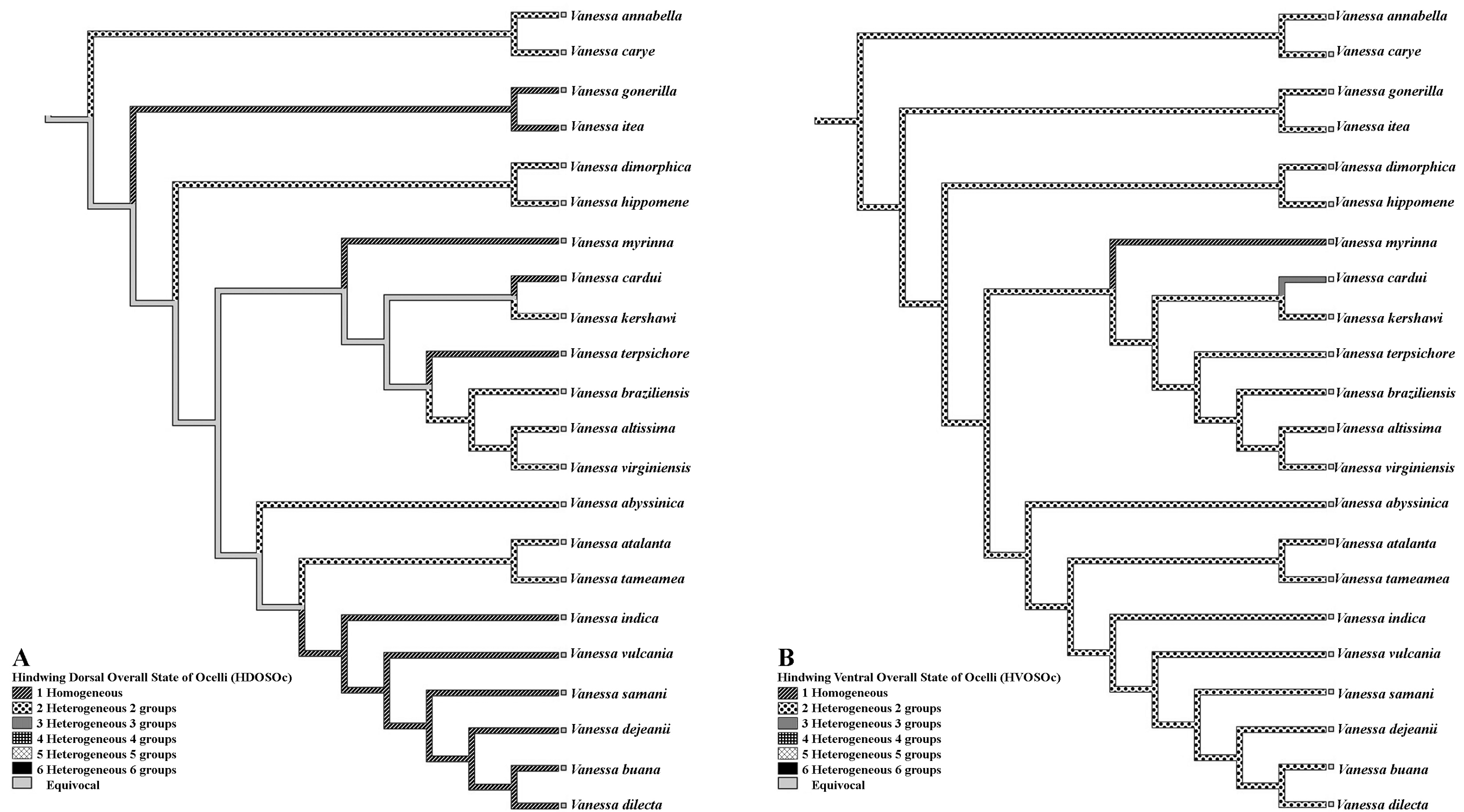

Figure 3-10. Maximum parsimony reconstruction of overall state of the ocelli (homogenous vs. heterogenous) on the dorsal (A) and ventral (B) surfaces of the hindwing in *Vanessa*.

Chapter 4 Multiple and independent lines of evidence support a new compartment boundary and organizer in the developing wings of holometabolous insects

Summary

I evaluated the organizational effects of compartment boundaries in holometabolous insects in butterflies and fruit flies. First, Independent Contrast (IC) analysis was applied to eyespot patterns in *Vanessa* butterflies. IC analysis of position and number of eyespot colour elements revealed significant positive correlations between eyespots 2 and 5 and between eyespots 3 and 4 on all wing surfaces. Examination of the wing sector patterns across all major families of butterflies revealed correspondence between wing sectors 1 and 4 and between wing sectors 2 and 3. Evaluation of published maps of butterfly and moth homeotic and gynandromorph clones reveals that there is a peak in the abundance of sharp smooth edges to clones along the wing vein M2, which divides wing sectors 2 and 3, and which is consistent to the presence of a compartment boundary in the vicinity of the vein. Finally, genetic crosses of *Drosophila* fruit flies including FLP/FRT mitotic recombination system shows clones with consistent edges beneath the L5 wing vein on the far posterior wing sector of fruit flies which is equivalent to vein M3 in butterflies. Combining together all of these findings, the existence of an additional compartment boundary associated with an organizer in wing sector 3 that is responsible for patterning the posterior region of the insect wings is proposed. The suspected patterns of overlapping gene expression associated with the two A-P organizers produce a unique combination of expressed genes, suggesting a mechanism by which butterfly eyespots in each wing sector can be individually regulated.

Keywords: Colour pattern evolution, *Vanessa*, eyespot development, butterfly wing, *Drosophila,* compartment boundary, mirror-image developmental organizer, serial homology

Abstract word count: 250 words

Introduction

Wing colour patterns in butterflies are visual communication devices that serve as sexual signals in courtship, aposematic signals to visual predators, and camouflage (Fig. 4-1) (Nijhout 1996). In particular, eyespots, also called border ocelli, which are concentric rings of colourful scales around an organizing center, are thought to serve as intimidating or deflecting elements in many different species of butterflies and moths (Stevens 2005; Kodandaramaiah et al. 2013). They represent an important model system for understanding the evolution of serial homology (Oliver et al. 2014; Abbasi and Marcus 2015b). In some species, all of the eyespots on a given wing surface are similar, while in other species (or sometimes even on different wing surfaces in the same species), eyespots in different wing sectors are substantially different (Kodandaramaiah 2009; Abbasi and Marcus 2015b). While there have been many studies conducted on the development of eyespots in general (e.g. (Brakefield et al. 1996; Evans and Marcus 2006; Marcus and Evans 2008; Monteiro et al. 2013)), few studies have focused on how eyespots in different wing sectors come to be different from each other, a process known as individuation (Keys et al. 1999; Monteiro et al. 2003; Monteiro et al. 2007; Monteiro 2008).

Butterfly eyespot development occurs in the late larval and pupal stages and is superimposed on top of a pre-existing molecular genetic coordinate system responsible for regulating the development of the wing as a whole (Keys et al. 1999; McMillan et al. 2002). Much of what is known about this coordinate system has come from research on

Drosophila (Biehs et al. 1998; Marcus 2001; Werner et al. 2010), which has established that the anterior-posterior (A-P) patterning of the wing is organized by a domain of expression of the *engrailed (en)* transcription factor in cells in the posterior portion of the wing. Cells in this region also secrete the short-range signal *hedgehog (hh),* which in turn stimulates the expression of the long-range signal *decapentaplegic* (*dpp*) in cells immediately anterior to the *engrailed* expression domain (Zecca et al. 1995; Gilbert 2000). The concentration of *dpp* received by cells in the wing establishes a set of nested domains of gene expression that define the placement of wing veins in *Drosophila* (Sturtevant et al. 1997; Biehs et al.1998; Cook et al. 2004). Veins are essential for the proper development of butterfly colour patterns and it appears that the developmental processes responsible for placing veins and eyespots on the wing are inter-related (Koch and Nijhout 2002; Evans and Marcus 2006; Marcus and Evans 2008). Wing veins also define the boundaries of entomological wing cells or sectors (fields of cytological cells bordered by the wing margin and a series of wing veins) (Comstock 1918; Evans and Marcus 2006).

Of the genes involved in A-P patterning in *Drosophila,* expression patterns for two of these genes have been studied in butterfly wings. *Engrailed* is expressed throughout the posterior of the wing (with the anterior boundary between butterfly wing sectors 1 and 2) (Fig. 4-2A) while the transcription factor *spalt* is expressed in the wing sectors immediately anterior and posterior to the *engrailed* domain boundary (Fig. 4-2B) (Keys et al. 1999; Brunetti et al. 2001; Beldade et al. 2005; Monteiro et al. 2006). *Invected* was also reported to have the same expression domain as *engrailed* (Carroll et al. 1994). Exactly how the A-P patterning mechanism of the butterfly wing interacts with

mechanisms of colour pattern development is not known, but understanding the connections between these two processes is essential for understanding the process of eyespot individuation.

Much of what is known about eyespot individuation comes from interactions between classical mutants with eyespot phenotypes. *Spotty* (Brakefield and French 1993) and *Missing* (Monteiro et al. 2007) are spontaneous mutations with effects on eyespots 3 and 4 in *Bicyclus anynana*. *Spotty* adds additional eyespots in sectors 3 and 4 on both surfaces of the forewing, which lack eyespots in wildtype *Bicyclus*. *Missing* removes eyespots from wing sectors 3 and 4 on the ventral hindwing. *Spotty/Missing* double mutants show that *Missing* also has pleiotropic effects on eyespots 3 and 4 in the forewing (making them smaller), but these effects are not apparent in wildtype individuals (which lack these eyespots entirely) (Monteiro et al. 2007).

An additional mutation (called *3+4*) produced using X-ray mutagenesis by Monteiro et al. (Monteiro et al. 2003) also causes eyespots 3 and 4 to disappear simultaneously in the ventral hindwing of *Bicyclus anynana*. A variety of other mutants produced in the same X-ray mutagenesis screen also resulted in loss of eyespot phenotypes involving varying numbers and combinations of eyespots, but all of these individuals proved to be sterile. Monteiro et al. (2003) suggested that the cis-regulatory region of a hypothetical gene involved in eyespot formation may be organized in modules (in a manner similar to the organization of cis-regulatory elements of the *even-skipped* gene in *Drosophila* (Ludwig et al. 1998)) and that the X-ray induced lesions produced in the screen may be repeatedly affecting this region. The cis-regulatory element evolution hypothesis for the individuation of butterfly eyespots was further elaborated by Monteiro

(2008), but the possible identity of *3+4* gene, and its mechanistic role in eyespot development remains unknown.

Kodandaramaiah (Kodandaramaiah 2009) took a very different approach to examining the evolution of individuation of butterfly eyespots in *Junonia.* He used a phylogeny of *Junonia* and related butterfly genera to trace the evolution of eyespot number and position on the dorsal hindwing within Junoniini (Nymphalidae). He categorized the arrangements of eyespots into two main types of patterns: serial (eyespots occur in 6 serial wing sectors) and individual (based on the absence and presence of eyespots in a subset of wing sectors). He then defined and scored 5 eyespot patterns found on the dorsal surface of the hindwing in the genus *Junonia*: one serial and four individual patterns. Among the individual arrangements, he found a strong association between the presence of eyespots 2 and 5 on this wing surface.

The generality of the associations found by these authors (Monteiro et al. 2003; Monteiro et al. 2007; Kodandaramaiah 2009) between eyespots 2 and 5 on the dorsal hindwing in *Junonia* and between eyespots 3 and 4 on the dorsal and ventral surfaces of the forewing and ventral surface of the hindwing in *Bicyclus* is currently unknown, as are the mechanisms that may be producing these associations. Here I determine whether these eyespot associations are maintained in other butterfly groups. I addressed this question by examining the eyespot morphology of 22 *Vanessa* species and determining whether these possible morphological associations could be generalized for all wing surfaces. This genus contains species that have a particularly diverse array of eyespot patterns on their wings (Cech and Tudor 2005; Otaki et al. 2006; Vane-Wright and Hughes 2007; Abbasi and Marcus 2015b). I took a phylogenetic approach to the study of

eyespot colour patterns (Kodandaramaiah 2009; Abbasi and Marcus 2015a) and conducted a statistical analysis using independent contrasts (Felsenstein 1985; Garland and Ives 2000) to detect eyespot morphology correlations in *Vanessa* butterflies. I also surveyed colour pattern associations in genera in all butterfly families. To explain the patterns that I detected, I hypothesized the existence of an additional compartment boundary, the Far-Posterior (F-P) compartment boundary, and a developmental organizer in the wing that is also responsible for the symmetrical associations found by previous authors (Monteiro et al. 2003; Monteiro et al. 2007; Kodandaramaiah 2009). Wing compartment boundaries are associated with organizers that produce symmetrical gene expression domains on either side of the boundary. I evaluated naturally occurring homeotic butterfly and moth clones to test the hypothesis. I then used the fruit fly *Drosophila melanogaster* as an experimental model to further test for the presence of the Far-Posterior (F-P) compartment boundary. Finally, I propose an expression model that establishes an A-P coordinate system that permits both the independent and coordinated regulation of eyespot and other color pattern phenotypes on insect wings.

Materials and Methods

Independent contrast analysis of the Vanessa eyespot elements

Specimens were acquired for 38 species of butterflies: 22 ingroup taxa from *Vanessa* and 16 outgroup taxa from tribe Nymphalini including *Aglais, Antanartia, Araschnia, Hypanartia, Kaniska, Mynes, Nymphalis, Polygonia,* and *Symbrenthia*. Outgroup taxa were selected with reference to (Wahlberg et al. 2005). Sequences for 10

genes from the 38 exemplar species (Wahlberg et al. 2005; Otaki et al. 2006; Wahlberg and Rubinoff 2011) were analyzed by Bayesian phylogenetic inference using MrBayes v3.2.1 (Ronquist and Huelsenbeck 2003) as described in Abbasi and Marcus (2015a).

Eyespot character states (Table 4-1) were primarily evaluated from specimens in the Marcus laboratory research collection (Abbasi and Marcus 2015a). For species with a small numbers of specimens, I supplemented my direct observations with published photographs (Layberry et al. 1998; Vane-Wright and Hughes 2007; Wahlberg and Rubinoff 2011; Dec 2012; Fric et al. 2012; Warren et al. 2012). Species were examined to assess probable polymorphisms among regional populations, sexes, and seasonal forms. Specimens examined for this study are listed in Chapter 2 and in Abbasi and Marcus (2015a).

Counting the number of parts within structures is a common way of measuring and comparing the non-hierarchical complexity of those structures (McShea 2000; Marcus 2005; Fleming and McShea 2013). The number of different colour elements within each eyespot is a quantitative approach to comparing the relative elaboration of these phenotypes. Correlations between the number of colour elements between eyespots on the same or different surfaces of the fore- and hindwings were evaluated by independent contrast (IC) analysis. IC analysis is a phylogenetic-based method that calculates differences of traits between two closely related taxa by eliminating similarities caused by common ancestry (contrasts) (Felsenstein 1985; Garland and Ives 2000). The Bayesian phylogenetic tree was manipulated to remove all outgroups except *Hypanartia kefersteini,* which was used to root the tree as required by IC analysis. The analysis was carried out using COMPARE 4.6b (Martins 2004). Statistical correlation

analysis in Microsoft Excel (Redmond, Washington, USA) was then used to analyze the contrasts and to determine relationships and correlation coefficients between each pair of traits.

Overall pattern similarity analysis in butterflies

Overall pattern similarity of the wing sectors in major families and subfamilies of butterflies were examined. In order to maximize the diversity included in my sample, I chose to evaluate 1 species per genus, for up to 25 genera per butterfly family (some butterfly families contain less than 25 genera). A total of 136 butterfly species representative of 136 different genera from families Hesperiidae (24 genera), Papilionidae (12 genera), Pieridae (25 genera), Riodinidae (25 genera), Lycaenidae (25 genera) and Nymphalidae (25 genera) were evaluated using images of pinned butterflies (Warren et al. 2012). Also, because the vast majority of the species did not have eyespots in their wing sectors, I looked at similarities between wing sectors in terms of all colour patterns found in the wing sectors. On each wing surface, adjacent and nonadjacent wing sectors were compared with each other , including comparisons between sectors 0+1, 0+3, 1+2, 1+4, 2+3, 2+5, 3+4, 3+6, 4+5, and 5+6 (Abbasi and Marcus 2015a, 2015b).

Clonal analysis of homeotic wing data in butterflies

Published homeotic butterfly wing data (Sibatani 1983a, 1983b) was evaluated to trace possible compartment boundaries on all four wing surfaces. A total of 392 wing surface maps belonging to the dorsal and ventral surfaces of the forewing (205 surfaces) and hindwing (187 surfaces) were evaluated. For each wing I examined two separate

maps of clones: one belonging to the clones on the discal cell, the wing sector that surrounds Discalis II (Fig. 4-1), on both wing surfaces of a given wing; and the other belonging to the remaining wing sectors (non-discal area) on a given wing. Fig. 4-3 shows two imaginary homeotic wing surfaces with different kind of clones on the forewing and hindwing reported in Sibatani (1983a and 1983b). Each cross line is a representative of a wing vein and the bottom triangle on each wing surface illustrates the discal wing sector. I was interested in whether homeotic clones cross or make an edge on wing veins and/or interveins on different wing surfaces.

Clonal analysis of fruit fly wings

Three experiments were designed in order to create clones (group of cells with specific molecular or morphological characters) on the wing surfaces of fruit flies *Drosophila melanogaster* labeled with either phenotypic markers or with fluorescence. Six fly stocks were acquired from the Bloomington *Drosophila* Stock Center at Indiana University (Bloomington, Indiana, USA). These fruit flies have a FLP (Flippase) /FRT (Flippase recognition target) mitotic recombination system integrated into different chromosomes in their genome (Xu and Rubin 1993). The overall strategy for producing mitotic clones (groups of cells homozygous for traceable phenotypic characters that stand in contrast to the background phenotype) using the FLP/FRT system is shown in Fig. 4-4. To produce mitotic clones in F1 females, a 2 hour heat shock with a 1 hour room temperature interval was applied between 72 to 96 hours after eggs were laid. The purpose of the heat shock was to induce the expression of the FLP enzyme. During the G2 phase of the cell cycle, the chromosomes undergo recombination at the FRT sites.

When the cell divides, chromosomes randomly go into each cell and if a cell happens to receive two copies of the mutant yellow gene, it expresses the mutant yellow phenotype. If there is a boundary in vicinity of these cells, however, the marked cells would not be expected to cross the boundary.

Three separate FLP/FRT crosses were initiated (Fig. 4-5). In cross A, when heat shock is applied to the F1 progeny, it causes FLP to remove a cassette containing an extra stop codon inserted into the GFP gene and once removed, the GFP gene is expressed in that cell. All of the progeny of this cell with normal GFP gene glow green under the appropriate fluorescent illumination (Fig. 4-5A). In cross B, there were FRT sites as well as a copy of mutant yellow allele y^1 on chromosome 1. As seen in Fig. 4-5B, the F1 females are heterozygous for alleles at the *yellow* locus (y^1/y^+) and FLP-induced recombination results in y^+/y^+ or y^1/y^1. The progeny of any cell that receives two copies of the mutant yellow allele (y^1/y^1) will show the yellow cuticle phenotype of the homozygous mutant allele. In cross C, there were two mutant yellow alleles on chromosome 1 (y^1/y^1) as well as one copy of the wild-type yellow allele y^+ and an FRT site on chromosome 2 (Fig. 4-5C). When the heat shock was applied to the F1 progeny in cross C, the Flippase enzyme was expressed, causes recombination at the FRT sites on chromosome 2. The 3 possible outcomes of FLP/FRT recombination on chromosome 2 were as follow; y^+/y^+, y^+/y^- and y^-/y^-. If a cell has the y^-/y^- genotype (no wild-type yellow alleles) on chromosome 2, the y^1/y^1 genotype of chromosome 1 will cause the expression of the yellow cuticle phenotype and all of the progeny of that cell will have the same phenotype.

Emerging flies were moved to new vials loaded with formula 4-24 instant *Drosophila* medium blue food (Carolina Biological Supply Company, Burlington, North Carolina, USA) and new colonies of flies were subsequently established. Virgin females were collected from dedicated stocks and crossed with males in separately labelled vials as outlined in Fig. 4-5. Eggs and larvae from the parental generation were heat shocked for 2 hours in a 38° C water bath with a 1 hour room temperature interval, 72 to 96 hours after cross vials were established to activate the heat shock inducible promoters of the FLP gene. To control the amount of moisture in the vials, the openings of the vials were covered with parafilm prior to being placed in the water bath. When the female flies emerged, they were screened for clones on their eyes, bodies, and wings with an Olympus SZ61 stereomicroscope (Olympus, Shinjuku, Tokyo, Japan) and a CO_2 anesthetization system. Selected flies from the screening process were put into 1.6 mL microcentrifuge tubes containing 70% ethanol for slide preparation at a later time. Wings from each specimen were removed and fixed on the slides using a drop of Euparal (Anglian Lepidopterist Supplies, Hindolveston, Norfolk, UK) and covered with a cover slip. Dried slides were examined on a Leica M205 C stereomicroscope (Leica, Wetzlar germany) equipped with a Nikon Digital Sight DS-Fi2 imaging system (Nikon, Chiyoda, Tokyo, Japan) under 160X magnification. Photographs were taken from the slides with clones under 63X magnification. The images were adjusted by means of rotation and clones in each image were subsequently outlined in Canvas 14 for Windows. Four landmarks (red dots) were placed on all four parts of the wings to help superimpose wing images of different sizes, shapes, and angles.

Immunohistochemistry

Wing imaginal discs were collected from 5th instar *Junonia coenia* larvae and immunohistochemistry experiments were performed with a mouse monoclonal primary antibody (4F11) that recognizes an epitope present in both *Engrailed and Invected* transcription factors (Patel et al. 1989) at 1:5 dilution. A donkey anti-mouse secondary antibody conjugated with Alexa Fluor® 488 fluorescent dye (Thermo Fisher, Waltham, Massachusetts, USA) was used at 1:400 dilution according to established protocols (Carroll et al. 1994). The imaginal discs were then visualized by fluorescence-microscopy on an Olympus IX71 inverted compound microscope.

Results

Independent contrast analysis of the Vanessa eyespot elements

Independent contrast (IC) analysis was used to evaluate correlations between characters on the same or different surfaces (dorsal, ventral) and on the same or different wings (forewing, hindwing). Significant positive correlations in terms of morphology were found between eyespots 2 and 5 (2+5 correlation) and between eyespots 3 and 4 (3+4 correlation) on all wing surfaces (Table 2), suggesting the existence of an axis of symmetry on vein M3, which runs between wing sectors 3 and 4. Of 28 comparisons investigated within each wing surface, additional significant correlations were detected, but these were generally limited to interactions on single wing surfaces (Table 4-2, Fig. 4-6). Most of the single-wing-surface correlations were between eyespots in adjacent

wing sectors, suggesting that these phenotypic relationships may be relatively plastic over evolutionary time. Comparing the number of elements in homologous eyespots between the two sides of the forewing revealed only one significant positive correlation (R = 0.57, p = 0.0045) in eyespot 5. On the two sides of the hindwing, there is also only one significant correlation (R = 0.46, p = 0.0272), again between the dorsal and ventral eyespots in wing sector 5. Comparing the number of elements in homologous eyespots on the same surfaces of the forewing and hindwing did not reveal any significant correlations. Comparing the number of eyespots between the two sides of the forewing revealed that they are significantly correlated (R = 0.46, p = 0.0272), but the same comparison on the hindwing did not reveal any significant correlations. Finally, comparing overall state (homogenous vs. heterogeneous) of ocelli between the two surfaces of each wing did not reveal any significant correlations.

Overall pattern similarity analysis in butterflies

Generally, colour patterns in adjacent wing sectors show very similar wing pattern characteristics relative to nonadjacent wing sectors (Data not shown). I also found that there are higher associations between the overall pattern of wing cells 2 and 3 (2+3) (Fig. 4-7A) as well as 1 and 4 (1+4) in resulting distribution (Fig. 4-7B). This finding suggests an axis of symmetry on vein M2 between sectors 2 and 3.

Clonal analysis of homeotic wing data in butterflies

I surveyed published data to determine whether spontaneously produced clones (patches of cells with distinct phenotype produced by mutations) in homeotic Lepidoptera

species form smooth edges along wing veins or at intervein regions associated with known or suspected compartment boundaries. I investigated mapped clones across the Lepidoptera (Sibatani 1983a, 1983b) and found that there is a peak abundance of smooth edges on vein M2 between wing sectors 2 and 3 on both discal and non-discal areas of the forewing and discal area of the hindwing. The non-discal area of the hindwing showed a peak aboundance of smooth edges on veins Cu1 and Cu2 (see Fig. 4-7D). Table 4-3 demonstrates the number of crosses and edges on all veins of the examined maps. This finding suggests an existence of a clonal boundary on vein M2.

Clonal analysis of fruit fly wings

The cross with fluorescent clones (cross A, (Fig. 4-5A)) was not successful due to the nature of experiment and rareness of the informative clones. While I was able to produce clones in other parts of the body with this cross, detecting clones in the wing required photographing female fly wings before the wing cells die shortly (less than 2 hours) after the imago emerges from the pupa. The frequency of wings with GFP clones was very low, so it was very difficult to find *Drosophila* at the right stage that also had wing clones. The two yellow crosses yielded better results. From the many flies that I produced and examined, I mounted wings from 889 female flies. Of 1778 mounted wings examined, I was able to find 44 wings with large clones. In cross B (Fig. 4-5B), I collected 327 female flies and of the 654 wings mounted and examined, I was able to find 12 wings with large clones. In cross C (Fig. 4-5C), I collected 448 female flies and of the 896 wings mounted and examined, I found 32 wings with large clones (Fig. 4-8). The wings with large clones from each cross were photographed and superimposed on top of

each other (Fig. 4-9A and 4-9B). Results in each cross independently showed that there is a clonal boundary posterior to vein L5 in *Drosophila* that is equivalent to vein M3 in butterflies (see Fig. 4-1 for vein homologies).

Immunohistochemistry

The A-P compartment boundary of butterfly wing imaginal discs is revealed by the anterior boundary of expression of Engrailed protein. This was visualized using the 4F11 monoclonal antibody, and is shown in light green in Fig. 4-2A.

Discussion

Independent contrast analysis of the Vanessa eyespot elements

Independent contrast (IC) analysis allowed us to evaluate correlations between eyespot characters on the same wing surface or on different wing surfaces (Table 4-2). Significant positive correlations were found between eyespots 2 and 5 (2+5 correlation) and eyespots 3 and 4 (3+4 correlation) across all wing surfaces. Many other significant positive correlations were also detected, but these were restricted to individual wing surfaces, rather than being consistent across all wing surfaces, suggesting that these phenotypic relationships may be relatively plastic over evolutionary time. Butterfly eyespots play a role in both mating systems and predator avoidance and these selective forces may drive the colour pattern phenotypes on different wing surfaces in different directions (Robertson and Monteiro 2005; Kodandaramaiah et al. 2013; Abbasi and

Marcus 2015a). Eyespots 2, 3, 4, and 5 are expected to be subject to these selective forces, yet the relationships among eyespots 2, 3, 4, and 5 are very consistent. The consistency of the 2+5 and 3+4 correlations across all wing surfaces in *Vanessa*, in combination with prior observations of similar eyespot correlation patterns in *Junonia* and *Bicyclus* butterflies (Monteiro et al. 2003; Monteiro et al. 2007; Kodandaramaiah 2009), suggests that these patterns of eyespot correlations may reflect the underlying developmental architecture of the insect wing.

Overall pattern similarity analysis in butterflies

Analysis of the wing patterns across all families of butterflies showed that there is an association between the overall pattern of wing sectors 1 and 4 (1+4) as well as 2 and 3 (2+3). Thus, there are 2 lines of evidence that are consistent with the existence of a colour pattern organizer located in the posterior region of all 4 wing surfaces. The independent contrast analysis of eyespots in *Vanessa* butterfly species places this boundary in the region centered on vein M3 that includes wing sectors 3 and 4. The broad survey of colour patterns in butterfly taxa suggests a region centered on vein M2 that includes wing sectors 2 and 3. The well-studied A-P organizer in the *Drosophila* wing that secretes the long-range signal *dpp* is found in an intervein region (and is not coincident with or immediately adjacent to a wing vein), so by analogy it would not be surprising if this organizer were also in an intervein region. Wing sector 3 was included in the interval identified by both approaches as a possible location of a colour pattern organizer.

Clonal analysis of homeotic wing data in butterflies

The peak abundance of smooth edges suggest the presence of a compartment boundary in the vicinity of vein M2 (see Fig. 4-7D) that aligns the clonal cells and produces an edge, and does not allow cells to cross the boundary. This places the compartment boundary in the same vicinity as the colour pattern organizer that I detected by other methods, which is also consistent with the organization of the well-studied A-P compartment boundary and organizer in *Drosophila.* Gynandromorphs (Fig. 4-7C) are another source of information but the mosaic gynandromorphs from sexually dimorphic species that could be used to test this hypothesis are very rare.

Clonal analysis of fruit fly wings

Using the three layers of information that had been collected, I hypothesized the existence of a second compartment boundary on the far posterior part of the wing (in an anonymous position to avoid any bias during scoring the results). I propose that if the A-P boundary exists in both butterflies and fruit flies, then the second compartment boundary may also exist in both insect groups. Given the convenience of working with the fruit fly *Drosophila melanogaster* and the availability of *Drosophila* genetic tools, I used *Drosophila melanogaster* as a model to test my hypothesis by generating and mapping mitotic clones (Garcia-Bellido 1977). In *Drosophila,* clones do not cross the boundary defined by *engrailed* expression (Blair 1992), so experimentally marked clones would not be expected to cross the compartment boundary in the far posterior part of the wing either. The results from FLP/FRT *Drosophila* crosses consistently demonstrate a

place where the clones formed a smooth edge just posterior to vein L5, which is homologous to vein M3 in butterfly wings. Collectively, these findings suggest that there is an additional compartment defined by a compartment boundary in the posterior part of the wing in holometabolous insects. This boundary may be called the Far-Posterior (F-P) compartment boundary. Given that the A-P compartment boundary was discovered approximately four decades ago and that *Drosophilla* is a well-studied model, the presence of an additional, previously undocumented compartment boundary can be considered a breakthrough in our understanding of wing development and patterning.

Proposed model to describe the data

Establishment of the A-P compartment boundary in the *Drosophila* wing disk begins with signals from the pair-rule genes, which are involved in specification of body segments in the *Drosophila* embryo (Howard and Ingham 1986; Lawrence et al. 1987; Lawrence and Pick 1998; Gilbert 2000). The pair-rule genes drive the expression of *engrailed (en)* in the posterior portion of each segment and in the posterior of the wing imaginal disk and suppresses the expression of *en* in the anterior portion of the wing disk. All of the daughter cells in the 2 compartments retain the presence or absence of *en* expression of their progenitors. *Engrailed* upregulates expression of the secreted *hedgehog (hh)* signal*,* which diffuses to the anterior compartment (Zecca et al. 1995; Gilbert 2000). In the anterior compartment, cells in a narrow band immediately anterior to the A-P compartment boundary respond to *hh* by expressing *decapentaplegic (dpp),* a second secreted signaling molecule.

The Dpp-secreting cells at the A-P compartment boundary act as a developmental organizer that is responsible for patterning the sectors anterior and posterior to the compartment boundary (Zecca et al. 1995). *Brinker (brk), spalt (sal)* and *optomotor-blind* (*omb*) are 3 genes that respond to Dpp (Cook et al. 2004). High concentrations of the Dpp morphogen repress expression of *brk*. In turn, the brk protein represses expression of *omb*, *sal* and *vestigial* (*vg*) in regions anterior and posterior to the *dpp* organizer in a dosage-dependent manner. In other words, Dpp and brk act in opposition and in a complementary fashion to one other (Cook et al. 2004). An interaction between sal and brk in the anterior region of the *Drosophila* wing disk, mediated by *knirps* (*kni*) and *rhomboid* (*rho*), defines the position of the L2 longitudinal wing vein (homologous to the R5 vein in other insects, Fig. 4-1) (Comstock 1918; Stark et al. 1999). Similarly, the interaction between omb and brk in the posterior region of the wing disk, mediated by abrupt (ab), defines the position of the *Drosophila* L5 wing vein (homologous to the Cu2 vein in other insects, Fig. 4-1) (Cook et al. 2004). Finally, the boundaries of the domain of cells responding to hh in the anterior compartment of *Drosophila* define the placement of the L3 and L4 wing veins (homologous to the M2 and Cu1 veins, respectively, Fig. 4-1) (Sturtevant et al. 1997; Biehs et al. 1998).

The region of the wing patterned by the *engrailed* compartment boundary organizer in *Drosophila* corresponds to a span of wing sectors along the A-P axis centered on the organizer, and arranged as a nested series of expression domains. The existence of the *en* A-P compartment boundary in butterfly wings has been documented by examination of *en* and *sal* expression patterns (Keys et al. 1999; Brunetti et al. 2001; Beldade et al. 2005; Monteiro et al. 2006). Engrailed expression in the wing imaginal

discs of 5th instar *Junonia coenia* larvae also shows the A-P compartment boundary in the region between veins M1 and M2 on the wing disc. The area in which the anti-*engrailed* antibody binds is posterior to the boundary and is depicted in light green (Fig. 4-2A). Expression of *Dpp* and *omb* has also been documented in transcriptome analysis of the wing disc of the moth *Ostrinia furnacalis* (Liu et al. 2014), but there is no data pertaining to the expression domains of *dpp* and *omb* in butterflies and moths. Fig. 4-2B is a projection of the expression of genes responsible for A-P patterning in *Drosophila* onto a butterfly wing. Positioning of the expected domains of gene expression is based on known expression domains of *en* and *sal* in butterflies.

Using *Vanessa* butterfly eyespots as morphological landmarks on the wing, my analysis shows that across all 4 wing surfaces, there is a span of 4 wing sectors, which may also be consistent with a nested series of expression domains corresponding to the 2+5 and 3+4 eyespot correlations. However, the span of wing sectors defined by the eyespot correlations is not centered on the known anterior limit of *en* expression (between butterfly wing sectors 1 and 2)(Fig 4-2A and 4-2B). This suggests that there may be an additional A-P organizer on butterfly wings located posterior to the organizer associated with *en* and *dpp* expression known from *Drosophila.* This hypothetical posterior organizer may drive gene expression in the posterior of the wing, producing a nested series of domains organized symmetrically around it, similar to the way in which known patterns of gene expression are arranged around the *dpp* organizer in the anterior of the wing (Fig. 4-2C). This would provide a mechanistic explanation for the consistent tendency in Nymphalid butterflies to produce eyespots in a mirror image arrangement around the wing vein that separates wing sectors 3 and 4. A ligand with properties similar

to *dpp* (*dpp-like*) may serve as the organizing signal, and genes with functional similarities to *sal, omb,* and *brk* (*sal-like, omb-like,* and *brk-like*) may establish the nested domains of gene expression. Whether any of these hypothetical genes have sequence homology to those that participate in the *en* compartment boundary and the *dpp*-dependent A-P wing organizer is a matter of speculation. In A-P body axis determination in the *Drosophila* embryo, many genes, primarily the *Hox* genes, responsible for regional specification of body segments share sequence homology, but other genes with very similar roles (e.g. *teashirt* (*tsh*)) have no sequence homology to the *Hox* genes (Dezulueta et al. 1994).

The next question to address is how the locations of the boundary and organizer are specified along the A-P axis. If we continue to use the *en* compartment boundary and *dpp* organizer in the anterior of the *Drosophila* wing as a model, this suggests that the placement of the posterior organizer may also be specified by a compartment boundary associated with the anterior limit of expression of a gene with a role similar to *en* (*en-like*), thereby defining a far-posterior compartment (Zecca et al. 1995; Gilbert 2000). Given the vast amount of experimental work that has been devoted to the development of the *Drosophila* wing, it is important to note that there are no published precedents for the existence of an additional wing compartment in that model system (Cook et al. 2004). However, the wings of *Drosophila,* like those of all flies, are highly derived in structure with vestigial hindwings (converted to halteres) and forewings that have reduced venation and are compressed (with most of the apparent tissue loss from the posterior portions of the wing) (Comstock 1918; Marcus 2001).

Other insects in which wing development has been studied, such as ants (Abouheif and Wray 2002), beetles (Lommen et al. 2009; Clark-Hachtel et al. 2013), aphids (Brisson et al. 2010), and treehoppers (Prud'homme et al. 2011), also have wings that are highly modified in structure and/or reduced in size. While Lepidopteran wings are unique among insect wings in that they are covered with scales, they are otherwise more representative of the wing structure of a typical insect than are the wings of ants, beetles, aphids, and treehoppers (Comstock 1918; Borror et al. 1989). It is possible that the hypothesized far-posterior wing compartment has not been detected in other insects because it has been lost over evolutionary time as their wings have been modified and reduced in size. Alternatively, it is possible that the far-posterior wing compartment is so reduced in these species that mutations that affect this compartment do not have large phenotypic effects in model systems like *Drosophila,* making mutants difficult to identify in mutagenesis screens (Terriente-Felix et al. 2010). Finally, it is also possible that the existence of a far-posterior compartment and its associated organizer is a novel developmental trait in the insect lineage that gave rise to the Lepidoptera.

A consequence of the A-P patterning mechanism that has been proposed is that the overlapping patterns of gene expression, driven by the *dpp* organizer and the newly hypothesized Far-Posterior (F-P) organizer, create distinct combinations of gene expression in each butterfly wing sector (Fig. 4-2D). When combined with the transcription factor loci *apterous* (*ap*), which defines dorsal cell fates in the wing (Weihe 2001), and *Ultrabithorax* (*Ubx*), which distinguishes the hindwing from the forewing (Weatherbee et al. 1999), the A-P patterning genes would create a unique combinatorial code or "address" for each wing sector on all 4 wing surfaces. This would allow

butterflies to independently determine the phenotypes of each wing sector through the regulation of downstream genes responsible for initiating colour pattern formation (Evans and Marcus 2006; Monteiro et al. 2006). In the case of eyespots, which show enormous diversity with respect to the number and degree of elaboration on any given wing surface (Allen et al. 2008; Oliver et al. 2014; Abbasi and Marcus 2015b), this suggests a potential mechanism by which eyespots in each wing sector can become individuated.

This hypothesis and model are related to a previously proposed cis-regulatory element evolution hypothesis for eyespot diversification (Monteiro et al. 2003; Monteiro et al. 2007; Monteiro 2008). Most of the gene regulation within each wing sector is likely mediated through the differential binding of transcription factors to regulatory elements associated with genes involved in eyespot formation. There is, however, no direct evidence that all of the cis-regulatory elements responsible for eyespot individuation are associated with a single master control gene and are organized in a manner similar to the regulatory elements of *Drosophila even-skipped* (*eve*) as suggested in the original model (Monteiro et al. 2003; Monteiro et al. 2007; Monteiro 2008). It is entirely possible that the eyespot regulatory elements may be associated with several different genes at the beginning of the eyespot genetic regulatory network such as *Distal-less* (*Dll*), *Notch* (*N*), and perhaps *Antennapedia* (*Antp*) (Evans and Marcus 2006; Saenko et al. 2011). This would permit independent control of the initiation of eyespot development in multiple wing sectors while also retaining the ability to produce different eyespot phenotypes in each wing sector by modulating multiple interactions between genes responding to the wing A-P organizers and genes responsible for regulating eyespot development.

Remarkably, if this model is correct, it not only offers a potential explanation for why certain eyespots (e.g. 3+4, 2+5) often have similar phenotypes on a given wing surface, but also a mechanism for how these phenotypic correlations can become dissociated to produce individuated eyespots as is seen, for example, on the ventral forewing of *V. braziliensis* (Fig. 4-1). All that may be required to change the phenotypic associations between eyespots on a wing surface is alteration of the binding sites for the gene products responsible for A-P patterning of the wing in the regulatory regions of genes in the eyespot development pathway. A detailed understanding of the mechanism by which eyespots are deployed in wing sectors greatly enhances the value of this system as a model for studying the evolution of serial homology (Allen 2008; Allen et al. 2008; Oliver et al. 2014; Abbasi and Marcus 2015b).

Conclusion

Butterflies and fruit flies appear to possess at least 3 A-P wing compartments. Overlapping gene expression established by A-P and F-P organizers may explain the observed phenotypic correlations and allow for the coordinated regulation and the individuation of butterfly colour patterns. Research in non-traditional model organisms reveals important aspects of development not revealed in 40 years of *Drosophila* wing research.

Future Directions

Discovering the gene network responsible for the establishment of the F-P compartment boundary and investigating their role on patterning of the wing by lose of

function mutation are the important future steps for the research in insect wing development. Transcriptome analysis of the wing disc may be a good way to find candidate genes that might contribute to establishing the boundary. A candidate gene approach to identify genetic components of the posterior A-P patterning mechanism may also be a productive strategy. Homologs of *en* (called *invected, in*) and *dpp* (called *glass-bottom boat, gbb*) are expressed in the *Drosophila* wing and have partially overlapping functions with their paralogs. Loss of function mutations in these genes does not however have severe wing phenotypes (Simmonds and Bell 1998; Bangi and Wharton 2006). Perhaps this can be attributed to the highly reduced wing morphology in wildtype *Drosophila.* Exploring the expression patterns of *in* and *gbb* in developing butterfly wings may shed light on the role of these gene products in A-P patterning. In the butterfly *Papilio dardanus, in* appears to play a role in colour pattern specification (Clark et al. 2008), which may be consistent with its role in A-P patterning. Additional candidate genes associated with the hypothesized organizer may be identified from homology searches of Lepidopteran genome projects with genes such as *omb, brk,* and *sal* (Xia et al. 2004; Heliconius Genome Consortium 2012) or from EST libraries generated from different parts of developing butterfly wings (Beldade et al. 2006).

Similar experimental clonal analysis could be done in butterflies in order to confirm the F-P boundary with homeotic mutantions such as *Hindsight* in *Junonia coenia* (which transforms clones of cells in the ventral hindwing such that they take on ventral forewing cell fates (Nijhout and Rountree 1995; Weatherbee et al. 1999)), in mosaic intermediates between the seasonal forms of *Precis octavia* (McLeod 2007)*,* or by somatic transformation of wing tissue with vectors carrying reporter constructs (Lewis

and Brunetti 2006; Golden et al. 2007). To test the generality of the 3-compartment model of wing development, the existence of the compartments should also be examined in other insects. I recommend examining hemimetabolous insects in the basal part of the insect phylogeny. There are examples of gynandromorphs/mosaics in basal insect orders such as Odonata, Homoptera (Fulgoridae), Orthoptera and Mantodea. It would also be helpful to be able to do clonal analysis with experimentally produced clones using CRISPR/Cas9 or other lineage markers in the these groups.

The 3-compartment, 2-organizer model for A-P patterning of insect wings is very specific in its predictions. This specificity makes it a powerful hypothesis in that it can be vigorously tested with conventional experiments in developmental biology and molecular genetics. *Vanessa* butterflies are an example of how phylogenetic reconstruction and analysis of adult phenotypes in a non-traditional model system can provide important insights into important developmental processes that may have gone undetected in more traditional experimental systems.

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Table 4-1. Morphological characters of the fore- and hindwing. Serial ocelli refer to the number of ocelli that are in adjacent wing sectors, with no intervening empty wing sectors. Individual ocelli refer to situations in which there is a sector without an eyespot between two sectors containing eyespots.

Wing	Position	Characters	Abbreviation	States and Their Codes
Forewing	Dorsal	Ocelli Quantity	**FDOcQt**	0, 1, 2, 3, 4, 5, 6, 7, 8
		Overall State of Ocelli on the Wing Surface	**FDOSOc**	0=No Ocelli, 1=Homogenous, 2=Heterogeneous 2 groups, 3=Heterogeneous 3 groups, 4=Heterogeneous 4 groups
		No. of Elements in Ocellus -1	**FDNEOc-1**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 0	**FDNEOc0**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 1	**FDNEOc1**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 2	**FDNEOc2**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 3	**FDNEOc3**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 4	**FDNEOc4**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 5	**FDNEOc5**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 6	**FDNEOc6**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 7	**FDNEOc7**	0, 1, 2, 3, 4, 5
	Ventral	Ocelli Quantity	**FVOcQt**	0, 1, 2, 3, 4, 5, 6, 7, 8
		Overall State of Ocelli on the Wing Surface	**FVOSOc**	0=No Ocelli, 1=Homogenous, 2=Heterogeneous 2 groups, 3=Heterogeneous 3 groups, 4=Heterogeneous 4 groups
		No. of Elements in Ocellus -1	**FVNEOc-1**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 0	**FVNEOc0**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 1	**FVNEOc1**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 2	**FVNEOc2**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 3	**FVNEOc3**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 4	**FVNEOc4**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 5	**FVNEOc5**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 6	**FVNEOc6**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 7	**FVNEOc7**	0, 1, 2, 3, 4, 5
Hindwing	Dorsal	Ocelli Quantity	**HDOcQt**	0, 1, 2, 3, 4, 5, 6, 7, 8
		Overall State of Ocelli on the Wing Surface	**HDOSOc**	0=No Ocelli, 1=Homogenous, 2=Heterogeneous 2 groups, 3=Heterogeneous 3 groups, 4=Heterogeneous 4 groups
		No. of Elements in Ocellus -1	**HDNEOc-1**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 0	**HDNEOc0**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 1	**HDNEOc1**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 2	**HDNEOc2**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 3	**HDNEOc3**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 4	**HDNEOc4**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 5	**HDNEOc5**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 6	**HDNEOc6**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 7	**HDNEOc7**	0, 1, 2, 3, 4, 5
	Ventral	Ocelli Quantity	**HVOcQt**	0, 1, 2, 3, 4, 5, 6, 7, 8
		Overall State of Ocelli on the Wing Surface	**HVOSOc**	0=No Ocelli, 1=Homogenous, 2=Heterogeneous 2 groups, 3=Heterogeneous 3 groups, 4=Heterogeneous 4 groups
		No. of Elements in Ocellus -1	**HVNEOc-1**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 0	**HVNEOc0**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 1	**HVNEOc1**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 2	**HVNEOc2**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 3	**HVNEOc3**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 4	**HVNEOc4**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 5	**HVNEOc5**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 6	**HVNEOc6**	0, 1, 2, 3, 4, 5
		No. of Elements in Ocellus 7	**HVNEOc7**	0, 1, 2, 3, 4, 5

Table 4-2. Significant independent contrast correlations detected among the 28 within-wing-surface comparisons. Eyespots 2+5 and 3+4 correlate on all wings that suggest the existence of an axis of symmetry within sectors 3 and 4.

	Dorsal surface	Ventral surface
Forewing	-1+0 (Invariant) -1+3 (R=0.81, P=2.82 x 10^{-6}) -1+4 (R=0.57, P=0.0045) 0+3 (R=0.81, P=2.82 x 10^{-6}) 0+4 (R=0.57, P=0.0045) 1+4 (R=0.58, P=0.0037) 1+5 (R=0.47, P=0.0236) 2+4 (R=0.58, P=0.0037) 2+5 (R=0.47, P=0.0236) 3+4 (R=0.49, P=0.0176)	-1+0 (R=0.95, P=4.37 x 10^{-12}) -1+1 (R=0.53, P=0.0093) -1+2 (R=0.53, P=0.0093) 0+1 (R=0.48, P=0.0204) 0+2 (R=0.48, P=0.0204) 1+5 (R=0.48, P=0.0204) 2+5 (R=0.48, P=0.0204) 3+4 (R=0.51, P=0.0129) 3+6 (R=0.46, P=0.0272) 4+5 (R=0.53, P=0.0093) 4+6 (R=0.51, P=0.0129) 5+6 (R=0.54, P=0.0078)
Hindwing	2+5 (R=0.48, P=0.0204) 3+4 (R=0.69, P=0.0003) 4+5 (R=0.79, P=7.373 x 10^{-6})	1+3 (R=0.66, P=0.0006) 1+4 (R=0.67, P=0.0005) 1+5 (R=0.52, P=0.0110) 2+5 (R=0.53, P=0.0093) 3+4 (R=0.82, P=1.675 x 10^{-6}) 3+5 (R=0.44, P=0.0356) 4+7 (R=0.54, P=0.0078) 6+7 (R=0.48, P=0.0204)

Table 4-3. Homeotic butterfly analysis results showing the position and number of cases that clones cross or make an edge on the examined homeosis maps.

Forewing	Found cases	Hindwing	Found cases
Sc Cross	9	SR Cross	5
Sc Edge	4	SR Edge	7
R1Cross	10	Rs Cross	10
R1Edge	3	Rs Edge	9
R1Discal Cross	6	Rs Discal Cross	9
R1Discal Edge	0	Rs Discal Edge	1
R2 Cross	8		
R2 Edge	2		
R2 Discal Cross	6		
R2 Discal Edge	0		
R3 Cross	8		
R3 Edge	7		
R3 Discal Cross	6		
R3 Discal Edge	0		
R5 Cross	9		
R5 Edge	5		
R5 Discal Cross	6		
R5 Discal Edge	1		
M1Cross	11	M1Cross	16
M1Edge	25	M1Edge	21
M1Discal Cross	8	M1Discal Cross	15
M1Discal Edge	3	M1Discal Edge	3
M1M2 Cross	22	M1M2 Cross	22
M1M2 Edge	32	M1M2 Edge	18
M1M2 Discal Cross	10	M1M2 Discal Cross	17
M1M2 Discal Edge	12	M1M2 Discal Edge	5
M2 Cross	26	M2 Cross	30
M2 Edge	83	M2 Edge	41
M2 Discal Cross	23	M2 Discal Cross	27
M2 Discal Edge	16	M2 Discal Edge	21
M2/M3 Cross	33	M2/M3 Cross	53
M2/M3 Edge	52	M2/M3 Edge	17
M2/M3 Discal Cross	27	M2/M3 Discal Cross	48
M2/M3 Discal Edge	7	M2/M3 Discal Edge	11
M3 Cross	21	M3 Cross	50
M3 Edge	55	M3 Edge	53
M3 Discal Cross	23	M3 Discal Cross	55
M3 Discal Edge	4	M3 Discal Edge	11
M3/Cu1Cross	33	M3/Cu1Cross	82
M3/Cu1Edge	31	M3/Cu1Edge	24
M3/Cu1Discal Cross	18	M3/Cu1Discal Cross	55
M3/Cu1Discal Edge	2	M3/Cu1Discal Edge	3
Cu1Cross	23	Cu1Cross	69
Cu1Edge	36	Cu1Edge	57
Cu1Discal Cross	17	Cu1Discal Cross	50
Cu1Discal Edge	3	Cu1Discal Edge	3
Cu2 Cross	24	Cu2 Cross	72
Cu2 Edge	31	Cu2 Edge	57
Cu2 Discal Cross	12	Cu2 Discal Cross	40
Cu2 Discal Edge	1	Cu2 Discal Edge	2

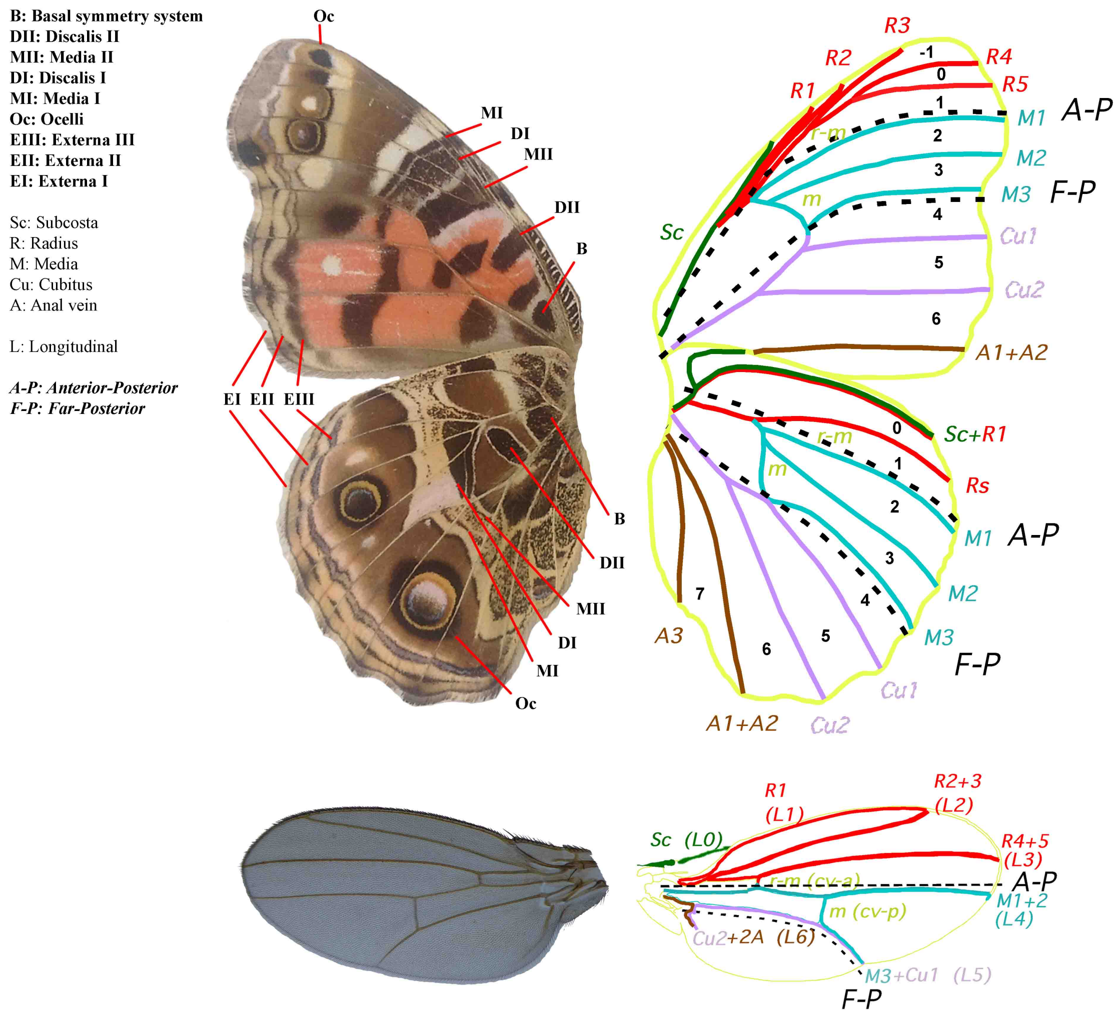

Figure 4-1. The Nymphalid Ground Plan as found across the Lepidoptera (Left) and wing vein homologies in fruit flies and butterflies (Right). The colour pattern phenotypes shown on the actual butterfly wing are based on those found on the ventral wing surfaces of *Vanessa braziliensis* and labeled using the nomenclature of Schwanwitsch (Schwanwitsch 1924). The major ground plan elements are: ‘‘Basalis’’ (B); ‘‘Discalis II’’ (DII); ‘‘Media II’’ (MII); ‘‘Discalis I’’ (DI); ‘‘Media I’’ (MI); ‘‘border ocelli’’ (Oc); and ‘‘Externa patterns’’ (E), which are the parafocal, submarginal, and marginal elements (EIII, EII, and EI, respectively) that border the wing (After (Nijhout 2001; Martin and

Reed 2010)). To the right, the cartoon shows wing venation system in adult *Vanessa* butterflies adapted from references, the veins are: ‘‘Subcosta’’ (Sc); ‘‘Radius’’ (R); ‘‘Media’’ (M); ‘‘Cubitus’’ (Cu); ‘‘Anal vein’’ (A) (Miller and Brown 1989; Martin and Reed 2010). Longitudinal (L) veins in fruit fly *Drosophila melanogaster* have been labelled by butterfly homologues (Stark et al. 1999). A-P and F-P compartment boundary have been demonstrated by black dotted lines in both cartoons.

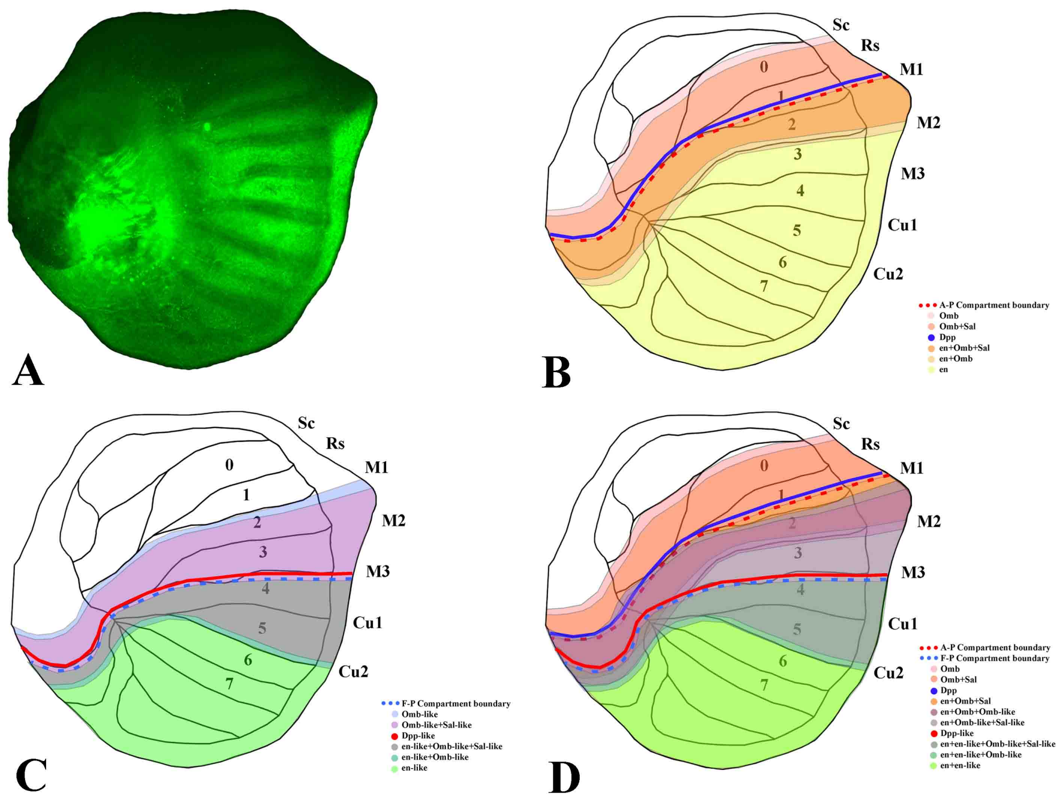

Figure 4-2. Proposed model for the A-P compartment boundaries on butterfly wings. (A) Expression pattern of *engrailed* on a *Junonia* wing disc. (B) Model of the well-studied A-P compartment boundary associated with *engrailed* expression (Keys et al. 1999; Brunetti et al. 2001; Beldade et al. 2005; Monteiro et al. 2006). (C) The additional proposed F-P compartment boundary based on this study. (D) Combining the patterns of gene expression from the two compartment boundaries. The model predicts that each wing sector has a unique combination of expressed genes, providing a mechanism by which the eyespot phenotypes can be coordinated or independently regulated.

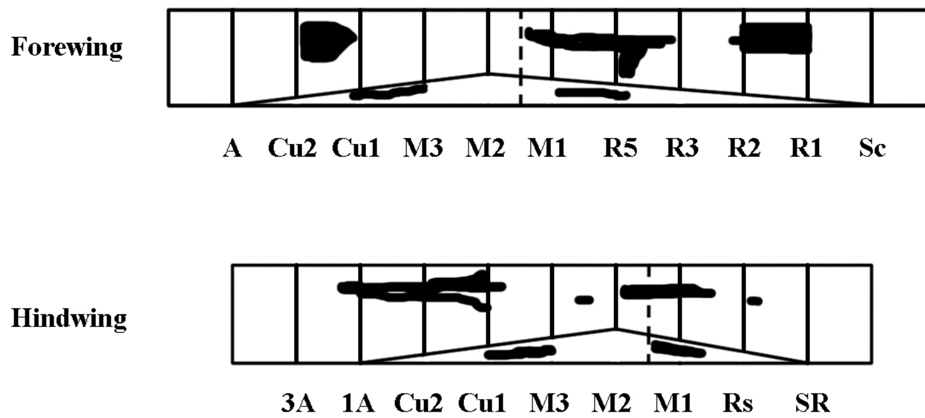

Figure 4-3. Key to the scoring method of Sibatani data (Sibatani 1983a, 1983b). Each cross line shows the butterfly wing vein and the dotted line shows the place of A-P compartment boundary. The triangle on the base of each illustration shows the discal wing sector. As an example to the scoring methodology, in the imaginary diagram above there are edges on Cu2, Cu1, M1/M2, R3 and R1 and crosses on M1, R5 and R2 on the forewing. There are edges on M3 and M1 and crosses on Cu1 and R5 on the discal wing sector of the forewing. Also there are edges on M2 and Rs and crosses on A1, Cu2, Cu1, M1/M2, M1 of the hindwing. Also there are edges on Cu1, M3 and M1/M2 and a cross on M1 of the discal wing.

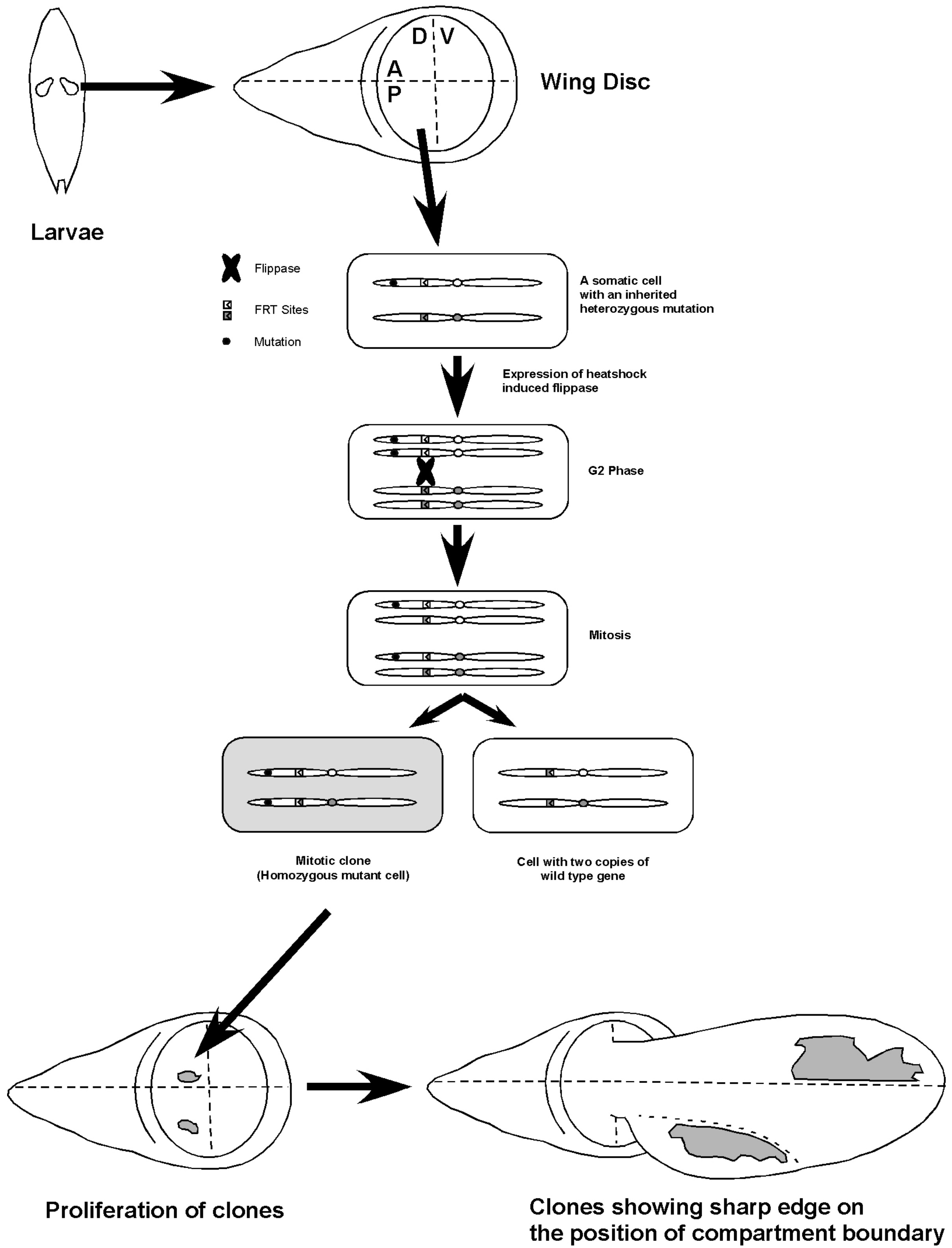

Figure 4-4. Producing mitotic clones using FLP/FRT system.

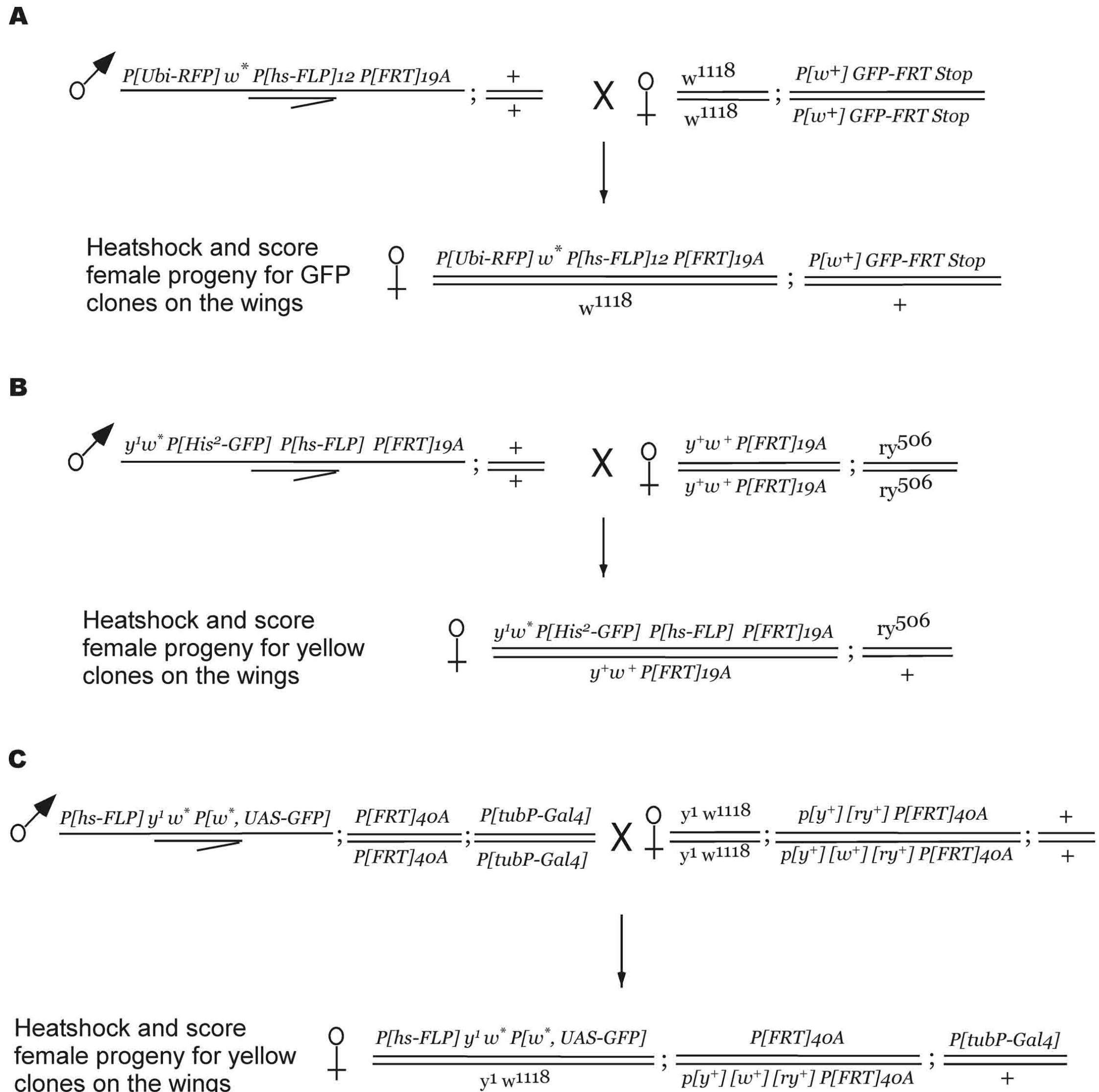

Figure 4-5. Diagram showing the genetic crosses made to examine the second hypothetical compartment boundary on the wings of fruit fly *Drosophilla melanogaster*. A) GFP-FRT cassette clonal analysis, B) Chromosome 1 clonal analysis and C) Chromosome 2 clonal analysis.

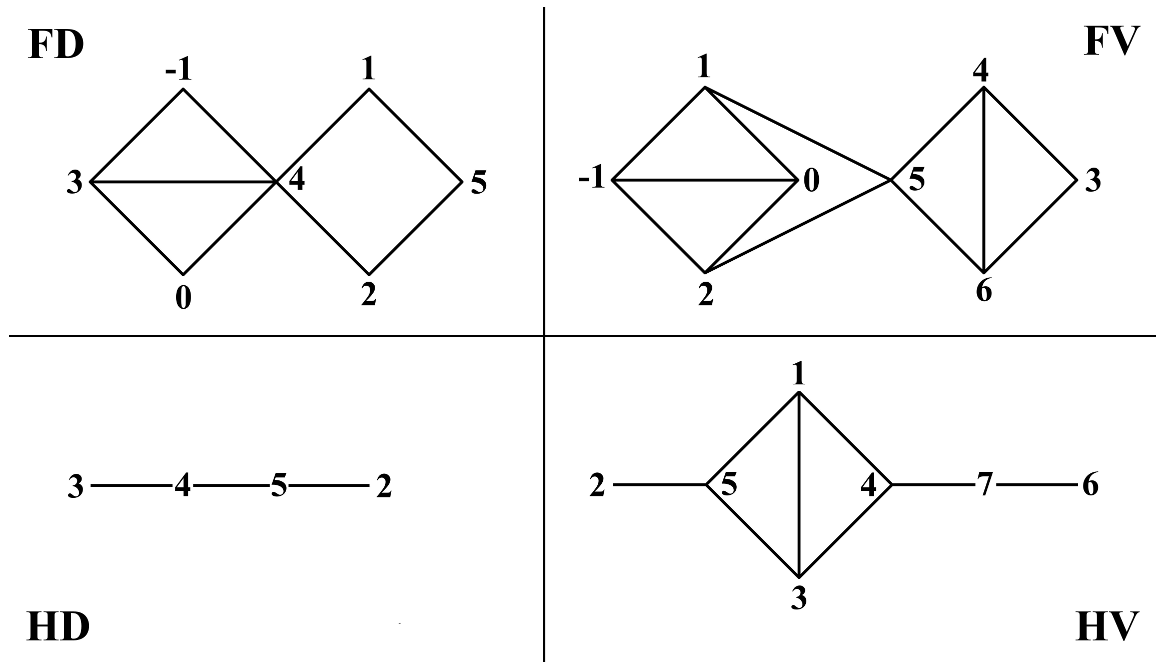

Figure 4-6. Diagrammatic representation of the significant correlations between the number of eyespot elements on each wing surface. Numbers indicate eyespots and their corresponding wing sectors on the wing disk. Lines indicate the significant correlations that we found. FD=forewing dorsal, FV=forewing ventral, HD=hindwing dorsal and HV=hindwing ventral.

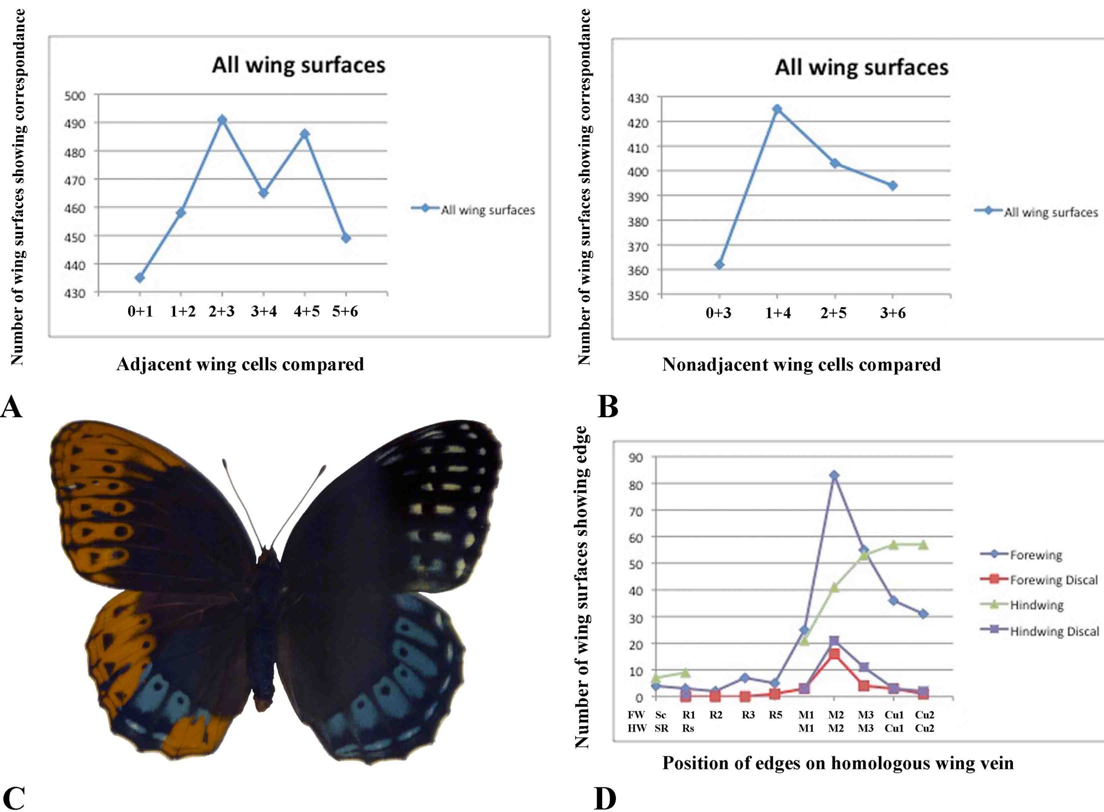

Figure 4-7. Results of the broad survey of overall colour pattern similarities and homeotic clonal data. (A) Overall pattern similarity on adjacent wing sectors. The X axis of the graph represents the wing sector comparisons. The Y axis shows the number of wing surfaces that show the correspondence. Wing sectors 2 and 3 demonstrate a peak showing the highest correspondence which suggest existence of an axis of symmetry near vein M2 that runs between the two wing sectors. (B) Overall pattern similarity on nonadjacent wing sectors. The X axis of the graph represents the wing sector comparisons. The Y axis shows the number of wing surfaces that show the correspondence. Wing sectors 1 and 4 demonstrate a peak showing the highest correspondence which emphasizes on the existence of the axis of symmetry near vein M2. (C) An example of a gynandromorph butterfly *Speyeria diana*. Female butterfly (ZW) has lost W chromosome and become haploid for Z chromosome in parts of its body

and the cells with haploid Z genotype shows the male phenotype (Orange area). The figure shows that the cells with orange colour on the left hindwing did not cross the vein M3 and show an smooth edge. The anterior edge of the blue clone on the left hindwing coincides with the position of the putative Far-Posterior compartment boundary. By marking patch of the cells genetically, gynandromorphs and homeotic mutants provide a good source of data to test the compartment boundary hypothesis. (D) Analysis of lepidopteran clones (Sibatani 1983a, 1983b). The X-axis shows the position of edges on homologous forewing and hindwing veins while the Y-axis shows the number of wing surfaces showing the edge. The graph shows a peak of sharp clonal boundaries within wing sectors 2 and 3. A peak in the number of clonal edges coincides with the presumptive compartment boundary.

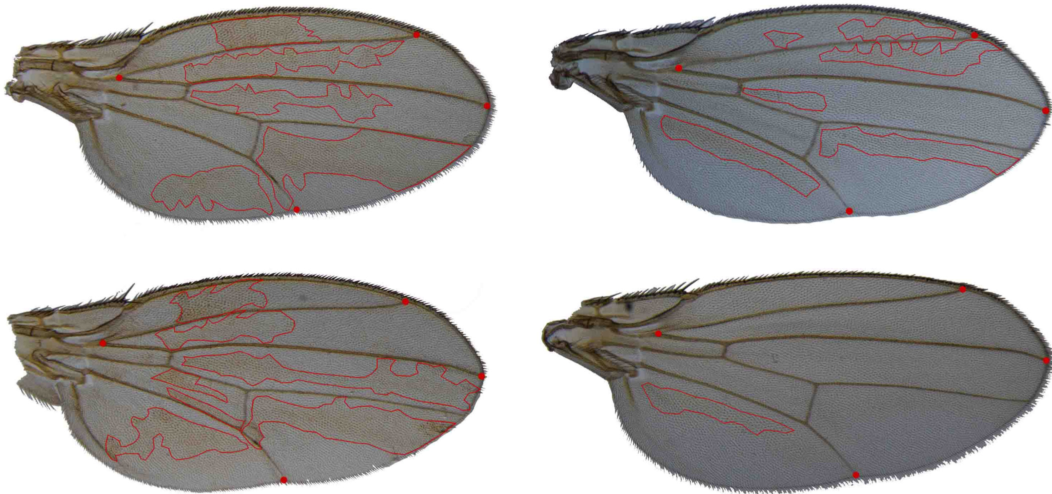

Figure 4-8. *Drosophila* wings showing clones of yellow coloured cells produced by FLP/FRT system and a WT copy of the mutant yellow (y) allele embedded on chromosome 2 (cross C). If there is a compartment boundary on the posterior part of the wing, then the produced yellow clones show a smooth edge; the clones are not expected to cross the boundary. Here we show 4 representative wings showing the position of the Far-Posterior (F-P) compartment boundary, posterior to vein L5. The positions of the A-P and F-P compartment boundaries on fruit fly and butterfly wings are indicated by black dotted lines in Fig 4-1 and 4-9.

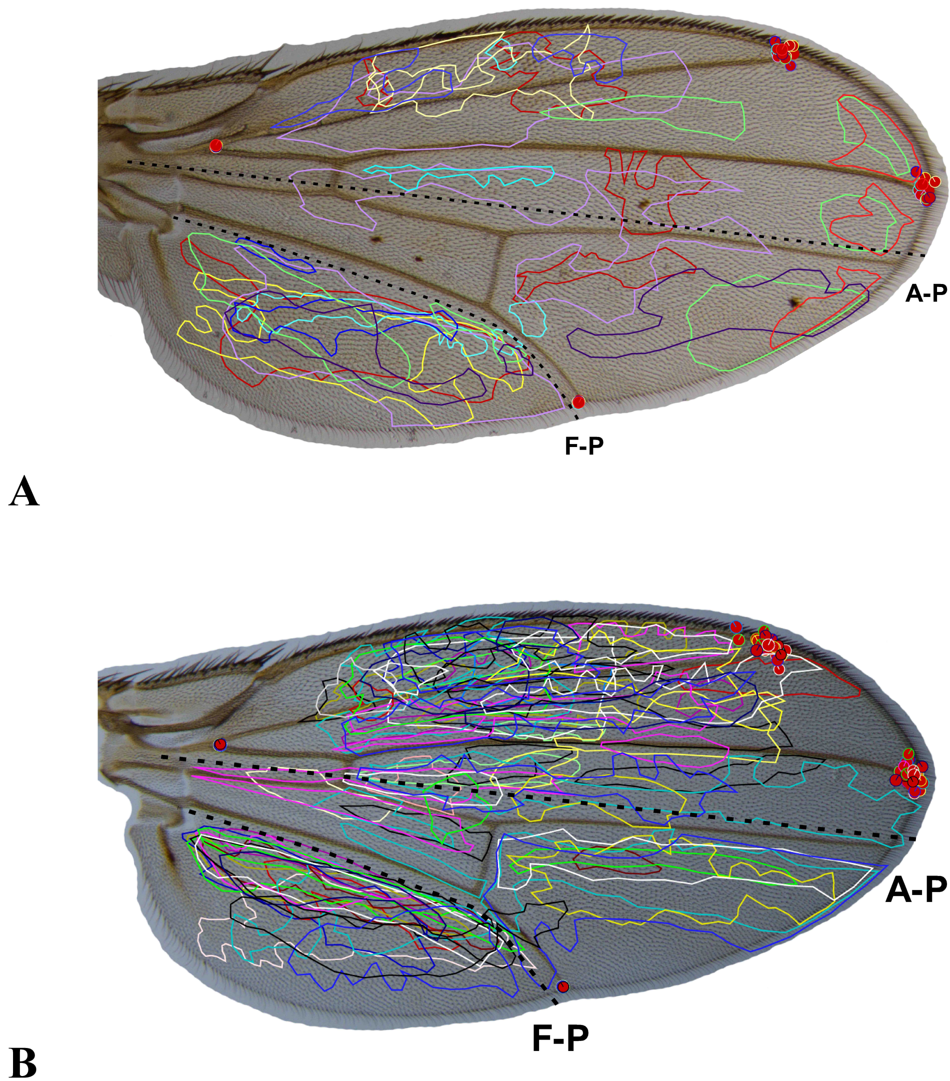

Figure 4-9. Superimposed images. Similar results were detected from different FLP/FRT crosses where a total of 1778 wings were evaluated. (A) Compartment boundary on cross B with FLP/FRT system on chromosome 1 (12 wings). (B) Compartment boundary on cross C with FLP/FRT system on chromosome 2 (30 wings). A-P and F-P compartment boundaries are indicated by black dotted lines. *Drosophila* has a F-P boundary in a position homologous to that of butterflies.

Chapter 5 General discussion

Evolution of non-eyespot (Abbasi and Marcus 2015a) and eyespot (Abbasi and Marcus 2015b) characters were investigated in the butterfly genus *Vanessa*. *Vanessa* is a small genus of butterflies with 22 species and very diverse array of eyespots on their wing surfaces which makes these butterflies ideal model to explore about serial homology (Abbasi and Marcus 2015b). Butterfly eyespots are an example of serially homologous structures that have been evolved recently compared to other serially homologous structures such as vertebrae, teeth and limbs (Oliver et al. 2014). Studying these patterns in butterflies may yield insights that will also help us understand older and more complex serially homologous structures. I have hypothesized the existence of, a Far-Posterior (F-P) compartment boundary and a developmental organizer to explain pervasive interspecific patterns of eyespot similarity on the wing surfaces of butterflies.

Evolution and development of non-eyespot characters in *Vanessa*

I generated a Bayesian phylogeny for 22 *Vanessa* species and 16 outgroups based on a combination of previously published data (Otaki et al. 2006; Wahlberg and Rubinoff 2011) and sequence data that I obtained myself. The phylogeny is well supported and shows a monophyletic status for *Vanessa* butterflies consistent with Wahlberg and Rubinoff (2011). I used this phylogeny to analyze the evolution of colour pattern elements within the genus. I divided the colour pattern elements into non-eyespot and eyespot elements for further analysis. I scored 6 non-eyespot pattern elements of the Nymphalid Groundplan from wing margin to wing hinge including Externa I, II and III, Discalis I and II, and Basal Symmetry System respectively on all wing surfaces. There were a total of 24 characters scored and I then used this data to reconstruct the evolution

of non-eyespot characters in this genus. I tested to see if there is directionality in the changes of characters over time in these characters in the genus *Vanessa*.

The reconstruction showed that 11 of the characters were uniform with regard to the predicted ancestral states, and 13 characters were variable. Of the 13 non-uniform characters, 7 showed ambiguous ancestral states making the directionality test difficult to perceive. Among the remaining 6 characters, only 3 characters including the forewing ventral Basal Symmetry System, hindwing ventral Externa III and hindwing dorsal Discalis I showed directional changes and among these 3, only dorsal Discalis I showed strong directionality towards evolving a united form of the character. Overall my data does not show any strong directionality in the evolution of non-eyespot characters among *Vanessa* species.

I found that homologous non-eyespot characters on the two sides of the forewing correspond better than the two sides of the hindwings in *Vanessa*. I also found that homologous non-eyespot characters on the ventral sides are more similar than the dorsal sides are to each other. The expression patterns of *wingless* (Carroll et al. 1994) and *WntA* (a paralogue of *wingless*) (Martin et al. 2012) as well as *aristaless 2* (Martin and Reed 2010) and *engrailed/invected* (Clark et al. 2008) on developing butterfly wings, suggests the possible association of these genes with the formation of non-eyespot colour patterns. Functional studies through gain of function (misexpression) and loss of function (knock out or knock down) are required to confirm their role on the formation of these characters. Gain and loss of function strategies has been developed and applied in butterflies through germline (Marcus et al. 2004; Monteiro et al. 2013; Beaudette et al. 2014) and somatic or ectopic transformation of butterflies (Lewis et al. 1999; Lewis and

Brunetti 2006; Dhungel et al. 2013). It seems that due to other developmental roles that most of these genes have, ectopic and tissue specific manipulations using techniques that already applied to butterflies such as viral transfection (Lewis et al. 1999; Lewis and Brunetti 2006) and RNA interference (Monteiro et al. 2013) and morpholinos would be the best ways to investigate their function.

Evolution and development of eyespot characters in *Vanessa*

Using the same approach as non-eyespot characters, I studied the evolution of eyespot characters in the genus *Vanessa*. I defined 12 eyespot characters on each wing surface for a total of 44 characters on all four wing surfaces. Forewings have a reduced wing sector number 7 and hindwings have a reduced wing sector number -1, therefore no eyespot occurs in these sectors. Nine out of the 12 characters deal with the number of coloured elements or rings in ocelli on each wing sector from wing sector number negative -1 to wing sector number 7. In the remaining 3 characters I examined the quality, quantity, and overall state of ocelli.

My reconstruction analysis showed the predicted ancestor of *Vanessa* had 5 serially arranged ocelli on all four wing surfaces. On the forewing dorsal and forewing ventral surfaces, the 5 serial ocelli were in wing sectors negative -1 to 3, while on the hindwing dorsal and hindwing ventral the 5 serial ocelli were in wing sectors 1 to 5. I found that the homologous ocelli on the two sides of the forewing are more similar than, the two sides of the hindwings in *Vanessa*. This is consistent with what I observed for non-eyespot characters (Abbasi and Marcus 2015a). I also found that homologous ocelli on the dorsal sides are more similar than the ventral sides which is in contrast to what I

found for non-eyespot characters (Abbasi and Marcus 2015a). Overall these findings suggest that the two surfaces of the two wings as well as non-eyespot and eyespot characters are probably under different selective pressures (natural and sexual selection), therefore they change in different directions. As an example dorsal surface of the butterfly wings are darker than the ventral side due to thermoregulation role. Also ventral sides of the wings play role in camouflage and deflection of the predators, therefore they seem more colourful with eyespots that has more colour pattern elements. However possible effect of developmental constraints in some cases prevents substantial changes on the characters. Developmental constraints act when the genes that produce or regulate one character also have other important roles; therefore it is not possible to lose the character by losing the genes.

Far-Posterior compartment boundary hypothesis for colour pattern determination and development

A Far-Posterior compartment boundary and a developmental organizer have been proposed as a mechanism for differential regulation of colour patterns between wing sectors. Several lines of evidence have been explored to test this hypothesis (Chapter 4). The possible existence of a previously undetected axis of symmetry on all butterfly wing surfaces was first suggested to me by independent contrast analysis of *Vanessa* eyespot patterns (Felsenstein 1985; Garland and Ives 2000). Independent contrast analysis showed that there are significant correlations between eyespots 2 and 5 (2+5) as well as 3 and 4 (3+4). This finding is consistent with previous findings about the co-variation of eyespots 3 and 4 in the ventral hindwing of *Bicyclus anynana* due to mutation and perturbation experiments (Monteiro et al. 2003), as well as correlation of eyespots 2 and

5 on the dorsal hindwing of the genus *Junonia* (Kodandaramaiah 2009). My findings demonstrate that there is an axis of symmetry in the vicinity of vein M3 between wing sectors 3 and 4 in *Vanessa* butterflies.

A broad survey of wing sector colour pattern similarities in each of the butterfly families demonstrated the existence of an axis of symmetry on all wing surfaces on vein M2 between wing sectors 2 and 3. Also, a review of published data for spontaneous butterfly homeotic clones (Sibatani 1983a, 1983b) showed a frequent sharp clonal edge near vein M2, which gives also suggests the existence of an additional compartment boundary posterior to the A-P compartment boundary.

The existence of the new Far-Posterior (F-P) compartment boundary has been demonstrated in the fruit fly *Drosophila melanogaster* experimentally on a place adjacent to the L5 vein, which is equivalent to the M3 vein in butterflies. Compared to findings from spontaneous clones in butterflies, experimentally produced clones in *Drosophila* are informative because they were produced in a genetically uniform background within a single species and because evidence for the F-P boundary in *Drosophila* suggests that the presence of this compartment boundary is not limited to the Lepidoptera.

The broad survey of colour patterns across wing sectors and reanalysis of published homeotic clone maps each suggest an axis of symmetry and a compartment boundary in the vicinity of vein M2. In contrast, independent contrast analysis of the *Vanessa* eyespot character states and clonal analysis in *Drosophila* suggest the existence of an axis of symmetry and a compartment boundary in the vicinity of vein M3. The clonal boundary in *Drosophila* and the reflection line for the F-P axis of symmetry (independent contrasts) do not appear to be precisely coincident. This lack of coincidence

of these two developmental features is also seen in the A-P boundary of *Drosophila* in which the clonal boundary is slightly posterior to the axis of symmetry that establishes the placement of the longitudinal wing veins (Garcia-Bellido 1968; Garcia-Bellido et al. 1973; Sturtevant et al. 1997; Cook et al. 2004). My findings also suggest that the wing sector number 3, which occurs between veins M2 and M3 in butterflies, is of central importantance in terms of anterior-posterior wing patterning.

In terms of the different place that has been suggested for the compartment boundary by butterfly homeotic clonal data (Sibatani 1983a, 1983b) and my *Drosophila* experimental data, I can argue that given how early the engrailed compartment boundary is established in insects (in the embryo), and how consistent the placement of this boundary in insect wings is across many insect orders, it may be that these boundaries do not shift very much. The F-P boundary may not behave in the same way as the A-P boundary, but there is no evidence to show one way or the other. The homeotic clonal analysis and the broad survey of butterfly wing patterns are the weakest part of the analysis in this study. It is because I did not have access to the homeotic butterfly materials (Sibatani 1983a, 1983b) and inconsistency of the examined colour patterns (non-eyespot patterns due to lack of eyespot) in butterfly images, therefore I place emphasis on the independent contrast analysis and the experimentally produced *Drosophila* clones and build my model based on those lines of evidence. The Anterior-Posterior (A-P) compartment boundary in *Drosophila* occurs between the veins L3 (equivalent to R4+5 in butterflies) and L4 (equivalent to M1+2 in butterflies) and right anterior to vein L4 on the A-P axis of the wing. In butterflies, also the A-P compartment boundary is in between the veins Rs and M1. My model proposes overlapping domains

of gene expression in each wing sector that produce a unique combinatorial code. When the combinatorial code for sectors is superimposed on expression domains of the hox genes Antennapedia (Antp) in thoracic segment 2 and Ultrabithorax (Ubx) in thoracic segment 3 as well as the transcription factor apterous (ap) which is expressed only in the dorsal epithelium of the wing discs (Fig. 5-1), it allows butterflies to individually regulate each eyespots and other patterns in specific sectors on the surfaces of each wing (Fig. 5-2 and 5-3).

Colour pattern formation in animals is among the last morophogenic events during development (Nijhout 1980). The determination of the eyespot focus happens early in the development of the wing disc during the larval stage, but the differentiation and pigmentation of eyespots happens during pupation (Nijhout 1980). Nijhout (1980) demonstrated that grafting the focus of an eyespot in *Junonia coenia* and transplanting it to another position that normally lacks an eyespot would produce an eyespot. He argued that the focal cells probably send out signals, known as morphogens, that diffuse radially and the response by surrounding cells to the different signal levels and gradient produce coloured rings of the eyespot (Nijhout 1980). French and Brakefield in their study on *Bicyclus anynana,* showed that in contrast with early damage (1-12 hours after pupation) on the focus that eliminates eyespot, late damage (12-24 hours after pupation) can mimic the focus and produce eyespot (French and Brakefield 1992). They proposed an alternative hypothesis to the morphogen source hypothesis, such that the focus eliminates morphogen, like a sink, to create a morphogen gradient through speculation that the late damage removes morphogens by leakage (French and Brakefield 1992). French and Brakefield showed that there is a certain position in the proximal-distal axis on the wing

surface where eyespots can occur and grafting and transplanting a focus beyond that would not induce formation of an eyespot (French and Brakefield 1995). Gene expression data suggests that there are at least 12 genes (listed in the chapter 1, page 12) that are expressed in the eyespot focus of different butterflies, but 5 of these genes including *engrailed, spalt, Distal-less, Notch* and *Antennapedia* seem to be common across Nymphalidae based on the current studies (Oliver et al. 2012; Shirai et al. 2012; Monteiro 2015), which makes them good candidates for the functional analysis. Both the morphogen source and sink hypothesis suggest that eventually there is a morphogen gradient around the focus that induces a cascade of events in the responding cells. Eventually this gradient produces eyespots, but none of them describe how similar signals produce different responses, and how subsequently different eyespot phenotypes appear in different wing sectors. My model, with its combinatorial layers of gene expression, provides a powerful model to explain the similarities and differences in the response of tissues.

Future directions

My data suggests that wing sector number 3 is very important in terms of wing patterning, as it is the likely location of both a compartment boundary and the source of a secreted Dpp-like morphogen that patterns other gene expression anterior and posterior to the axis of symmetry that it creates. Therefore, I suggest transcriptome analysis of wing sectors in a wing disc be done separately from one another (possibly by tagging with different oligonucleotide) for differential analysis to find candidate genes that might establish the F-P boundary and morphogen gradients. Transcriptome analysis of the wing

disc of Asian corn borer moth, *Ostrinia furnacalis,* shows that 91,907 unigenes with average length of 610 nucleotide are expressed on the entire wing disc (Liu et al. 2014). They listed 46 potential unigenes for wing development most of which belong to the Hedgehog (Hh), Decapentaplegic (Dpp), Wingless (Wg), and Notch (N) signaling pathways (Liu et al. 2014). Obtaining the transcriptome data from fourth/fifth instar larvae of a *Vanessa* butterfly with diverse colour patterns and heterogenous eyespots (i.e. *V. cardui* or *V. virginiensis*) and comparing that to the Asian corn borer moth data (Liu et al. 2014) might identify promising candidate genes and level of their expression. I also suggest to identify the expression pattern using in situ hybridization and to test the function of homologues of *en* (called *invected, in*) and *dpp* (called *glass-bottom boat, gbb*) in butterflies. *gbb* is expressed in the wing disk in the long range similar to *dpp* and required for *Drosophila* wing development in posterior most are of the wing disk (Khalsa et al. 1998; Ray and Wharton 2001). Also it is shown that *gbb* acts in longer range than *dpp* (Bangi and Wharton 2006) and mutants show similar phenotypes like *dpp* mutants in *Drosophila* (Khalsa et al. 1998).

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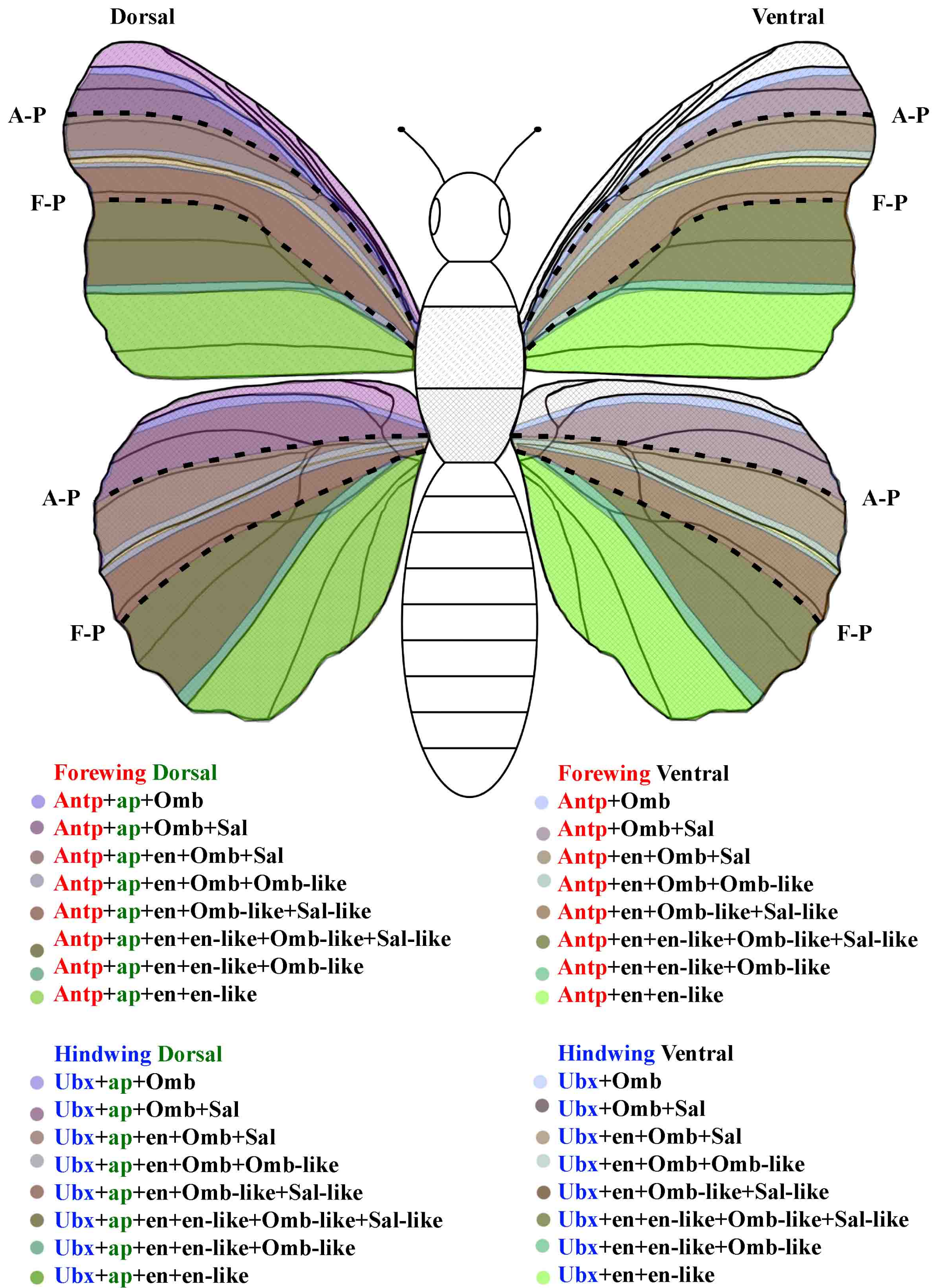

Figure 5-1. Superimposition of proposed gene expression domains on the expression domains of Antp and Ubx as well as ap creates a unique combinatorial code for each wing sector on each wing surface. This allows differential regulation of eyespots and

other patterns on each wing sector. Dorsal surfaces and ventral surfaces are shown in left and right hand side respectively.

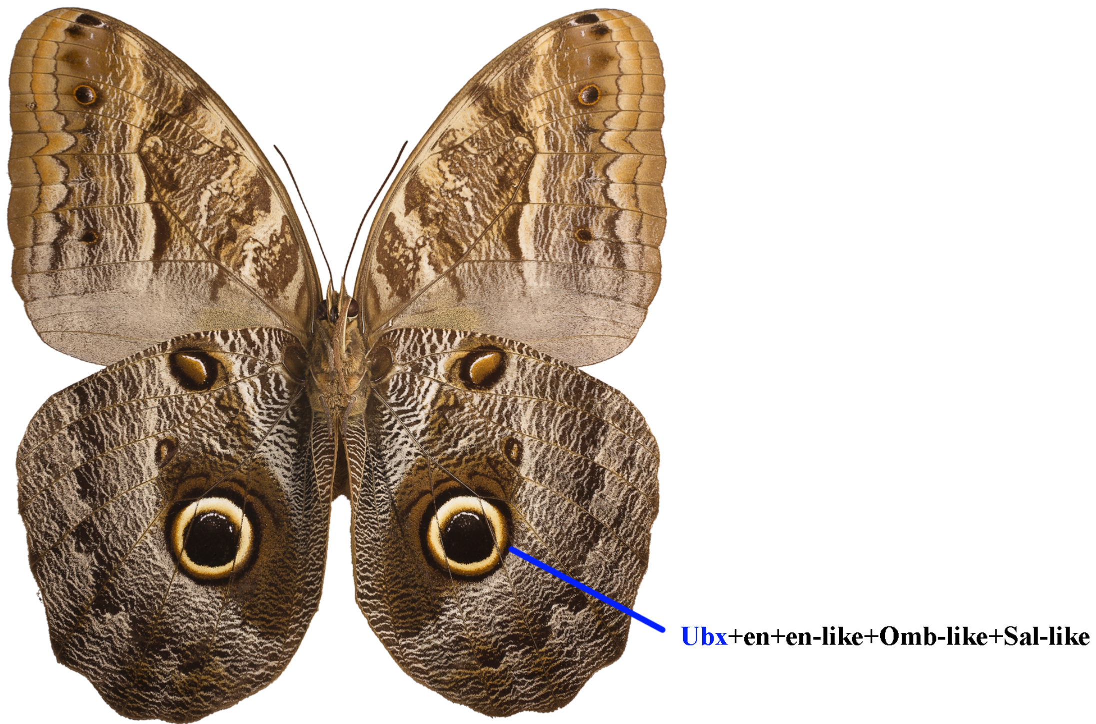

Figure 5-2. Given the proposed combinatorial code model of gene expression, the Teucer Owl butterfly (*Caligo teucer*) is predicted to employ a specific set of transcription factors to regulate position and identity of its prominent eyespot on the sector 5 of ventral hindwing. Image modified from: (Descouens 2011).

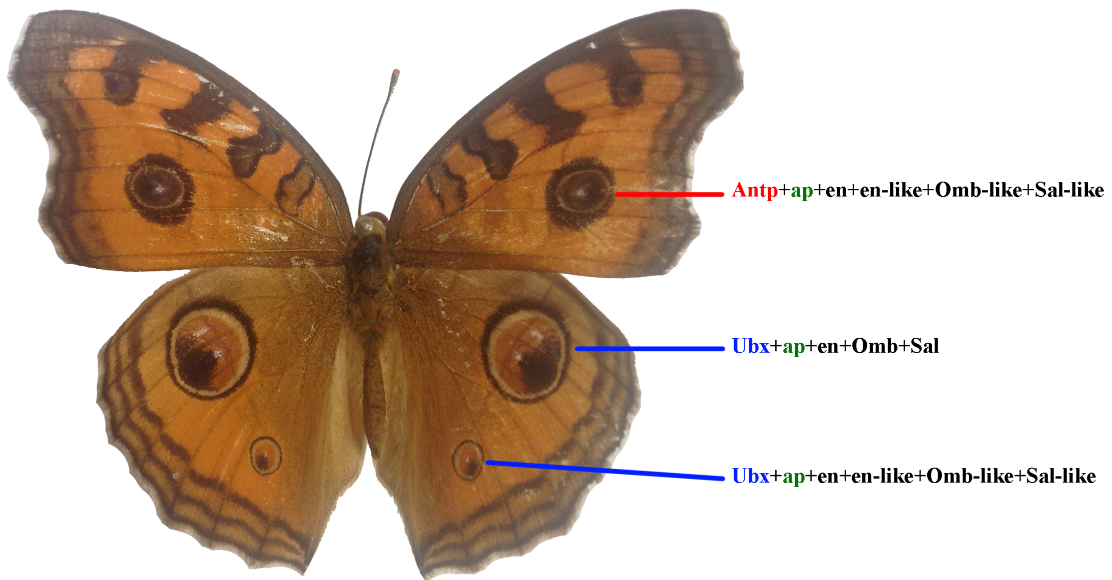

Figure 5-3. Given the proposed combinatorial code model of gene expression, the peacock pansy butterfly (*Junonia almana*) is predicted to employ a specific set of transcription factors in order to regulate position and identity of its prominent eyespot in sector 5 of the dorsal forewing and dorsal hindwing as well as in sectors 1 and 2 of the dorsal hindwing.

Appendix 1

Table S2-1. Genbank Accession numbers for the sequences used for phylogenetic analysis. Sequences in red were generated specifically for this study. NA indicates Not Applicable/missing sequence.

Species	ArgKin	CAD	COI	EF1a	GAPDH	IDH	MDH	RpS5	Wg	ND5
Aglais urticae	GQ864509	GQ864599	AY248786	AY248811	FJ639522	GQ865055	GQ865161	FJ639577	AF412777	**KJ649100**
Antanartia delius	GQ864513	GQ864606	AY788610	AY788712	GQ864922	GQ865062	GQ865167	GQ865387	AY788473	**KJ649047**
Antanartia schaeneia	NA	**KJ648960**	HQ734932.1	HQ734952.1	HQ734979.1	NA	**KJ649033**	HQ735055.1	**KJ649139**	**KJ649104**
Araschnia levana	GQ864516	GQ864610	GQ864735	GQ864829	GQ864925	GQ865064	GQ865171	GQ865391	GQ864423	**KJ649101**
Hypanartia bella	HQ734860.1	HQ734881.1	AY788638	AY788757	HQ734981.1	NA	HQ735023.1	HQ735057.1	AF246590	NA
Hypanartia charon	NA	HQ734883.1	AY788639	AY788758	HQ734983.1	HQ735003.1	HQ735025.1	HQ735059.1	AY788518	NA
Hypanartia dione	HQ734862.1	HQ734884.1	HQ734933.1	HQ734953.1	HQ734984.1	HQ735004.1	HQ735026.1	HQ735060.1	HQ734843.1	**KJ649036**
Hypanartia kefersteini	HQ734863.1	HQ734885.1	AY788640	AY788759	HQ734985.1	HQ735005.1	HQ735027.1	HQ735061.1	AY788519	**KJ649038**
Hypanartia lethe	HQ734859.1	HQ734880.1	AF187774	AY788760	HQ734980.1	HQ735001.1	HQ735022.1	HQ735056.1	AY788520	**KJ649092**
Hypanartia lindigii	HQ734861.1	HQ734882.1	AY248781	AY248806	HQ734982.1	HQ735002.1	HQ735024.1	HQ735058.1	AF412759	NA
Hypanartia paulus	EU141268	EU141320	EU141365	EU136672	EU141499	EU141557	EU141621	EU141398	EU141246	**KJ649056**
Kaniska canace	NA	NA	AY248792	AY248817	FJ639528	NA	NA	FJ639583	AY248833	**KJ649084**
Mynes geoffroyi	EU141273	EU141327	AY248778	AY248803	EU141505	EU141564	EU141628	EU141405	AF412760	NA
Nymphalis polychloros	EU141270	EU141323	AY248788	AY248813	EU141502	EU141560	EU141624	EU141401	AY248829	**KJ649055**
Polygonia c-album	NA	HQ734879.1	AY090222	AY090188	FJ639514	HQ735006.1	HQ735028.1	FJ639569	AY090154	**KJ649040**
Symbrenthia lilea	GQ864582	GQ864713	AY788679	AY788817	GQ865034	GQ865150	GQ865266	GQ865497	AY788577	**KJ649085**
Vanessa abyssinica	HQ734857.1	HQ734877.1	AY788609	AY788711	HQ734977.1	HQ734999.1	HQ735020.1	HQ735053.1	AY788472	**KJ649078**
Vanessa altissima	NA	HQ734866.1	HQ734900.1	HQ734943.1	HQ734963.1	HQ734987.1	HQ735009.1	HQ735038.1	HQ734835.1	**KJ649093**
Vanessa annabella	HQ734850.1	HQ734872.1	AY788685	AY788823	HQ734970.1	HQ734992.1	HQ735014.1	HQ735046.1	AY788583	**KJ649045**
Vanessa atalanta	GQ864589	GQ864722	AY090221	AY090187	GQ865045	GQ865155	GQ865275	GQ865508	AF412772	**KJ649099**
Vanessa braziliensis	HQ734858.1	HQ734878.1	AY788686	AY788824	HQ734978.1	HQ735000.1	HQ735021.1	HQ735054.1	AY788584	**KJ649094**
Vanessa buana	HQ734846.1	NA	HQ734902.1	HQ734944.1	HQ734964.1	NA	HQ735010.1	HQ735039.1	HQ734836.1	**KJ649063**
Vanessa cardui	HQ734851.1	HQ734873.1	HQ734908.1	HQ734947.1	HQ734971.1	HQ734993.1	HQ735015.1	HQ735047.1	HQ734838.1	**KJ649097**
Vanessa carye	HQ734845.1	HQ734865.1	HQ734899.1	HQ734942.1	HQ734962.1	HQ734986.1	HQ735008.1	HQ735037.1	HQ734834.1	**KJ649053**
Vanessa dejeanii	HQ734855.1	HQ734876.1	HQ734913.1	HQ734949.1	HQ734975.1	HQ734997.1	HQ735018.1	HQ735051.1	HQ734840.1	**KJ649071**
Vanessa dilecta	**KJ648948**	**KJ648955**	**KJ649021**	**KJ648969**	**KJ649024**	NA	**KJ649028**	**KJ649117**	**KJ649128**	**KJ649073**
Vanessa dimorphica	HQ734847.1	HQ734868.1	HQ734904.1	HQ734946.1	HQ734965.1	HQ734988.1	NA	HQ735041.1	HQ734837.1	**KJ649082**
Vanessa gonerilla	HQ734848.1	HQ734870.1	AY248784	AY248809	HQ734968.1	HQ734990.1	HQ735012.1	HQ735044.1	AF412782	**KJ649086**
Vanessa hippomene	NA	HQ734867.1	HQ734903.1	HQ734945.1	NA	NA	NA	HQ735040.1	**KJ649124**	**KJ649051**
Vanessa indica	HQ734849.1	HQ734871.1	AY788687	AY788825	HQ734969.1	HQ734991.1	HQ735013.1	HQ735045.1	AY788585	**KJ649113**
Vanessa itea	**KJ648950**	HQ734869.1	AY788688	AY788826	HQ734967.1	HQ734989.1	HQ735011.1	HQ735043.1	AY788586	**KJ649089**
Vanessa kershawi	HQ734854.1	HQ734875.1	AY788689	AY788827	HQ734974.1	HQ734996.1	HQ735017.1	HQ735050.1	AY788587	GQ922169.1
Vanessa myrinna	NA	**KJ648957**	AY788690	AY788828	HQ734966.1	NA	**KJ649030**	HQ735042.1	AY788588	**KJ649096**
Vanessa samani	HQ734856.1	NA	HQ734915.1	HQ734951.1	HQ734976.1	HQ734998.1	HQ735019.1	HQ735052.1	HQ734842.1	**KJ649076**
Vanessa tameamea	**KJ648951**	**KJ648963**	HQ734889.1	HQ734935.1	HQ734955.1	NA	**KJ649035**	HQ735030.1	HQ734827.1	**KJ649110**
Vanessa terpsichore	HQ734844.1	HQ734864.1	HQ734897.1	HQ734941.1	HQ734961.1	NA	HQ735007.1	HQ735036.1	HQ734833.1	NA
Vanessa virginiensis	HQ734853.1	**KJ648964**	AY248783	AY248808	HQ734973.1	HQ734995.1	**KJ649034**	HQ735049.1	AY248827	**KJ649046**
Vanessa vulcania	HQ734852.1	HQ734874.1	HQ734911.1	HQ734948.1	HQ734972.1	HQ734994.1	HQ735016.1	HQ735048.1	HQ734839.1	**KJ649058**

Appendix 2

Table S2-2. Specimens consulted for the study of colour pattern evolution and for DNA extractions. Key to the specimen laboratory code: the first 2 letters are an abbreviation for the country of origin, the second 2 letters the first 2 letters of the genus, the third 2 letters stand for first two letters of species name, M/F stands for male and female, and the final number stands for the sample number in case of multiple specimens per species.

Species	Collection Locality	Collection Date	Collector	Specimen Laboratory Code
Aglais urticae	Kamikawa-Hokkaido, Japan	20-Jul-92		JPAgur
Aglais urticae	D-Gutersloh, Germany	Jun-73		DEAgurM
Aglais urticae	Cevahua, Romania	19-Jul-98		ROAgur1
Aglais urticae	Cevahua, Romania	01-Aug-98		ROAgur2
Aglais urticae	Lasko, Poland	14-Aug-01	Dobiegniew	PLAgur
Aglais urticae braz	Andover hants, England	Jun-92		GBVabr1
Aglais urticae braz	Andover hants, England	Jun-92		GBVabr2
Aglais urticae ichnusa	West Gennargentu, Sardina, Italy	12-Jun-05		ITAgur
Aglais urticae turcica	Sayinesti, 700 m Alt., Romania	03-Jun-05		ROAgurF
Antanartia delius	Central African Republic	No Data		CFAndeM
Antanartia delius	Mbalmayo Forest, Southern Region, Cameroon	24-Sep-07	E. Victorien	CMAndeM
Antanartia delius	Mount Hoyo, Democratic Republic of the Congo	Feb-11		CDAnde2
Antanartia delius	Mount Hoyo, Democratic Republic of the Congo	Feb-11		CDAnde1
Antanartia delius	Democratic Republic of the Congo	No Data		CDAnde3
Antanartia delius	Democratic Republic of the Congo	No Data		CDAnde4
Antanartia delius	No Data	No Data		NDAnde
Antanartia schaenia dubia	Kilindi Forest, Nguu Mountains, Tanzania	06-Jun-07	Keith Stiff	TZAnscF
Antanartia schaenia dubia	Mtunguru Forest, Amani, Tanga Region, Tanzania	19-May-07	Keith Stiff	TZAnscM
Antanartia schaenia dubia	Echuya Forest, Uganda	Jul-12	Alberto Martinez Pola	UGAnscM
Antanartia schaenia dubia	Echuya Forest, Uganda	Jul-12	Alberto Martinez Pola	UGAnscF
Araschnia burejana	Sichuan, China	Apr-04		CNArbu
Araschnia burejana	Shomaru Saitama, Japan	Apr-96		JPArbu
Araschnia levana	Jocelu, Gooj, Romania	Jul-98		ROArle1
Araschnia levana	Puciacte, Romania	26-Jun-03		ROArle2
Araschnia levana	Lichterveide, Belgium	Jun-96	F. Prosa	BEArle1
Araschnia levana	Lichterveide, Belgium	Jun-96	F. Prosa	BEArle2
Araschnia levana	Brosteni, 290 m Alt., Romania	17-Apr-06		ROArleM
Araschnia levana	Pipiris, 540 m Alt., Romania	11-May-06		ROArleF
Hypanaria kefersteini	Junin, Satipo, San Antonio, 1450m, Peru	17-Jul-07		PEHyke
Hypanaria kefersteini	North Yungas, Bolivia	Aug-03		BOHyke1
Hypanaria kefersteini	North Yungas, Bolivia	Aug-03		BOHyke2
Hypanartia bella	Corupá, Santa Catarina, Brazil	31-Dec-84		BRHybe
Hypanartia bella	Eldorado, Misiones, Argentina	15-Apr-99		ARHybe1
Hypanartia bella	Obera', Misiones, Argentina	16-Mar-99		ARHybe2
Hypanartia dione	North Yungas, Bolivia	Aug-03		BOHydi1
Hypanartia dione	North Yungas, Bolivia	Aug-03		BOHydi2
Hypanartia dione	Junin, Satipo, San Antonio, 1450m, Peru	17-Jul-07		PEHydi
Hypanartia godmanii	Chuchuvi, 550mts, Prov. Esmeraldas, Ecuador	Jan-12		ECHygo
Hypanartia lethe	Upper Huallaga Valley, Peru	Nov-02		PEHyle
Hypanartia lethe	Argentina	11-Nov-94		ARHyle
Hypanartia lethe	North Yungas, Bolivia	Aug-03		BOHyle1
Hypanartia lethe	North Yungas, Bolivia	Aug-03		BOHyle2
Hypanartia paulla	Santo Domingo, Dominican Republic	25-Nov-00		DOHypa
Hypanartia paulla	Azua, Dominican Republic	Feb-04		DOHypa2
Kaniska canace canace	Chiang Mai, Thailand	May-96		THKaca
Kaniska canace canace	Xam Neua, Laos	May-04		LAKacaM
Mynes geoffroyi	Bulolo MP, Papua New Guinea	Sep-86		PGMyge
Mynes websteri	Arfak Mountain, Indonesia	Aug-96		IDMyweF
Mynes websteri	Arfak Mountain, Indonesia	Aug-96		IDMyweM
Nymphalis polychloros	Southern China	No Data		CNNypo
Nymphalis xanthomelas japonica	Wakutama -P -Tawn, Nati Katwra, Japan	May-93		JPNyxa

Polygonia c-album	Norland, Ontario, Canada	04-Jul-91		CAPocaF
Polygonia c-album	Norland, Ontario, Canada	10-Jul-91		CAPoca
Polygonia c-album	Swaton Village; Sleaford Lincs, England	2011	JJE Wright	GBPoca1
Polygonia c-album	Swaton Village; Sleaford Lincs, England	2011	JJE Wright	GBPoca2
Polygonia c-album	Far East of Russia, Ussuri Region	22-Jun-03	Igor Udod	RUPoca1
Polygonia c-album	Far East of Russia, Ussuri Region	22-Jun-03	Igor Udod	RUPoca2
Polygonia interrogationis	Miners Bay, Ontario, Canada	Sep-04		CAPoin
Polygonia interrogationis Male	Cape Ceir, Maryland, USA	02-Aug-01		USPoinM
Polygonia satyrus	Sourdough Canyen, Gallatin co., Montana, USA	18-Aug-92		USPosa
Polygonia satyrus	Vic. Of Hudson Bay, Saskatchewan, Canada	30-Jul-03		CAPosaM
Symbrenthia lilea	HaGiong, Vietnam	Apr-03		VNSyli
Symbrenthia lilea lilea	Xam Neua, Laos	Apr-04		LASyliM
Vanessa abyssinica	Kabaru f. Nyeri, Kenya	14-May-06	Tim Wafula	KEVaabF
Vanessa abyssinica	Kabaru f. Nyeri, Kenya	14-May-06	Tim Wafula	KEVaabM
Vanessa altissima	Cuzco, Cosnipata Valley, Paucartambo/Pilcopata Rd., Wayqecha, 3100m, Peru	07-Aug-05	L. Gibson	PEVaal1
Vanessa altissima	Cuzco, Cosnipata Valley, Paucartambo/Pilcopata Rd., Wayqecha, 3100m, Peru	18-Aug-05	L. Gibson	PEVaal2
Vanessa altissima	Pob. Toldopampa, Prov. Satipo, dep. Junin, Peru, 3380 m.	24-Sep-12	A S.L.	PEVaalM
Vanessa annabella	Santa Barbara County, Santa Barbara, California, USA	Oct-01		USVaca1
Vanessa annabella	Santa Barbara County, Santa Barbara, California, USA	Nov-01		USVaca2
Vanessa annabella	Upper Twin Lake, Mono County, California, USA, N3809003/W11922206	28-Sep-10	Robert V. Dowell	CAVan1
Vanessa annabella	El Dorado County, Along 1-80, California, USA N3919397/W12024578	13-Oct-10	Robert V. Dowell	CAVan2
Vanessa annabella	El Dorado County, Along 1-80, California, USA N3919380/W12025138	13-Oct-10	Robert V. Dowell	CAVan3
Vanessa atalanta	Keswich, Ontario, Canada	03-Jul-06		CAVaat1
Vanessa atalanta	Keswich, Ontario, Canada	03-Jul-06		CAVaat2
Vanessa atalanta	Keswich, Ontario, Canada	03-Jul-06		CAVaat3
Vanessa atalanta	Lasko, Poland	15-Aug-01	Dobiegniew	PLVaat1
Vanessa atalanta	Bialowiezaj, Poland	08-Jul-03		PLVaat2
Vanessa atalanta	Poland	08-Jul-03		PLVaat3
Vanessa atalanta	Crevatuis, Romania	19-Jul-96		ROVaat1
Vanessa atalanta	Romania	08-Aug-97	Joolu Gooj	ROVaat2
Vanessa atalanta	Leucreatiutai, Cueuieva, Romania	18-Apr-98		ROVaat3
Vanessa atalanta	Siueris, Pualhava, Romania	16-Oct-98		ROVaat4
Vanessa atalanta	Siluoria, Puahava, Romania	17-Oct-98		ROVaat5
Vanessa atalanta	Taylor county, RM: N.E. of Gilman, Jerry lake Rd. Wisconsin, USA	22-Jun-03		USVaat1
Vanessa atalanta	Taylor county, RM: N.E. of Gilman, Jerry lake Rd. Wisconsin, USA	23-Jun-03		USVaat2
Vanessa atalanta	University of Manitoba campous, Winnipeg, Manitoba, Canada	23-May-12	Jeffrey Marcus	CAVaat4
Vanessa atalanta	University of Manitoba, Fort Gary Campus, Winnipeg, Canada	11-May-12	Jeffrey Marcus	CAVaat5
Vanessa atalanta	University of Manitoba, Fort Gary Campus, Winnipeg, Canada	Jul-12	Jeffrey Marcus	CAVaat6
Vanessa atalanta	ORCA Verco Beach, Florida, USA, N27º 35.339'/W080º21.938'	23-Jan-11	Jeffrey Marcus	FLVat
Vanessa braziliensis	Cuzco, Cosnipata Valley, Paucartambo/Pilcopata Rd., Wayqecha, 3100m, Peru	02-Feb-06	L. Gibson	PEVabr1
Vanessa braziliensis	Cuzco, Cosnipata Valley, Qbda. Buenos Aires 2400-2500m, Peru	13-Aug-05	L. Gibson	PEVabr2
Vanessa braziliensis	Cuzco, Paucartambo/Pilcopata Rd. 1.6 km W of Tres Cruces Rd., 3490m, Peru	15-Jun-04	L. Gibson	PEVabr3
Vanessa braziliensis	Cuzco, Cosnipata Valley, Qbda. Morro Leguia 2135-2300m, Peru	07-Jun-04	L. Gibson	PEVabr4
Vanessa braziliensis	Posadas, Argentina	11-Nov-94		ARVabr1
Vanessa braziliensis	Repiri, Argentina	31-Aug-94		ARVabr2
Vanessa braziliensis	Satipo, Peru	Oct-06		PEVabrM1
Vanessa braziliensis	Satipo, Peru	Oct-06		PEVabrM2
Vanessa braziliensis	Satipo, Peru	Oct-06		PEVabrF
Vanessa brazilliensis	San A.Cobrea 3.774 Mts; Salta Rep., Argentina	No Data	JJE Wright	ARVabr3
Vanessa buana	Mt. Lompobattang, Sulawesi, Indonesia	Dec-00		IDVabuM
Vanessa buana	Mt. Lompobattang, Sulawesi, Indonesia	Apr-03		IDVabuF2
Vanessa buana	Mt. Lompobattang, Sulawesi, Indonesia	Apr-03		IDVabuF3
Vanessa buana	Mt. Lompobattang, Sulawesi, Indonesia	Apr-03		IDVabuF4
Vanessa buana	Mt. Lompobattang, Sulawesi, Indonesia	Apr-03		IDVabuM2
Vanessa buana	Mt. Lompobattang, Sulawesi, Indonesia	Apr-03		IDVabuM3
Vanessa buana	Mt. Lompobattang, Sulawesi, Indonesia	Apr-03		IDVabuM4
Vanessa buana	Mt. Lompobattang, Sulawesi, Indonesia	Feb-94		IDVabuF
Vanessa cardui	Rusalka Ostenik, Poland	10-Jul-02	Bieszczady	PLVaca
Vanessa cardui	Leucrea, Tiutui, Cueuieva, Romania	06-Sep-98		ROVaca1
Vanessa cardui	Pucrieata, Romania	20-Jun-99		ROVaca2
Vanessa cardui	HaGiong, Vietnam	Apr-03		VNVaca1
Vanessa cardui	HaGiong, Vietnam	Apr-03		VNVaca2
Vanessa cardui	HaGiong, Vietnam	Apr-03		VNVaca3
Vanessa cardui	HaGiong, Vietnam	Apr-03		VNVaca4
Vanessa cardui	Lorgues, France	12-Jul-96		FRVaca1
Vanessa cardui	Lorgues, France	12-Jul-96		FRVaca2
Vanessa cardui	University of Manitoba, Fort Gary Campus, Winnipeg, Canada	23-Jul-12	Jeffrey Marcus	CAVaca1

Vanessa cardui	University of Manitoba, Fort Gary Campus, Winnipeg, Canada	03-Aug-12	Jeffrey Marcus	CAVaca2
Vanessa cardui	University of Manitoba, Fort Gary Campus, Winnipeg, Canada	03-Aug-12	Jeffrey Marcus	CAVaca3
Vanessa cardui	Upper Twin Lake, Mono County, California, USA, N3809003/W11922206	28-Sep-10	Robert V. Dowell	CAVca
Vanessa cardui	6 kms NE of Peesane, Saskatchewan, Canada	06-Aug-01		CAVacaF1
Vanessa cardui	6 kms NE of Peesane, Saskatchewan, Canada	06-Aug-01		CAVacaF2
Vanessa cardui	6 kms NE of Peesane, Saskatchewan, Canada	06-Aug-01		CAVacaF3
Vanessa cardui	6 kms NE of Peesane, Saskatchewan, Canada	06-Aug-01		CAVacaM1
Vanessa cardui	6 kms NE of Peesane, Saskatchewan, Canada	06-Aug-01		CAVacaM2
Vanessa cardui	6 kms NE of Peesane, Saskatchewan, Canada	06-Aug-01		CAVacaM3
Vanessa cardui	Mt. Jirisan, South Korea	05-Sep-04		KRVaca
Vanessa carye	Rancagua, VI region, Chile	23-Oct-99		CLVacaM
Vanessa carye	Rio Matanza, Buenos Aires, Argentina	05-Mar-11	Kiss Szilard	ARVaca
Vanessa carye	Rancaqua, VI region, Chile	24-Oct-03	Igor Udod	CLVacaM2
Vanessa dejeanii dejeanii	MT. Ijen, East Java, Indonesia	Aug-93		IDVade
Vanessa dejeanii dejeanii	Mt. Argopuro, East Java, Indonesia	Oct-94		IDVadeM1
Vanessa dejeanii dejeanii	Gunung Gede, West Java, Indonesia	Jul-00		IDVadeM2
Vanessa dejeanii dejeanii	Mt. Argopuro, East Java, Indonesia	Oct-05		IDVade2
Vanessa dejeanii dejeanii	Mt. Argopuro, East Java, Indonesia	Nov-99		IDVadeF
Vanessa dejeanii dejeanii	Mt. Argopuro, East Java, Indonesia	May-94		IDVadeM4
Vanessa dejeanii dejeanii	Java, Indonesia	No Data		IDVadeM5
Vanessa dejeanii samabaluna	Bali, Indonesia	Jun-93		IDVadeM3
Vanessa dejeanii samabaluna	Bali, Indonesia	Nov-99		IDVadeF2
Vanessa dejeanii samabaluna	Bali, Indonesia	Jun-93		IDVadeM6
Vanessa dejeanii samabaluna	Bali, Indonesia	Aug-99		IDVadeM7
Vanessa dilecta	Timor, Indonesia	May-04		IDVadiM1
Vanessa dilecta	Timor, Indonesia	Jul-04		IDVadiM2
Vanessa dilecta	Timor, Indonesia	Feb-04		IDVadiF1
Vanessa dilecta	Timor, Indonesia	May-04		IDVadiF2
Vanessa dimorphica	Mbololo Forest, Teita Hills, Kenya	Dec-91	Wfula Harris	KEVadimF1
Vanessa dimorphica	Mbololo Forest, Teita Hills, Kenya	19-28-Feb-1996	Wfula Harris	KEVadimF2
Vanessa dimorphica	Mbololo Forest, Teita Hills, Kenya	19-28-Feb-1996	Wfula Harris	KEVadimM
Vanessa dimorphica	Nabkoi, Kenya	01-Sep-06	John Morrall	KEVadimF3
Vanessa dimorphica	Kabaru f. Nyeri, Kenya	14-May-06	Tim Wafula	KEVadimM2
Vanessa dimorphica	Nabkoi, Kenya	2006/02/08	John Morrall	KEVadimM3
Vanessa dimorphica dimorphica	Gatamayu F. Kenya	31-Jan-12	Kennedy Walmalwa	KEVadimM4
Vanessa dimorphica dimorphica	Gatamayu F. Kenya	31-Jan-12	Kennedy Walmalwa	KEVadimF4
Vanessa gonerilla	Hutt Valley, North of Wellington, North Island, New Zealand	Jan-04	R. Moore	NZVagoM1
Vanessa gonerilla	Hutt Valley, North of Wellington, North Island, New Zealand	Jan-04	R. Moore	NZVagoM2
Vanessa gonerilla	Hutt Valley, North of Wellington, North Island, New Zealand	Jan-04	R. Moore	NZVagoM3
Vanessa gonerilla	Hutt Valley, North of Wellington, North Island, New Zealand	Jan-04	R. Moore	NZVagoM4
Vanessa gonerilla	Wellington, New Zealand	23-Apr-07		NZVago1
Vanessa gonerilla	Wellington, New Zealand	23-Apr-07		NZVago2
Vanessa hippomene	Mount Hoyo, Democratic Republic of the Congo	Aug-11		CDVahi1
Vanessa hippomene	Mount Hoyo, Democratic Republic of the Congo	Aug-11		CDVahi2
Vanessa hippomene	Mount Hoyo, Democratic Republic of the Congo	Aug-11		CDVahi3
Vanessa hippomene	Mount Hoyo, Democratic Republic of the Congo	Aug-11		CDVahi4
Vanessa hippomene	Nguu forest, Tanga, Tanzania	Nov-07		TZVahi
Vanessa hippomene	Echuya Forest, Uganda	Jul-12	Alberto Martinez Pola	UGVahiM
Vanessa hippomene	Echuya Forest, Uganda	Jul-12	Alberto Martinez Pola	UGVahiF
Vanessa indica	HaGiong, Vietnam	Apr-03		VNVain1
Vanessa indica	HaGiong, Vietnam	Apr-03		VNVain2
Vanessa indica	HaGiong, Vietnam	Apr-03		VNVain3
Vanessa indica	HaGiong, Vietnam	Apr-03		VNVain4
Vanessa indica	Sicwa, China	May-98		CNVain1
Vanessa indica	Sicwa, China	May-98		CNVain2
Vanessa itea	Wellington, New Zealand	23-Apr-07		NZVait1
Vanessa itea	Wellington, New Zealand	23-Apr-07		NZVait2
Vanessa itea	Katoomba, Blue Mounts, Sydney, Australia	14-Dec-79	Goergens	AUVait
Vanessa kershawi	Katoomba, Blue Mounts, Sydney, Australia	12-Dec-79	Goergens	AUVake
Vanessa myrinna	Upper Huallaga Valley, Peru	Nov-00		PEVamy1
Vanessa myrinna	Upper Huallaga Valley, Peru	Nov-00		PEVamy2
Vanessa myrinna	Upper Huallaga Valley, Peru	Nov-00		PEVamy3
Vanessa myrinna	Upper Huallaga Valley, Peru	Nov-00		PEVamy4
Vanessa samanni	Aceh, Sumatra, Indonesia	Jun-05		IDVasaM1
Vanessa samanni	Aceh, Sumatra, Indonesia	Jun-05		IDVasaM2
Vanessa tameamea	Hanolo montain, 700m, Hilo, Hawaii, USA	28-Feb-10		USVataM
Vanessa virginiensis	Depauw IN; Harrison Co. B.R.T., USA	Sep-11	Jay Timberlake	USVavi1
Vanessa virginiensis	Bowling Green Kentucky, USA	24-May-07	Mollie R. Johnson	USVavi2
Vanessa virginiensis	Bowling Green Kentucky, USA	14-Jun-07	Mollie R. Johnson	USVavi3
Vanessa virginiensis	ORCA, Vero Beach, Florida, USA, N27º 35.339'/W080º21.938'	11-Jul-05	Jeffrey Marcus	USVavi4
Vanessa virginiensis	University of Manitoba, Fort Gary Campus, Winnipeg, Canada	26-Jul-12	Jeffrey Marcus	CAVavi1
Vanessa virginiensis	University of Manitoba, Fort Gary Campus, Winnipeg, Canada	30-Jul-12	Jeffrey Marcus	CAVavi2
Vanessa virginiensis	University of Manitoba, Fort Gary Campus, Winnipeg, Canada	12/Feb/08	Jeffrey Marcus	CAVavi3

Vanessa virginiensis	University of Manitoba, Fort Gary Campus, Winnipeg, Canada	8/Aug/12	Jeffrey Marcus	CAVavi4
Vanessa virginiensis	University of Manitoba, Fort Gary Campus, Winnipeg, Canada	13/Aug/12	Jeffrey Marcus	CAVavi5
Vanessa virginiensis	Franklin, Tennessee, USA	14/Jun/13		USVavi5
Vanessa virginiensis	El Dorado County, Along 1-80, California, USA N3919380/W12025138	13-Oct-10	Robert V. Dowell	CAVvi
Vanessa vulcania	Tenerife, Canary Islands Los Realejos, Calle del Medio de Arriba, Spain. 505m. 28º22'6.41"N/16º35'11.57"W	12-Sep-12	Jan Petersen	ESVavuF1
Vanessa vulcania	Tenerife, Canary Islands Los Realejos, Calle del Medio de Arriba, Spain. 505m. 28º22'6.41"N/16º35'11.57"W	12-Sep-12	Jan Petersen	ESVavuM1
Vanessa vulcania	Tenerife, Canary Islands Los Realejos, Calle del Medio de Arriba, Spain. 505m. 28º22'6.41"N/16º35'11.57"W	12-Sep-12	Jan Petersen	ESVavuF2
Vanessa vulcania	Tenerife, Canary Islands Los Realejos, Calle del Medio de Arriba, Spain. 505m. 28º22'6.41"N/16º35'11.57"W	12-Sep-12	Jan Petersen	ESVavuM2
Vanessa vulcanica	Puerto de la Cruz, Canary Islands, Spain	16-Dec-97		ESVavu1
Vanessa vulcanica	Puerto de la Cruz, Canary Islands, Spain	16-Dec-97		ESVavu2

Appendix 3

Table S2-3. Scored values for the non-eyespot colour pattern characters found on the four wing surfaces of *Vanessa* butterflies and outgroups.

	Forewing Dorsal						Forewing Ventral						Hindwing Dorsal						Hindwing Ventral					
Species	**FDB**	**FDDII**	**FDDI**	**FDEIII**	**FDEII**	**FDEI**	**FVB**	**FVDII**	**FVDI**	**FVEIII**	**FVEII**	**FVEI**	**HDB**	**HDDII**	**HDDI**	**HDEIII**	**HDEII**	**HDEI**	**HVB**	**HVDII**	**HVDI**	**HVEIII**	**HVEII**	**HVEI**
Ingroups																								
Vanessa abyssinica	2	2	2	2	2	1	2	2	2	2	2	1	3	0	0	2	2	1	1	2	2	2	2	1
Vanessa altissima	1	1	2	2	2	1	1	1	2	2	1	1	2	2	2	2	2	1	1	2	2	2	2	1
Vanessa annabella	1	2	2	2	2	1	1	2	2	2	2	1	2	0	2	1	1	1	2	2	2	2	2	1
Vanessa atalanta	2	1	2	2	2	1	1	1	2	2	2	1	3	0	0	0	1	1	1	2	2	2	1	1
Vanessa braziliensis	1	1	2	2	2	1	1	1	2	2	2	1	2	2	2	2	1	1	1	2	2	2	2	1
Vanessa buana	2	2	2	2	2	1	1	1	2	2	2	1	3	0	0	2	2	1	2	2	2	2	1	1
Vanessa cardui	1	1	2	2	2	1	1	1	2	2	2	1	2	0	2	1	1	1	1	2	2	2	2	1
Vanessa carye	1	2	2	2	2	1	1	1	2	2	2	1	2	0	2	1	1	1	2	2	2	2	2	1
Vanessa dejeanii	2	2	2	2	2	1	1	1	2	2	2	1	3	0	0	1	2	1	2	2	2	2	2	1
Vanessa dilecta	2	2	2	2	2	1	1	1	2	2	2	1	3	2	2	1	2	1	2	2	2	2	2	1
Vanessa dimorphica	2	2	2	2	2	1	2	2	2	2	2	1	3	0	0	2	2	1	1	2	2	2	2	1
Vanessa gonerilla	2	2	2	2	2	1	2	2	2	2	2	1	3	0	0	2	2	1	2	2	2	2	2	1
Vanessa hippomene	2	2	2	2	2	1	2	2	2	2	2	1	3	0	0	2	2	1	1	2	2	1	2	1
Vanessa indica	1	1	2	2	2	1	1	1	2	2	2	1	3	0	0	1	2	1	2	2	2	2	1	1
Vanessa itea	2	2	2	2	2	1	2	2	2	2	2	1	2	0	0	2	2	1	2	2	2	2	2	1
Vanessa kershawi	1	1	2	2	2	1	1	1	2	2	2	1	2	2	2	1	2	1	1	2	2	2	2	1
Vanessa myrinna	1	1	2	2	2	1	1	1	2	2	2	1	2	2	2	2	2	1	1	2	2	2	2	1
Vanessa samani	1	1	2	2	2	1	1	1	2	2	2	1	2	0	0	1	1	1	2	2	2	2	2	1
Vanessa tameamea	1	1	2	2	2	1	1	1	2	2	2	1	3	0	0	1	2	1	2	2	2	1	2	1
Vanessa terpsichore	1	1	2	2	2	1	1	1	2	2	2	1	2	0	2	2	1	1	1	2	2	2	1	1
Vanessa virginiensis	1	2	2	2	2	1	1	1	2	2	2	1	2	0	2	2	2	1	1	2	2	2	2	1
Vanessa vulcania	1	1	2	2	2	1	1	1	2	2	2	1	3	0	0	1	2	1	2	2	2	2	1	1
Outgroups																								
Aglais urticae	1	2	2	2	2	2	1	2	2	2	2	2	2	0	0	2	2	1	1	2	2	2	2	2
Antanartia delius	1	2	2	2	2	1	1	2	2	2	2	1	2	2	2	2	2	1	1	2	2	2	2	2
Antanartia schaeneia	2	2	2	2	2	1	2	2	2	2	2	1	3	0	0	2	2	1	1	2	2	1	1	1
Araschnia levana	2	2	2	1	2	1	2	2	2	2	2	1	2	2	2	2	2	1	1	2	2	2	1	1
Hypanartia bella	1	2	2	2	2	1	1	2	2	2	2	1	2	2	2	2	2	1	1	2	2	2	2	1
Hypanartia charon	2	2	2	2	2	1	1	2	2	1	2	1	3	0	0	2	2	1	2	2	2	1	2	2
Hypanartia dione	2	2	2	2	2	1	1	2	2	1	1	1	0	0	1	2	2	1	1	1	1	2	2	1
Hypanartia kefersteini	1	1	2	2	2	1	1	2	2	2	2	1	2	0	2	2	2	2	1	2	2	2	2	2
Hypanartia lethe	2	2	2	2	2	1	1	2	2	2	1	1	0	0	0	2	2	1	1	2	2	2	2	2
Hypanartia lindigii	1	2	2	2	2	1	1	2	2	2	2	1	0	0	2	1	2	2	1	2	2	2	2	2
Hypanartia paulus	2	2	2	2	2	1	1	2	2	2	2	2	0	0	2	1	2	2	2	2	2	1	2	2
Kaniska canace	2	2	2	2	2	1	1	2	2	2	2	1	3	0	0	2	2	2	1	2	2	2	2	1
Mynes geoffroyi	0	0	0	2	2	2	1	0	0	2	2	2	0	0	0	2	2	1	2	0	0	1	2	2
Nymphalis polychloros	0	2	2	2	2	2	1	2	2	2	2	2	2	0	0	2	2	2	2	2	2	2	2	2
Polygonia c-album	2	2	2	2	2	1	1	2	2	2	2	1	2	0	0	1	0	1	1	2	2	1	1	1
Symbrenthia lilea	2	2	2	2	2	1	1	2	1	1	2	2	2	0	0	2	2	1	0	2	2	2	2	2

Appendix 4

Table S3-1. Number of colour components found in the ocellus of each wing cell on the four wing surfaces of *Vanessa* butterflies and outgroups.

	Forewing Dorsal								Forewing Ventral								Hindwing Dorsal								Hindwing Ventral							
Wing Cell	**-1**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**-1**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Species	**FDNEOc-1**	**FDNEOc0**	**FDNEOc1**	**FDNEOc2**	**FDNEOc3**	**FDNEOc4**	**FDNEOc5**	**FDNEOc6**	**FVNEOc-1**	**FVNEOc0**	**FVNEOc1**	**FVNEOc2**	**FVNEOc3**	**FVNEOc4**	**FVNEOc5**	**FVNEOc6**	**HDNEOc0**	**HDNEOc1**	**HDNEOc2**	**HDNEOc3**	**HDNEOc4**	**HDNEOc5**	**HDNEOc6**	**HDNEOc7**	**HVNEOc0**	**HVNEOc1**	**HVNEOc2**	**HVNEOc3**	**HVNEOc4**	**HVNEOc5**	**HVNEOc6**	**HVNEOc7**
Ingroups																																
Vanessa abyssinica	1	1	1	1	1	0	0	0	1	1	3	3	2	0	0	0	0	1	1	2	2	2	0	0	0	3	4	2	2	4	0	0
Vanessa altissima	1	1	2	2	1	0	1	0	3	3	5	5	2	0	1	0	0	0	2	1	1	2	0	0	0	1	5	1	0	5	0	0
Vanessa annabella	1	1	1	1	1	0	0	0	1	1	3	3	1	0	0	0	0	1	1	2	2	2	0	0	0	3	4	4	3	4	0	0
Vanessa atalanta	1	1	1	1	1	1	0	0	3	2	5	5	1	1	1	0	0	1	1	1	1	1	0	0	0	3	4	3	3	4	2	0
Vanessa braziliensis	1	1	3	3	1	1	1	0	3	3	5	5	2	1	1	0	0	0	2	1	1	2	0	0	0	1	5	1	0	5	0	0
Vanessa buana	1	1	1	1	1	0	0	0	2	2	4	4	1	0	1	0	0	0	1	1	1	1	0	0	0	4	3	3	3	3	0	0
Vanessa cardui	1	1	1	1	1	0	0	0	2	2	3	3	2	0	0	0	0	1	1	2	2	3	0	0	0	3	4	3	3	5	0	0
Vanessa carye	1	1	1	1	1	0	0	0	3	2	4	4	2	0	0	0	0	1	3	3	4	4	0	0	0	3	4	4	3	5	0	0
Vanessa dejeanii	1	1	1	1	1	0	0	0	2	2	4	4	2	2	1	0	0	1	1	1	1	1	0	0	0	3	4	3	3	4	0	0
Vanessa dilecta	1	1	1	1	1	0	0	0	2	2	4	4	1	0	1	0	0	1	1	1	1	1	0	0	0	3	3	3	3	4	0	0
Vanessa dimorphica	1	1	1	1	1	1	0	0	1	1	4	4	1	1	0	0	0	0	0	0	3	4	0	0	0	3	3	2	2	5	0	0
Vanessa gonerilla	2	2	1	1	2	1	0	0	3	3	4	4	2	0	0	0	0	0	2	2	2	2	0	0	0	3	4	4	5	5	1	1
Vanessa hippomene	1	1	1	1	1	0	0	0	1	1	2	2	1	1	0	0	0	0	0	0	3	4	0	0	0	4	4	2	2	5	0	0
Vanessa indica	1	1	1	1	1	0	0	0	2	2	4	4	1	0	0	0	0	1	1	1	1	1	0	0	0	3	4	3	3	4	0	0
Vanessa itea	1	1	1	1	1	0	0	0	2	2	4	4	3	0	0	0	0	1	2	2	2	2	0	0	0	3	5	3	3	5	0	0
Vanessa kershawi	1	1	1	1	1	0	0	0	2	2	2	2	2	0	0	0	0	1	1	2	2	2	0	0	0	2	5	3	3	5	0	0
Vanessa myrinna	2	2	3	3	1	1	1	0	3	3	5	5	2	1	1	0	0	1	3	1	1	3	0	0	0	0	4	0	0	4	0	0
Vanessa samani	1	1	1	1	1	0	0	0	1	1	2	2	1	0	0	0	0	1	1	1	1	1	0	0	0	1	3	2	2	3	0	0
Vanessa tameamea	1	1	0	0	1	0	0	0	2	2	3	3	1	0	0	0	0	0	1	1	1	1	0	0	0	3	3	2	3	3	0	0
Vanessa terpsichore	1	1	1	1	1	0	1	0	1	1	5	5	1	0	3	0	0	0	2	0	0	2	0	0	0	2	5	1	1	5	0	0
Vanessa virginiensis	1	1	2	2	1	0	1	0	4	4	5	5	2	1	2	0	0	1	2	1	1	2	0	0	0	1	5	2	0	5	0	0
Vanessa vulcania	1	1	1	1	1	0	0	0	2	2	4	4	2	0	0	0	0	0	1	1	1	1	0	0	0	3	4	3	3	4	0	0
Outgroups																																
Aglais urticae	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Antanartia delius	1	1	1	1	2	1	1	1	1	1	5	5	5	5	5	4	0	1	1	1	1	1	1	0	1	2	3	3	3	4	3	0
Antanartia schaeneia	1	1	1	1	1	1	0	0	3	2	5	5	1	1	1	0	0	1	2	1	1	2	0	0	0	2	2	2	4	5	3	0
Araschnia levana	1	1	1	1	1	1	0	0	2	2	4	4	2	2	1	0	0	1	1	2	2	3	0	0	0	1	1	2	2	3	2	0
Hypanartia bella	1	1	0	0	1	0	1	0	2	2	4	4	2	0	0	0	0	0	0	0	3	3	2	0	0	4	3	4	4	5	0	0
Hypanartia charon	1	1	2	2	1	0	1	0	2	2	2	2	2	0	0	0	0	0	1	1	1	1	0	0	0	1	1	1	1	1	0	0
Hypanartia dione	2	2	3	3	1	1	1	0	1	1	2	2	2	2	1	0	0	1	1	2	2	2	0	0	0	4	4	4	4	4	3	3
Hypanartia kefersteini	1	1	1	1	1	1	0	0	4	4	5	5	2	1	2	0	0	1	1	2	2	2	0	0	0	4	4	3	4	4	2	0
Hypanartia lethe	1	1	1	1	0	0	0	0	2	2	3	3	2	0	0	0	0	0	2	0	0	2	0	0	0	3	3	2	4	3	0	0
Hypanartia lindigii	1	1	1	1	1	1	1	0	3	3	5	5	2	1	1	0	0	1	3	1	1	3	0	0	0	3	3	3	4	4	3	0
Hypanartia paulus	1	1	0	0	2	0	2	0	2	2	4	4	1	0	0	0	0	0	0	0	4	4	2	0	0	2	3	4	4	3	0	0
Kaniska canace	1	1	1	1	2	2	2	2	2	2	2	2	2	2	3	3	2	2	2	2	2	2	2	0	2	2	2	2	2	2	2	0
Mynes geoffroyi	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Nymphalis polychloros	1	1	0	0	1	0	0	0	1	1	1	0	1	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Polygonia c-album	1	1	1	1	1	0	1	0	1	1	2	2	1	1	0	0	0	1	1	1	1	1	0	0	0	1	1	1	1	1	0	0
Symbrenthia lilea	1	1	2	2	2	1	2	1	1	1	3	3	3	3	4	3	1	1	1	1	1	1	0	0	3	3	3	2	2	2	2	0