Chapter 2

GENETIC STUDIES

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2.1 INTRODUCTION

Coccinellids have been used as genetic models since the beginning of the 20th century. Interest arose initially because of the genetically polymorphic colour patterns possessed by a number of species, and historically the majority of genetic work has been on coccinellid colour pattern polymorphism. However, studies of coccinellid genetics have by no means been limited to colour pattern. A considerable number of studies exist on coccinellid cytology and on the genetics of other traits. More recent molecular genetic work has examined not only the evolution of specific gene regions, but has been used to make evolutionary and ecological inferences about diverse aspects of coccinellid biology including sexual and population biology and phylogenetic relationships. In this chapter we begin by discussing whole genome and cytogenetic aspects of coccinellid biology (Sections 2.2 and 2.3), before moving on to discuss the vast body of work on coccinellid colour pattern (2.4), and genetics studies of non-colour pattern traits (2.5). Finally, we consider the rapidly burgeoning number of molecular studies of coccinellids and what they tell us, not only about the coccinellid genome, but also more generally about coccinellid biology (2.6).

2.2 GENOME SIZE

Genome size, measured as haploid nuclear DNA content or **C-value**, has been studied in 31 species of coccinellid, primarily within the Coccinellinae, but overall representing six subfamilies and eight tribes (Gregory et al. 2003): this makes the Coccinellidae the third best studied beetle family after the Tenebrionidae and Chrysomelidae. The C-values of coccinellids vary between 0.19 pg and 1.71 pg, with a mean of 0.53 pg. By comparison with the two other beetle families, the Coccinellidae appear to be **more variable in genome size**, although their mean C-value is between that of tenebrionids and chrysomelids. There appears to be **very little intraspecific variation** in genome size. Most C-value variation occurs between coccinellid subfamilies, although even congeneric species can exhibit up to two-fold variation. Genome size is not correlated with body size, nor does it appear to be related to chromosome number, but larger genomes do appear to be associated with longer development. In comparison with fast-developing aphidophagous species, two slower developing coccidophagous species have some of the largest coccinellid genomes. Due to the small number of species from many coccinellid subfamilies and ecotypes included, most of the conclusions of the C-value study remain tentative and require further verification (Gregory et al. 2003).

2.3 CHROMOSOMES AND CYTOLOGY

2.3.1 Chromosome numbers and banding

The **chromosomal complements** of something under 200 coccinellid species are now known (Smith & Virkki 1978, Lyapunova et al. 1984, Rozek & Holecova 2002). The reported **diploid number of chromosomes varies between 12 and 28** (Smith 1960, 1962a, Yadav & Gahlawat 1994). The distribution of species' chromosome numbers varies across subfamilies and tribes (Fig. 2.1); the most common number, comprising a diploid number of 18 autosomes and a Xy_p sex chromosome pair, is that which is often considered ancestral for the Coleoptera (Smith & Virkki 1978, Lyapunova et al. 1984).

Studies on chromosome numbers have been supplemented by an increasing amount of work on **chromosome structure and banding**. Differential staining of chromosomal regions gives banding patterns that allow different chromosomes of equal size to be uniquely identified, as well as potentially identifying changes in chromosome structure such as inversions. Most work has focused on chromosome **C-banding** patterns, which arise from intense staining of **heterochromatic** (genetically inactive) chromosome regions. In ladybirds, as in other beetles, these are mainly associated with regions near the centromere of a chromosome and in the short arms of chromosomes (Ennis 1974, Drets et al. 1983, Maffei et al. 2000, 2004, Rozek & Holecova 2002, Beauchamp & Angus 2006). A number of other banding techniques have also been investigated (Ennis 1974, 1975, Maffei & Pompolo 2007). Homology of chromosome banding patterns has been used to infer a monophyletic origin for the coccinellids *Chilocorus orbus*, *Chil. tricyclus* and *Chil. hexacyclus* (Ennis 1974, 1975, 1976).

2.3.2 Sex determination

The commonest form of sex determination system in coccinellids is the so-called **Xy parachute (Xy_p)**

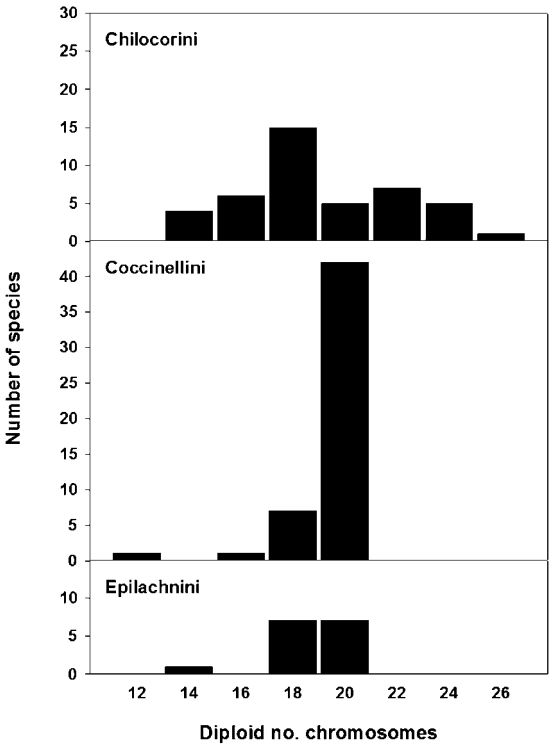

Figure 2.1 Diploid chromosome numbers of coccinellid species in the tribes Chilocorini, Coccinellini and Epilachnini. Female diploid chromosome numbers are used. From data in Smith & Virkki (1978), amended to take into account current views on the taxa included in these tribes.

system: females are XX, while males are Xy. The y chromosome is very small (which is why it is typically written as a lower case rather than an upper case Y) and it is paired with the X chromosome at metaphase I in a 'parachute' configuration, hence the name (Stevens 1906, Smith 1950). In some coccinellid species the y chromosome has been completely lost, giving an **X0 system**: females possess two X chromosomes and males one. The absence of a y chromosome in such species suggests that, more generally in the Coccinellidae, it is the ratio of X chromosomes to haploid sets of autosomes (A) [2X:2A = female; 1X:2A = male] that determines sex rather than the occurrence of specific genes on the y chromosome which code for 'maleness' (Majerus 1994): both systems are known from other insects (Sanchez 2008).

In other species the X chromosome has fused with an autosome to form a **neo-XY** chromosome system: this is typical for the Chilocorini, although it also occurs in other groups (Smith & Virkki 1978).

Maffei and colleagues have investigated the special mechanism by which Xy_p sex chromosomes are associated during mitosis and meiosis. Their studies indicate that, depending on species, nucleolar proteins are either synthesized within or imported into the sex bivalent for this purpose. In the former case **fluorescent *in situ* hybridization (FISH)** techniques showed that **ribosomal DNA (rDNA)** genes mapped to the sex vesicle in *Olla v-nigrum* (Maffei et al. 2001a). In the latter case, in *Cycloneda sanguinea*, rDNA genes mapped to autosomes outside the sex vesicle; however, silver staining revealed that nucleolar material, which is typically associated with rDNA, was also associated with the sex chromosomes (Maffei et al. 2001b, 2004).

2.3.3 Supernumerary (B) chromosomes

A number of coccinellid groups contain species in which some individuals possess one or more **supernumerary chromosomes**, often called **B-chromosomes**, in addition to their normal chromosome complement. Populations containing such individuals are generally polymorphic, containing both individuals with and without B-chromosomes (e.g. Smith & Virkki 1978, Maffei et al. 2000, Tsurusaki et al. 2001). In a few known cases, such as in *Chilocorus stigma* and *Chil. rubidus*, all individuals bear at least one B-chromosome (Smith & Virkki 1978). The number of B-chromosomes carried by individuals can also vary. Tsurusaki et al. (2001) recorded between zero and four in one member of the *Henosepilachna* (= *Epilachna*) *vigintioctopunctata* species complex. The highest number of B-chromosomes recorded from a single individual is 13 from a male *Chil. rubidus* (Smith & Virkki 1978).

B-chromosomes are derived from the other chromosomes of the species that possess them or, more rarely, from the chromosomes of a related species through hybridization events (Jones & Rees 1982, Camacho et al. 2000). In *Henosepilachna* (= *Epilachna*) *pustulosa* some males possess a **supernumerary y chromosome**, probably derived from y chromosome duplication. During meiosis, both y chromosomes join with the X, producing a Xyy parachute, and ultimately gametes with either an X chromosome or two y

chromosomes (Tsurusaki et al. 1993). Similar supernumerary y chromosomes are also known from *Coccinella quinquepunctata* (Lyapunova et al. 1984). Some of the B-chromosomes of coccinellids appear to be restricted to or to predominate in only one sex and might also have **originated from sex chromosomes** (Maffei et al. 2000, Tsurusaki et al. 2001). The formation of a neo-XY sex determination system from the fusion of an X chromosome with an autosome is also likely to give rise to B-chromosomes, arising both from the former y chromosome and from a centric fragment lost during the fusion (Smith & Virkki 1978). More generally B-chromosomes may arise as a result of **chromosomal fusion** or from the **loss of genetically inert, heterochromatic chromosomal arms** (Smith & Virkki 1978). In *Chil. stigma*, which is polymorphic for a number of chromosomal fusions (2.3.4), the mean number and range of B-chromosomes increases with an increase in the frequency of fusions across populations (Smith & Virkki 1978). In this species, as throughout the Chilocorini, unfused chromosomes have one fully heterochromatic arm. Centric fusion occurs between the other **euchromatic** parts of chromosomes, leaving the lost heterochromatic arms as supernumerary chromosomes (Smith 1959). Heterochromatic arms can also sometimes be lost and become B-chromosomes without chromosomal fusion (Smith & Virkki 1978).

In many cases after their genesis, supernumerary chromosomes are lost in subsequent generations. Based on the number of chromosomal fusions observed in *Chil. stigma*, and thus the number of related B-chromosomes expected to be present, only 22% of potential B-chromosomes survive as such in this species (Smith & Virkki 1978). In other organisms, both positive and negative direct effects on fitness have been recorded for B-chromosomes, as well as biased transmission rates to offspring that are in excess of the rates expected under simple Mendelian inheritance (Jones & Rees 1982, Camacho et al. 2000); however, no such studies yet exist for coccinellids. Henderson (1988) found a **negative correlation between male B-chromosome frequency and the proportion of males** in populations of British *Exochomus quadripustulatus*. He concluded that this correlation was a consequence of a third factor that affected both B-chromosome frequency and sex ratio. This conclusion was based on the observation that other coccinellids with a neo-XY sex determining system like that of *E. quadripustulatus* showed similar sex ratio biases across populations without possessing B-chromosomes (Henderson & Albrecht 1988). It is perhaps worth noting that the studies pre-date detailed investigations on bacterial male-killing in coccinellids (Chapter 8), although none of the species studied by Henderson and Albrecht have been shown to possess male-killers (G.D.D. Hurst, personal communication). It is also now clear that B-chromosomes sometimes **directly affect the sex-ratio of the offspring** of individuals carrying them, although unlike in *E. quadripustulatus*, generally increasing the heterogametic sex (Beladjal et al. 2002, Werren & Stouthamer 2003, Underwood et al. 2005). Nonetheless the possibility that the correlation between *E. quadripustulatus* B-chromosome frequency and population sex ratio results from a direct relationship cannot be ruled out.

2.3.4 Cytogenetic changes, intraspecific cytogenetic variation and speciation

Relatively little **speciation** in the Coccinellidae appears to be directly related to cytogenetic changes. Most subfamilies and tribes exhibit a relatively limited variation in chromosome number and structure; consequently the majority of speciation in these groups is unlikely to be linked to cytological differences. The most variable group is the Chilocorini where diploid numbers of chromosomes can vary between 14 and 26 (Smith 1959, Lyapunova et al. 1984; Fig. 2.1). Studies of this group in North America demonstrate that the relationship between cytology and reproductive isolation is complex, and that changes in cytology do not necessarily lead to reproductive isolation (Smith 1959, Smith & Virkki 1978).

Chilocorus stigma exhibits up to six intraspecific **chromosomal fusion polymorphisms** in a genome fundamentally of diploid number 26 (Smith 1959, 1962b, Smith & Virkki 1978). The frequency of fusions increases westwards and northwards, through the range of *Chil. stigma*, which covers most of North America except the extreme west (Fig. 2.2). In the east, from Florida to New York and Connecticut, no individuals exhibit chromosomal fusions. However, moving northwestwards, three fusions appear sequentially in Maine, eastern Ontario and central Ontario (Smith 1959, 1962b). Three further fusions have been found in more northerly parts of Ontario (Smith & Virkki

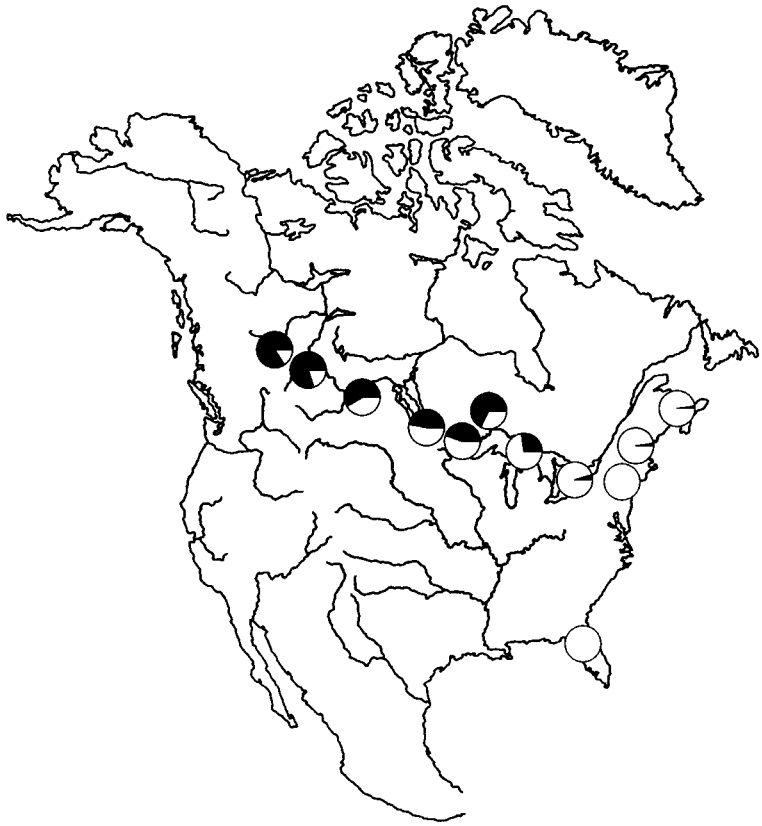

Figure 2.2 Geographic distribution of chromosome fusions in *Chilocorus stigma*. Circles show the average percentage of the maximum number of six fusions per individual (black). Thus the number of fusions increases to the north and west. From data in Smith & Virkki (1978).

1978). The clear distributional pattern of fusions suggests they are adaptive (Smith 1957, 1959, 1962b), although the nature of this adaptation is unknown. During chromosome pairing, individuals that are heterozygotic for fusions form trivalents, comprising one fusion chromosome paired with two homologous unfused ones. Fusion homozygotes form ring bivalents. In one case two fusions are semi-homologous (i.e. both contain one shared predecessor chromosome, in each cased fused to a different second chromosome): in this case heterozygotes for these fusions form a chain-of-four configuration. Meiosis involving all these configurations produces chromosomally balanced gametes (Smith 1966, Smith & Virkki 1978), making **fusion heterozygotes fully fertile**; thus *Chil. stigma* forms a **single species**.

In western North America, a parallel situation exists with respect to chromosomal fusions. Three monophyletic *Chilocorus* species, known as the *cyclus* complex, exhibit an increasing number of chromosomal fusions moving northwards. The most southerly species, *Chil. orbus*, has a diploid number of 22 chromosomes (two fusions). The more northerly *Chil. tricyclus* has 20 chromosomes (three fusions) and *Chil. hexacyclus* has 14 chromosomes (six fusions) (Smith 1959). However, unlike the chromosomal forms of *Chil. stigma*, the three species exhibit high levels of **hybrid sterility** as in heterozygote trivalents the

chromosomes are often orientated incorrectly (Smith 1959, 1966). Thus while some gene flow occurs between species in the restricted areas where they overlap, it is limited (Smith 1966). It is unclear why parallel processes of chromosomal evolution have given rise to a single polymorphic species in the east but a complex of three separate species in the west, although a number of possibilities have been suggested. Smith (1959) indicated that *Chil. stigma* might represent an earlier stage than the *cyclus*-complex in the evolution of new species through cytogenetic change. This seems unlikely, and two further, non-exclusive possibilities were proposed by Smith (1966). The first is that *Chil. stigma* fusions are advantageous in the heterozygous form, maintaining the polymorphism in this species in a balanced state. The second is that fusions in the western *cyclus* complex evolved in isolated marginal populations in which they rapidly became homozygous; *cyclus* complex hybrids therefore lost the ability to form correctly orientated trivalents.

From these two examples, particularly that of the chromosomally polymorphic *Chil. stigma*, we can conclude that complete **reproductive isolation rarely arises as a consequence of cytogenetic changes alone**. Nonetheless, such changes can certainly play a role. In a cytogenetic study of the *Henosepilachna* (= *Epilachna*) *vigintioctomaculata* species complex, Tsurusaki et al. (1993) found that the species complex fell into two groups: group A, comprising *Henosepilachna* (= *Epilachna*) *vigintioctomaculata*, possessed relatively small heterochromatic segments in their chromosomes, whereas group B, comprising *H. pustulosa*, *Henosepilachna* (= *Epilachna*) *niponica* and *Henosepilachna* (= *Epilachna*) *yasutomii* possessed long heterochromatic segments in seven autosomes. Hybrids between group A and B members exhibited high mortality during embryogenesis. Tsurusaki et al. hypothesize that in hybrids replication of the different chromosomes will occur at a highly heterogeneous rate, because of the widely differing amounts of heterochromatin in the chromosomes from the two parental species. Consequently in the early stages of embryogenesis, the rate of cell division, which is probably maternally determined at this stage, is poorly synchronized with chromosome replication, leading to low embryonic survival (Fig. 2.3). Tsurusaki et al. thus provide a **cytogenetic basis for reproductive isolation** between group A and B species. It is worth noting, however, that even in this example additional factors appear to play a role, most notably incompatibility between the female genital tract and allospecific sperm (Katakura 1986, Katakura & Sobu 1986). More generally, much work is still needed on the non-cytogenetic causes of reproductive isolation between closely related species, which overall remain poorly understood.

2.4 COLOUR PATTERN VARIATION

Coccinellid colouration has long attracted the attention of professional and amateur entomologists, probably because its high variation provided the opportunity of 'taxonomic description' of morphs to authors that did not come in contact with new species. These efforts peaked in the 1930s with Mader's (1926–37) catalogue which named both existing and theoretically possible morphs of Central European species.

A second and ultimately more important reason to study colour pattern variability was the insight it gave into evolutionary processes. Colour pattern studies first flourished at the time of the development of the synthetic theory of evolution. The authors hoped to observe the speciation process in action. 'If the genes that are unlike in two of these low ranking groups (subspecies and species) can be ascertained, there is some chance of obtaining a picture of the evolution process which produced the dissimilarity. This measure is of particular interest when it relates to those characters which competent taxonomists regard as the specific distinctions' (Shull 1949).

Conspicuous colour pattern polymorphisms played an important part in the debate surrounding natural selection and evolution. Early researchers believed that co-existing colour patterns were selectively neutral, and that their frequencies were consequently subject to genetic drift. Later studies showed that, in fact, they were under strong selection, which determined relative morph frequencies. However, the relative importance of selection and drift in the maintenance of conspicuous polymorphisms remained hotly debated for a long time (Ford 1964). In many polymorphic species, including ladybirds, there was consequently great interest not only in the genetic determination of alternative forms, but (i) their temporal and spatial variability, (ii) their ecological significance and (iii) the ultimate selective factors underlying the polymorphism. An enormous body of work on ladybirds was accomplished in the 20th century: its results are still significant for current biology and remain far from complete, even in well-investigated species (Majerus 1994).

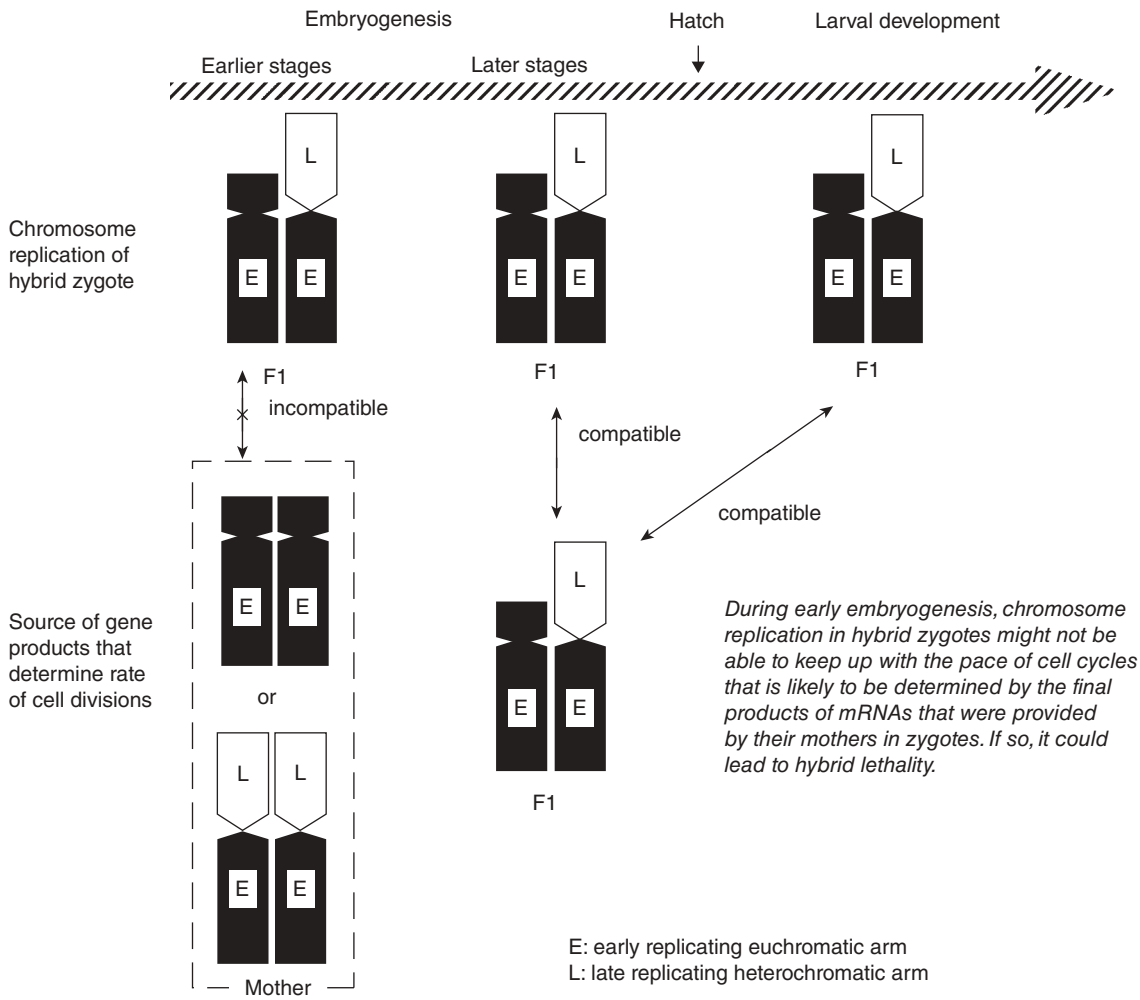

Figure 2.3 A hypothetical model of cytogenetic incompatibility between *Henosepilachna vigintioctomaculata*, with chromosomes with a small amount of heterochromatin, and species B members of the *Henosepilachna vigintioctomaculata* species complex, with chromosomes with a large amount of heterochromatin causing late replication (L). Redrawn from Tsurusaki et al. 1993, with permission.

2.4.1 The nature of colour patterns

The **elytral pattern** usually consists of dark spots on a light background (light or **non-melanic** morphs) or light spots on a dark background (dark or **melanic** morphs). The light areas (usually yellowish, reddish to brownish) are coloured by **carotenes**, the dark areas by **melanins** (Cromartie 1959). The spots always appear in predetermined positions and differ in presence, size and shape (Zimmermann 1931, Timofeeff-Ressovsky et al. 1965). The best method of describing variation of spot presence in 'light' morphs is probably that of Schilder (Schilder & Schilder 1951/2, Schilder 1952/3), illustrated in Fig. 2.4.

Several studies have concerned **morphological 'laws' of spot organization** and the ontogeny of the pattern. In particular species, not all possible combinations of spots are realized (Zarapkin 1938b, Filippov

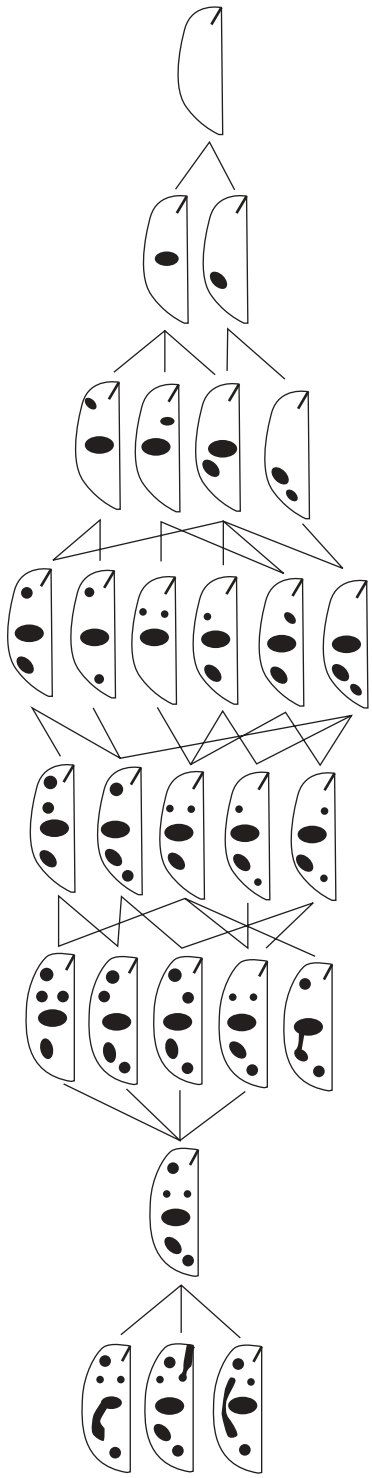

Figure 2.4 A classification of variation in elytral spot pattern using the example of *Hippodamia variegata*. The beetles are classified according to the cumulative number of spots and fusions. Each spot or fusion is a 'unit of melanic surface' and their sum roughly characterizes the degree of melanization. The horizontal series are defined by the particular number of spots and fusions, for example, the elytra shown in the lower series (bottom row) have 6 spots + 1 fusion (= 7 units of melanic surface), the elytron above has only 6 spots (= 6 units of melanic surface). Modified from Schilder & Schilder (1951/2).

1961). Principles of spot pattern variation were investigated by Smirnov (1927, 1932). In some species there exist several typical sequences in which particular spots appear after adult eclosion. This sequence of melanin deposition in particular spots was studied in *Adalia bipunctata* (Zarapkin 1930, 1938a, Majerus 1994). After spots are pigmented, the process of pigment deposition may continue so that the individuals become completely melanic. This process can occur within a few days, as in *Olla v-nigrum* (Vandenberg 1992), or over several months, as in *Anatis labiculata* (Majerus 1994).

Individuals can also differ in **scutum colouration**, which is also variable in pattern and degree of melanization. Particular patterns are associated with differing elytral patterns, so that light-spotted morphs have a light pronotum with dark marks of different shape while melanized individuals have dark elytra and pronota (Lusis 1932, Majerus 1994, Blehman 2007, 2009). The colour pattern of the head (frons) may also vary and the colouration is frequently typical of the sex (Table 2.1). Moreover the ventral side, thorax and abdomen also vary in the degree of melanization.

2.4.2 Genetic determination of colour patterns

Among many coccinellid species polymorphic for colour patterns, few have been the subject of detailed examination. Experimental studies are available for 15 species which have pronounced but not very complicated colour patterns and that are abundant and easy to collect and rear (Table 2.1). The studies were mostly accomplished in the first half of the 20th

Table 2.1 Experimental evidence of inheritance of and environmental effects of colour patterns: reports of crossing experiments and genotype x environment interactions of particular species, and their references.

Subject of the paper	Reference
Adalia bipunctata	
Melanic morphs dominant over light morph	Schroeder 1909
Multi-spot light morphs dominant over a melanic morph	Palmer 1911
Inheritance of multi-spot and melanic morphs in relation to typical (single spotted) morph	Palmer 1917
Inheritance of typical and dominant melanic morphs	Hawkes 1920
Allelomorphic series of three morphs, two melanic morphs dominant to typical (single spotted) morph	Fiori 1928
Dominance in allelomorphic series of morphs	Lusis 1928
Inheritance and dominance of 12 elytral colour morphs and pronotum pattern	Lusis 1932
Experiments on local differences in inheritance of colour morphs	Majerus 1994
Morph *tigrina* dominant over light *typica* and recessive to melanic morphs	Zakharov 1996
Adalia decempunctata	
Dominance of morphs in allelomorphic series	Lusis 1928
Inheritance of an allelic series, multi-spotted morph dominant to chequered and melanic morphs	Majerus 1994
Experiments on local variation in inheritance of colour morphs	Majerus 1994
Inheritance of slow and fast (dominant) deposition of carotenoids and melanin on elytra	Majerus 1994
Aphidecta obliterata	
Sex differences in head colouration on frons	Witter & Amman 1969
Sex differences in elytral colour pattern	Eichhorn & Graf 1971
Calvia sp.	
Allelomorphic series and order of dominance of three morphs (originally considered separate species)	Lusis 1971
Coelophora inaequalis	
Inheritance of colour morphs	Hales 1976; Houston 1979
Eight allelic morphs of which non-melanic is dominant	Houston & Hales 1980
Coelophora quadrivittata	
Inheritance of three allelomorphs with pale morph dominant over spotted and striped morphs	Chazeau 1980
Coccinella septempunctata	
Low temperature during pre-imaginal development increases spot size, in three populations of Japan	Okuda et al. 1997
***Henosepilachna elaterii* (=*Epilachna chrysomelina*)**	
Selection of strains with different size of spots	Tennenbaum 1931
Spot size is influenced by selection as well as temperature	Zimmermann 1931
Increasing development temperature decreases spot size, different reaction in three geographically distant populations	Timofeeff-Ressovsky 1932, 1941
Inheritance of the size and form of an elytral spot	Tennenbaum 1933
Harmonia axyridis	
Inheritance of four alleles of elytral colour determination	Tan & Li 1934
Inheritance of six elytral colour and pattern morphs and elytral ridge	Hosino 1936
Inheritance of *forficula* and *transversifascia* morphs	Hosino 1939
Inheritance of *aulica* and *gutta* morphs	Hosino 1940a
Establishing allelomorphic series and order of dominance of seven morphs	Hosino 1940b
Rare melanic morph *distincta* described and order of its dominance determined	Hosino 1941
Inheritance of alleles of *succinea* morph with different numbers of spots	Hosino 1942
Inheritance of three alleles of *axyridis* and two alleles of *aulica* morphs	Hosino 1943a
Inheritance of *succinea* and *axyridis* morphs	Hosino 1943b

(*Continued*)

Table 2.1 (*Continued*)

Subject of the paper	Reference
Sixteen alleles determining colour polymorphism and order of their dominance	Tan 1946
Genetic variation of spot size in *succinea* morph	Hosino 1948
Distinguishing alleles of elytral colour pattern morphs	Komai 1956
Decreasing developmental temperature increases spot size in *succinea* morph	Sakai et al. 1974
Inheritance of rare *supercilia* and *interduo* morphs	Tan & Hu 1980
Sexual differences in labrum and prosternum colour	McCornack et al. 2007
Crosses between phenotypes illustrated	Seo et al. 2007
Temperature influences melanization in *succinea* morph	Michie et al. 2010
Hippodamia sinuata	
Inheritance of spotted and spotless (dominant) morph	Shull 1943
Hippodamia convergens	
Inheritance of spotted and spotless (dominant) morph	Shull 1944
Hippodamia quinquesignata	
Inheritance of spotted and spotless (dominant) morph	Shull 1945
Olla v-nigrum	
Inheritance of spotted and melanic (dominant) morphs	Vandenberg 1992
Propylea japonica	
Inheritance of three elytral colour and pattern morphs	Miyazawa & Ito 1921
Propylea quatuordecimpunctata	
Sexual differences in head (frons) and pronotum colour	Rogers et al. 1971
Polygenic inheritance of spot size	Majerus 1994

century and have been reviewed by Komai (1956). Table 2.1 shows that fewer crossing experiments have been made recently, except for the enormous but unfortunately largely unpublished work of the late Mike Majerus (Majerus 1994). His results indicate that the study of colour patterns requires further refinement, even in the most intensively studied species like *A. bipunctata*.

The published data on crossing experiments led to several generalizations. In most cases it was demonstrated that the morphs are determined by a series of **multiple alleles localized at the same locus**. However, species in which colour morphs are determined by two or more non-allelic genes might also exist (Komai 1956). Further ideas were proposed by Ford (1964) and advocated by Majerus (1994), who consider it more likely that such a multiple allele effect, as has been demonstrated in coccinellids, is caused by a sufficiently close juxtaposition of the loci of the genes so that crossing-over is most unlikely to separate them. Ford designated such a complex of loci as a '**supergene**'. Minor variation of **spot size** and shape is controlled **polygenically**, for example in *Henosepilachna elaterii* (= *Epilachna chrysomelina*) (Timofeeff-Ressovsky 1932, Timofeeff-Ressovsky et al. 1965) or *Propylea quatuordecimpunctata* (Majerus 1994). For individual species, the **number of allelomorphs per locus** roughly correlates with the amount of experimental work invested in its analysis. Thus the number of established alleles for elytral colour pattern exceeded 22 in *Harmonia axyridis* (Tan 1946, Komai 1956) while for *A. bipunctata*, Majerus (1994) greatly increased this number above the 12 determined by Lusis (1928, 1932, Fig. 2.5). Patterns determined by particular alleles are frequently similar and difficult to distinguish. An example is the *succinea* group of morphs in *Har. axyridis* (light elytra with up to nine black spots), where morphs with particular numbers of dark spots are determined by particular alleles. Consequently some 15 alleles, each with a particular expression of the *succinea* morph in a homozygous state, may exist (Tan 1946). On the other hand, **modifiers** may vary the patterns determined by particular alleles. This became evident in crossing *A. bipunctata*

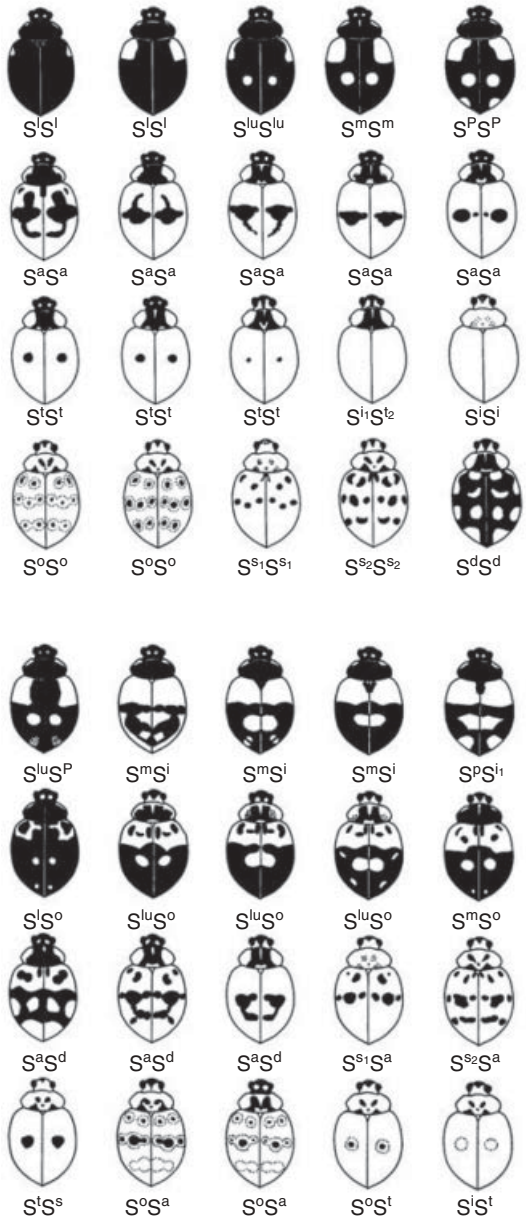

Figure 2.5 Variability of colour pattern of *Adalia bipunctata*. Homozygous morphs (top four rows) are determined by 12 alleles arranged in the order of dominance starting from top left. Some heterozygotes are shown in the bottom four rows. After Lusis (1932).

males and females originating from distant geographic populations. Unexpected colour morphs appeared in the progeny, probably because the mixing of different sets of modifiers resulted in the normal dominance relationship breaking down (Majerus 1994). Heterozygous forms further increase intraspecific variation in colour morphs. In many species and populations, alleles determining melanic morphs are dominant to those determining light morphs. This led Tan (Tan 1946, Tan & Hu 1980) to formulate the '**mosaic dominance**' hypothesis which supposes that the heterozygote develops black pigmentation on any part of the elytron which is black in either homozygote (Tan 1946). Dominance of the melanic form is not typical for all species of coccinellids, however. This is because the order of dominance is determined by genetic environment of the allele for colour morph. Consequently species and populations exist where light morphs are dominant. Thus the allele determining the light morph is dominant over that for the melanic morph in *Hippodamia* (Shull 1949) and *Coelophora* (Houston 1979, Chazeau 1980).

2.4.3 Geographic variation

Geographic variation in morph frequencies was first systematically studied in *A. bipunctata* and *A. decempunctata* (Dobzhansky 1924b), and then in other species including *Anatis ocellata*, *Anisosticta novemdecimpunctata*, *Coccinella magnifica* (= *C. divaricata*), *C. septempunctata*, *C. transversoguttata*, *C. quinquepunctata*, *Coccinula quatuordecimpustulata*, *Hippodamia* (= *Adonia*) *variegata* and *Oenopia* (= *Synharmonia*) *conglobata* (Dobzhansky 1925, 1933, Dobzhansky & Sivertzew-Dobzhansky 1927). For all species, **geographic centres exist for less heavily pigmented populations**, with more distant areas inhabited by more heavily pigmented ones. Moreover, such centres for the different species roughly coincide in geographical location. The centre for light morphs lies in Central Asia for the eastern hemisphere and in California for the western hemisphere. The proportion of pigmented morphs increases radially in all directions from each 'light' centre.

Fine-scale patterns of geographic differentiation of proportions of colour morphs in local populations have been particularly well studied in two species,

A. bipunctata and *Har. axyridis*. Proportions of light morphs (mostly *typica* with one black spot on each of the reddish elytra) and melanic morphs (with one, two or three reddish spots on each black elytron) were studied in **local populations of *A. bipunctata***. In areas with an oceanic climate, the proportion of melanic morphs may vary from 0–97% in the British Isles (Creed 1966, 1971a, b), 2–86% in the Netherlands (Brakefield 1984a, b, c), 0–83% in Norway (Bengtson & Hagen 1975, 1977), 6–88% in central Italy (Scali & Creed 1975) and 9–88% in some areas of the Baltic sea coast near St. Petersburg (Zakharov & Sergievsky 1978, 1980). Other populations generally have low proportions of melanics, for example 8–21% in Germany (Klausnitzer & Schummer 1983), 0–20% in Bohemia (Honek 1975), 0–15% on the Baltic coast of southern Finland (Mikkola & Albrecht 1988) and 3% in Stockholm (Zakharov & Shaikevich 2001). As one moves to Central Asia, the populations become more polymorphic than in Europe and the proportions of melanic morphs in local populations of Central Asia vary from 1–96% (Lusis 1973).

Several studies have attempted to find a **correlation between local environmental conditions and the proportion of melanics**. In Great Britain, melanic populations are concentrated in industrial areas (Creed 1966, 1971b) and around sources of air pollution in rural areas (Creed 1974). The same applies in the Baltic region (Lusis 1961, Zakharov & Sergievsky 1978, Mikkola & Albrecht 1988). The proportion of melanics also varies with distance from the sea, in central Italy (Scali & Creed 1975) and Norway (Bengtson & Hagen 1977): it is greatest near the sea, and decreases moving inland. However, contrasting trends were observed in the Netherlands (Brakefield 1984a, b, c). Local differences were observed in Ukraine, where Emetz (1984) in the course of eight years observed an increasing proportion of melanics (from 0 to 68%) on a burned site, while in the surrounding oak forest the proportion of melanics remained zero.

Variation in the proportions of particular **colour morphs in local *Har. axyridis* populations** have been studied by a number of authors (Dobzhansky 1924a, 1933, Komai et al. 1950, Komai & Hosino 1951, Komai 1956, Komai & Chino 1969, Vorontsov & Blehman 2001, Korsun 2004, Blehman 2007). The melanic morph *axyridis* is limited naturally to western Siberia where its distribution approximately coincides with the Jenisei river basin. Dobzhansky (1924a) and Vorontsov and Blehman (2001) supposed that this morph occurs as a subspecies, probably differentiated from other populations by genetic changes at several loci. This hypothesis was supported by Blekhman (2008), who demonstrated the near-absence of a dominant elytral ridge allele (see also 2.5.1) in populations of *axyridis* morphs in western Siberia, as well as in more recent molecular genetic analyses (Blekhman et al. 2010). In the rest of the natural range of *Har. axyridis* (Pacific coast of Russia, China, Korea and Japan) all morphs occur together, but their proportions vary geographically. These populations consist of a mixture of light *succinea* morphs, melanic *axyridis*, *spectabilis* and *conspicua* morphs and a number of rare morphs. In Japan there is a marked cline, with decreasing proportions of *succinea* and increasing proportions of melanics, as one moves from the north to the south. The proportion of morphs is different in the continental populations of eastern Siberia, Korea and northern China where the proportions of melanic *spectabilis* and *conspicua* are higher than in Japan.

Other species have been less intensively studied. Studies of the geographic variation of *C. septempunctata* have concerned the size of spots. The maximum spot size occurs in ssp. *brucki* living on the Pacific coasts of Russia, Korea and Japan; while the centre of occurrence of small-spotted populations is in Central Asia. (Dobzhansky & Sivertzew-Dobzhansky 1927). Local trends for decreasing spot size in populations of warm areas compared to those of cooler areas were established by Tolunay (1939) in Turkey. The *Coccinella* species of North America are more variable. *Coccinella transversoguttata richardsoni* has several morphs that occur together, but whose frequency varies among local populations. By contrast, in *C. monticola*, *C. novemnotata* and *C. hieroglyphica,* particular morphs dominate in a particular area (Brown 1962). *Hip. variegata* can have 0–6 spots on each elytron. Six forms are found more frequently than the other 21 minority forms. The dominant forms are the same in different geographic populations of Central Europe (Schilder 1928, Strouhal 1939, Balthasarova-Hrubantova 1950, Schilder & Schilder 1951/2), but other spot forms occur in different proportions.

Variation in the extent of melanization between local populations of several species living in two relatively close localities in Central Asia, the warm Chuiskaya region and the cooler and overcast areas around Lake Issyk Kul, was described by Kryltzov (1956).

Melanization was on average greater in the Issyk Kul than the Chuiskaya region. The species exhibit either increases in spot size (*Psyllobora* (= *Thea*) *vigintiduopunctata*, *Bulaea lichatschovi*, *Coccinula sinuatomarginata*, *C. septempunctata*, *Coccinula quatuordecimpustulata*) or in the number of spots and confluences between them (*P. quatuordecimpunctata*, *Hippodamia tredecimpunctata*, *Hip. variegata*). The changing degree of melanization with climatic conditions is less pronounced in species with a dark ground colour than in species with a light ground colour (Kryltzov 1953).

Variation in the proportion of morphs on particular host plant/aphid systems represents a kind of micro-geographic variation. While no differences were established for populations of *A. bipunctata* and *A. decempunctata* on more than 20 host plants (Honek et al. 2005), significant variation was established between populations of *Har. axyridis* on coniferous and broad leaved plants (Komai & Hosino 1951). However, this variation could well be confounded by the presence of a sibling species, *Har. yedoensis*, which colonizes only conifers (Sasaji 1980).

2.4.4 Temporal variation

Temporal variation in the proportion of morphs at the same locality includes seasonal variation and long term changes over a span of years. **Seasonal variation** in morph frequency was first recorded in *A. bipunctata* by Meissner (1907a, b) and studied in detail by Timofeeff-Ressovsky (1940). During the 1930s, in Potsdam near Berlin he observed a regular increase in the proportion of melanic morphs over the summer breeding period and a decrease in the proportion of melanics during overwintering. The contrasting processes maintained an average proportion of melanics in the population. However, several studies from other geographic areas revealed no similar significant differences in the proportion of melanics early and late in the season (Lusis 1961, Bengtson & Hagen 1975, Honek 1975, Zakharov & Sergievsky 1980, Klausnitzer & Schummer 1983, Majerus and Zakharov 2000, Honek et al. 2005). No seasonal change was also found in *A. decempunctata* (Honek et al. 2005). Significant seasonal trends in the proportions of *Har. axyridis* colour morphs were the opposite of *A. bipunctata*: an increase in the light *succinea* morph through the vegetative season and lower melanic mortality in the winter was observed in populations of northern China and Japan (Osawa & Nishida 1992, Wang et al. 2009). In central China, the frequency of the *succinea* morph was 48% in the spring, decreased to 22% in the summer and increased to 59% again in the autumn, while dark morphs exhibited a reciprocal change (Tan 1949). However, even in *Har. axyridis,* the temporal variation is area-specific and Kholin (1990) found no seasonal variation in populations of the Maritime Province of Russia.

In addition to seasonal fluctuations, **long-term changes** also have been observed in coccinellids. A decrease in the proportion of melanic *A. bipunctata* was observed in industrial areas of Birmingham in Great Britain from the 1960s onwards. From an initial 40–50%, the proportion of melanics decreased, between 1960 and the late 1970s, to about 10% and remained at this low level (Creed 1971a, Brakefield & Lees 1987). A similar decrease may have occurred during this period at Potsdam, where there were about 37–59% of melanics in the 1930s (Timofeeff-Ressovsky 1940), 15% in 1973 (Creed 1975) and only 5% in 1981 (Honek et al. 2005). Elsewhere the proportion of melanics has risen with increased industrial pollution. A large increase was observed in Gatchina, near St. Petersburg, where the proportion of melanics increased from 9% in the 1930s to 43% in 1975 (Zakharov & Sergievsky 1978). The proportion probably also increased in central Italy (Bologna) from 41% in 1926 (Fiori 1928) to 56% in 1974 (Scali & Creed 1975). The proportion of the melanic morph *sublunata* substantially increased at several localities in Central Asia, for example in Tashkent, from 47% in 1908 to 96% in 1972 (Lusis 1973). Examples of no change were observed in populations with low melanic frequencies (Klausnitzer & Schummer 1983). In *Har. axyridis* only slight long term changes were observed in a population in central Japan where the frequency of *succinea* was stable (43%) between 1912 and 1920 but decreased by 10% by the mid-1940s (Komai et al. 1950). In southern Slovakia, a population of *Hip. variegata* was sampled from 1937 to 2009 (Strouhal 1939, Balthasarova-Hrubantova 1950, Honek unpubl.): the dominant morphs remained the same and their frequencies varied little over the entire 70-year period.

2.4.5 Significance and evolution

The main interest of authors studying intraspecific colour polymorphism has been to explain its

significance and origin. Because the patterns, body size and repellency/toxicity of coccinellid species differ substantially, it is likely that **the explanation will not be the same for all**. Majerus (1994) suggested that the significance of colour patterns would differ for (i) 'smaller species', (ii) the Chilocorini, (iii) host plant/habitat generalists and (iv) host plant/habitat specialists. For each group of species, light and melanic morphs have different significances and particular patterns may serve as a 'warning' in poisonous species and their mimics; 'cryptic colouration' could be adapted to particular environments, to signal for mates or as a means of thermoregulation.

In ***A. bipunctata*** four explanations of differences in local morph proportions were proposed: industrial melanism, (micro)climatic selection, assortative mating and Müllerian mimicry. The first two explanations are in fact complementary and well documented in some areas at least (Sergievsky & Zakharov 1981, 1983, 1989, Majerus & Zakharov 2000); the latter two represent distinct factors in the evolution of *A. bipunctata* polymorphism. **Industrial melanism** and its evolution were best documented in Britain (Creed 1966, 1971a) and northern Russia (Majerus & Zakharov 2000, Zakharov 2003), where a positive relationship between **smoke (small particle) pollution** of the air and the proportion of melanics observed exists (Creed 1971b, 1975). There was close correspondence between the proportion of melanics and the level of smoke pollution, but a less significant one or none with **sulphur dioxide pollution** (Lees et al. 1973, Brakefield & Lees 1987). However, industrial melanism does not exist in some areas, such as Bohemia and eastern Germany, where pollution was very high in the past (Honek 1975, Klausnitzer & Schummer 1983, Honek et al. 2005). This apparent discrepancy may be explained by another factor in melanism, **climate**. There is only a weak negative relationship between the proportion of melanics and **temperature**, but more important is a negative relationship between melanic proportion and **duration of sunshine** (Nefedov 1959, Lusis 1961). A statistically significant relationship was demonstrated for British populations where the correlation was $r = -0.59$ (Benham et al. 1974); after omitting sites for which sunshine hours were only estimated, it rose to $r = -0.75$ (Muggleton et al. 1975). Brakefield (1984a, b, c) also found a significant, negative correlation between sunshine hours and melanic frequency. However, the correlation between sunshine hours and melanism may not apply on a very local scale in Britain (Creed 1975), to populations of warm Mediterranean areas (Scali & Creed 1975) and to populations of Central Asia with up to 96% of melanics (Lusis 1973) where sunshine is perhaps in excess. Climatic factors may explain the variation in frequency of melanic morphs in areas where industrial pollution fails to explain the situation. This applies to Norway where a coastal climate, i.e. the joint effects of temperature, humidity and sunshine, favours the occurrence of melanics (Bengtson & Hagen 1975, 1977). The authors found the best correlation ($r = 0.93$) was between percent melanics and an '**index of oceanity**', which combines the effects of temperature and rainfall. In the Netherlands, besides the expected negative correlation between melanic proportion and sunshine hours ($r = -0.45$, $p < 0.05$), there was also a correlation with humidity and the index of oceanity. The latter correlation was very high but, in contrast to Norway, a negative one ($r = -0.90$) (Brakefield 1984a, b, c). This suggests caution in interpreting correlations between climatic data and melanic proportions.

For the localities where seasonal differences in the proportion of melanic morphs of *A. bipunctata* exist, i.e. Germany (Timofeeff-Ressovsky 1940, Timofeeff-Ressovsky & Svirezhev 1966, 1967), Great Britain (Creed 1966, 1975) and the Netherlands (Brakefield 1985a), the mechanism of change probably comprises **balanced selection**, favouring melanics during the breeding period and non-melanics during the winter. One may calculate the selection coefficients s against black morphs in winter and t against red morphs in the summer (Creed 1975). This value is a quantitative measure of the intensity of selection which indicates the proportional reduction of the gametic contribution of a genotype compared to the favoured genotype. The average coefficients were $s = 0.52$ and $t = 0.33$ for the Potsdam population in the 1930s, and lower $s = 0.24$ and $t = 0.09$ for Birmingham in the 1960s. The magnitude of s increases with the lowest mean monthly temperature, and the values of t increased with the highest monthly maximum temperature (Creed 1975).

A **mating** advantage for melanic morphs in the breeding period of spring and summer may result from their **thermal properties**. The elytra of red morphs have a greater reflectance than those of the melanic morphs, and correspondingly absorb less radiant

energy. As a consequence, dark animals exposed to sunlight have a greater temperature excess over ambient (by 2.1°C) and their initial rates of heating are 50% greater than non-melanics (Brakefield & Willmer 1985). The magnitude of temperature excess also increases with body size. The higher body temperatures of melanic individuals may increase activity during the breeding period. Melanic individuals then copulate more readily than the red ones. Lusis (1961) counted the proportion of red and dark copulating individuals on several days when the weather was convenient for breeding. The proportion of reds in copula at the time of the census (23.0 ± 6.5% of the all red individuals recorded during the count) was significantly lower than the proportion of dark individuals (31.8 ± 7.1%). The same was true for Dutch populations, where melanics also gained a copulatory advantage (Brakefield 1984c). Melanic morphs also dispersed earlier to breeding sites in the spring, mated earlier and emerged from pupae earlier (Brakefield 1984b) which may all be a consequence of their thermal properties. Colour morphs of *Har. axyridis* also differ in several characters determining fitness including developmental rate at some stages, predation activity, longevity and fecundity (Soares et al. 2001, 2003).

In *A. bipunctata* **sexual selection and assortative mating** (preference for the mate of a particular morph) have also been supposed to maintain the variation in morph proportions observed in natural populations (O'Donald & Muggleton 1979). Some experiments indicated a **female preference** for males of the melanic morph *quadrimaculata*. This preference was observed in females regardless of their colour. It was frequency dependent and its intensity increased as the proportion of *quadrimaculata* males decreased (Majerus et al. 1982). After 14 generations of selection, O'Donald et al. (1984) and Majerus et al. (1986) were able to increase the degree of this preference in several isofemale lines. However, later studies revealed that even the combined effect of sexual and climatic selection cannot provide a general explanation for observed variation in morph frequency. In several British populations females had no mating preferences, or the melanic advantage was not frequency dependent (Kearns et al. 1990). Moreover, female preferences for melanic males in artificially selected lines disappeared after prolonged maintenance of these strains in the laboratory, and an attempt at selecting new isofemale lines with similar preferences failed (Kearns et al. 1992). Similar preferences in both sexes for melanic mates were found in *C. septempunctata* (Srivastava & Omkar 2005) while the reverse was observed in Japanese *Har. axyridis* where females preferentially choose non-melanic males in spring, but non-melanics are less successful at mating than melanics in summer (Osawa & Nishida 1992). In this species, colour morph preference may be confounded by body size since in non-melanic males the mating individuals were larger than unmated individuals, while in melanic males there was no difference (Ueno et al. 1998).

Müllerian mimicry has also been proposed for maintaining colour polymorphism. Species whose adults have bright red or yellow colouration on the upper surface are thought to be aposematically coloured to prevent attacks by visual predators, mostly birds. The repellency is a consequence of previous negative experience of the predator with a distasteful prey, which has been demonstrated a number of times for coccinellids (e.g. Whitmore & Pruess 1982) (also Chapter 9). Several species of similar colouration may represent a Müllerian complex, with predators not distinguishing between the individual component species. The negative experience of the first attack on a member of this complex becomes generalized and all its members are protected in the future. Brakefield (1985b) hypothesized that polymorphic and relatively non-toxic *A. bipunctata* and *A. decempunctata* were mimicking two Müllerian models, the red *C. septempunctata* and black *Exochomus quadripustulatus*, both of which are highly distasteful and toxic to predators (Marples et al. 1989, Marples 1993). More recently, it has also been suggested that intraspecific variation in colour pattern might be an 'honest indicator' of chemical defensive strength (Bezzerides et al. 2007).

2.5 THE INHERITANCE OF OTHER TRAITS

2.5.1 Morphological characters: wing polymorphism

In addition to studies of colour pattern, there has been a diversity of other genetic studies on other biological characteristics of coccinellids. Studies of **morphological characters** include those of a transverse ridge

occurring on the hind part of the elytra of *Har. axyridis*, which is polymorphic for this character. The ridge is thought to be encoded by a single dominant gene (Hosino 1936) and, although the function of the ridge is unknown, geographic variation in its occurrence appears to be clinal, suggesting it is under selection (Komai 1956, Sasaji 1980, Blekhman 2008, 2.4.3). The majority of morphological studies have been on **wing polymorphism** in coccinellids, which has been intensively studied in other insect groups (Zera & Denno 1997). In some species, such as *Subcoccinella vigintiquatuorpunctata* and *Rhizobius* spp., genetically controlled wing reduction occurs commonly in natural populations (Pope 1977, Hammond 1985). A more recent focus of such studies has been *A. bipunctata*, in which naturally occurring wingless individuals are very rare (Majerus & Kearns 1989, Marples et al. 1993). In this species, winglessness is controlled by a single recessive allele, although the extent of its development is determined by genetic background, modifier genes and environmental influences, notably temperature (Marples et al. 1993, Ueno et al. 2004, Lommen et al. 2005, 2009). Genetically controlled flightlessness has also been found in *Har. axyridis*: in this case the ladybirds appear morphologically normal, but structural modifications are present in the wing muscles (Tourniaire et al. 2000). Flightless strains of *Har. axyridis* have been used as a means of biological control, being largely unable to disperse away from the target crop (Gil et al. 2004, Seko et al. 2008, but see Seko & Miura 2009); a similar biological control function has been proposed for flightless *A. bipunctata* (Lommen et al. 2008; see also Chapter 11).

For wingless morphs of *A. bipunctata*, the **pattern of wing development** is similar to that of fully winged individuals, but is slower, making the wing discs smaller at pupation. Additionally the wings are truncated at adulthood, although this does not appear to arise from apoptosis (cell death). Expression of the gene *Distal-less* is limited to the proximal anterior margin of the wing discs in the larvae of wingless *A. bipunctata* morphs, while in winged morphs it is expressed all round the wing disc margin. The expression of this gene may determine the extent of winglessness (Lommen et al. 2009). Two other genes, *vestigial* and *scalloped*, have also been shown to play a role in ladybird wing development similar to the roles they play in *Drosophila melanogaster*. Interestingly, *scalloped* also plays a role in pupal ecdysis. It has been suggested that disruption of *vestigial* and *scalloped* expression could be used as a means to produce wingless *Har. axyridis* for biological control purposes (Ohde et al. 2009).

2.5.2 Life history characters: heritability, selection experiments and genetic trade-offs

The majority of **life history characters**, such as body size, developmental and reproductive characteristics, and the requirement for diapause before reproduction, exhibit complex polygenic inheritance. There are two main approaches used to study genetic variation in such characters. **Heritability studies** give a measure of the contribution of genetic variation to total observed phenotypic variation in a species. For example, Ueno (1994a) reared *Har. axyridis* from different male–female parental combinations to estimate the heritabilities of body size and developmental characters; because males were mated to more than one female (although females were singly mated), he was able to make these estimates separately for the sexes. The estimates were moderate, varying between 0.24 and 0.56 of total phenotypic variance resulting from genetic variance. An alternative approach is to apply **directional selection** to a particular character, in order to demonstrate underlying genetic variation in the character. Thus, for example, it proved possible to select against the tendency for an obligate diapause before oviposition in *C. septempunctata* with eggs collected after short preovipositional period, resulting in a general decline in the proportion of females requiring a diapause before reproduction (Hodek & Cerkasov 1961). This suggested that variation in the requirement for diapause in this species had an underlying genetic component (Chapter 6).

Linked to these sorts of studies are estimates of **genetic correlations** and **trade-offs** between traits. Selection on one particular trait may have correlated effects on other traits (**pleiotropy**) if the genes involved affect both traits. The traits may be obviously related: for example, in his study of *Har. axyridis*, Ueno (1994a) found high positive genetic correlations between body weight and body length (i.e. ladybirds that are longer are also heavier). Of much greater interest are the relationships between less closely related traits, since these give an idea of how genetic constraints can mould the

overall phenotype of an organism. In his study, Ueno (1994a) found a possible negative correlation between body size and developmental time and a positive one between body size and developmental rate: thus, a longer developmental time and a slower developmental rate were apparently linked to smaller body size. This finding is in part supported by a study in which fast development was selected for in *Hippodamia convergens*: the fast developing larvae consumed more prey per unit time and exhibited a higher feeding efficiency. In selected lines there was a negative correlation between developmental time and body weight, even though body weight was apparently not affected by intense selection (Rodriguez-Saona & Miller 1995).

Of particular interest has been the relationship between traits related to the ability to exploit different types of food. Here, genetic trade-offs are seen as providing a basis for **dietary breadth and specialization**, with specialists being seen as having evolved very high efficiency on one type of food at the expense of an ability to exploit other types. The general focus has been on the suitability of food mediated by its allelochemical and nutrient content. Interestingly the majority of evidence from both phytophagous and aphidophagous coccinellids supports a view that, although genetic variation exists in the ability to process particular food species, genetic trade-offs in their nutritional suitability are either very weak or non-existent in ladybird consumers (Ueno et al. 1999, 2003, Ueno 2003, Fukunaga & Akimoto 2007; see also Rana et al. 2002, Sloggett 2008).

2.6 MOLECULAR GENETIC STUDIES

The first molecular genetic studies of the Coccinellidae were published in Japan in the 1970s (Sasaji & Ohnishi 1973a, b, Sasaji & Hisano 1977, Kuboki 1978); however, it is only in the last 20 years that molecular techniques for studying the Coccinellidae have come into regular use, mirroring the wider availability and lower cost of a diversity of methodological approaches. With the exception of the work discussed earlier on chromosomes (2.3.2) and wing development (2.5.1), there is little work providing insight into the biochemical, regulatory and developmental pathways connecting specific genes to particular phenotypic characteristics of coccinellids. However, coccinellids have been used as **models for the evolution of specific genomic regions** and **molecular markers** have played a significant role in ecological and evolutionary studies of coccinellid biology.

A useful feature of coccinellids is that they can be **non-destructively sampled** for molecular genetic studies. Both the adults and larvae of coccinellids exude alkaloid-bearing haemolymph as a chemical defence, a process known as reflex bleeding (Happ & Eisner 1961, Kendall 1971; Chapters 8 and 9). The reflex blood contains enough protein for isozyme or allozyme analysis (Kuboki 1978, Ransford 1997) and it is also possible to amplify DNA from reflex blood using the polymerase chain reaction (PCR) (Karystinou et al. 2004). It is therefore not necessary to kill or damage live coccinellids in order to obtain samples for genetic analysis. This is a particularly valuable attribute for behavioural studies. For example, in a study of mating behaviour, sperm competition and paternity, Ransford (1997) was able to genotype adult *A. bipunctata* using allozymes from reflex blood extracts. This left the beetles alive for subsequent breeding and use in mating experiments.

2.6.1 Sequence evolution

2.6.1.1 Mitochondrial DNA and the inference of evolutionary history

Many studies of evolutionary history utilize **mitochondrial DNA (mtDNA)** as a molecular marker, because of its ease of PCR amplification, rarity of recombination and uniparental cytoplasmic inheritance (i.e. only through the female line). These factors have made mtDNA attractive when compared to nuclear molecular markers for phylogenetics and studies of genetic structure and phylogeography (Harrison 1989, Simon et al. 2006). Additionally more recently mtDNA has been proposed as a universal means for identifying species using a specific characteristic 'barcode' DNA sequence (Hebert et al. 2003, Hajibabaei et al. 2007). The universal utility of mtDNA has been questioned on a number of grounds, however (Ballard & Whitlock 2004, Hurst & Jiggins 2005). One objection has been that **mtDNA is often not selectively neutral**, as should be the case for molecular markers, because it is effectively **linked to endosymbiotic bacteria** such as male-killers, which are also inherited cytoplasmically and are likely to be under strong selection. In consequence interpretations of population structure or evolutionary history based on mtDNA are confounded by selection (Turelli et al.

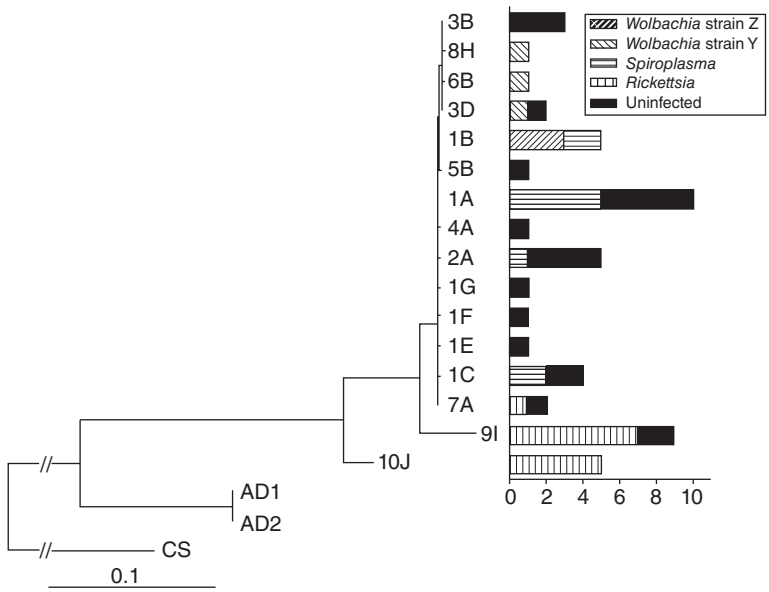

Figure 2.6 Phylogeny of *Adalia bipunctata* mtDNA sequences and the number and male-killing infection status of individuals bearing that mitotype. In the phylogeny CS, AD1 and AD2 are the mitotypes from one specimen of *Coccinella septempunctata* and two of *Adalia decempunctata*; all other terminal branches are *A. bipunctata* mitotypes. Scale bar = 0.1 substitutions per nucleotide for tree branch lengths. Redrawn from Schulenburg et al. (2002), with permission.

1992, Johnstone & Hurst 1996, Hurst & Jiggins 2005). The wide prevalence of male-killing bacteria in aphidophagous coccinellids (Chapter 8) has made them particularly suitable for studying this issue. Male-killing symbionts clearly affect host fitness, increasing female fitness at the cost of the dead males (Chapter 8). The consequent increase in infected daughters in a population will lead to a related increase in the mtDNA variant associated with the male-killer; furthermore because male-killers are rarely transmitted perfectly between generations, so that a proportion of uninfected individuals is produced, the mtDNA variant will also reach significant levels in uninfected individuals (Johnstone & Hurst 1996).

Schulenburg et al. (2002) investigated **mtDNA diversity and sequence divergence in relation to male-killing** in *A. bipunctata*. This coccinellid was known to harbour four different male-killers throughout its range: a *Rickettsia*, a *Spiroplasma* and two strains of *Wolbachia*. Schulenburg et al. sequenced two variable mtDNA regions (parts of the cytochrome oxidase subunit I (COI) and NADH dehydrogenase 5 (ND5) genes) from 52 infected and uninfected ladybirds from nine populations extending from Britain to Russia. They then compared the distributions of different mitochondrial variants to the distributions of the male-killers across individuals and populations. Of a total of 10 mitotypes found associated with male-killing bacteria, nine were found to occur with only one bacterial type (Fig. 2.6). There was no significant differentiation of mitotypes from different geographic regions. Instead, **mitotypes were differentiated according to bacterial infection**, particularly between ladybirds with different such infections; differentiation between uninfected ladybirds and those with *Spiroplasma* and *Wolbachia* infections was much lower. A higher degree of mitotype differentiation among *Rickettsia*-infected individuals supported the idea that this bacterium had been present in *A. bipunctata* for longer than the others, allowing for greater subsequent mitochondrial sequence divergence.

Schulenburg et al.'s findings very clearly support the view that the distribution of DNA mitotypes is strongly correlated with the occurrence of the different

male-killing symbionts: mtDNA variants and cytoplasmic male-killers are inherited together as a single unit and selection on one will result in a 'hitch-hiking' effect on the other. Building on this earlier study, Jiggins & Tinsley (2005) addressed the question of **selection on mitochondrial and nuclear DNA**. Concentrating exclusively on *Rickettsia*-bearing *A. bipunctata*, they showed that three different strains of the bacterium were associated with three different mtDNA variants. The occurrence of the three haplotypes varied geographically and, more equivocally, temporally. Tests on the haplotype frequency and genealogy of the mtDNA variants indicated that **mtDNA was under balancing selection** (i.e. selection maintaining stable frequencies of the mitotypes). Jiggins & Tinsley compared nuclear and mitochondrial genes by sequencing a nuclear gene (the glucose-6-phosphate dehydrogenase gene (*g6pd*)) in individuals from many of the same populations as those studied by Schulenburg et al. (2002) and comparing their data to Schulenburg et al.'s mtDNA data. In contrast to the mtDNA data, the **nuclear *g6pd* data was consistent with neutral evolution**. Furthermore, the gene diversity of mtDNA was much higher than that expected under neutrality when compared to *g6pd*. Jiggins & Tinsley also found strong differences in haplotype frequencies in infected and uninfected beetles: they were therefore able to conclude that selection was acting on the mtDNA through the male-killer rather than directly on the mtDNA; the latter would be more likely to result in similar haplotype frequencies in infected and uninfected beetles.

It is clear that in aphidophagous coccinellids the presence of cytoplasmically inherited male-killing symbionts and their linkage to mtDNA means that the assumptions of mtDNA selective neutrality required are violated. Hurst & Jiggins (2005) discuss the implications of this for studies using mtDNA. First, the **mtDNA diversity will be affected through selection on the symbiont**, confounding the use of mtDNA diversity in phylogeographic studies of population history and demography. Second, selection maintaining different symbionts in different populations, and the mitotypes associated with them, may lead to **increased estimates of population differentiation**, even if migration occurs between populations. Third, in the case of very closely related species, hybridization can lead to transfer of endosymbionts and associated mitotypes from one species to another; if positive selection occurs for the symbiont in the new species, this can lead to **homogenization of the mtDNA of the two species.** This will make them indistinguishable in phylogenies, attempts to delimit species boundaries or when using mtDNA-based barcoding techniques. It is worth noting that none of these problems are limited to coccinellids: Hurst & Jiggins point out that symbiotic or parasitic inherited endosymbionts are very common in arthropods, meaning problems in the use of mtDNA markers may be extremely widespread.

Until now, much of the work using mtDNA in coccinellids has been phylogenetic (2.6.2.2). Most of the species in these phylogenies are too distantly related for there to be serious problems in the construction of a phylogeny. In most known cases, even sibling coccinellid species do not produce viable, fertile offspring (e.g. Zaslavskii 1963, Sasaji 1980, Ireland et al. 1986, Kobayashi et al. 2000, but see Sasaji et al. 1975); consequently cases of mtDNA introgression are likely to be very rare. Nonetheless, a lack of mtDNA neutrality could compromise calculations of times since species diverged based on the assumption of a neutral molecular clock (e.g. Palenko et al. 2004). The implications for population genetic and phylogeographic research of using mtDNA as a marker are more serious. However, allozymes or, more recently, microsatellites have generally been preferred to mtDNA for such purposes in studies of the Coccinellidae (see 2.6.2.3), and although mtDNA has been used in some studies (e.g. Blekhman et al. 2010), this is often in concert with additional nuclear molecular genetic data (e.g. Marin et al. 2010, C.E. Thomas et al. 2010). As our knowledge of the coccinellid genome increases, the potential of nuclear markers for population and evolutionary studies is becoming better characterized. For example, Jiggins & Tinsley's work suggests that the *g6pd* sequence is a suitable marker for population or phylogeographic studies; an increasing number of nuclear genomic regions have now also been characterized in molecular phylogenetic studies (2.6.2.2).

2.6.1.2 The ITS1 region

The **first ribosomal internal transcribed spacer (ITS1)** region is a non-coding region between the 18S and 5.8S ribosomal RNA genes. It is relatively fast-evolving due to its non-coding nature, and is easily

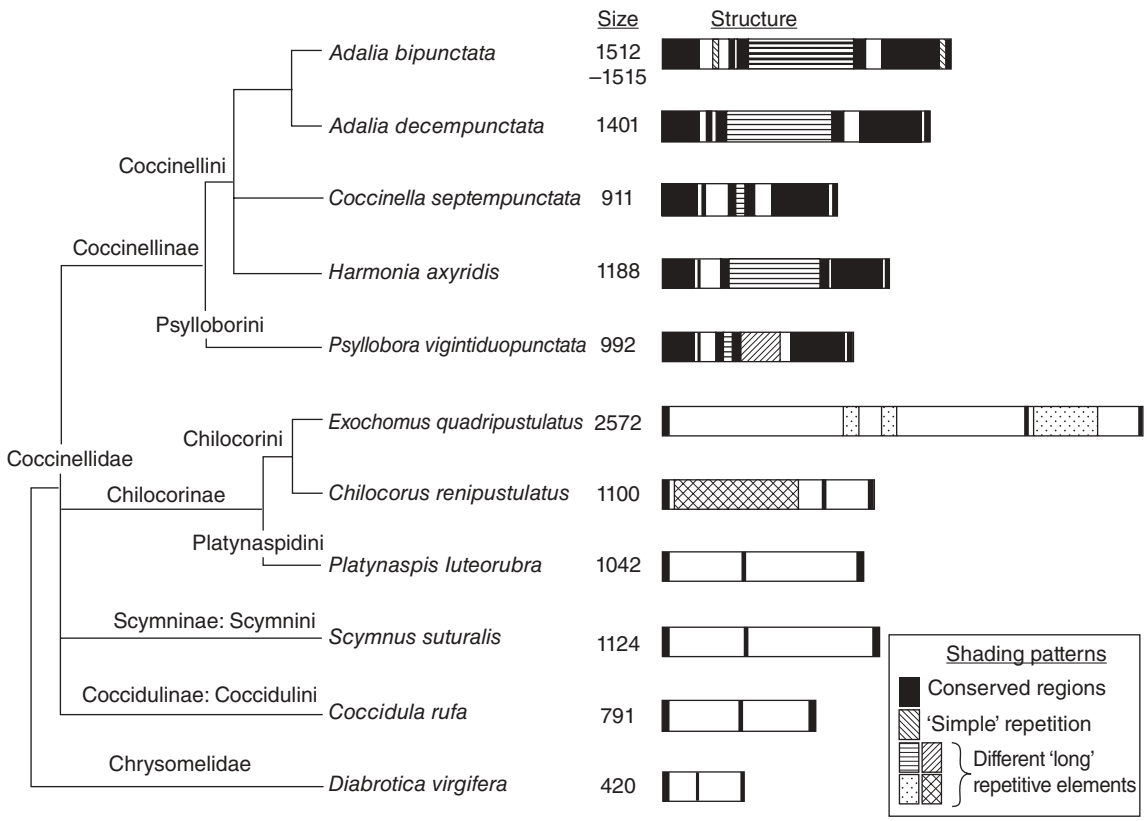

Figure 2.7 Summary of ITS1 sequence data from the Coccinellidae. From left to right, phylogenetic tree of ITS1 sequences, ITS1 sequence size in bp, and the structure of the ITS1 region. Redrawn from Schulenburg et al. (2001), with permission.

amplified from diverse taxa by PCR, due to the conserved gene regions lying on either of it, and the high copy number of rDNA arrays within the genome (Hillis & Dixon 1991). These characteristics have made it of value in phylogenetic and population studies of a diversity of taxa including insects (e.g. Schloetterer et al. 1994, Roehrdanz et al. 2003, Kawamura et al. 2007). The high number of copies of rDNA scattered across the nuclear genome of organisms means that heteroplasmy can occur (i.e. different rDNA copies have different sequences, e.g. Vogler & DeSalle 1994, Harris & Crandall 2000). Nonetheless, many taxa are believed to exhibit a high degree of rDNA copy homogeneity, as a result of concerted evolution across rDNA repeats (Hillis & Dixon 1991).

The ITS1 region has been particularly well studied in the Coccinellidae, by Schulenburg et al. (2001): they sequenced the ITS1 regions of 10 coccinellid species in the subfamilies Coccinellinae, Chilocorinae, Scymninae and Coccidulinae. Both the **ITS1 size and interspecific size variation were extremely large**, with sizes ranging between 791 and 2572 base pairs (bp) (Fig. 2.7), compared to values of less than 550 bp for other polyphagous beetles. Even within tribes size variation remained high, being over 1.6-fold in the Coccinellini and 2.3-fold in the Chilocorini. The **ITS1 sequences of different species were strongly divergent**, and clearly homologous sequences could only be identified across all species at three very small regions of about 20 bp or less. Homology was higher within the subfamily Coccinellinae, comprising six regions of in total about 550 bp: it was possible to use these regions for phylogenetic reconstruction within the Coccinellinae. *Adalia bipunctata* specimens from

Britain, Germany and Russia exhibited minimal inter-individual ITS1 variation, this being limited to three nucleotide changes and two copy number differences in simple repetitive elements of two or three nucleotides. There was no indication of ITS1 heteroplasmy (within-individual variation between different ITS1 copies) in any of the coccinellid species studied.

Six coccinellid species exhibited **large units, of 26–212 bp, that were repeated more than once** within their ITS1 sequences (Fig. 2.7). Within the Coccinellinae, there was a high degree of sequence similarity in a given type of repeated element both intra- and interspecifically. This was not invariably the case for all repeated elements, however: sequence divergence varied both intraspecifically and, when elements were homologous, interspecifically. Schulenburg et al. (2001) argue that these long repetitive elements have played a significant role in the evolution of the large size of the coccinellid ITS1 region and the interspecific size variation exhibited by coccinellids. The repetition observed indicates that the evolution of new repeats is a common occurrence in the coccinellid ITS1 region, while a high level of sequence divergence in homologous repetitive elements means that many older repetitions may not be detected due to the effects of high nucleotide substitution rates. Schulenburg et al. suggest that the observed interspecific size variability in the ITS1 region potentially makes it an ideal molecular marker for coccinellid species in ecological studies (see also 2.6.2.1).

2.6.2 Molecular studies of coccinellid biology

Molecular studies now play a considerable role in studies of coccinellid ecology and evolutionary biology, and have already provided significant insights into the evolutionary history of members of the Coccinellidae, their population structure and behaviour. The areas where molecular studies have been or are likely to be of greatest value can be broadly divided into four areas: (i) molecular identification of species, populations or strains; (ii) phylogenetic studies; (iii) studies of population genetics and phylogeography, and (iv) studies of paternity and sperm competition. Clearly there is some overlap between these areas: for example, sequence data from studies aimed at providing molecular identification of coccinellid species may often be suitable for use in phylogenetic work (cf. Hajibabaei et al. 2007). A number of different methodological approaches have been used, which are summarized in Table 2.2, although this is not an exhaustive list of the potential techniques available. The reader is referred to textbooks by Avise (2004), Beebee and Rowe (2008) and Freeland et al. (2011) for further background on the use of molecular genetic techniques in ecological and evolutionary studies.

2.6.2.1 Species, population and strain identification

Markers specific to species, populations or strains possess great potential in ecological work. Species-specific markers could be used to identify problematic life-history stages such as eggs or first instar larvae, which may otherwise require further rearing for unambiguous identification (Schulenburg et al. 2001). They are also likely to be of particular value in gut content analysis of potential predators of ladybirds, to identify ladybird prey consumed in the field; in particular field studies of intraguild predation may benefit from this approach (Gagnon et al. 2005, Weber & Lundgren 2009; Chapter 10). Population- or strain-specific markers could potentially have utility in the monitoring of the fate of released strains in an area where a natural population of the same species is already present, in competition experiments between strains or in the monitoring of multiple laboratory colonies with different origins for cross-contamination (Roehrdanz 1992, Roehrdanz & Flanders 1993). Although as yet there has been relatively little work published using genetic markers any of these ways, a number of studies have focused on the methodological approaches that would be most appropriate for such work.

Early work showed that both the **Restriction Fragment Length Polymorphism (RFLP)** and **Random Amplification of Polymorphic DNA (RAPD)** methods could be used to generate species-specific electrophoretic banding or fragmentation patterns (Roehrdanz 1992, Roehrdanz & Flanders 1993). RFLP analysis was carried out on a PCR-amplified 1200–1300 base pair mitochondrial region spanning parts of the 12S and 16S rDNA genes and the region between them. Using this method it was possible to distinguish *C. septempunctata*, *C. transversoguttata*, *Hip. variegata* and *P. quatuordecimpunctata* (Roehrdanz 1992). The proportion of shared restriction fragments varied from 97% between the congeneric *C. septempunctata* and

Table 2.2 Coccinellid studies using molecular genetic markers. Studies marked with an asterisk were preliminary studies that demonstrated that a particular methodological approach was suitable for a given use in coccinellid studies; however the method was not further utilized in such work.

Methodological approach	Description	Uses in coccinellid studies	References
Isozyme/ allozyme analysis	Variants of the same enzyme are separated by gel electrophoresis; they are visualized by adding the specific substrate with which the enzyme reacts and a stain specific to the product produced when the enzyme and substrate are present together. Allozymes are enzyme variants that are the product of a single genetic locus; isozymes are enzymes that that perform the same reaction, but occur at multiple loci.	Phylogenetics and phylogeography (species delimitation)	Sasaji & Ohnishi 1973b; Kuboki 1978; Sasaji & Nishide 1994
		Analysis of population genetic structure	Eggington 1986; Krafsur et al. 1992, 1995, 1996a, 1996b, 1997, 2005; Steiner & Grasela 1993; Coll et al. 1994; Krafsur & Obrycki 1996; Obrycki et al. 2001
		Paternity analysis	Ransford 1997
Restriction Fragment Length Polymorphism (RFLP) analysis	Detects genomic variation by cutting up the genome using restriction enzymes and using electrophoresis to examine the size of the resulting fragments. Polymorphisms at restriction enzyme cutting sites, as well as DNA sequence length polymorphisms, result in differing fragment lengths which can be separated using electrophoresis.	Species identification	Roehrdanz 1992*
		Analysis of population genetic structure	Haddrill 2001*
		Paternity analysis	Haddrill 2001*
Amplified Fragment Length Polymorphism (AFLP) analysis	Total genomic DNA is cut up using restriction enzymes and adaptor sequences joined to the ends of the resulting DNA fragments. A subset of the fragments is amplified using PCR with primers complementary to the adaptor and part of the fragment. The amplified fragments are visualized by electrophoresis.	Analysis of population genetic structure	Haddrill 2001*
		Paternity analysis	Haddrill 2001*
Random Amplification of Polymorphic DNA (RAPD) analysis	Uses PCR with short, relatively unspecific primers (typically 8–12 nucleotides) is to amplify small (300–2000 base pair) fragments of genomic DNA which are then resolved by electrophoresis. The resultant patterns of amplified DNA fragments, from a number of such primers, can be used to generate a characteristic DNA profile for a species, population or strain of an organism.	Species, population and strain identification	Roehrdanz 1992*; Roehrdanz & Flanders 1993*

Table 2.2 (*Continued*)

Methodological approach	Description	Uses in coccinellid studies	References
Microsatellite analysis	Uses tandemly repeated sequences of typically one to six nucleotides (e.g. CACACACA . . .), which are amplified using PCR primers designed to bind either side of the repeated sequence. The number of times the sequence is repeated, and thus the length of the microsatellite region, is frequently highly variable intraspecifically, making microsatellites suitable markers for population genetic analysis; in combination they can give a near-unique individual specific DNA fingerprint, making them of value in studies of individual reproductive success and paternity.	Species identification (single locus)	A. Thomas et al. 2010
		Analysis of population genetic structure or phylogeography	Haddrill 2001; Haddrill et al. 2008; Lombaert et al. 2010
		Paternity analysis	Haddrill et al. 2008
Inter-Simple Sequence Repeat (ISSR) analysis	Uses length polymorphism in genomic regions between microsatellite loci, which are amplified using primers complementary to two neighbouring microsatellites. As ISSRs are often more conserved than microsatellites they are used in phylogeographic studies, rather than studies of individuals.	Phylogeography (species delimitation)	Marin et al. 2010
Use of DNA sequences	PCR and sequencing of specific gene regions; the sequence is used directly in analyses	Phylogenetics	Howland & Hewitt 1995; Kobayashi et al. 1998; Palenko et al. 2004; Hunt et al. 2007; Robertson et al. 2008; Weinert 2008; Giorgi et al. 2009; Magro et al. 2010; Sloggett et al. in press
		Phylogeography (including species delimitation)	Kobayashi et al. 2000, 2011; Blekhman et al. 2010; Marin et al. 2010; C.E. Thomas et al. 2010
Use of specific PCR primers	Target-specific primers designed on the basis of sequence data. PCR of samples, followed by electrophoresis. Presence of a PCR amplification band indicates target DNA present.	Species identification	Gagnon et al. 2005*; Harwood et al. 2007
DNA microarray analysis	Labelled amplified DNA is simultaneously probed with many distinct gene- or organism-specific DNA oligonucleotides arranged together as microscopic spots on a solid matrix. Hybridization to the probes is detected through labelling, and conclusions drawn on the basis of which probes have hybridized to the DNA.	Species identification	Pasquer et al. 2009*

C. transversoguttata to 25% between the *Coccinella* species and *Hip. variegata*, suggesting a phylogenetic signal. The RAPD method was able to distinguish not only the four species, but intraspecifically between laboratory colonies with widely different geographic origins. Combinations of three or four specific primers were sufficient to completely differentiate the colonies, although many more primers were tested to find the most suitable (Roehrdanz 1992, Roehrdanz & Flanders 1993). With RAPD markers, as with RFLPs, although *C. septempunctata* and *C. transversoguttata* were distinct, they were more similar than *C. septempunctata*, *Hip. variegata* and *P. quatuordecimpunctata* were to each other. Although the authors suggested a number of uses for such markers, neither the RAPD nor RFLP methods appear to have been used in any practical application. The use of RAPD markers has in general declined, in large part due to the relatively low reproducibility of results with small variations in initial PCR conditions; both methods are now considered somewhat outmoded by comparison with the use of DNA sequences, either directly or, more often, to design PCR primers or probes specific to the entity being studied.

The direct use of DNA sequences for species identification has found its fullest expression in the idea of **DNA barcoding**, whereby short standardized sequences are used to distinguish species (Hebert et al. 2003, Hajibabaei et al. 2007). The barcode sequence of an unknown specimen, amplified using conserved primers, is compared to a library of such sequences obtained from specimens of known species identity; the specimen can then be identified if its sequence closely matches one in the library. A 650 bp fragment of the 5′ end of the mitochondrial COI gene has been adopted as a universal standard barcode region for animals. A number of such barcodes already exist for coccinellids in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/); however, coccinellid barcodes have not yet been used in ecological studies, although it seems likely they will be used in the future. The reservations of Hurst & Jiggins (2005) about the potential for mtDNA to cross species boundaries must be borne in mind if very closely related species are studied using COI barcodes, although the risk is quite small (2.6.1.1). A potential alternative to using mtDNA barcodes is the use of the nuclear ITS1 sequences (Schulenburg et al. 2001; 2.6.1.2). These genomic regions exhibit high evolutionary rates in coccinellids, making them species specific; furthermore they appear to be relatively homogeneous, both within and between individuals of a species.

Sequence data from identified target individuals can also be used to design **specific PCR primers or probes**. Gagnon et al. (2005) designed four sets of **species-specific primers** to amplify parts of the ITS1 regions of *C. septempunctata*, *P. quatuordecimpunctata* and *Har. axyridis* and the COI region of *Coleomegilla maculata*. Overall, the species-specificity of the primers was high, although there were two cases of cross-priming to other coccinellid species. After electrophoresis of the PCR product, it was possible to detect a PCR band from a target species' DNA when in a 10% mixture with that of another species, mimicking the proportion of prey DNA expected to occur in an intraguild predator. PCR of a **single microsatellite marker** specific to *A. bipunctata* has recently been used to identify *Har. axyridis* intraguild predation of this species (A. Thomas et al. 2010).

Microarray chips with COI DNA probes were used by Pasquer et al. (2009) to differentiate a number of different beneficial insects, including the coccinellids *A. bipunctata*, *C. septempunctata*, *Chilocorus nigritus*, *Cryptolaemus montrouzieri* and *Rhizobius lophanthae*. The chip, carrying a number of distinct **species-specific probes**, was exposed to fluorescence-labelled, PCR-amplified DNA from a single insect. The insect species was identified by which probes **hybridized** to the DNA. Pasquer et al. used at least four different probes for each beneficial insect species they wished to identify, to avoid a higher error rate consequent on the use of single probes (through false positives or negatives). The authors point out that, particularly for a species like *A. bipunctata* with a very high level of polymorphism in the COI gene (see 2.6.1.1), reliable identification can only be accomplished through the use of multiple probes. It seems likely that, as our knowledge of coccinellid genomes and markers increases, microarray chips will be increasingly used to identify not only species, but specific populations and strains as well.

It is worth noting that the **methods described in this section for coccinellids can be equally applied to the organisms with which they interact**. Laboratory- and field-based work already exists in which molecular techniques have been used to identify or quantify **prey in coccinellid diets**, (Weber & Lundgren 2009; Chapter 10). A less obvious

application of molecular genetic methods is as a means to identify and study **coccinellid symbionts, pathogens and parasites**. Molecular methods are routinely used in the identification of male-killing endosymbionts (Chapter 8), but hold great and as yet untapped potential as an investigative tool with which to study other parasitic and pathogenic organisms of coccinellids, which in many cases have been poorly studied.

2.6.2.2 Phylogenetics (see also Chapter 1)

Phylogenetic studies of the Coccinellidae, like all such studies, originally utilized phenotypic characteristics, particularly **morphology**, and until recently our view of coccinellid phylogeny and consequent taxonomy has been almost exclusively morphologically based (e.g. Sasaji 1968, Fuersch 1996, Kovar 1996). The advent of molecular methods has not only made it possible to re-address questions about overall broad scale relationships between subfamilies and tribes, but also to address in detail smaller scale questions about the exact relationships between closely related species within the larger groupings. Although a number of methods have been shown to provide phylogenetic information, including studies of isozyme variation in closely related species (Sasaji & Ohnishi 1973b, Kuboki 1978, Sasaji & Nishide 1994), RFLP and RAPD studies (Roehrdanz 1992, Roehrdanz & Flanders 1993), the vast majority of molecular phylogenetic studies of coccinellids have been based on **DNA sequence data**. They include studies addressing the monophyly of the Coccinellidae and its position within the Coleoptera (Howland & Hewitt 1995, Hunt et al. 2007, Robertson et al. 2008), relationships between coccinellid subfamilies and tribes (Giorgi et al. 2009, Magro et al. 2010) and the phylogeny of closely related genera or species (Kobayashi et al. 1998, Palenko et al. 2004, Sloggett et al. in press).

Studies focused on **closely related genera or species** have been exclusively limited to mitochondrial COI sequence data. This sequence appears to exhibit the optimum level of divergence for phylogenetic analysis at the level of distinct species within genera or very closely related genera (Kobayashi et al. 1998, Palenko et al. 2004), although the issue of mtDNA linkage to endosymbionts must be considered for sibling species (Hurst & Jiggins 2005; 2.6.1.1). Above this taxonomic level, the phylogenetic utility of the sequence declines. Mawdsley (2001) found that a COI sequence phylogeny of *C. septempunctata*, *A. bipunctata* and *Calvia quatuordecimguttata* from the Coccinellinae and *E. quadripustulatus* from the Chilocorinae varied markedly in its topology depending on the outgroup and tree-searching algorithm used. Sloggett et al. (in press) in comparing a number of genera of Coccinellini, found that the inferred amino acid sequences were more informative than DNA sequences for reconstruction of the relationship among genera; DNA sequence information was likely confounded by a high degree of homoplasy (i.e. identical bases in different species not occurring by common descent). In attempting a COI-based phylogeny of the Coleoptera, Howland and Hewitt (1995) found sequence variation within beetle families in some cases to be a great as that between beetle families, and concluded that a more conserved sequence was necessary. Studies of the phylogenetic relationships of the whole family Coccinellidae or of subfamilies or tribes within the Coccinellidae have used more slowly evolving sequences, particularly 18S ribosomal DNA (18S rDNA) and other rDNA regions (Hunt et al. 2007, Robertson et al. 2008, Giorgi et al. 2009, Magro et al. 2010); unlike studies of close relatives they have also all used more than one gene region.

DNA sequence-based studies of the **relationships of the Coccinellidae to other beetle groups** all agree that the Coccinellidae is a monophyletic group. Studies at **the subfamilial and tribal level** within the Coccinellidae have indicated that some traditionally recognized groups are paraphyletic, that is they do not include all the descendants of the most recent common ancestor and thus do not form natural groups. Giorgi et al. (2009) suggest that this is the case for the Sticholotidinae, Chilocorinae, Scymninae and Coccidulinae, although the Coccinellinae was supported as monophyletic. Their conclusions are broadly mirrored by Magro et al. (2010), who also considered the Epilachninae as a paraphyletic group. In the near future we are likely to have a much improved view of the relationships at all taxonomic levels within the group, which is currently being intensively investigated as part of the US National Science Foundation Partnerships for Enhancing Expertise in Taxonomy (PEET) and Assembling the Tree of Life (AToL) initiatives. For this work a broad range of gene regions are being used (mitochondrial 12S rDNA, 16S rDNA, COI and COII; nuclear 18S rDNA, 28S rDNA and histone

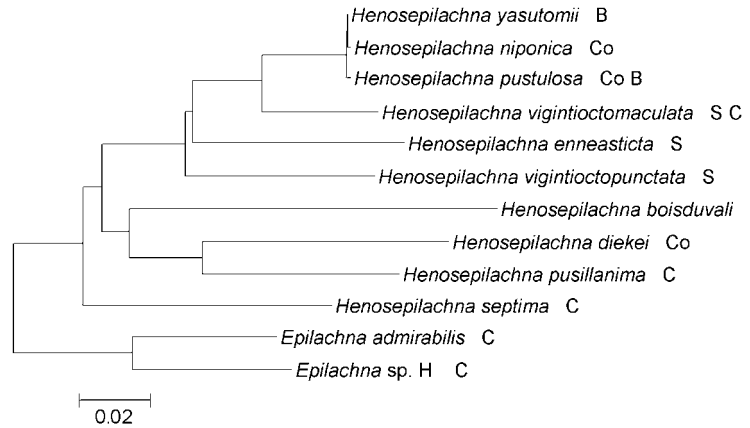

Figure 2.8 Phylogenetic tree of twelve Epilachninae, showing plant host shifts in this phytophagous group. The tree was constructed using 1000 bp cytochrome oxidase I (COI) mitochondrial sequences. Scale bar = 0.02 substitutions per nucleotide for tree branch lengths. Host plants: B, Berberidaceae; Co, Compositae; S, Solanaceae; C, Cucurbitaceae. Redrawn from Kobayashi et al. (1998) taking into account revised taxonomy, with permission.

H3 genes). Some of these regions are slowly evolving (18S and 28S), whereas others are fast evolving regions (12S, 16S, COI, COII): in this way it is hoped to provide complementary data on both older and more recent evolutionary events (J.A. Giorgi & J.V. McHugh, pers. comm.).

There are also a number of phylogenetic studies that have not aimed purely at providing phylogenetic clarity as an end in itself, but rather have aimed to address a variety of questions about the **evolution of other characteristics in a phylogenetic context** (Kobayashi et al. 1998, Weinert 2008, Giorgi et al. 2009, Sloggett et al. in press). For example, Giorgi et al. (2009) have addressed how **food preferences** have evolved within the Coccinellidae by mapping feeding preferences onto a DNA sequence phylogeny of subfamilies and tribes. From this they were able to conclude that coccidophagy is ancestral, with most other feeding preferences having evolved directly from coccidophagy. At a lower phylogenetic level, Kobayashi et al. (1998) used COI sequences to examine host plant shifts in the Epilachninae (Fig. 2.8). Sloggett et al. (in press) used COI sequences to construct a phylogeny of Coccinellini bearing different **chemical defences**. Using the phylogeny, they were able to show that the myrmecophilous *C. magnifica*, which is unusual amongst *Coccinella* species in possessing convergine–hippodamine chemical defences, is derived within the *Coccinella* genus and is not in fact more closely related to other species with the same alkaloids, as had previously been suggested. They concluded that this change of chemical defence type was linked to the preference of *C. magnifica* for living with ants, which is also unique within the genus.

An interesting further development of the phylogenetic technique is provided by Weinert (2008), who studied **evolution of the endosymbiotic bacteria *Wolbachia* and *Rickettsia***, which can both cause male-killing in coccinellids. In her work, Weinert utilized DNA sequences and consequent phylogenies of both ladybird hosts and the bacteria that they harbour. Thus, she was able to show that both types of bacteria have invaded the Coccinellidae multiple times and that bacterial infection of new coccinellid species is most common when they are closely related to the ancestral host (i.e. decreases with increasing genetic distance). Nonetheless, within individual *Rickettsia* and *Wolbachia* clades that were exclusively coccinellid symbionts, only the *Rickettsia* clade exhibited close congruence with host phylogeny (Fig. 2.9).

2.6.2.3 Population genetic and phylogeographic studies

Population genetic studies are concerned with allele distributions and change, and **phylogeographic**

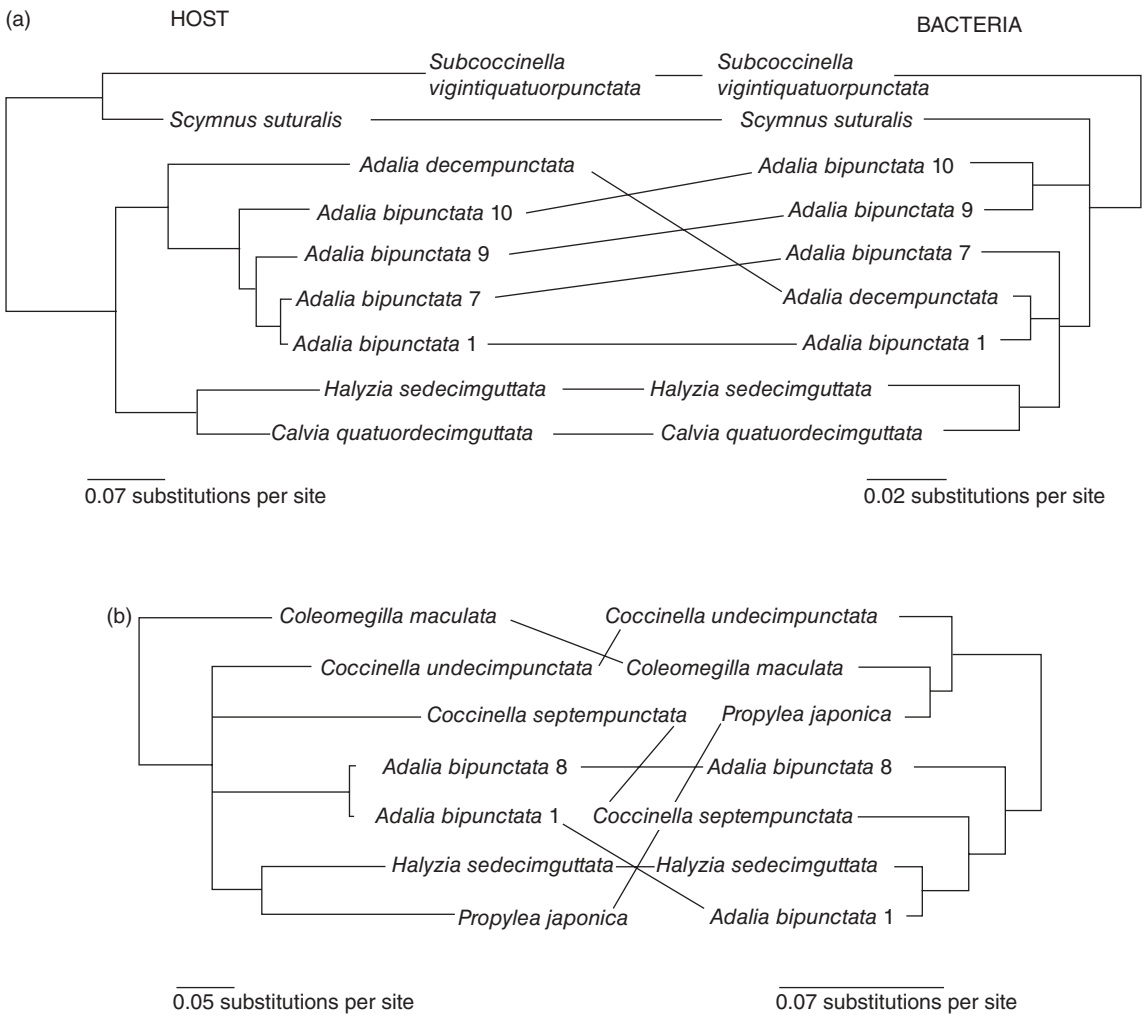

Figure 2.9 Tanglegrams of coccinellid host phylogeny with (a) an individual clade of *Rickettsia* coccinellid endosymbionts and (b) an individual clade of *Wolbachia* coccinellid endosymbionts. Scale bars represent substitutions per nucleotide for tree branch lengths. Connecting lines are drawn to connect bacteria strains to the host species they infect (or mitochondrial haplotypes within the host species *Adalia bipunctata*). While members of the *Rickettsia* clade exhibit close congruence with host phylogeny, members of the *Wolbachia* clade do not; consequently, a higher amount of horizontal transmission between more distantly related hosts can be inferred for members of the *Wolbachia* clade. Redrawn from Weinert (2008), with permission.

studies are concerned with the role that historical and biogeographical factors play in the current genetic structure of populations and species. Molecular studies across and between populations generally contain overlapping population genetic and phylogeographic components and therefore they are considered together here.

Although the majority of pre-molecular population genetic and evolutionary studies were concerned with colour pattern polymorphism (2.4), work away from this subject included chromosomal studies (2.3.4), and investigations of inbreeding (Lusis 1947) and hybridization (reviewed in Komai 1956). Arguably the most significant studies in the context of population genetic

architecture were those of Lusis (1947), who investigated **inbreeding** in *A. bipunctata*. This species was found to exhibit very high levels of **inbreeding depression** consistent with a high number of deleterious recessive alleles occurring naturally in the heterozygous form. Similar results were obtained more recently by Morjan et al. (1999) for *P. quatuordecimpunctata* introduced to North America. The results suggest that mating between close relatives in the field is rare. Hurst et al. (1996), addressing this issue, showed that the low proportion of developed but dead eggs in field-collected *A. bipunctata* egg clutches was more similar to that of outbred than inbred clutches from the laboratory, the latter exhibiting very high egg mortality. Unfortunately, Hurst et al. did not consider the effect of multiple mating and sperm mixis (2.6.2.4). With multiple paternity of field-collected egg clutches, clutches would not be expected to exhibit equivalent high mortality to those from single inbred laboratory matings of virgin beetles, even if inbred matings do sometimes occur naturally.

More recently, the population genetics of *A. bipunctata* populations in Cambridge in the UK and Paris in France have been investigated using 10 polymorphic **microsatellite** loci (Haddrill 2001). This study found a **deficit of heterozygotes** at a number of loci examined in both populations, suggesting population substructuring or inbreeding. Estimates of inbreeding were particularly high, with mean relatedness in the two populations being 0.31 and 0.37, i.e. greater than that between half siblings. The results are particularly curious, given the earlier evidence of high inbreeding depression in *A. bipunctata*. A possible explanation is that the ladybirds sampled were part of the second generation that year: their parents may not have had to move too far to find food and mates after emergence, leading to matings between related individuals. It is worth noting that Hurst et al.'s (1996) inbreeding study, described above, used first generation eggs to estimate inbreeding in the field. Estimates of *A. bipunctata* **genetic differentiation** between the populations were low but significant, comparable to those obtained for *A. bipunctata* in earlier allozyme studies (Eggington 1986, Krafsur et al. 1996a). This is consistent with a relatively high level of gene flow between populations of this species, as well as with a large effective population size.

Prior to Haddrill's (2001) study, the majority of work on coccinellid genetic architecture had used **allozymes**. Most notably, in a long series of papers, Krafsur, Obrycki and co-workers described extensive allozyme-based population genetic analyses of native and introduced aphidophagous ladybirds of North America: these include *Hip. convergens*, *Col. maculata*, *A. bipunctata*, *C. septempunctata*, *P. quatuordecimpunctata*, *Hip. variegata* and *Har. axyridis* (Krafsur et al. 1992, 1995, 1996a, b, 1997, 2005, Krafsur & Obrycki 1996, Obrycki et al. 2001). These studies, which used a large number of putative allozyme loci (27–52, depending on species), showed that measures of genetic variation were similar to those for other beetles and that measures of population differentiation were generally low. Considered together with ecological studies, work relating to population genetic structure is consistent with populations of aphidophagous coccinellids being **near-panmictic**. This is unsurprising for a group of beetles that frequently move between habitats seeking aphids, as well as dispersing to and from overwintering sites on a yearly basis. While aphidophagous coccinellids might remain in the same place for more then one generation, as suggested by Haddrill for *A. bipunctata*, yearly movements between habitats will ultimately lead to genetic mixing between subpopulations. It is worth noting, however, that the aforementioned studies were all on aphidophagous habitat and dietary generalist species; more sedentary specialists might exhibit a lower degree of genetic mixis (Sloggett 2005). Similarly it is unclear to what extent these results could be extended to ladybirds with other diets, such as coccidophagous or phytophagous species.

A central aim of the allozyme studies was to investigate **genetic diversity in relation to the successful establishment of exotic biocontrol agents**. Measures of genetic diversity were similar for both native and exotic species, leading the authors to conclude that there is no obvious relationship between genetic diversity and successful colonization of new areas. More recent work by Lombaert et al. (2010) has thrown more light on the establishment by exotic ladybirds outside their native range, in a phylogeographic study examining the **origins of invasive *Har. axyridis* populations**. The authors used microsatellites and modern Bayesian computational techniques to compare different colonization scenarios. For each step of the worldwide spread of *Har. axyridis* (spread into eastern North America, western North America, Europe, South America and Africa), the Bayesian analysis gave a highest probability scenario for the

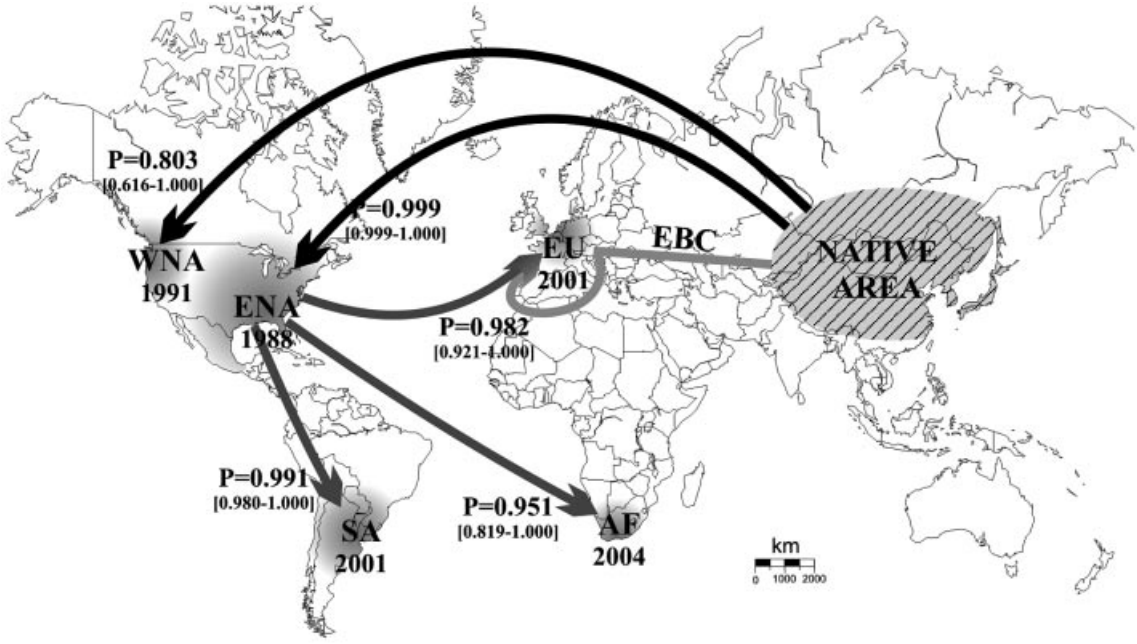

Figure 2.10 The most likely *Harmonia axyridis* invasion routes into eastern North America (ENA), western North America (WNA), South America (SA), Europe (EU) and Africa (AF), based on a study using microsatellites and Bayesian statistics. For each outbreak, the arrow indicates the most likely invasion pathway and the associated posterior probability value (P), with 95% confidence intervals in brackets. The years that invasive populations were first observed are also indicated. EBC is the European biocontrol strain, originally collected in 1982, which probably forms an additional source for European populations along with with ladybirds from eastern North America. From Lombaert et al. (2010), courtesy of the authors.

population origin (Fig. 2.10). The results showed that the first established non-native population, in eastern North America, acted as a 'bridgehead' source for the majority of the other non-native populations that subsequently established (Fig. 2.10). It may be inferred that some evolutionary change occurred in the eastern North American population that made *Har. axyridis* a much more successful colonizer of new regions than before.

Another phylogeographic use of molecular markers is to **delimit cryptic species** within complexes of close relatives that, on morphological grounds, can appear to be a single species. Work in this area has been carried out on phytophagous Epilachninae, where cryptic species can be specialized on different host plants. Very early on in the history of coccinellid molecular studies Kuboki (1978) used isozymes as a way of delimiting species within the *Henosepilachna vigintioctomaculata* complex and examining the phylogenetic relationships between them. More recently, using a combination of breeding work, karyological studies and mitochondrial COI sequence analysis, Kobayashi et al. (2000) were able to show that the species *Henosepilachna* (= *Epilachna*) *vigintioctopunctata* was divided into two cryptic species. Conversely molecular studies can also show that species separated on morphological grounds, are in fact, a single entity. Marin et al. (2010), using crossing experiments, morphological analysis, analysis of COI sequences and **inter-simple sequence repeat (ISSR)** markers, showed that *C. septempunctata*, including the putative *C. septempunctata brucki* and *C. algerica*, were a single species and not a complex. The results from ISSR markers in this work are of particular interest. Due to linkage to endosymbiotic bacteria such as male killers, which are known in *C. septempunctata* (Majerus &

Hurst 1997), mtDNA may be a much less reliable genetic marker for such studies (2.6.1.1).

2.6.2.4 Reproductive success, paternity and sperm competition

Distinctive genetic markers are especially valuable in studies which aim to measure the success of particular strains or individuals in subsequent generations. The most important use of coccinellid genetic markers in this context has been in studying sperm competition; that is, to **deduce patterns of paternity when females mate multiply** and sperm from different males compete to fertilize a female's eggs, as is typically the case for coccinellids (Hodek & Ceryngier 2000; Chapter 3). If males carrying different genetic markers are used in experiments, the offspring can then be genotyped to deduce which male is the father.

Studies of sperm competition and paternity in coccinellids have included the Coccinellini *A. bipunctata*, *A. decempunctata* and *Har. axyridis* and the Epilachnini *Epilachna varivestris* and *Henosepilachna pustulosa* (Webb & Smith 1968, Nakano 1985, de Jong et al. 1993, 1998, Ueno 1994b, Ransford 1997, Haddrill et al. 2008); molecular studies have, however, been limited to *A. bipunctata*. The first studies of *A. bipunctata* were non-molecular, using its **polymorphic colour pattern** as a genetic marker. Virgin non-melanic females (i.e. homozygous recessive for colour pattern) were mated to non-melanic and melanic males (the latter being homozygote or heterozygote for the dominant melanic gene). By rearing the larvae to adulthood and scoring their colour pattern, it was possible to work out which male had fathered the offspring. Initial experiments, in which virgin females were mated to one male of each phenotype, concluded that *A. bipunctata* exhibited almost complete last male sperm precedence (de Jong et al. 1993). In a later series of experiments, however, de Jong et al. (1998) found much greater **variation in the proportion of eggs fertilized** by a melanic male, both when the female had previously been singly or multiply mated to non-melanic males. This was supported by evidence from the field, which showed that matings between heterozygote melanic and non-melanic individuals rarely resulted in a 1:1 proportion of the colour forms in the offspring, as would be expected with last male sperm precedence. In mating treatments consisting of two single matings, de Jong et al. (1998) observed a positive correlation between the proportion of eggs fertilized by the second male and the **ratio of the duration of the second mating relative to the first**. They considered that manipulation of the first male's sperm by the second male was more successful the longer a male mated for; however, they did not discuss in detail by what mechanism sperm displacement occurred.

Colour pattern markers have been also used also to study sperm competition in *Har. axyridis* (Ueno 1994b) and, in the light of that study, to estimate how many males a female mates with in the field (Ueno 1996). However, there are some problems associated with the use of colour pattern markers. It is necessary to carry out time- and resource-intensive rearing of large numbers of offspring so that the colour patterns can be scored: de Jong et al. (1998) reared a total of 3108 *A. bipunctata* eggs to adults in their laboratory experiments and a further 5077 individuals from eggs obtained from field-collected adults. During the rearing process, it is important to ensure that there is no potential for selection to occur between larvae of different genotypes, which could potentially lead to biased estimates of morph frequencies: this in itself makes the rearing process more arduous, requiring the experimenter to avoid problems commonly encountered in the mass rearing of ladybirds such as crowding, food shortage or cannibalism. Furthermore, additional rearing may be required to verify parental genotypes: de Jong et al. (1993, 1998) had to mate all experimental melanic males to additional non-melanic virgin females, and rear resulting offspring to adult to verify whether the males were homozygote or heterozygote melanics.

Noting these types of problem, Ransford (1997) used an **allozyme**-based approach to study sperm competition in *A. bipunctata*. He used four allozyme variants at the isocitrate dehydrogenase 1 (*Idh1*) locus, of which one was common (92%) and three were rare (≤5%). The genotypes of individual ladybirds were deduced using electrophoretic analysis of reflex blood extracts, leaving the ladybirds alive for breeding and mating experiments. Neonate offspring were used whole to score their genotype. Therefore it was not necessary to rear them through to adult, as with colour pattern markers. Matings of males heterozygous for the different allozyme variants to homozygous virgin females produced 1:1 distributions of the paternal allozyme variants in the offspring, indicating that the variants were equivalent in their fertilization success. Two experiments on sperm precedence were carried

out. The first used homozygous laboratory-bred virgin females mated to two males homozygous for different alleles. The second used males with rare alleles mated to field collected, non-virgin females: in this case the rarity of the experimental male alleles meant that many field-collected females had not mated previously with such males. In both experiments very **high variation in the proportion of offspring sired by the last-mating male was found**; with the only correlate of this variation being **mating duration**. Interestingly, among offspring not sired by the last mating, the relative precedence of a male mating over earlier matings persisted even after a further mating had occurred. Ransford's work suggests that sperm mixing occurs, and that the relative abundance of the sperm of different males determines how likely they are to fertilize the eggs. Although Ransford's allozyme study of paternity represents a significant advance over colour pattern-based paternity studies, a substantial amount of laboratory breeding was still required to produce *A. bipunctata* males homozygous for low frequency *Idh1* alleles, which are exceedingly scarce naturally.

Also studying *A. bipunctata*, Haddrill et al. (2008) used three extremely variable **microsatellite** loci to study mating frequency and paternity. All larvae from two egg clutches laid by individual field-collected females were genotyped. The females were also genotyped for exclusion purposes and the number of paternal microsatellite alleles present in each clutch determined for the three loci. Using this method, Haddrill et al. were able to estimate that individual *A. bipunctata* egg clutches were fathered by 2.5–3.5 males. Additionally, laboratory paternity studies indicated that **all males mating with a female then father at least a few offspring**; this indicates that females possess a limited ability, if any, for post-copulatory mate discrimination, for example by rejecting the sperm of certain males. Like other researchers, Haddrill et al. also found that **longer matings resulted in higher paternity of subsequent egg clutches**.

The majority of work on *A. bipunctata* is consistent with the conclusions of Ransford (1997), that *A. bipunctata* exhibits a mechanism of **instantaneous mixing during sperm displacement**. As a male transfers sperm he will displace earlier sperm in direct proportion to their instantaneous occurrence in the spermatheca of the female, including any he has already transferred (Parker et al. 1990). Consequently the more sperm a male transfers, the higher the proportion of subsequent eggs he fertilizes. In *A. bipunctata* and *A. decempunctata*, males that mate for longer transfer more sperm due to a mating mechanism in which individual spermatophores are transferred cyclically, with males undergoing up to three cycles in a single mating. Males that mate for longer undergo more cycles and transfer more spermatophores (Ransford 1997). Although the cyclical *Adalia* mating mechanism does not appear to be shared by most coccinellids (e.g. see Obata & Johki 1991), at least in *Har. axyridis* the mechanism of sperm displacement appears to be similar (Ueno 1994b). The availability of molecular methods makes it much easier to extend studies to coccinellids that are not polymorphic for colour pattern; thus in the future the generality of instantaneous mixing during sperm displacement across the Coccinellidae can be established. Because microsatellites provide a unique genetic fingerprint for each individual studied, they possess great potential for investigating multiple mating in coccinellids and to compare levels of promiscuity across individuals, populations and species. They are particularly promising markers for studies of populations from the field (cf. Haddrill et al. 2008).

2.7 CONCLUSIONS

Coccinellid genetic studies possess as much potential for the future as they have manifested in the past. In particular, due to the wealth of ecological data already available on coccinellids, the high visibility of the beetles in the field and their relative ease of maintenance in the laboratory, coccinellids are well suited for studies uniting both ecological and molecular approaches. This has already been shown, for example, in work on male-killing and on sperm competition, but as more genomic information on coccinellids becomes available, the approach is likely to be extended to other areas (Chapter 12).

This potential is best illustrated by reference to that genetic phenomenon which remains the best known for the Coccinellidae, colour pattern polymorphism. In spite of the vast body of knowledge on the inheritance of coccinellid colour patterns and on the selective pressures acting on them, as yet **we know very little about the genetic and developmental pathways that underlie colour pattern production**. Such knowledge would undoubtedly enhance our understanding of colour pattern polymorphism and its

evolution, especially when integrated with what is already known from work in the field and laboratory. Coccinellid colour patterns, through studies of their variability, inheritance and maintenance, were of great importance to our understanding of evolution in the twentieth century. Molecular studies of their genetics and development could ensure that they remain at the forefront of evolutionary research in the twenty-first.

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This chapter is dedicated to the late Professor Mike Majerus (1954–2009). Through his own research, and through his influence on many of his students who have gone on to study various aspects of ladybird genetics themselves, Professor Majerus made an enormous contribution to our current understanding of ladybird genetics. This is reflected in the large number of papers cited here that were authored or co-authored by himself and/or his students.

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