Range expansion, population structure and life history of the small red-eyed damselfly *Erythromma viridulum* (Charpentier, 1840) at its northwest margin.

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Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

September, 2007

Abstract

Non-native species can alter ecosystem processes, act as vectors of diseases and diminish biological diversity. The threat posed by non-native species is expected to increase as a result of global climate change. Climate change has already been linked to poleward shifts in range across a wide range of taxa. The small red-eyed damselfly *Erythromma viridulum* has expanded its range in a north westerly direction across northern Europe at remarkable rate over the last 30 years and in 1999 became the first recorded example of a migrant damselfly to colonise the UK. Since then it has expanded its range in the UK by and average of 32 km per year and is currently distributed between Devon and East Yorkshire and as far inland as Warwickshire. A partial genomic microsatellite library enriched for CA microsatellites was constructed for *E. viridulum* and generated 10 polymorphic loci, which typically have short stretches of uninterrupted repeat units and low levels of expected heterozygosity. These loci were used to conduct a population genetic study of *E. viridulum* across its UK range and northern Europe. Samples were collected 2002-2006 from 28 sites in the British Isles and 11 sites from continental Europe. *E. viridulum* showed a slight but clear trend of reduced genetic diversity from east to west following its invasion path from continental Europe into the UK. Overall genetic differentiation among the UK and continental European populations was considered moderate or low. There was a lack of local genetic isolation by distance caused by high levels of gene flow and recent establishment of populations. There was genetic differentiation between southern and eastern UK populations indicative of different putative source populations, or an extreme founder effect. The source of the eastern UK populations was within the range sampled across continental Europe, however the southern UK source remains unknown. There was little difference in the patterns of genetic structure over time and the small differences observed were ascribed to discontinuities in the invasion process. In a larval growth study *E. viridulum* was found to be predominantly semivoltine in the UK, though under favorable environmental conditions some individuals may complete development in one year.

Acknowledgements

This thesis could not have been written without the assistance of many people and I have much pleasure in acknowledging their help. In particular I would like to thank the inspirational Phill Watts for his tireless enthusiasm and effort over the course of this thesis. I am heavily indebted to the help provided by Dave Thompson whose vast experience and guidance been invaluable. Special thanks must also go to Steve Cham, Graham French and the British Dragonfly Recording Network on which much of this thesis is dependent.

I would like to thank the following people for their help in the collection of samples and assistance around the lab: Lorna Bousfield, Caroline Daguet, Dave Dana, Laurent Gavory, John Horne, Samantha Jacobs, Sebastien Legris, Paul Richardson, Robby Stoks, Frank Sühling, Cédric Vanappelghem, Peter De Knijff, Anthony Nixon, Drew Wolfenden, Chris Lowe, Harry Noyes, Tom Heyes, Errol Newman and Arjen Van't Hof. Big thanks also to the people I've met whilst doing fieldwork and who have let me onto their land, especially Chris Slatcher at Wrest Park. Thanks to NERC for funding this PhD.

Finally I'd like to recognise the outside inspiration has come from the relentless nagging of Mum and Dad, Katie and all my other friends and family.

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Thesis plan

This thesis is concerned with the first successful invasion and establishment of a damselfly to the British Isles in living memory. The species concerned is *Erythromma viridulum* (Charpentier, 1840) known in English as the small red-eyed damselfly. Its arrival in Britain and its rapid spread in southern England are discussed in the context of global climate change from a population genetical perspective. The aims of this thesis are (1) to document the range expansion of this species in recent years, (2) measure levels of genetic variability in the UK and Europe over a number of years, (3) analyse the genetic data for spatial and temporal structure, (4) investigate life history traits of this species in the UK and (5) bring together this data to make inferences about the nature of the invasion of the UK. The first part of the introductory chapter describes in more detail how the thesis is arranged and the topics covered chapter by chapter.

The second part of the introductory chapter brings together the current knowledge of the range expansion and biology of *Erythromma viridulum* in the light of recent climate change. It describes the north-westerly range expansion of this species in Europe over the last 30 years, including the colonisation of the British Isles. Detailed records from the British Dragonfly Recording Network on the distribution of this species in the UK since its arrival in 1999 are presented and discussed. Information from this database is further used to illustrate the rate of range expansion and the phenology of *E. viridulum* in the UK. Patterns of colonisation in the UK and Europe are described and used to hypothesise about future range distribution scenarios. This chapter also covers the behaviour and ecology of *E. viridulum*, including its physical and environmental requirements and preferences. Critical factors such as habitat type, water chemistry and aquatic macrophytes associated with this species are reported.

Chapter 2 is a methodological chapter which describes the isolation and characterisation of a panel of ten microsatellite loci for *E. viridulum*, used in Chapters 3 and 4 for population genetic purposes. Microsatellites are popular and convenient population genetic markers; however they must be isolated

de novo from the target species. The chapter details the processes used to create a partial genomic library enriched for CA microsatellites including DNA extraction, microsatellite capture, ligation into *E. coli*, screening for microsatellites, and the design and characterisation of primers. Loci that proved polymorphic are analysed for levels of genetic diversity, which are compared with other odonate species for which microsatellite loci have been isolated.

In Chapter 3 the microsatellite loci characterised in Chapter 2 are used for an analysis of the population genetic structure of *E. viridulum* in the UK and northern Europe. It describes the fieldwork carried out between 2004 and 2006 at 28 sites across the range of *E. viridulum* in the UK and also from 11 sites from northern Europe. This chapter covers the statistical analysis of the genetic data produced from these samples. The first aim is to analyse variation in genetic diversity between the UK and continental European samples and to look for evidence of a genetic bottleneck caused by the colonisation process. The second aim is to look for genetic structure within the UK population and from this infer likely continental European sources. Finally temporal variation in genetic structure is briefly investigated; a subject covered in greater detail in Chapter 4. These analyses are then discussed with reference to (i) the range expansion described in Chapter 1, (ii) other odonate studies and (iii) patterns of population genetic structure found in other invasive and range expanding species.

Following on from the initial work on temporal genetic variation in Chapter 3, Chapter 4 investigates genetic variation between successive cohorts of *E. viridulum* in the UK. Samples collected from 9 sites between 2004 and 2006 are used to quantify level of genetic differentiation between cohorts. Temporal genetic data is used to estimate effective population size and migration rate based on the premise that allele frequencies drift apart more rapidly in smaller populations than in larger ones. The results of this chapter are discussed in the light of the range expansion of *E. viridulum* and patterns of voltinism identified in Chapter 5.

Chapter 5 presents a study of larval growth of *E. viridulum*, with the aim of identifying the pattern of voltinism in the UK. As odonates typically exhibit a gradient of voltinism with latitude, it is of particular interest to quantify voltinism in this species at its northwest range margin. This chapter describes how larvae were collected, identified and measured monthly over the summer growth period at 4 sites in the UK. The results are considered alongside current knowledge of phenology in the UK and voltinism in continental Europe.

In the final chapter the findings of Chapters 1-5 are described and brought together into a general discussion on the range expansion of *E. viridulum.* Each chapter is summarised with reference to other relevant conclusions from this investigation and prior predictions. The responses of odonates to global temperature increase and predictions about future patterns of range change are discussed. Also in this chapter, outstanding questions about *E. viridulum* are considered in the light of the findings of this study and future areas of interest for investigation are suggested.

Chapter 1

A review of the colonisation history in northern Europe and the British Isles, and the biology and ecology of the small red-eyed damselfly *Erythromma viridulum* (Charpentier, 1840).

1.1. Invasion of the small red-eyed damselfly

Proxy data indicates that the increase in temperature observed in the Northern Hemisphere during the 20th century is the largest in the last 1,000 years (Hulme *et al.*, 2002), and is predicted to rise a further 3 to 5°C in the next half century in the UK and most parts of Europe (Houghton *et al.*, 2001; Schiermeier, 2004; IPCC, 2007). This change is predicted to have profound influences on natural systems. Climate change is expected to affect organisms causing physiological, phenological, community interaction, range and distributional changes (Walther *et al.*, 2002). Rapid shifts in range margins would be expected in species that are highly mobile with good dispersal ability or in those that are strongly temperature limited (Pearson & Dawson, 2003; Pearson, 2006). Life history plasticity may offer some flexibility in the response to climate change for some species (Bale *et al.*, 2002; Parmesan, 2006). Poleward shifts in range margins, linked to climate change, have already been documented in a wide range of taxa (Allen & Breshears, 1998; Thomas & Lennon, 1999; Hickling, 2006; Mieszkowska *et al.*, 2006) most notably butterflies (Parmesan *et al.*, 1999; Warren *et al.*, 2001), and it would be expected that other winged insects may exhibit a similar response.

There is evidence that odonates are shifting their range margins northward (Ott, 1996; Aoki, 1997) as well as exhibiting phenological changes (Hassall *et al.*, 2007) in response to climate change. British odonates in particular appear to be reacting to climate changes with 37 species having shifted their range margin northward over the last 40 years, apparently in response to climate warming (Hickling *et al.*, 2005). The average increase in northerly range margin for non migratory British odonates (1960-1999) was 74 km with

a maximum of 346 km for *Sympetrum striolatum* (Hickling *et al.*, 2005). Alternative or contributing factors to this expansion are that habitat quality has improved allowing species to increase their range and that recording has increased.

The small red-eyed damselfly, *Erythromma viridulum*, has seen significant north-westerly range expansion in Europe in the last 30 years (Ketelaar, 2002) and is the first recorded example of a migrant damselfly establishing colonies in the British Isles (Dewick & Gerussi, 2000). As such the range expansion of *E. viridulum* sits among the broader context of poleward shifts in distribution occurring across many taxa, apparently in response to global climate change. Therefore *E. viridulum* represents an excellent model with which to investigate the causes, effects and implications of rapid range expansion. This aims of this chapter are (1) to present an account of the biology and ecology of this species and (2) describe it's recent range expansion, both in continental Europe and the UK.

1.2. Appearance

Two species of red-eyed damselfly exist in Europe, the small red-eyed *Erythromma viridulum* and the red-eyed *Erythromma najas*. *E. viridulum* is similar in appearance to *E. najas,* though smaller with an abdominal length of 22-25 mm compared to 25-35 mm (D'Aquilar *et al.*, 1986). The extent of the blue markings on the male's abdomen is the most distinctive identifier, with segments 2 and 8 being almost entirely blue when viewed from the side in *E. viridulum*. In addition, the tenth abdominal segment of *E. viridulum* viewed from above appears to have a black "x" mark. There is a sub-species, *E. viridulum orientale,* which has different coloration of thoracic antihumeral stripes. This sub-species is found in Syria and is closely resembles *E. viridulum viridulum* from eastern France (Boudot & Jacquemin, 1988). Tenerals appear a light green/brown colour with a red tint to their eyes. They can usually be identified from the faint cross mark on the tenth abdominal segment, which becomes more apparent as maturation occurs.

E. viridulum larvae have rounded, unpigmented lamellae and come to a slight point (Brook, 2003). *E. najas* has larger lamellae than *E. viridulum* with pigmented vertical stripes (Gerken & Sternberg, 1999). Larvae can be definitively identified by the presence of setae on the ventral surface of the posterior margin of the first abdominal segment, but absence of setae on the mastesternum (Carchini, 1983). Setae are present even on smaller instars of both *E. viridulum* and *E. najas*. Other characteristics of *E. viridulum* larvae include a pale stripe running along the caudal surface of the abdomen in later instars. Colour varies between leaf-green and an earthy brown colour (S. Keat, pers. obs.).

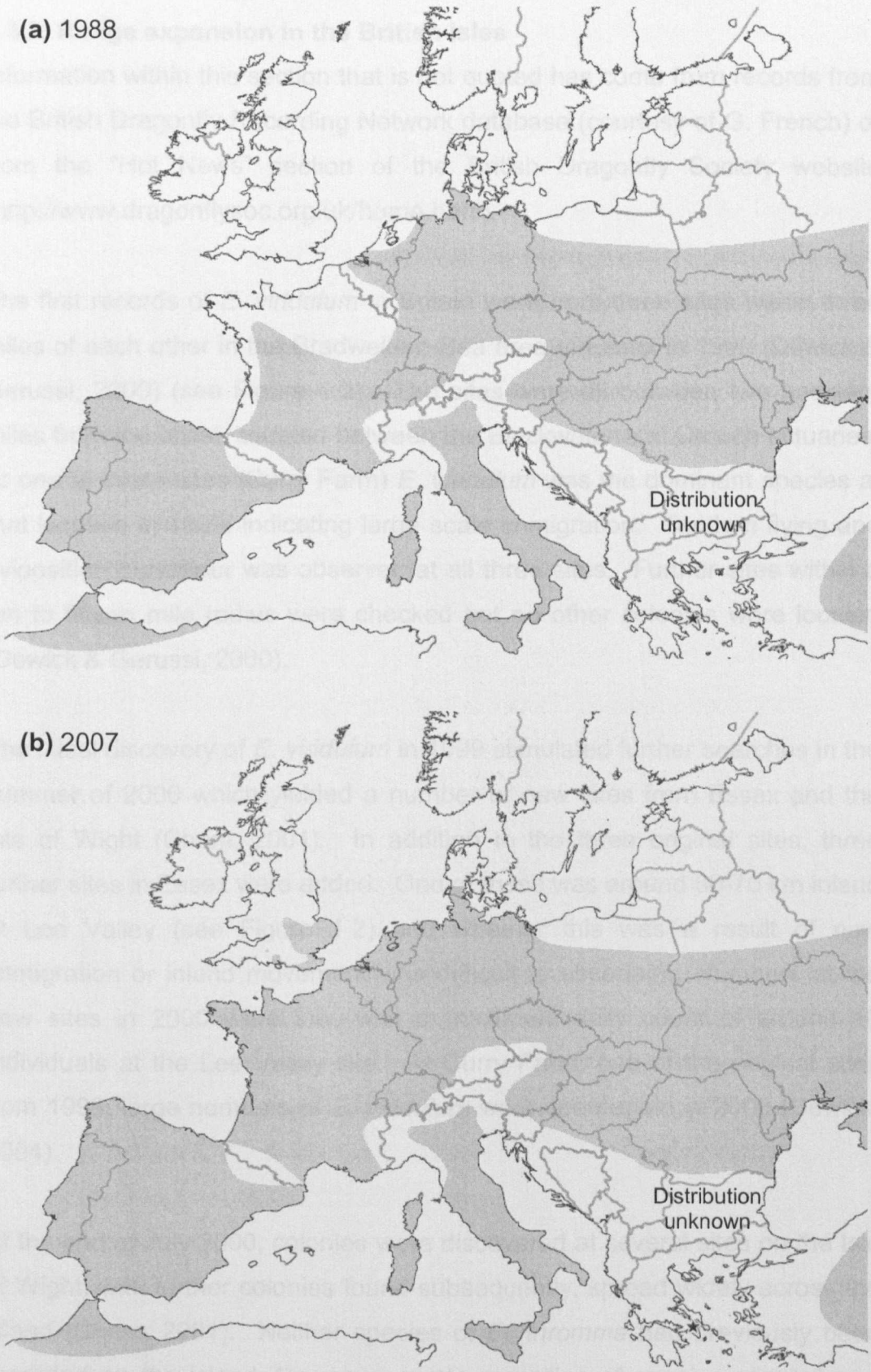
1.3. Recent distributional changes

1.3.1. Range expansion in Europe

E. viridulum has undergone a significant north-westerly range expansion in northern Europe in the last 30 years. A comparison of the distribution in 1988 (redrawn from Askew, 1988) compared with the 2006 distribution is presented in Figures 1.1a, b. It is now a common species in the Netherlands but up until the seventies it was considered a rare visitor. The first record of *E. viridulum* in the Netherlands was from 1917 in the south east of the country, followed by infrequent sightings over the next six decades (Ketelaar, 2002). In 1971 the first population of seven males and two females was documented and by the late seventies it was clear that *E. viridulum* was expanding its range in the Netherlands, including populations near Amsterdam and Naarden (Schoorl & Verdonk, 1978). Wasscher (1999) suggests that *E. viridulum* may not have been as rare as believed before the seventies and may have been overlooked or misidentified. During the nineties the expansion undoubtedly accelerated rapidly and by 1995 *E. viridulum* had become the most abundant species in some parts of the country. In 1995 and 1996, *E. viridulum* was recorded from six of the seven Waddensea Islands, where dune pools and agricultural ditches were occupied. Overall it was the ninth most abundant odonate species in the Netherlands during the nineties, with some 75,938 individuals recorded

(Ketelaar, 2002 – source National Dragonfly Database; Dutch Society for Dragonfly Studies (NVL), EIS-Netherlands and Dutch Butterfly Conservation).

The presence of *E. viridulum* is recorded in central Europe between 1963 and 1984 (Jacquemin *et al.*, 1987). Records of from Switzerland and France during this period indicate that it was spreading north and it had reached Lorraine in north-east France by 1982. Askew (1988) states that the range of *E. viridulum* extends northwards, in scattered colonies, to the Netherlands and north Germany, possibly expanding its range, but it does not reach England or Scandinavia (see Figures 1.1a, b). In Belgium, *E. viridulum* was one of only three species that had significantly expanded it range in the period 1990-2000 (De Kniff *et al.*, 2001). The southern limit of the range of *E. viridulum* is North Africa (Morocco and north-west Algeria) and in the east to Kazakhstan, Kyrgyzstan, Tajikistan, Turkmenistan, and Uzbekistan. It is not known if and how the southern range limit has changed in recent years.



Figures 1.1a, b. The European distribution of *E. viridulum* indicated by the shaded area in (a) 1998 and (b) 2007 (adapted from Askew, 1988).

1.3.2. Range expansion in the British Isles

Information within this section that is not quoted has come from records from the British Dragonfly Recording Network database (courtesy of G. French) or from the "Hot News" section of the British Dragonfly Society website (http://www.dragonflysoc.org.uk/home.html).

The first records of *E. viridulum* in Britain were from three sites within three miles of each other in the Bradwell-on-Sea area of Essex in 1999 (Dewick & Gerussi, 2000) (see Figure 1.2). The sites were all between two and four miles from the coast, situated between the Blackwater and Crouch estuaries. At one of these sites (Curry Farm) *E. viridulum* was the dominant species at that location in 1999, indicating large scale immigration. Tandem flying and oviposition behaviour was observed at all three sites. Further sites within a ten to fifteen mile radius were checked but no other colonies were located (Dewick & Gerussi, 2000).

The initial discovery of *E. viridulum* in 1999 stimulated further searches in the summer of 2000 which yielded a number of new sites from Essex and the Isle of Wight (Cham, 2001). In addition to the three original sites, three further sites in Essex were added. One of these was around 60-70 km inland at Lee Valley (see Figure 1.2) and whether this was a result of new immigration or inland movement was difficult to ascertain. Numbers at the new sites in 2000 were low, with a maximum daily count of around 10 individuals at the Lee Valley site. At Curry Farm, one of the original sites from 1999, large numbers of *E. viridulum* were seen again in 2000 (Dewick, 2004).

At the end of July 2000, colonies were discovered at several sites on the Isle of Wight, with further colonies found subsequently, spread widely across the island (Cham, 2001). Neither species of *Erythromma* had previously been recorded on the island, though accurate recording of species present was last carried out in 1996 and for this reason it is impossible to know the exact year in which *E. viridulum* arrived there. The widespread colonies and daily

counts in the upper twenties at many locations indicated that the species only arrived on the Isle of Wight in recent years (Cham, 2001).

In 2001 the largest immigration of *E. viridulum* into the British Isles yet seen was recorded, with sites added along the south east coast including arrivals in Norfolk, Suffolk, Essex, Kent and the Isle of Wight as well as inland expansion stemming from the core population in south Essex (Cham, 2002) (see Figure 1.3). At Curry Farm, one of the original sites, numbers crashed dramatically in 2001 compared with the huge numbers seen the previous year (Dewick, 2004), however it was seen at a number of nearby sites, suggesting that it was then well established in that area of Essex. A large number of new sites were recorded in Essex in 2001, both along the coast and further inland. Further north up the coast from Essex, in Suffolk, four new sites were added, all close to the coast.

Also in 2001 new sites appeared along the north Norfolk coast, thought be the result of a large scale migration from the continent, most likely the Netherlands. Observations at coastal sites in Norfolk suggested waves of damselflies were coming in from the sea and then moving inland. On 14 August at least 100 individuals were seen in dune pools at Winterton-on-Sea (see Figure 1.3). These individuals dispersed over the next few days, only to be replaced by a second wave on the 17 August, which again dispersed by the 25 August. Sightings inland occurred between 22-28 August, suggesting westerly movement following their arrival on the coast (Cham, 2002). The influx into Norfolk coincided with the arrival of a large number of Migrant Hawkers *Aeshna mixta* at a well known site for migrant birds and dragonflies in Great Yarmouth (Cham, 2002).

Further south from the main influx in East Anglia, 2001 saw the first records for Kent at Folkestone racecourse (see Figure 1.3). A number of sightings at three Kent locations were made between 13 August and 15 September, though low numbers compared with East Anglia indicated that Kent was either on the edge of the main influx from the continent, or lacked the number of observers present in East Anglia (Cham, 2002).

On the Isle of Wight, *E. viridulum* was recorded at 11 sites in 2001, compared to 13 in 2000 and was absent from some sites where it had been present the previous year as well as present at some new sites. Daily counts increased from the previous year, reaching 155, including 38 ovipositing pairs, at one site. These records indicated that this species had become established on the Isle of Wight, with individuals attempting to colonise new sites (Cham, 2002).

As well as new immigrants from the continent 2001 saw significant inland movement originating from the original Essex populations and heading west, north of the Thames estuary. *E. viridulum* was recorded in late August at Priory Country Park, near Bedford (see Figure 1.3), Sundon Quarry, near Luton and Pitstone, Buckinghamshire. These sites are around 100 km from the sea and represented the most inland records at this time (Cham, 2002).

In 2002 *E. viridulum* was recorded further inland from many new sites in south-east England (see Figure 1.4). However, coastal regions of Norfolk, Suffolk and Essex recorded fewer sightings of *E. viridulum* in 2002 than 2001, suggesting little immigration from the continent and possible failure of eggs to hatch in sub-optimal environments (Cham, 2003), though see Chapter 5 where voltinism trends are discussed. As such there remained no definitive proof that *E. viridulum* was breeding successfully in the British Isles at this time. Essex maintained its high concentration of sites in 2002, especially in the area between Basildon and Hadleigh and it was assumed that it would be found at most suitable sites in south Essex. New sites were added in Essex and the numbers present at many of the old sites increased. Numbers at Curry Farm, one of the original sites in 1999, showed a dramatic resurgence with daily counts peaking at 1,011 on 24 July (Dewick, 2004).

Westerly spread was seen in Suffolk in 2002 from the original coastal sites of the previous year. Four new sites were added in West Suffolk and some of the sites from 2001 remained occupied, though daily counts remained only just into double figures. Westerly spread was also reported in Norfolk and a

sizeable colony was discovered in Thomas Water, Breckland (see Figure1.4), representing the most westerly population in the county. *E. viridulum* was reported from some, but not all of the coastal sites of 2001 (Cham, 2003).

Surprisingly Hertfordshire and Cambridgeshire only yielded one record between them in 2002, despite more sites being discovered in Bedfordshire, which is further north-west. Five further sites were added in Bedfordshire including a large colony at Wrest Park (see Figure 1.4) while the original Bedfordshire site at Priory County Park remained a stronghold and the most inland site at that time. Sites in this area were closely monitored by the UK National Dragonfly Recording Network coordinator, Steve Cham who lives in close proximity.

The Isle of Wight yielded more sites during 2002, now totalling 20, and higher counts were recorded at the existing sites. The number of sites in Kent increased to 13, though these all remained near the coast. Proof of breeding was confirmed in Kent by the finding of exuviae at Bluewater. The first county records were reported for East Sussex from sites near Icklesham (see Figure 1.4) (Cham, 2003).

By 2003 *E. viridulum* had become a well established species in the UK, with a widespread distribution at suitable sites between north-east Norfolk and Kent, with the Isle of Wight population remaining apparently isolated (see Figure 1.5). Frequently *E. viridulum* was the dominant species at suitable sites and at two sites in East Anglia reached numbers into the thousands (Parr, 2004). With the Essex population forming the core, westerly range expansion continued in 2003.

2003 saw high occupation of suitable sites and considerable daily counts in Essex. There was some evidence that sites where numbers were high were contributing to inland range expansion. Indeed, at Curry Farm the population gathered into unusually high densities, where at one stage 65 individuals had settled on two water lilies and considerable numbers were alighting high upon the reeds. The suggestion that they were preparing to migrate *en*

masse was confirmed after a few days when daily counts dropped to tens of individuals (Dewick, 2004).

After its arrival in Sussex in 2002, *E. viridulum* continued expanding in 2003 with five further sites being added throughout the eastern part of the county. Surrey also produced its first records in 2003, with small numbers seen at Mitcham Common and Blindley Heath (see Figure1.5). Further inland in Hertfordshire, further reports were added to the previous year's records, close to the known sites in Bedfordshire (Parr, 2004). Evidence of breeding at the Bedford sites gave *E. viridulum* a firm base from which to continue its expansion further westward (Cham, 2004a).

The well established resident population meant that it was hard to estimate immigration from the continent in 2003; however individuals seen at Winterton-on-Sea Dunes, Norfolk, on 20 July were likely to be immigrants because previous attempts to breed at this site had been unsuccessful (Nobes, 2003). Elsewhere in Norfolk, the resident population at Eccles-on-Sea maintained high daily counts, with sightings between 7July and 23 August and possible fresh immigrants arriving on 21 September (Bowman, 2004) (see Figure 1.5).

2003 brought the first record of *E. viridulum* for the Channel Isles, with the first record coming from Guernsey on 13 June. It was seen at several sites, with ovipositing observed later in the summer (Parr, 2004). It is possible that a paucity of observers caused this species to be overlooked previously.

Range expansion and fresh immigration continued in 2004 and included many new county records (see Figure 1.6). Most notably the first records from Hampshire at Southampton Common finally occurred, presumably as a result of immigration from the nearby Isle of Wight population (see Figure 1.6). The first week of August saw many new inland sightings, including first county records for Cambridgeshire at sites including Croxton Heath, Longthorpe in Northamptonshire, Tring Reservoir, Buckinghamshire and the Swift Valley Reserve, Warwickshire, all shown on Figure 1.6. The arrival of

E. viridulum in Warwickshire, at the Swift Valley reserve near Rugby, made this the most north westerly site at that time (Parr, 2005). The first records were also received for Jersey, Channel Islands, in late July.

In addition to range expansion, fresh immigration was again recorded in coastal Norfolk in 2004. As in previous years, immigration was noted at Winterton-on-Sea and on August 11 individuals were seen roosting "several to every bush or tree" in scrub along a 3-4 km stretch of dunes (Parr, 2005). These individuals were presumably resting at the first available landfall following migration from continental Europe. Waves of immigration were noted at Eccles-on-Sea with the largest occurring on September 4 (Parr, 2004). By now there was a common pattern of immigration occurring in late August and early September at these sites, presumably when numbers in continental Europe had reached their climax and weather conditions were favourable.

There was much less range expansion in 2005 than in previous years (see Figure 1.7). There was consolidation of the area around the Thames valley and westerly movement by 40.1 km at the northwest range margin. There was some inland movement in Norfolk, Suffolk and Kent. The most westerly site to be added in 2005 was at Hampton-in-Arden, Warwickshire, close to the Swift Valley Reserve. Steve Cham noted in 2005 that *E. viridulum* was emerging earlier each year at the Bedfordshire sites and was on the wing during much of June compared to its first emergence during July a few years before. The period of emergence was a little longer in 2005 than in previous years, with the earliest record on 14 June and the last on 2 October, suggesting that the British phenology was becoming more similar to that in the Netherlands (Wasscher, 1999).

After a quieter year in 2005, *E. viridulum* made significant westerly and northerly range expansions again in 2006, with many new county records (see Figure 1.8). First county records came from Leicestershire where seven sites were discovered between July and early August. New county records also came from Lincolnshire at the most northerly records to date from Oak

Road Lake, Hull, East Yorkshire (see Figure 1.8) and the nearby canal at Spurn. As these sites were >100 km from the nearest British population, it seems probable that these were new immigrants from continental Europe. Proof of *E. viridulum* breeding in Northamptonshire was recorded at Lyveden New Bield, where in July larvae were identified and later up to 40 adults were seen ovipositing.

In late July and early August 2006 there was a notable westerly migration with new records coming from Coombe Hill Meadows near Swindon in Wiltshire and as far west as Bath University in Avon and Severn Beach pools in Gloucestershire, all shown in Figure 1.8. There were also new sightings further west along the south coast, with the first Somerset record coming from Bennett's Water Gardens near Weymouth (James Bennett, pers. comm.) (see Figure 1.8). However the most westerly sighting in 2006 came from Lower Bruckland Farm lakes in south Devon (see Figure 1.8). As these records were close to the coast it begs the question as to whether these were immigrants from continental Europe or migrants from the Isle of Wight and Hampshire populations. If either of these populations were the source, it represents a sizeable migration as the nearest French coast is around 150 km away and the Isle of Wight is 120 km west of the Devon site, though it is possible they originated from an undiscovered site.

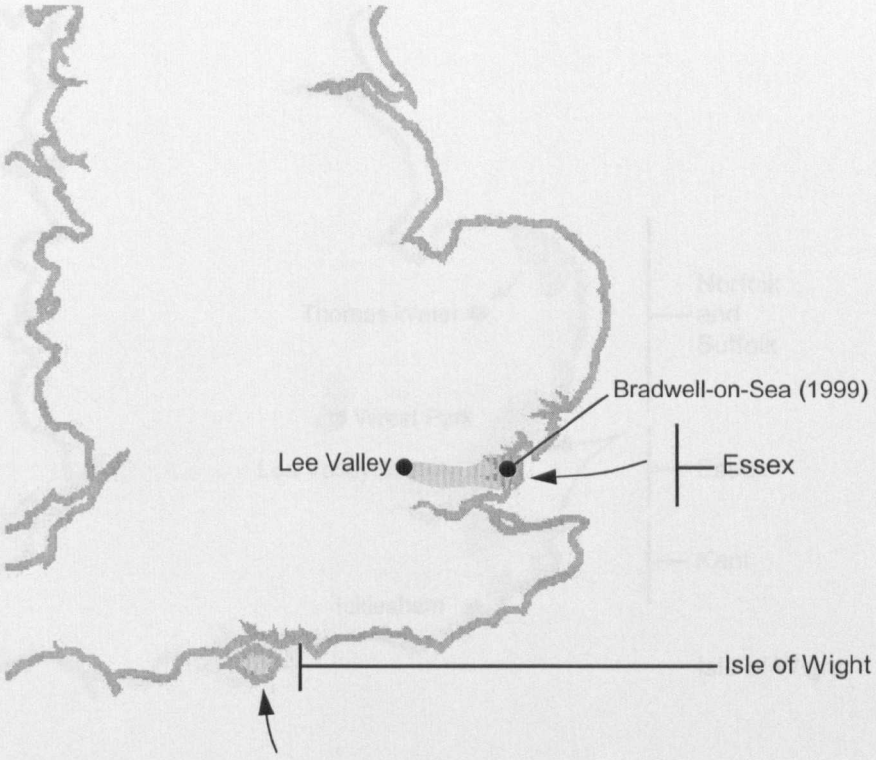


Figure 1.2. Distribution of *Erythromma viridulum* in the British Isles in 2000 (source: British Dragonfly Recording Network).

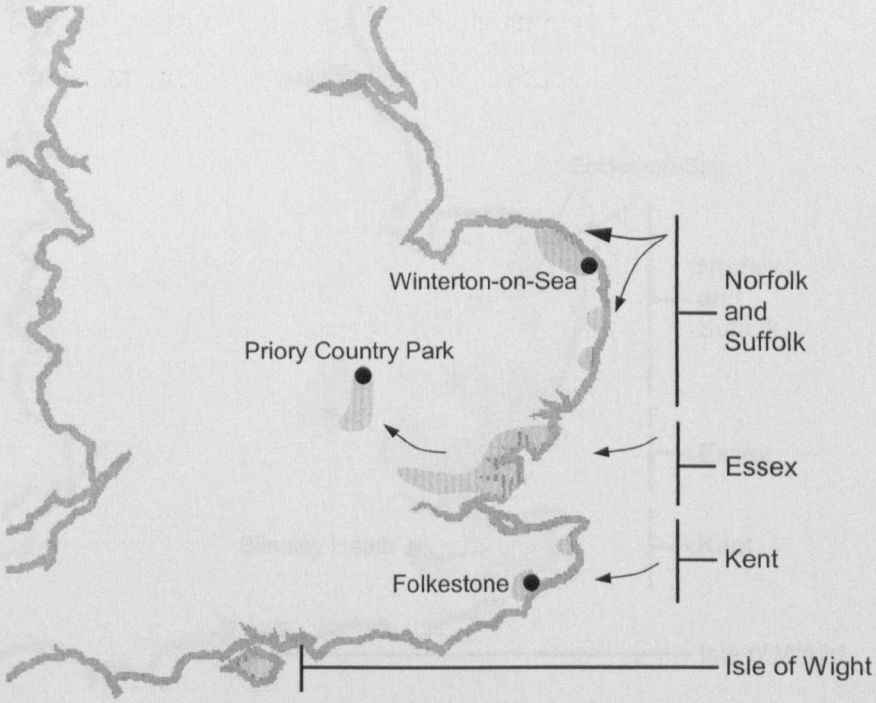


Figure 1.3. Distribution of *Erythromma viridulum* in the British Isles in 2001 (source: British Dragonfly Recording Network).

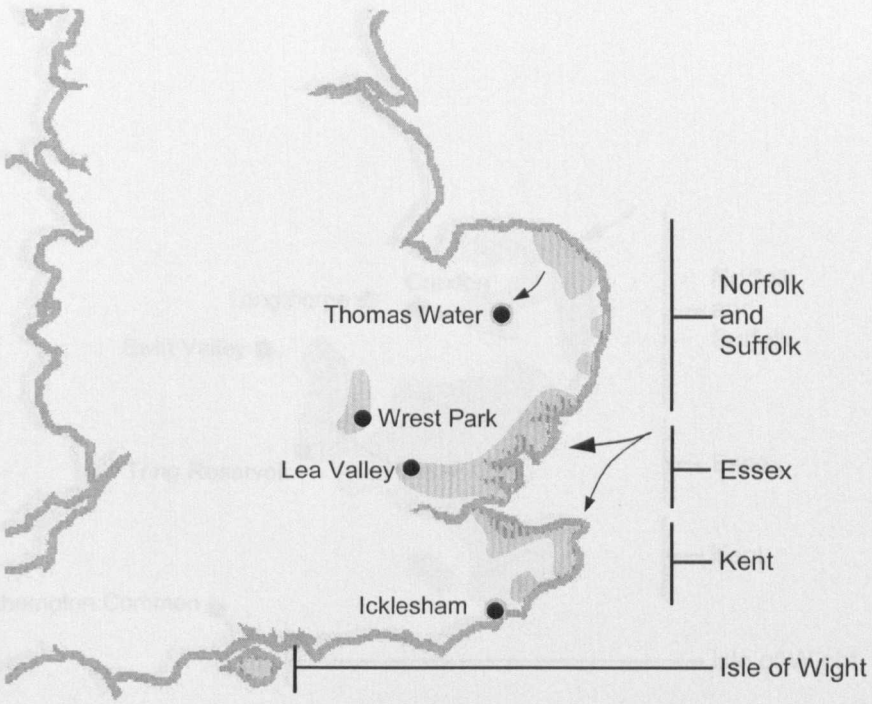


Figure 1.4. Distribution of *Erythromma viridulum* in the British Isles in 2002 (source: British Dragonfly Recording Network).

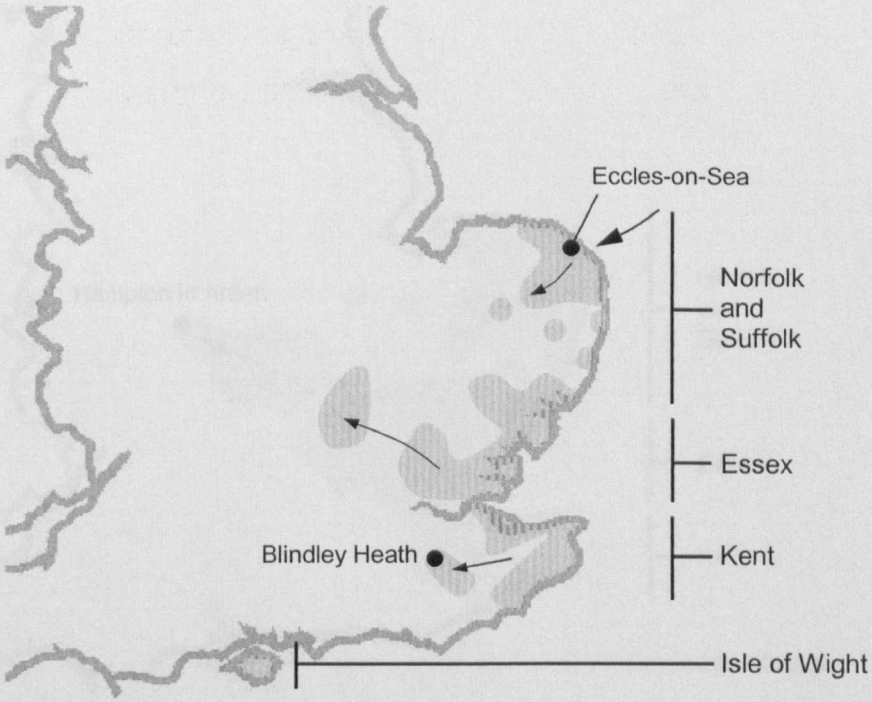


Figure 1.5. Distribution of *Erythromma viridulum* in the British Isles in 2003 (source: British Dragonfly Recording Network).

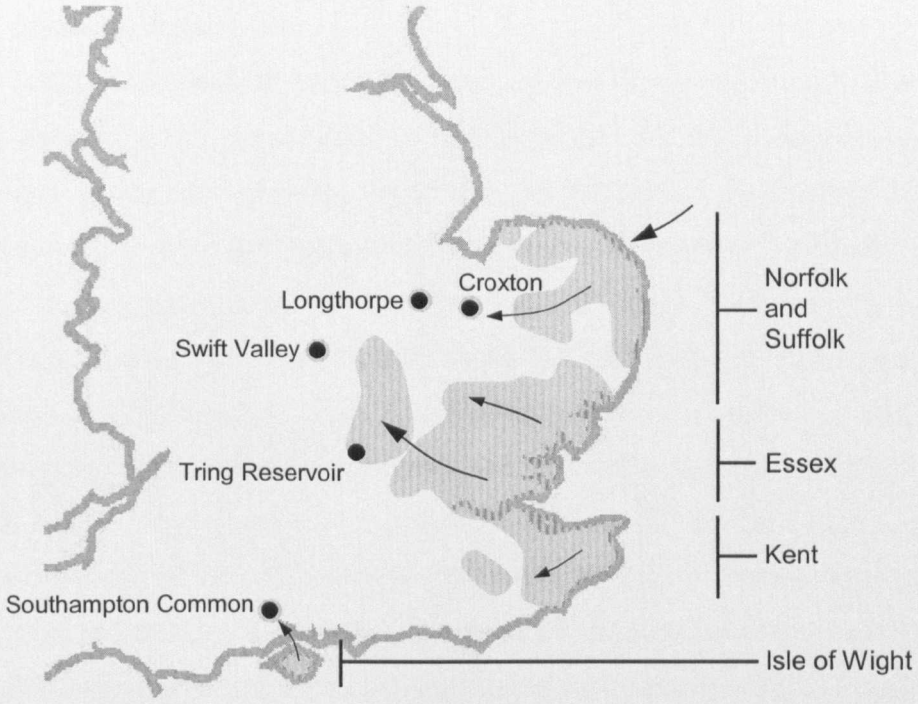


Figure 1.6. Distribution of *Erythromma viridulum* in the British Isles in 2004 (source: British Dragonfly Recording Network).

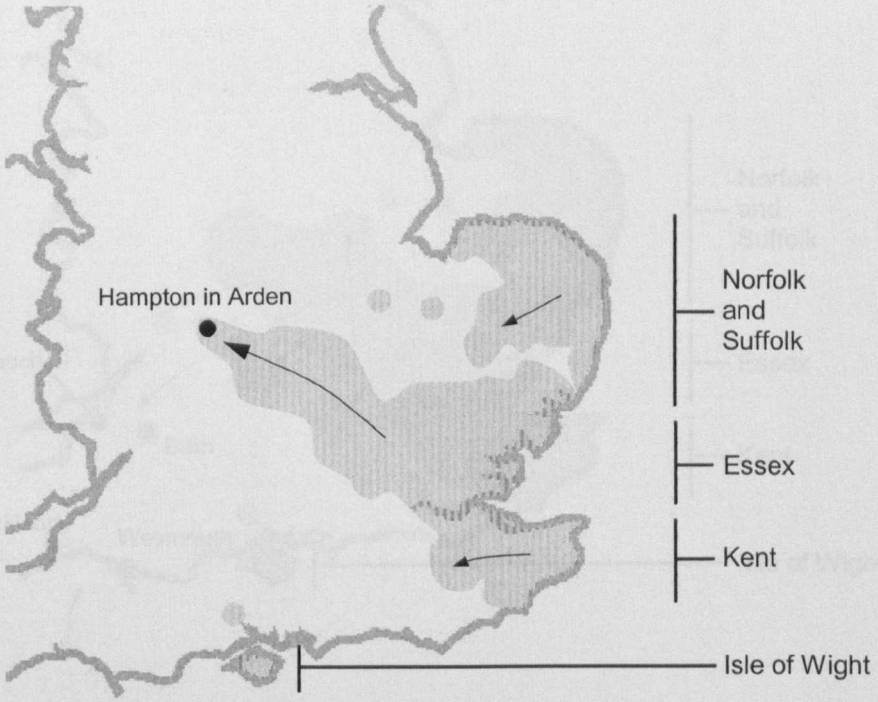


Figure 1.7. Distribution of *Erythromma viridulum* in the British Isles in 2005 (source: British Dragonfly Recording Network).

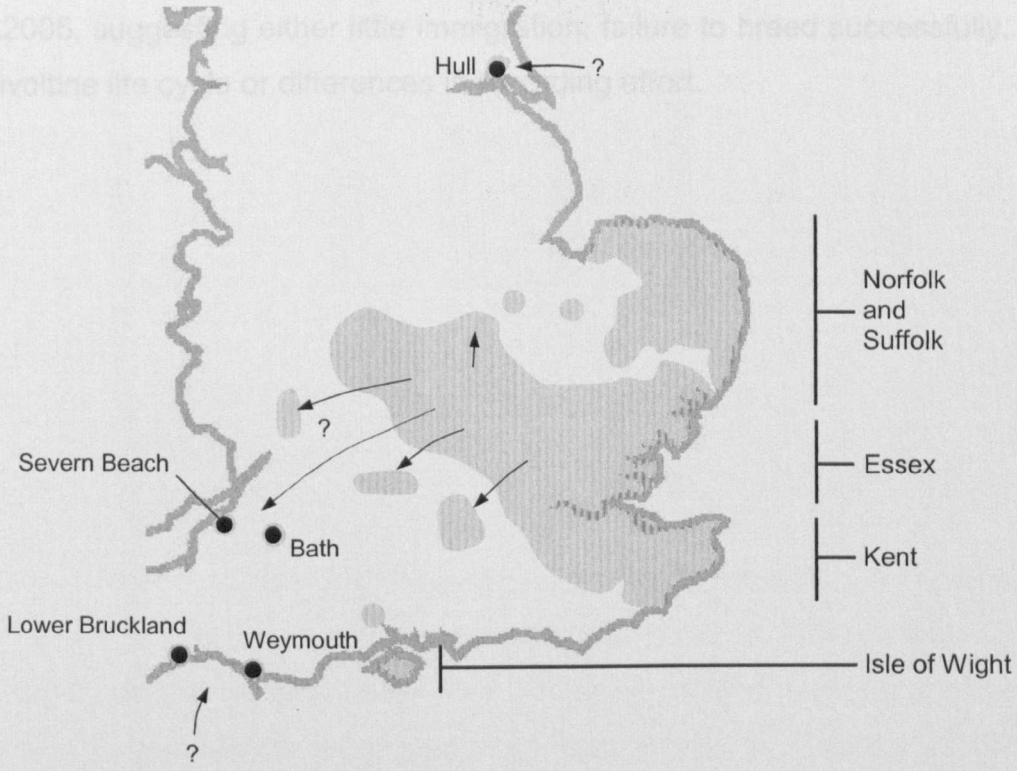
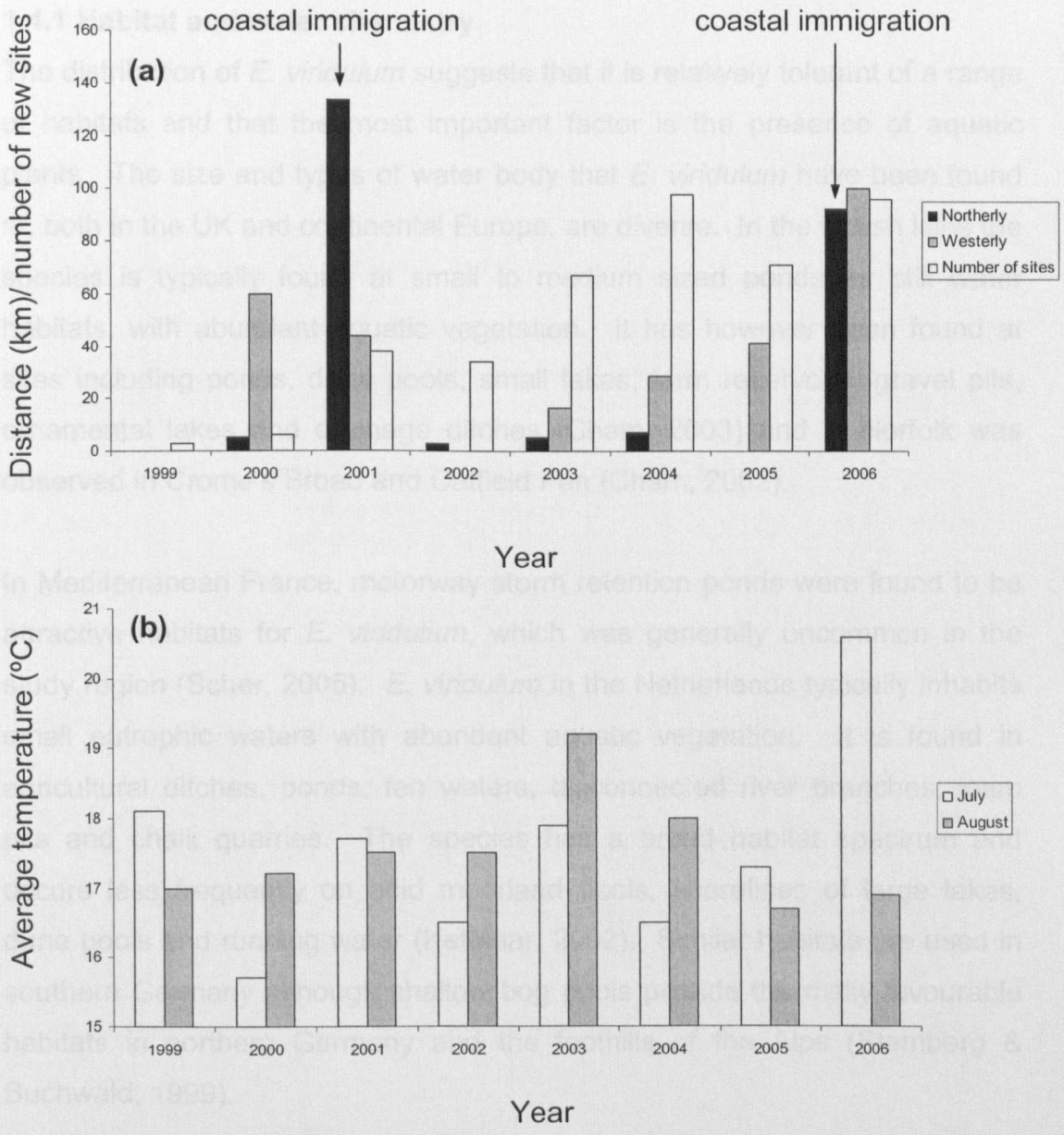


Figure 1.8. Distribution of *Erythromma viridulum* in the British Isles in 2006 (source: British Dragonfly Recording Network).

1.3.3. Rate of range expansion

The rate of range expansion over the years since *E. viridulum* has been recorded in the UK does not appear to be constant. Figure 1.9a presents three measures of the rate of range expansion in the British Isles using data from the Dragonfly Recording Network (courtesy of G. French). It shows that the greatest northerly expansions occurred in 2001 and 2006, which was likely to be because of new immigration from the continent in those years which was observed at coastal sites (Cham, 2002, http://www.dragonflysoc.org.uk/home.html). The large range expansions recorded in 2006 may have been partially attributable to the high July average temperature of 20.3°C (see Figure 1.9b). Westerly movement has occurred at a steadier rate, though the greatest expansion occurred in 2006, again probably because of fresh range expansion from continental Europe at sites in Somerset and Devon. Westerly expansion has proceeded at an average rate of 31.68 km per year. The number of sites at which *E. viridulum* has been recorded has grown steadily since its arrival, though dipped in 2002 and 2005, suggesting either little immigration, failure to breed successfully, a semivoltine life cycle or differences in recording effort.



Figures 1.9a, b. (a) Rate of range expansion of *E. viridulum* in the British Isles, where range expansion is measured by northerly range extension, westerly range extension (excluding the Isle of Wight) and the number of sites at which *E. viridulum* was recorded by the British Dragonfly Recording Network (data courtesy of G. French). (b) Monthly average temperature in south-east England for July and August (source: Met Office website http://www.metoffice.gov.uk/index.html).

1.4. Physical and environmental requirements

1.4.1 Habitat and water chemistry

The distribution of *E. viridulum* suggests that it is relatively tolerant of a range of habitats and that the most important factor is the presence of aquatic plants. The size and types of water body that *E. viridulum* have been found at, both in the UK and continental Europe, are diverse. In the British Isles the species is typically found at small to medium sized ponds, or still water habitats, with abundant aquatic vegetation. It has however been found at sites including ponds, dune pools, small lakes, farm reservoirs, gravel pits, ornamental lakes and drainage ditches (Cham, 2003) and in Norfolk was observed in Crome's Broad and Catfield Fen (Cham, 2002).

In Mediterranean France, motorway storm retention ponds were found to be attractive habitats for *E. viridulum*, which was generally uncommon in the study region (Scher, 2005). *E. viridulum* in the Netherlands typically inhabits small eutrophic waters with abundant aquatic vegetation. It is found in agricultural ditches, ponds, fen waters, disconnected river branches, loam pits and chalk quarries. The species has a broad habitat spectrum and occurs less frequently on acid moorland pools, shorelines of large lakes, dune pools and running water (Ketelaar, 2002). Similar habitats are used in southern Germany although shallow bog pools provide thermally favourable habitats in northern Germany and the foothills of the Alps (Sternberg & Buchwald, 1999).

Wasscher (1999) revealed in a study of water phosphate levels that *E. viridulum* tends to be rare in waters with very low phosphate content and tends to prefer eutrophic habitats. The species is occasionally present in mesotrophic waters and hypertrophic waters support fewer colonies than eutrophic (Sternberg & Buchwald, 1999). A detailed account of the environmental factors important to *E. viridulum* are summarised in Table 1.1.

Table 1.1. Summary of physical and environmental factors known to affect distribution of *E. viridulum*.

Factor	Comment	Reference
Air temperature	Adult activity only above 16°C	Sternberg & Buchwald (1999)
Water temperature	Extremes of surface freezing in winter to summer temperature higher than 30°C in southern Germany	Sternberg & Buchwald (1999)
Mean July temperature	In study area, E. viridulum confined to areas with average July temperature >17°C	Sternberg & Buchwald (1999)
Size of water body	Small ponds to lakes	Sternberg & Buchwald (1999) Cham (2002)
Water flow	Larvae restricted to areas with little or no flow	Sternberg & Buchwald (1999)
Wave action	High wave action impacts oviposition and emergence, continual disturbance not tolerated	Hunger (1998) Sternberg & Buchwald (1999)
Water depth	Tens of centimetres to tens of metres	Sternberg & Buchwald (1999)
Water nutrients	Generally eutrophic waters	Sternberg & Buchwald (1999) Wasscher (1999)
Water phosphate	Rare in waters with low phosphate	Wasscher (1999)
Shading	Adults and larvae prefer sunny conditions	Sternberg & Buchwald (1999)
Trees	Not necessary but provide shelter for adults	Sternberg & Buchwald (1999)

1.4.2. Aquatic macrophytes

E. viridulum is characterised by its ability to rapidly colonise as well as its adaptability to novel habitats. Nonetheless it seems to have distinct habitat requirements and preferences for long term success at a given site, the most important of which being the presence of aquatic macrophytes on the water surface. Dense rafts of aquatic macrophytes that reach the surface at the time when oviposition occurs and that make up a mosaic-like pattern on the water surface being preferred. This was confirmed by multivariate analysis, which showed that *E. viridulum* was more positively correlated with floating macrophytes than all other environmental variables looked at, including bank openness, pioneer plants, low growing vegetation, rushes and reeds and water persistence (Schindler *et al.*, 2003). It is believed that these macrophytes are preferred because they act as a site for oviposition, a safe larval habitat and as a site for emergence (Hunger, 1998).

The species of plant associated with *E. viridulum* in the UK are most notably *Ceratophyllum demersum* (Rigid Hornwort) and *Myriophyllum spicatum* (Spiked Water-milfoil) (Raab, 1996; Hunger, 1998; Cham, 2001, 2004; Ketelaar, 2002). These species provide the fine-leaved, submerged vegetation preferred. Algal mats are another commonly used perch and oviposition site, which may come about from *E. viridulum*'s apparent preference for eutrophic waters (Wasscher, 1999). Plants with floating leaves, such as lilies, are used as perches by adult males but are unsuitable for oviposition and on their own are not sufficient (Hunger, 1998). The emergent plant *Crassula helmsii* (New Zealand Water Stonecrop) is used as a perch and ovipositing site at East Ruston, Norfolk (pers. obs.). It must be noted that preference for particular plants species can only be estimated because the availability of each has not been quantified. Plant species found in association with *E. viridulum* are presented in Table 1.2.

Analysis of the distribution of *E. viridulum* larvae showed that they were most abundant on submerged macrophytes, occasionally found in the littoral zone and rarely found on the bottom (Solimini *et al.*, 2003). Little is known of the macrophyte species preferred by *E. viridulum* larvae, though from field work,

they have most frequently been caught among the species preferred as adult oviposition sites (*Ceratophyllum demersum* and *Myriophyllum spicatum*).

Table 1.2. Plant species found in association with *E. viridulum*.

Species	Common Name	Reference
Ceratophyllum demersum	Rigid Hornwort	Raab (1996)
		Hunger (1998)
		Bonsel (1999)
		Cham (2001)
		Ketelaar (2002)
		Cham (2004b)
Myriophyllum spicatum	Spiked Water-milfoil	(as above)
Hippurus spp.	Marestail	Cham (2003)
Potamogeton natans	Broad-leaved Pondweed	Cham (2002)
Potamogeton spp.		Hunger (1998)
Polygonum amphibian	Amphibious Bistort	Cham (2002)
Elodea nuttallii	Nuttall's Waterweed	Cham (2001)
Nymphaea alba	White Water Lily	Hunger (1998)
		Cham (2004a)
Nuphar lutea	Yellow Water Lily	Hunger (1998)
		Cham (2004b)
Sphagnum spp.	Sphagnum moss	Wasscher (1999)
Crassula helmsii	New Zealand Water Stonecrop	Pers. obs.

1.5. Adult behaviour

At temperatures above 16°C adult male *E. viridulum* are typically seen perching on floating aquatic vegetation or emergent plants away from the waters edge. When resting the end of the tail of male *E. viridulum* often curls upwards in contrast to *E. najas*, whose tails remain straight (Cham, 2000). When not resting on vegetation, they tend to fly low over the water with a strong direct flight. *E. viridulum* typically remain around areas of aquatic vegetation, though can occasionally be seen traversing expanses of open water. Males tend to adopt a temporary territory that they will defend inter and intra-specifically. Territories are defended with aerial battles and seized by hovering on or above competitors.

During periods of low light and cool weather *E. viridulum* rests on emergent and marginal plants around the edge of the pond such as rush and iris species (family: Juncaceae and family: Iridaceae respectively). If the weather further deteriorates *E. viridulum* will move higher up the vegetation surrounding the pond. In the event of rain *E. viridulum* can be seen making flights to roost in the tops of trees. When populations reach very high densities they can be seen resting in trees and bushes. Dewick (2004) reported from Curry Farm, Essex, "In the early years *E. viridulum* seemed strictly confined to the immediate surroundings of the various ponds and lakes, but in 2003 large numbers were sometimes to be found well away from the water. This was particularly so on sunny evenings, when 100 or more would be found basking in the shelter of a suitable length of hedge". Newly arriving immigrants to the British Isles have also been known to rest in trees and bushes as they make first landfall (Parr, 2005).

European species of Zygoptera and Gomphidae tend to emerge in an upright position and are thus able to emerge on horizontal substrates (Schmidt, 1991). All species show a preference for emergence on vertical stems though both UK *Erythromma* species show an ability to emerge horizontally on plants on the water surface. It is thought that this may be an adaptation for species that live on the open water. It is postulated that the ability to emerge horizontally on floating vegetation is an advantage because it means

it is unnecessary for larvae to travel to the shore, thereby reducing their exposure to fish or frog predation (Schmidt, 1991). Typical species of plants used for emergence are members of the genera *Nymphaea* and *Hydrocharis*, and also from algal mats (S Keat, pers. obs.). During emergence sometimes only the head and thorax are visible above the water surface.

Following emergence and expansion and maturation of teneral's body and wings, the first flight of *E. viridulum* is typically a matter of a few metres into the vegetation surrounding the pond. During evening *E. viridulum* tenerals make a steep flight, often high into trees and bushes surrounding the pond, where presumably they rest until capable of stronger flight the next day.

Odonate genetic sex determining mechanisms is almost always X0 and XX, with male heterogametic sex (Kiauta, 1969) and should produce an even sex ratio. However, populations of adult odonates often have temporarily or permanently biased sex ratios (*e.g.* Stoks, 2001a, b; reviewed by Corbet, 1999) , though the biological significance of this is not yet fully understood. In my total sample there were 1,564 males and 216 females giving an approximately 7:1 male:female ratio, though this result is likely to have been affected by behaviourally mediated differences in probability of capture between the sexes (Garrison & Hafernik, 1981). Sex ratios at breeding sites are male biased, because mature males are present all the time, whereas females tend only to visit breeding sites when they have a clutch of eggs to lay, which may be every 2-3 days. As a result of this sex bias, female *E. viridulum* are almost always seen flying in-tandem with a male, often pursued by groups of competitors and tend to arrive at the breeding site in tandem.

E. viridulum oviposits in tandem, with the male in the sentinel position, immediately after copulation but, unlike *E. najas* does not submerge when doing so. Mating takes place over the water, or in bankside vegetation, including trees. Eggs are typically laid into the undersides of floating vegetation away from the edge of the pond. When populations are large they tend to oviposit in groups, sometimes forming interspecific groups with *E. najas* (Cham, 2004b). Group ovipositing is thought to increase breeding

success and survival (Corbet, 1999). Ovipositing *E. viridulum* are sensitive to approaching fish and fly away when they detect either underwater movement, or the ripples they cause (Cham, 2004b).

1.6. Colonisation characteristics

E. viridulum is typically an early coloniser of new habitats and was one of the first species to arrive at a newly created pond in Vienna, Austria (Raab *et al.*, 1996). The rapid rate at which *E. viridulum* has spread northwards in Europe and its ability to cross the English Channel (a distance of >30 km from continental Europe) in large numbers demonstrates its long range dispersal capability. How does *E. viridulum* go about successfully dispersing in large enough numbers to start viable new colonies? It seems that migration events occur when populations reach critically high densities, as observed for many odonate species (reviewed by Corbet, 1999) although inverse density dependent movement has been recorded in other odonates (*e.g.* Rouquette & Thompson, 2007). Dewick (2004) observed individuals, at a highly populated site, gathering together uncharacteristically when at high densities, then in the following few days leaving within a short period. It appears that when populations reach a certain threshold density, they will group together and emigrate. Despite a potential cost to dispersal, this is potentially an effective strategy because the likelihood of a viable new population being created is increased with mass migration and will tend to ameliorate problems associated with small populations such as inbreeding depression (Charlesworth & Charlesworth, 1987; Frankham *et al.*, 2002; Allendorf & Luikart, 2007).

Odonates are typically accomplished fliers, however smaller species, such as most zygopterans, are expected to take advantage of prevailing weather conditions to migrate long distances (reviewed by Corbet, 1999). An insight into the strategy used for dispersal by *E. viridulum* came from a light trap normally used for catching moths. On 29 July 2003 an *E. viridulum* male was caught in a light trap that had been set in the dark and taken in about half and hour before there was any light in the sky at Icklesham, East Sussex (Jones, 2004). This individual was most likely a migrant that had been

travelling by night; a common migratory technique by insects (Drake, 1984; Beerwinkle *et al.*, 1994). Migrants taking off at or after dusk ascend under their own power to exploit the steady, fast winds at altitudes of 100–2,000 m, which are capable of transporting them rapidly over very large distances (Gatehouse, 1997). The potential scale of displacement by wind-borne migration is generally substantially greater for nocturnal than for diurnal migrants (Drake & Farrow, 1988) and species that must track changes in habitat availability on a regional or geographic scale generally fly by night (Gatehouse, 1997). Large scale seasonal migrations by night have been documented in one of the most globally common species of dragonfly, *Pantala flavescens*, in China. Radar observations indicated this species regularly makes journeys of 150-400 km in a single flight at altitudes of up to 1,000 km over open sea (Feng *et al.*, 2006). Long distance and/or one-way migrations appear to be typical of species that occupy temporary habitats or have to disperse to find seasonal refuges from severe weather conditions (*e.g.* monsoon) (Corbet, 1999).

Patterns of colonisation in the Netherlands did not follow a simple northerly expansion but show an apparent tendency to follow the course of the Rhine-valley and its branches (Ketelaar, 2002). Thus it is expected that the expansion of *E. viridulum* in the UK may also be dependent on connectivity between water courses. Indeed, the current distribution of *E. viridulum* indicates that the Thames valley has been an important feature in the colonisation of the British Isles.

1.7. Phenology

The flight period in the British Isles, using all recorded data 1999-2005 from the British Dragonfly Recoding Network (courtesy of S. Cham) is shown in Figure 1.10. It shows the flight period in the British Isles extends from the beginning of June until the start of September. The earliest recorded sightings in the British Isles were on 6 June at a number of sites including Wrest Park, Bedfordshire, Priory Park, Bedfordshire, and Marvel Farm, Isle of Wight. There was also an unconfirmed, but likely, sighting on 31 May at

Curry Farm Essex (Dewick, 2004). The peak period of adult activity occurs around mid August. The latest recorded sighting was on 2 October at Earls Barton Gravel Pit, Northamptonshire. The first adult emergences recorded in Britain, were during June in Essex. The first emergences on the continent are usually about 2-3 weeks later, with numbers peaking between the end of July and the middle of August (Wasscher, 1999). In the Netherlands, the flight period is currently longer than in the British Isles and extends from the end of May to the first week of October (Wasscher, 1999).

1.8. Community effects of invasion

Interspecific competition is likely to be most crucial at the larval stage of development because competition for food and predation on conspecifics can occur. The effect of the arrival of *E. viridulum* on freshwater communities in the UK is hard to predict with the current limited knowledge of its ecology and behaviour. However, the sheer numbers of individuals present at some sites would suggest that it has the potential to bring about community level perturbations. When the Golden-ringed dragonfly, *Cordulegaster boltonii* "invaded" a well documented stream community, niche space became more tightly packed and it was predicted that this might in cause species existing in similar niche space to go extinct (Woodward & Hildrew, 2001). However, unlike *C. botonii*, *E. viridulum* is unlikely to become the top predator in aquatic communities because larger odonate larvae or teleosts are already present in many of these ecosystems.

1.9. Summary

A wide range of taxa appear to be shifting their range margins northward, apparently in response to global climate change. The range expansion of *E. viridulum*, both in northern Europe in the last 30 years and the British Isles since 1999 has occurred at a rapid rate. Large numbers of migrants have crossed the English Channel, arriving at the British coast between Devon and East Yorkshire. This has been facilitated by *E. viridulum*'s high migration rate and ability to locate and colonise suitable new habitats. This species is

relatively eurytolerant, though shows specific habitat preferences, the most important of which is the presence of abundant floating aquatic macrophytes. Connectivity between freshwater systems is another important factor in the spread of *E. viridulum*, with the Rhine valley in Europe and the Thames valley in the UK acting as colonisations routes. The rapid expansion of this species throws up many questions about origins of the British population and the causes and effects of such an event, some of which will be addressed in later chapters.

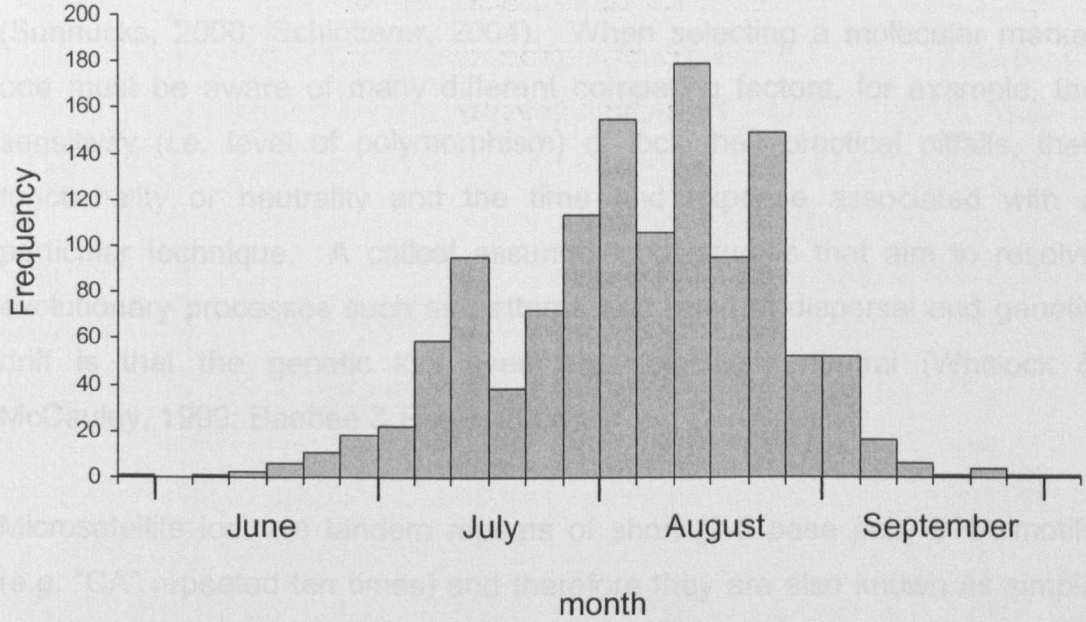


Figure 1.10. *E. viridulum* flight period in UK using 1999-2006 data from British Dragonfly Recording Network (courtesy of G. French).

Chapter 2

Isolation of microsatellite loci in the small red-eyed damselfly, *Erythromma viridulum*, using an enrichment approach.

2.1. Introduction

Genetic approaches to answering fundamental problems in ecology are becoming increasingly more efficient, powerful and flexible. For example, for natural populations it is now possible to accurately determine parentage and reproductive success, dispersal rates, effective population size and quantify the signal of historic events from contemporary spatial genetic structure (reviewed by Jarne & Lagoda, 1996; Sunnucks, 2000; Frankham *et al.*, 2002; Beebee & Rowe, 2004; Excoffier & Heckel, 2006; Selkoe & Toonen, 2006; Allendorf & Luikart, 2007). However, there are considerable differences in the characteristics of different types of molecular-genetic markers and it is crucial that the choice of marker is appropriate to the problem being tackled (Sunnucks, 2000; Schlötterer, 2004). When selecting a molecular marker one must be aware of many different competing factors, for example, the sensitivity (*i.e.* level of polymorphism) of loci, their practical pitfalls, their functionality or neutrality and the time and expense associated with a particular technique. A critical assumption of studies that aim to resolve evolutionary processes such as patterns and rates of dispersal and genetic drift is that the genetic loci used are selectively neutral (Whitlock & McCauley, 1999; Beebee & Rowe, 2004).

Microsatellite loci are tandem repeats of short (2-6 base pair) DNA motifs (*e.g.* "CA" repeated ten times) and therefore they are also known as simple sequence repeats (SSR), variable number tandem repeats (VNTR) and short tandem repeats (STR). This class of genetic markers has been found to be typically abundant in the genomes of all prokaryotic and eukaryotic organisms analysed to date, occurring in both coding and non-coding regions. Different alleles at a microsatellite locus vary in the number of core

repeat motifs, typically between 5 and 40 units, although longer arrays of repeat units appear typical of certain taxa such as teleosts, while many insect species appear to have rather short, and infrequently distributed, microsatellite loci (see Amos, 1999). Dinucleotide (2 bp motifs), trinucleotide (3 bp motifs) and tetranucleotide (4 bp motifs) repeats are the most frequently employed categories of repeat unit for molecular genetic studies. Dinucleotide repeats account for the majority of microsatellites isolated for many species (Li *et al.*, 2002), probably because they are the most abundant class of marker in the genome, although they can often suffer from problems when resolving alleles because of slippage during PCR that causes stutter banding (see Figure 2.2 for example of stutter bands). Trinucleotide and hexanucleotide (6 bp) repeats are the most likely repeat classes to appear in coding regions because they do not cause a frameshift when alleles expand or contract (Toth *et al.*, 2000).

Mutation at microsatellite alleles, which occurs in the form of slippage and proofreading errors during DNA replication, primarily changes the number of repeats (*i.e.* the length) of the microsatellite array (Jarne & Lagoda, 1996). Typically new alleles are formed by gain or loss of a single repeat motif, which is characterised by the stepwise model of mutation (SMM, Ohta & Kimura, 1973). However, multi-step mutations (*i.e.* expansion or contraction of >1 repeat motifs) have also been documented (Amos, 1999; Xu *et al.*, 2000; Harr *et al.*, 2002; Huang *et al.*, 2002). This type of mutation is addressed by the two-phase model (TPM; DiRienzo *et al.*, 1994) that allows mutations of 1 repeat (one-phase) with probability p and mutations of $\geq$ unit(s) (two-phase) with probability of $1-p$. Irrespective of the appropriate mutational model, because alleles vary in length, rather than an alteration to the underlying sequence, they can be readily distinguished by acrylamide gel electrophoresis, thereby permitting high-throughput genotyping for a fraction of the cost and time compared with direct sequencing. Because most microsatellite loci are situated in non-coding genomic regions they can tolerate high rates of mutation (*e.g.* between 10^{-2} and 10^{-6} mutations per locus per generation, and on average 5×10^{-4}), which generates the high levels of polymorphism necessary for genetic studies of processes acting on

ecological time scales (Schlötterer, 2000). In short, their high levels of variability and relative ease of survey mean that they are the genetic marker of choice for studies of population genetic structure or parentage (Selkoe & Toonen, 2006). One potential drawback with using microsatellite loci, however, is that typically they must first be isolated *de novo* from the target species.

Until recently, panels of microsatellite loci have been published for relatively few species of odonate: *Ischnura elegans* (Cooper *et al.*, 1996), *Megaloprepus coerulatus* (Fincke & Hadrys, 2001), *Nehalennia irene* (Wong *et al.*, 2003), *Coenagrion mercuriale* (Watts *et al.*, 2004b, c), *Trithemis arteriosa* (Giere & Hadrys, 2006), *Anax imperator* (Hadrys *et al.*, 2007a), *Orthetrum coerulescens* (Hadrys *et al.*, 2007b) and *Coenagrion puella* (Lowe *et al.*, 2007). Given that cross-species amplification of microsatellite loci is certainly possible in various taxa (*e.g.* Kemp *et al.*, 1995; Ellegren *et al.*, 1997; Galbusera *et al.*, 2000; Watts *et al.*, 2001; Cunha & Watts, 2007), including odonates (Watts *et al.*, 2004b), it is possible that genetic studies may be feasible for many other odonate species without the need for further microsatellite development. However a drawback of this approach is the characteristic reduction in the level of polymorphism observed in the non-target species (Ellegren *et al.*, 1997; Galbusera *et al.*, 2000; Watts *et al.*, 2004a). Moreover, given the relatively poor-amplification success of loci developed for *C. mercuriale* in other members of the Coenagrionidae (Watts *et al.*, 2004b) it is clear that only a panel of microsatellite loci isolated specifically from the genome of the small red-eyed damselfly *Erythromma viridulum* will provide the necessary numbers of polymorphic loci for meaningful population-genetic analyses.

A wide range of techniques have been developed to improve the efficacy of isolation of microsatellite loci (reviewed by Zane *et al.*, 2002; Selkoe & Toonen, 2006). The method I employed was taken from a guide to microsatellite library enrichment provided by Bloor *et al.* (2001), which is based on protocols developed by Refseth *et al.* (1997), Fischer & Bachmann (1998), Gardner *et al.* (1999) and Hamilton *et al.* (1999).

The aims of this chapter are (1) to isolate and characterise a panel of microsatellite loci from the genome of *E. viridulum* and (2) contrast levels of genetic diversity in microsatellite loci isolated from a variety of odonate species. The short publication in *Molecular Ecology Notes* that describes the microsatellite loci that were found to be polymorphic in *E. viridulum* is provided in Appendix 2.1.

2.2. Methods

2.2.1. Sample collection

Adult *E. viridulum* were collected using a dragonfly net with a 2 m extendable pole. To implement a general policy of non-destructive sampling where possible, hind tibias were removed from most samples and the adults released, as removal of a single leg has been shown not to measurably affect fitness in damselflies (Finke & Hadrys, 2001). However, to obtain enough DNA for microsatellite library construction (Sections 2.2.3 onwards) 2 adults were caught and killed in 2003 in Essex. Whole bodies were stored in 50 ml centrifuge tubes and leg samples in individual 1.5 ml microcentrifuge tubes in excess 70 % ethanol and maintained at 4°C until DNA extraction.

2.2.2. DNA extraction

Total genomic DNA was extracted using the high salt protocol of Sunnucks & Hales (1996). Briefly, for library construction thoracic muscle tissue was dissected from the thorax and blotted dry until the all traces of ethanol had evaporated. Leg or tissue samples were incubated in 300 or 500 µl of TNES buffer (50 mM Tris pH 7.5, 400 mM NaCl, 20 mM EDTA, 0.5 % SDS) respectively with 20 or 15 µl of 10 $mgml^{-1}$ Proteinase-K (Promega) at 50°C for at least 4 hrs. Following digestion, samples were then spun in a microcentrifuge at 13,000 rpm for 6 mins and the supernatant poured into a fresh 1.5 ml microcentrifuge tube. Two volumes of absolute ethanol (-20°C) was added to the supernatant which was then allowed to precipitate overnight at -20°C. Samples were spun at 13,000 rpm for 30 mins at 4°C and the supernatant removed carefully so as not to dislodge the precipitated

DNA pellet. DNA Pellets were gently washed twice with 500 µl ice-cold 70 % ethanol and allowed to air dry. DNAs from thorax muscle or legs were then re-suspended in 30 or 20 µl 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA) respectively.

2.2.3. Digestion of DNA and adapter ligation

Genomic DNA from the flight muscle of two adult *E. viridulum* was combined. Nine µg of this DNA was digested at 37°C for 2 hours using 40 u *Sau*3A restriction enzyme (Boehringer-Mannheim) in a final volume of 90 µl. The DNA fragments were then ligated to 50 pmol of phosphorylated linkers (SauLA -> 5'-GGC CAG AGA CCC CAA GCT TCG -3' annealed to SauLB -> 5'-PO_4-GAT CCG AAG CTT GGG GTC TCT GGC C-3'; Refseth *et al.*, 1997) using 40 u T4 DNA ligase (Promega) and incubation at 4°C overnight. The enzymes were then inactivated by heating to 65°C for 10 mins.

2.2.4. Size selection and PCR-amplification of adaptor-ligated DNA

All digested DNA was electrophoresed for 20 mins at 100 v on a 2 % agarose gel containing 0.5 $\mu g ml^{-1}$ ethidium bromide and then run alongside a 100 bp PCR ladder (Promega). Under ultraviolet light the fraction of digested DNA between 300 and 1,100 bp was excised using a sterile scalpel and placed into a pre-weighed 1.5 ml microfuge tube. DNA was then purified using QIAquick gel extraction kit (Qiagen) according to the manufacturer's protocol. The size-selected DNA was concentrated to a final volume of 15 µl using a Microcon YM-100 spin column (Millipore). Confirmation of successful ligation was achieved by 10 µl PCR that contained: 1 µl DNA, 75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20®, 0.2mM each dNTP, 1.5 mM $MgCl_2$, 250 pmol primer SauLA and 0.625 u *Taq* polymerase (ABgene). Thermal cycling conditions were: 95°C for 5 min, followed by 30 cycles of 95°C for 50 s, 56°C for 1 min, and 72°C for 2 min, and finally 72°C for 10 min. PCR success was determined by running 5 µl of PCR product on a 2 % agarose gel alongside a 100 bp DNA ladder (Promega) with success indicated by a smear between 300 and 1,100 bp.

2.2.5. Capture of microsatellite DNA-containing fragments

100 µl of streptavadin-coated magnetic beads (10 $mgml^{-1}$) (M-280 Dynabeads, Dynal) were washed twice with 100 µl of 1X Washing/Binding (W/B) buffer (1 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) in a 0.5 ml microcentrifuge tube. Magnetic beads were then resuspended in 200 µl of 2X W/B buffer to which 200 pmol of 3'-biotinylated oligonucleotide (100 mM) (CA_{12}) was added and then the mixture made up to a final volume of 400 µl. The sample was incubated at room temperature for 30 min with gentle agitation by pipetting every 5-10 min. Next, the beads were washed once in 400µl 1X W/B, twice in 400 µl of 6X SSC, and then re-suspended in 50 µl 6X SSC and incubated at 60°C. In a separate 0.5 ml microcentrifuge tube 10 µl of the ligated DNA, 20 pmol of SauLA and 20X SSC (*i.e.* a final concentration 6X) were made up to 50 µl final volume and gently mixed with a pipette and denatured by incubation at 95°C for 5 min. After this, the temperature was ramped down to 60°C and the contents of the resuspended bead mixture added to the single-stranded DNA sample and gently mixed. The adaptor-ligated / bead-probe mixture was then incubated at 60°C for 30 min, with gentle agitation every 5 min. Next, the magnetic beads were separated from the supernatant using a magnet and the supernatant removed. Magnetic beads were re-suspended in 100 µl of 2X SSC and washed a further four times with 1 ml 2X SSC, incubating the sample for 5 min at room temperature between each wash. Following this, the bead mixture was washed an additional four times in 1X SSC, with incubation for 5 min at room temperature. After the final wash, the bead mixture was re-suspended in 100 µl of 1X SSC and aliquotted into four 25 µl samples. 250 µl of 1X SSC was added to each aliquot which was then incubated at 60°C for 10 min. The supernatant was removed and the beads rinsed for 30 s at room temperature in 400 µl of 1X TE. The supernatant was again removed and the beads rinsed for 30 s at room temperature in 400 µl 50 mM NaCl. Finally, aliquots were re-suspended in 50 µl PCR-grade water giving a final bead concentration of 5 $\mu g\mu l^{-1}$.

To check quantity of enriched DNA, a PCR was set up in a 10 μl final reaction volume consisting of 40 μg bead suspension, 75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20®, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 30 pmol SauLA and 0.625 u *Taq* polymerase (ABgene). PCR conditions were 95°C for 3 mins, 3 cycles of 95°C 30 s, 55°C 30 s, 72°C 45 s, followed by 30 cycles of 92°C 30 s, 55°C 30 s, 72°C 55 s, and finally 72°C for 10 min. 5 μl of the PCR product was run on a 2 % agarose gel for 20 min at 100 v alongside a 100 bp ladder (Promega), with successful capture and PCR indicated by a smear between approximately 0.3 and 1.1 Kbp. PCR products were then purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol.

2.2.6. Ligation and transformation

Approximately 50 ng of PCR purified products were ligated into 50 ng of pGEM-T vector (Promega) using 3 Weiss units of T4 DNA ligase in a 10 μl final volume following the manufacturer's instructions. The ligation mixture was incubated overnight at 4°C to provide the maximum number of transformants.

Several aliquots of 2 μl of the ligation mixture were then each transformed into 100 μl JM109 high efficiency competent *Escherischia coli* cells (Promega), again following the manufacturer's exact protocol. 50 μl of each transformation reaction was plated onto S-gal agar (Sigma) plates containing 100 $\mu g ml^{-1}$ ampicillin (Sigma) and incubated overnight at 37°C; bacterial colonies with a vector and an insert were identified because of their white colour.

2.2.7. Library construction and microsatellite screening

Using a sterile toothpick, white colonies were picked and swirled in one well (of a 96-well plate) containing 100 μl LB media and ampicillin (final concentration of 100 $\mu g ml^{-1}$). For microsatellite screening, the same toothpick was then swirled into a corresponding well of a 96-well plate containing 20 μl PCR consisting of 75 mM Tris-HCl (pH 8.8), 20 mM

$(NH4)_2SO_4$, 0.01 % (v/v) Tween20, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 10 pmol SauLA, 10 pmol CA_{15} oligonucleotide and 0.25 U *Taq* polymerase (ABgene). LB/ampicillin plates were incubated at 37°C for 3-4 hours and then 100 µl of sterile LB/30 % glycerol was added to each well; these LB/glycerol plates are then stored indefinitely at -80°C. Thermal cycling conditions for the microsatellite-screening PCR were: 95°C 3 min, 3 cycles of 95°C 30 s, 56°C 30 s, 72°C 45 s, followed by 30 cycles of 92°C 30 s, 56°C 30 s, 72°C for 55 s, and finally 72°C for 10 min. 5 µl of the PCR product was run alongside a 100 bp PCR ladder (Promega) on a 2 % agarose gel containing ethidium bromide (at a final concentration of 0.5 μgml^{-1}) at 100 v for 20 min. When visualized under UV light, a double banded PCR product indicated the presence of a microsatellite containing insert.

2.2.8. Sequencing microsatellite containing inserts

From the libraries constructed above, *E. coli* bearing plasmids with microsatellite inserts were streaked onto single LA/ampicillin (100 μgml^{-1}) plates and incubated at 37°C overnight. Single colonies were inoculated into individual 50 ml Falcon tubes containing 10 ml of LB/ampicillin and grown overnight at 37°C with gentle shaking (at 150 rpm). Plasmids were prepared from 2 ml of this culture using a Qiagen plasmid mini kit according the manufacturer's instructions. I then cycle-sequenced 178 positive clones using standard M13 forward primer (5'- TGT AAA ACG ACG GCC AGT 3') and Big Dye™ chemistry (Applied Biosystems) and electrophoresis on an ABI3100 (Applied Biosystems). Sequences were analysed using SEQSCAPE v 2.0 (Applied Biosystems) to identify microsatellite repeat motifs. Samples containing five or more microsatellite repeat units were reverse sequenced using M13 reverse primer (5'- CAG GAA ACA GCT ATG ACC 3') and the reverse and forward sequences aligned with SEQSCAPE v. 2.0 (Applied Biosystems) to check for consistency.

2.2.9. Primer design and PCR optimisation

Primers flanking microsatellite sequences were designed using PRIMER 2 software (S.J. Kemp, unpublished). PCR conditions for each primer pair

were optimised for primer annealing temperature (T_a) and $MgCl_2$ concentration using a gradient PCR that spanned the predicted T_a by ±5°C. PCR conditions for primer optimisation were: 95°C 3 min, 30 cycles of 95°C 30 s, a gradient of T_a °C 30 s, 72°C 45 s, and finally 72°C 10 min. Each PCR contained 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20, 0.2 mM each dNTP, either 1.5 or 3.0 mM $MgCl_2$, 5-50 ng template DNA, 10 pmol each primer and 0.25 U *Taq* polymerase (ABgene).

Genbank (http://www.ncbi.nlm.nih.gov/) accession numbers and primers designed for all of the microsatellite sequences that provided single, interpretable bands (see section 2.3 below), along with optimal annealing temperatures for PCRs, are provided in Table 2.1. The majority of PCRs produced single PCR products at 3.0 mM $MgCl_2$, so this concentration was used for PCR at all loci.

2.2.10. Analysis of microsatellite polymorphism

Levels of polymorphism in microsatellite 10 loci were assessed from 43 adult *E. viridulum* collected from two populations: Yarbridge (n = 23) on the Isle of Wight and East Ruston (n = 20) in Norfolk both in UK (see Table 3.1 and Figure 3.1 for further details of site locations). The online version of GENEPOP v. 3.1d (Raymond & Rousset, 1995) was used to calculate basic measures of genetic diversity (numbers of alleles, observed heterozygosity, expected heterozygosity - over all samples), the significance of any deviations from expected Hardy-Weinberg conditions (within each site) and also for linkage disequilibrium between all pairs of loci (making 2,000 permutations of alleles among individuals within samples).

For comparison with other odonates, I constructed a scatterplot of the longest number of uninterrupted repeat motifs against (1) the expected heterozygosities (H_e) and (2) the numbers of alleles for all odonate microsatellite loci that have been characterised in primer notes (*i.e.* where > 5 loci were isolated).

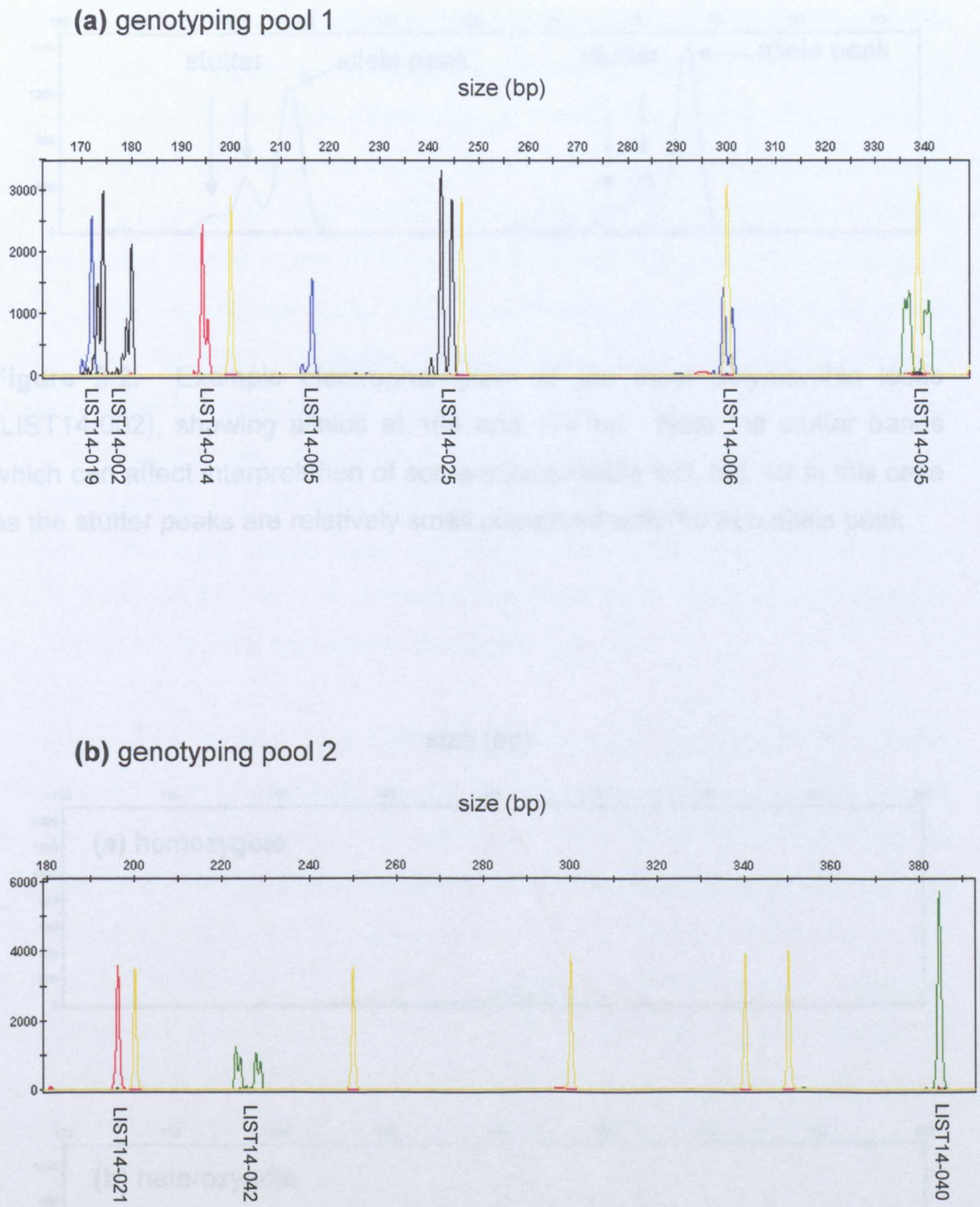
2.3. Results.

Of the 43 microsatellite sequences that I was able to design primers around (see Appendix 2.2), 20 loci amplified spurious bands and 13 loci were monomorphic (primers and PCR conditions for these loci are characterised in Appendix 2.3), leaving 10 polymorphic loci that resolved distinct alleles within the expected size range (see Table 2.1). These 10 loci were separated into two genotyping pools so that no two loci with the same fluorescent dye had overlapping allelic size ranges. Seven loci, LIST14-002, LIST14-005, LIST14-006, LIST14-014, LIST14-019, LIST14-025 and LIST14-035, were assigned to Pool 1, an example electropherogram of which is shown in Figure 2.1a, while just three loci, LIST14-021, LIST14-040 and LIST14-042, were assigned to genotyping Pool 2, as shown in the electropherogram in Figure 2.1b.

The number of alleles ranged between two (LIST14-021 and LIST14-40) and eight (LIST14-002); observed and expected heterozygosities varied between 0.000 and 0.698 and 0.045–0.688, respectively (Table 2.2). LIST14-005 was monomorphic in the Isle of Wight population but alternate alleles were found in the Norfolk population. An example electropherogram of the most polymorphic locus, LIST14-002, is shown in Figure 2.2, which demonstrates the size range at this locus.

Table 2.1. Summary characteristics and primer sequences for 10 polymorphic microsatellite loci isolated from the small red-eyed damselfly *Erythromma viridulum*; dye, 5' fluorescent label (Applied Biosystems); T_a, annealing temperature.

Locus	Primer sequence (5' -> 3')	Core motif	Dye	T_a (°C)	GenBank Accession no.
LIST14-002	F: TTT CGT CTC AAG GAC CGC R: GAG AGT GGT TTT TGC TTC G	$(AC)_5(AC)_4(AC)_{10}$	NED	56	BV448262
LIST14-005	F: CCA TAC CCA TGG ATA ATA GC R: AAG CTT CGG ATC ATT TTC GG	$(AC)_8$	6-FAM	56	BV448263
LIST14-006	F: GGC TCA GTC TCA CAT TTC C R: CAA AGG ACC TAT CCC AAC G	$(AC)_{10}$	6-FAM	56	BV448264
LIST14-014	F: CTT AAC CCA CCT CAC GAC CAC C R: TTC GCG CCC ATT CTC ACT CTC G	$(AC)_7$	PET	60	BV448265
LIST14-019	F: ACG TCC TCA TGC TGC ATT CGC R: CGA CTC CTG GTT ATG TCC TCC	$(GT)_{10}$	6-FAM	56	BV448266
LIST14-021	F: GGA GAT AAG CGA GGA TGA GG R: ACC CCA CTT TTA GGA GGT CC	$(GT)_2(GT)_5$	PET	48	BV448267
LIST14-025	F: ATC TCA CCC CAT CTT GTG C R: GTT CTC GGA AAA CTG ACC G	$(TG)_5(GT)_3$	NED	48	BV448268
LIST14-035	F: GTG TTG TCT GCT GAA TGG C R: TAC AGG GAA GAG GAC TAC C	$(GT)_9$	VIC	55	BV448269
LIST14-040	F: TAT GCG ACA AGT TAG CCG R: ATC CCA AGG TTA CAA CGC	$(GCT)_4(GCT)_2$	VIC	45	BV448270
LIST14-042	F: CAG CGG TAT CAA ACT TCG R: AAA AGG CGA GAA ATC CCG	$(GT)_9$	VIC	50	BV448271



Figures 2.1a, b. Example electropherograms of the ten polymorphic microsatellite loci assigned to genotyping pools 1 (**a**, seven loci) and 2 (**b**, three loci). Orange peaks correspond to the 500 bp LIZ size standard (Applied Biosystems).

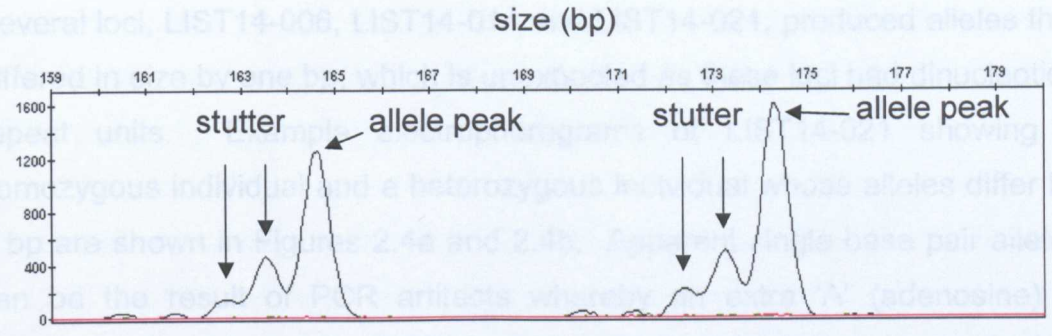
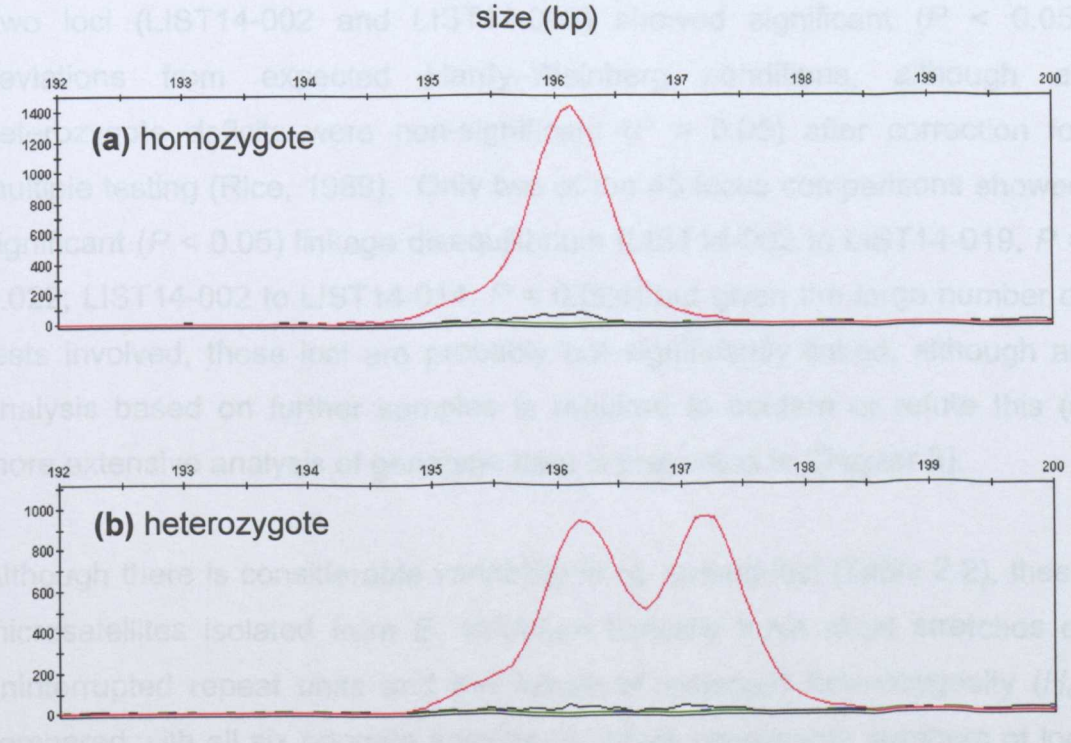


Figure 2.2. Example electropherogram of the most polymorphic locus (LIST14-002), showing alleles at 164 and 174 bp. Note the stutter bands which can affect interpretation of some microsatellite loci, but not in this case as the stutter peaks are relatively small compared with the true allele peak.



Figures 2.3a, b. Example electropherogram of alleles at LIST14-021 showing (a) a typical homozygous individual with a single allele at 196 bp and (b) a heterozygous individual with 196 and 197 bp alleles.

Several loci, LIST14-006, LIST14-014 and LIST14-021, produced alleles that differed in size by one bp, which is unexpected as these loci had dinucleotide repeat units. Example electropherograms of LIST14-021 showing a homozygous individual and a heterozygous individual whose alleles differ by 1 bp are shown in Figures 2.4a and 2.4b. Apparent single base pair alleles can be the result of PCR artifacts whereby an extra 'A' (adenosine) is inconsistently added to the PCR product or stutter banding, *i.e.* loss of allele during PCR (see Hauge & Litt, 1993; Dewoody *et al.*, 2006). However, both factors can be ruled out as the cause of these single bp alleles for these microsatellite because (i) little or no stutter was observed for homozygotes and (ii) heterozygotes were observed during repeat genotyping, which would not be expected if variable PCR-amplification was generating apparent alleles. Therefore, single base pair alleles were retained within the data – single base alleles are not uncommonly observed in microsatellite studies and represent a point mutation in the target sequence.

Two loci (LIST14-002 and LIST14-005) showed significant ($P < 0.05$) deviations from expected Hardy–Weinberg conditions, although all heterozygote deficits were non-significant ($P > 0.05$) after correction for multiple testing (Rice, 1989). Only two of the 45 locus comparisons showed significant ($P < 0.05$) linkage disequilibrium (LIST14-002 to LIST14-019, $P = 0.022$; LIST14-002 to LIST14-014, $P = 0.024$) but given the large number of tests involved, these loci are probably not significantly linked, although an analysis based on further samples is required to confirm or refute this (a more extensive analysis of genotype data is presented in Chapter 3).

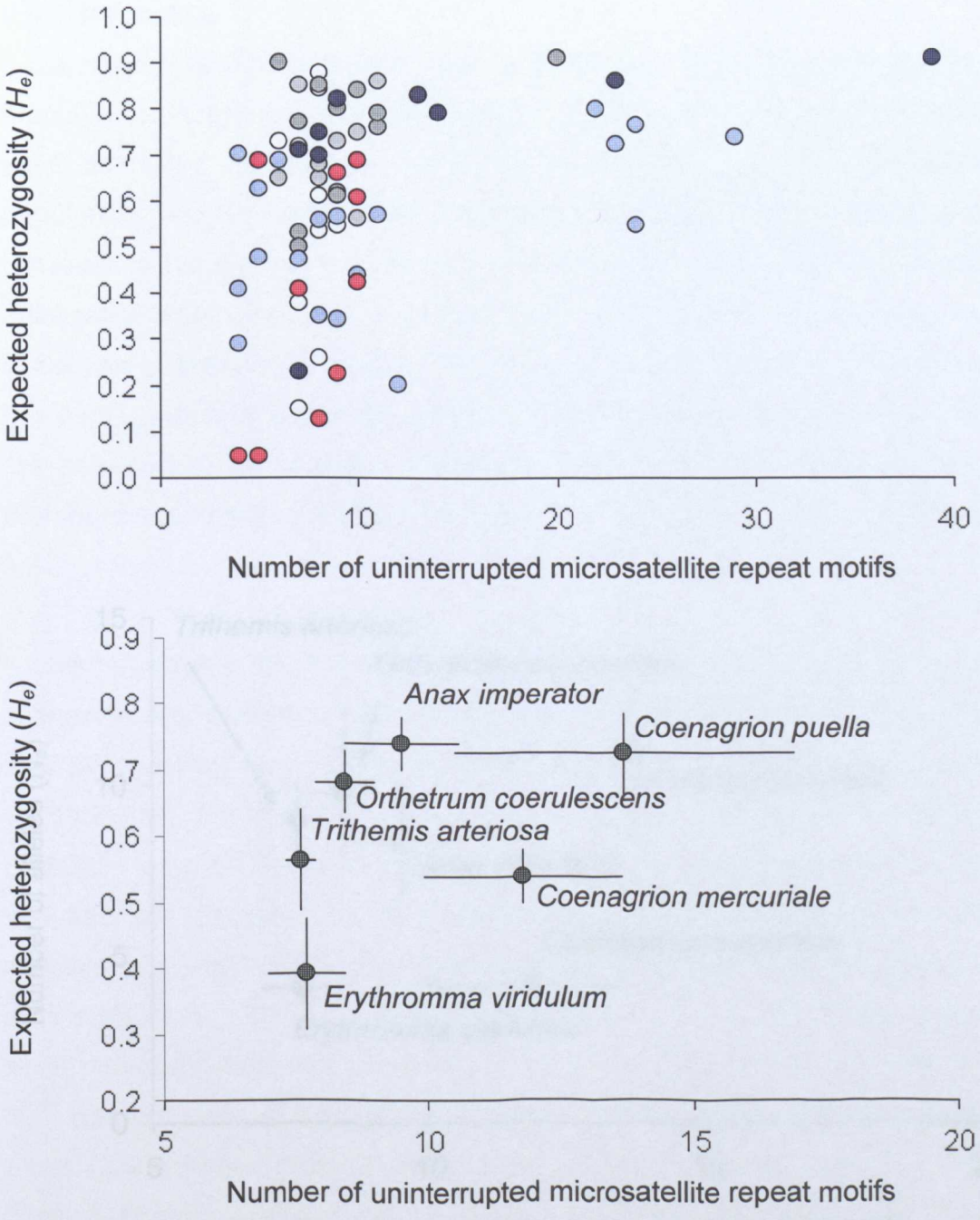
Although there is considerable variability in H_e among loci (Table 2.2), these microsatellites isolated from *E. viridulum* typically have short stretches of uninterrupted repeat units and low levels of expected heterozygosity (H_e) compared with all six odonate species for which reasonable numbers of loci have been characterised (Figures 2.5a, b).

A similar pattern of low genetic diversity is observed for the relationship between the mean number of uninterrupted repeat units and number of alleles present at odonate microsatellite loci (Figure 2.2).

The largest microsatellite found in *E. viridulum* consisted of 31 repeat units for LIST14-010 (see Appendix 2.2) though primer design for this locus was unsuccessful. Typically, none of the *E. viridulum* microsatellites have more than 10 uninterrupted repeats, while the two *Coenagrion* species generally had longer microsatellite loci. In general, the number of uninterrupted repeats for all odonates is low, with the majority of microsatellite loci having fewer than 15 tandem repeats (see Figures 2.5a, b).

Table 2.2. Levels of variability at 10 polymorphic microsatellite loci in adult small red-eyed damselflies *Erythromma viridulum* from Yarbridge, Isle of Wight (n = 23) and East Ruston, Norfolk (n = 20), both in the UK. N_a, number of alleles observed; H_o, observed heterozygosity; H_e, expected heterozygosity. *indicates a significant departure ($P < 0.05$) from expected (within site) Hardy–Weinberg equilibrium conditions.

Locus	Size range	N_a	H_o	H_e
LIST14-002	147–186	8	0.512*	0.687
LIST14-005	206–220	3	0.000*	0.126
LIST14-006	297–303	5	0.488	0.605
LIST14-014	194–198	3	0.419	0.408
LIST14-019	168–172	3	0.442	0.424
LIST14-021	196–200	2	0.000	0.045
LIST14-025	246–256	6	0.419	0.688
LIST14-035	333–343	5	0.698	0.662
LIST14-040	384–386	2	0.000	0.045
LIST14-042	223–230	3	0.163	0.225



Figures 2.5a, b. (a) individual locus variation in expected heterozygosity (H_e) and the maximum number of uninterrupted repeat motifs at microsatellite loci isolated from six odonates species: small red-eyed damselfly *Erythromma viridulum* (red circles); light blue, *Coenagrion mercuriale* (Watts *et al.*, 2004b, c); white, *Trithemis arteriosa* (Giere & Hadrys, 2006); dark grey, *Anax imperator* (Hadrys *et al.*, 2007a); light grey, *Orthetrum coerulescens* (Hadrys *et al.*, 2007b); dark blue, *Coenagrion puella* (Lowe *et al.*, 2007). (b) above data plotted as mean ±95 % CI.

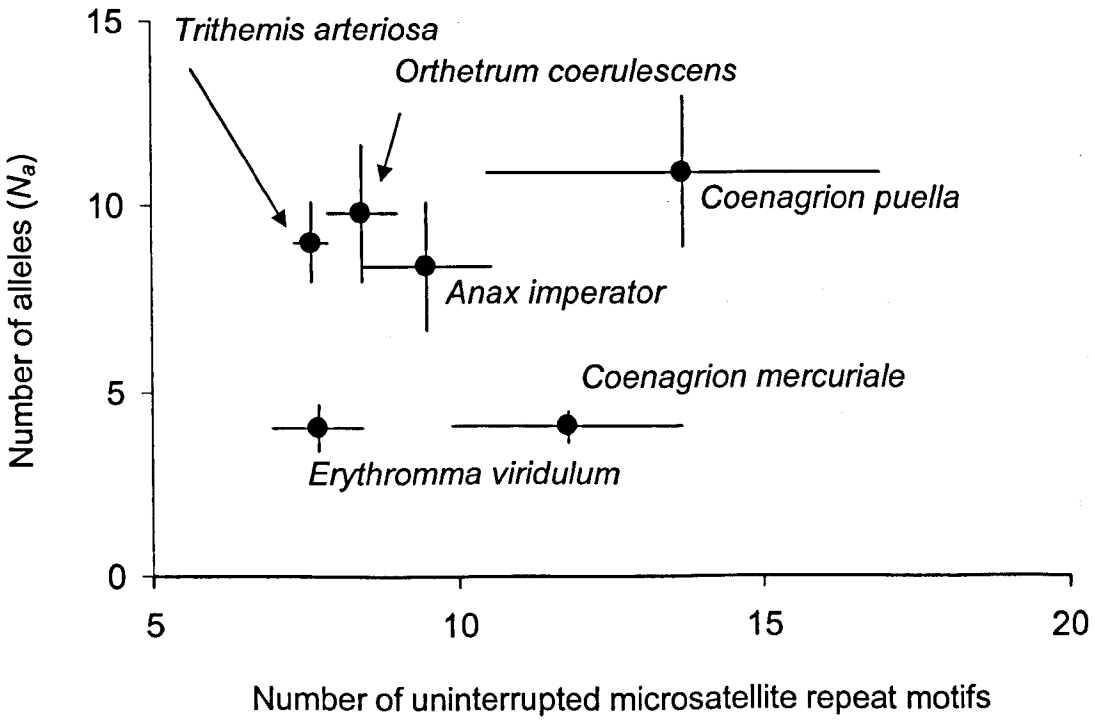


Figure 2.6. Variation in mean (±95 % CI) number of uninterrupted repeat motifs against mean (±95 % CI) numbers of alleles (N_a) at microsatellite loci isolated from six odonates species: small red-eyed damselfly *Erythromma viridulum*, *Coenagrion mercuriale* (Watts *et al.*, 2004b, c), *Trithemis arteriosa* (Giere & Hadrys, 2006), *Anax imperator* (Hadrys *et al.*, 2007a), *Orthetrum coerulescens* (Hadrys *et al.*, 2007b), *Coenagrion puella* (Lowe *et al.*, 2007).

2.4. Discussion

I developed ten polymorphic microsatellite loci that were subsequently employed to characterise spatio-temporal genetic structure in the small red-eyed damselfly, *E. viridulum* (see Chapters 3 and 4). To be useful for population genetic studies these loci must be unlinked (*i.e.* independent). However, while two pairs of loci were linked in this preliminary loci, further analysis of greater numbers of samples (see section 3.3.1) revealed only one locus pair in significant linkage disequilibrium (LIST14-021 and LIST14-042, $P < 0.001$). Since this pair of loci differed from those identified above, all loci are assumed to be unlinked. Further analyses of fits to expected Hardy-Weinberg equilibrium conditions are provided in section 3.3.1 and Appendix 3.3.

A striking result is the low levels of diversity in *E. viridulum* microsatellites compared with that described for other odonates. Data on levels of genetic diversity must not be over-interpreted when (1) descriptions of microsatellite variability are based on different sample sizes and (2) there is a distinct contrast to the environmental conditions experienced by the focus populations. Sample size is positively correlated with genetic diversity. However, any correlation is expected to be more pronounced for numbers of alleles (N_a) rather than expected heterozygosity (H_e), because rare alleles, which are uncovered with increasingly larger sample sizes, contribute little to H_e. Sample sizes of studies used to characterise microsatellite variability were: *C. mercuriale* (n = 44; Watts *et al.*, 2004b, c), *T. arteriosa* (n = 122; Giere & Hadrys, 2006), *A. imperator* (n = 14-90; Hadrys *et al.*, 2007a), *O. coerulescens* (n = 23-209; Hadrys *et al.*, 2007b) and *C. puella* (n = 50; Lowe *et al.*, 2007). Clearly, variation in sample size *per se* cannot explain the contrast in diversity between *E. viridulum* and *Coenagrion* species, and since the reduction in diversity is apparent for H_e (Figures 2.5a, b) as well as N_a (Figure 2.6) the signal of generally low genetic diversity in *E. viridulum* is almost certainly real rather than an artefact of poor sampling effort.

Indeed, further analysis of samples reveals that generally low levels of genetic variability in *E. viridulum* are typical of other populations from

northern Europe (see Figure 3.3a). This pattern is likely to represent a signature of recent and rapid range expansion (described in Chapter 1.3.1 and 1.3.2 and discussed in Chapter 3.4.1-3.4.3), rather than a more general signature of *E. viridulum*'s genomic evolution, although clearly further characterisation of additional *E. viridulum* populations from southern Europe is required to confirm or refute this. Thus, while I reported that 13 microsatellite loci were monomorphic (see Appendix 2.3), they may yet prove to be variable when used to genotype a range of individuals from populations towards the centre of *E. viridulum*'s distribution.

All published odonate loci fall into the characteristic mean length reported for insects of ~12 repeat units (Amos, 1999). Since microsatellite length (*i.e.* the number of uninterrupted repeats) generally correlates with allelic diversity, insect microsatellites are expected to be generally be less polymorphic than other taxa, though this has not yet been adequately investigated (Note: since this analysis, additional *Primer Notes* describing microsatellites in odonates have been published (Carballa *et al.*, 2007; Matthews *et al.*, 2007)], thus slightly increasing the power to detect trends in odonate microsatellite/genome structure). Relative abundance of microsatellite loci throughout genome cannot be determined as the odonate loci were isolated using enrichment techniques, but anecdotal evidence (*i.e.* difficulties in isolating large numbers of highly polymorphic loci from odonates – and hence the relative few species that have been studied) suggests that they are relatively infrequent compared with mammals and teleosts.

Chapter 3

Patterns of genetic diversity and spatial structure in the small red-eyed damselfly *Erythromma viridulum* at its north-west European range margin: evidence for two distinct origins of invasion in the UK.

3.1. Introduction

Species naturally respond to climate fluctuations through changes in physiology, phenology, community interactions and, in particular, their geographic distributions (Walther, 2002; Gilman *et al.*, 2006; Parmesan, 2006; Hassall *et al.*, 2007). Global warming has been associated with a directed, northward expansion of many insect species (Parmesan *et al.*, 1999; Parmesan & Yohe, 2003; Yukawa *et al.*, 2007), with rapid shifts in range expected for those taxa with good dispersal capabilities or whose distribution is temperature limited (Pearson & Dawson, 2003; Pearson *et al.*, 2004). Since global temperatures are predicted to rise by between 1.4 and 5.8°C over the next century (IPCC, 2007), many more species are expected to alter their distributions substantially in the near future, often expanding into new areas.

Introduction and spread of non-native species presents a global ecological and conservation crisis as invasive organisms are increasingly altering terrestrial and aquatic communities worldwide (Gurevitch & Padilla, 2004; Hobbs *et al.*, 2006; Nogales *et al.*, 2006). Non-native species can have serious effects on the ecosystems they invade, for example, by hybridisation with resident species, reducing biodiversity, altering demographic processes and impacting on ecosystem processes (Parker *et al.*, 1999; Batten *et al.*, 2006; Parker *et al.*, 2006; Gabbard & Fowler, 2007). From a conservation point of view, species' invasions, either through anthropogenic introduction or by range expansion, may be of particular concern because they often reduce the niche space of native taxa, causing their local extirpation (Yan & Pawson, 1997; Suarez *et al.*, 1998; Mergeay *et al.*, 2006). Anthropogenic introductions often have more severe impacts than range expansions

because they may involve transportation to a novel ecosystem where interactions with native species are less predictable (Cox, 2004). There is also a substantial body of evidence that is not frequently addressed in invasion literature that biological invasions may facilitate native species, particularly through habitat modification (reviewed in Rodriguez, 2006). Identifying the patterns and processes of colonisation of invasive species thus provides essential information for biodiversity management.

Molecular genetic markers offer a convenient and powerful method to trace rates and routes of dispersal since the pattern of spatial genetic structure is determined by the relative influences of migration between populations, selection, random genetic drift, mutation and the mating system (Wright, 1931; reviewed by Frankham *et al.*, 2002; Allendorf & Luikart, 2007). Colonisation in particular leaves a distinct genetic signature in the new population, either at neutral genes through the action of genetic drift, or at adaptive regions of the genome in response to selection in a novel environment. The degree to which the genetic structure of the colonising population differs from the source depends on a number of factors, such as the number of founders, the strength and direction of selective pressures in the new environment and the time since colonisation (Hassan *et al.*, 2003; Grapputo, 2005). Typically, rapid colonisation, by a relatively small population, results in loss of genetic intra-population diversity relative to the source, which may persist for several generations if the initial colonisers establish themselves sufficiently to lessen the genetic impact of subsequent arrivals (Hewitt, 1996; Hawley *et al.*, 2007). Moreover, since newly founded populations have insufficient time to attain migration-drift equilibrium, weak or atypical patterns of spatial genetic structure may result. As would be expected, many studies have found that invasive species have undergone a bottleneck resulting in reduced genetic variability in the introduced range (Lebois *et al.*, 2000; Tsutsui & Case, 2001; Grapputo, 2005). In other cases however, genetic diversity is maintained in the introduced population as a consequence of multiple introductions or large numbers of colonisers (for example, Johnson & Starks, 2004; Kolbe *et al.*, 2004; Korman & Pashley, 1991, 2004; Holland, 2001; Hassan *et al.*, 2003).

Odonates are an important component of freshwater and terrestrial ecosystems and are key bioindicator species. They are perceived as strong fliers, capable of wide dispersal. Many species are undergoing northward range expansion, apparently in response to climate change (Ott, 1996; Akoi, 1997; Hassall *et al.*, 2007), like many other insect species (Hill *et al.*, 1999; Carroll *et al.*, 2003; Crozier, 2003; Parmesan & Yohe, 2003; Hickling, 2006); British odonates are no exception, with 37 species having shifted their range margin northward over the last 40 years (Hickling *et al.*, 2005). Northward range expansion has meant that successful colonisation of the British Isles by formerly continental European odonates has been documented for two species in the last 8 years; the southern emerald damselfly *Lestes barbarus* (Fabricius, 1798) (Nobes, 2003) and the small red-eyed damselfly *Erythromma viridulum* (Charpentier, 1840) (Cham, 2002).

This chapter presents a study of the distribution of genetic variation and spatial genetic structure of the small red-eyed damselfly, *Erythromma viridulum* (Odonata: Zygoptera) across its range in the British Isles and in nearby continental European populations. *E. viridulum* is a thermophilic, holomediterranean species that has undergone significant northward range expansion in Europe in the last 30 years (Ketelaar, 2002), apparently exploiting the Rhine valley as a colonization route through northern Germany and the Netherlands whilst a slower sweep of range expansion has occurred across central Europe into northwest-France. Since 1999 *E. viridulum* has been recorded in the British Isles and is the first recorded example of a migrant damselfly establishing colonies in the British Isles (Dewick & Gerussi, 2000). From initial colonies along the south-east coast between Norfolk and Kent, *E. viridulum* has moved in a north-westerly direction at an average rate of 31.68 km per year (source: British dragonfly recording network, courtesy S. Cham) with continuing waves of immigration from the continent at coastal sites (Cham, 2001, 2002, 2004a, b). There is also an apparently isolated colony on the Isle of Wight that was first recorded in 2000 (Cham, 2001), that has since 2004 spread into neighbouring Hampshire. In 2007 the most northerly sites in the British Isles were in East Yorkshire and the most

westerly counties were Gloucestershire, Somerset and Devon (source: British Dragonfly Society website http://www.britishdragonflysoc.org.uk and the Dragonfly Recording Network database, courtesy of G. French). The rapid range expansion of *E. viridulum* in the British Isles, described in detail in Chapter 1, means that *E. viridulum* presents a unique model with which to investigate the genetic consequences of range expansion in a damselfly as colonisation occurs.

The aims of this chapter are to (1) examine the evidence for a population bottleneck in recently founded populations, (2) contrast levels of genetic variation in UK and continental European populations of *E. viridulum* and (3) determine trends in spatial genetic structure of newly-colonised populations in the UK. It would be expected that populations in the UK will be characterised by population bottlenecks, generally low levels of gene diversity relative to putative source populations from continental Europe and weak spatial genetic structure that is characteristic of recent gene flow and non-equilibrium genetic conditions.

3. 2. Materials and Methods

3.2.1. Study sites

The current range of *E. viridulum* extends from the north Norfolk coast, along the coast of East Anglia including Suffolk, throughout Essex and in more scattered colonies along the Kent coast. A significant westerly range expansion has occurred since the initial colonisation in 1999 that extends from the core of the range in southern Essex, running along the north of the Thames estuary, through Bedfordshire and currently as far inland as Rugby in Warwickshire. There has been a second, isolated colonisation event recorded on the Isle of Wight since 2000 that has slowly expanded its range into the surrounding area and is assumed to be the source of populations in nearby Hampshire that were first recorded around 2004 onwards.

Adult samples were collected during the summers of 2002-2006 from 28 sites in the British Isles (see Figure 3.1) and 11 sites from continental Europe (see

Figure 3.2). Sampling this species is difficult because of its habit of remaining over the water for the majority of its time and its high sensitivity to human disturbance; this meant good access to the water's edge was paramount to sampling success. The majority of samples came from small to medium sized ponds (with an area <1,000 m^{-2}) supporting abundant aquatic vegetation, with the exception of Silver End (SVE), East Ruston (EAR), Priory Country Park (PCP), and Thorpeness Meare (THM), which were larger bodies of water and Strumpshaw Fen (SSF), which is made up of a network of ditches among reed beds. Samples from continental Europe were collected from Braunschweig (BRN) in northern Germany, Koudekerke (KDK) and Alphen Ann Den Rijn (UAD) in south-west Holland, Zonhoven in Belgium at Wijvenheide Eutroof Ven (WEV), Wijvenheide Grote Vijer (WGV) and Het Vinne (HET) near Zoutleeuw and French samples came from an area north of Paris at Courtemont Varennes (CRT), Boves (BVS), Blangy-Tronville (BLT) and Le Marais de Guines (LMD) and (MGG). Locations and grid references for all sites are provided in Figures 3.1 and 3.2, and Table 3.1.

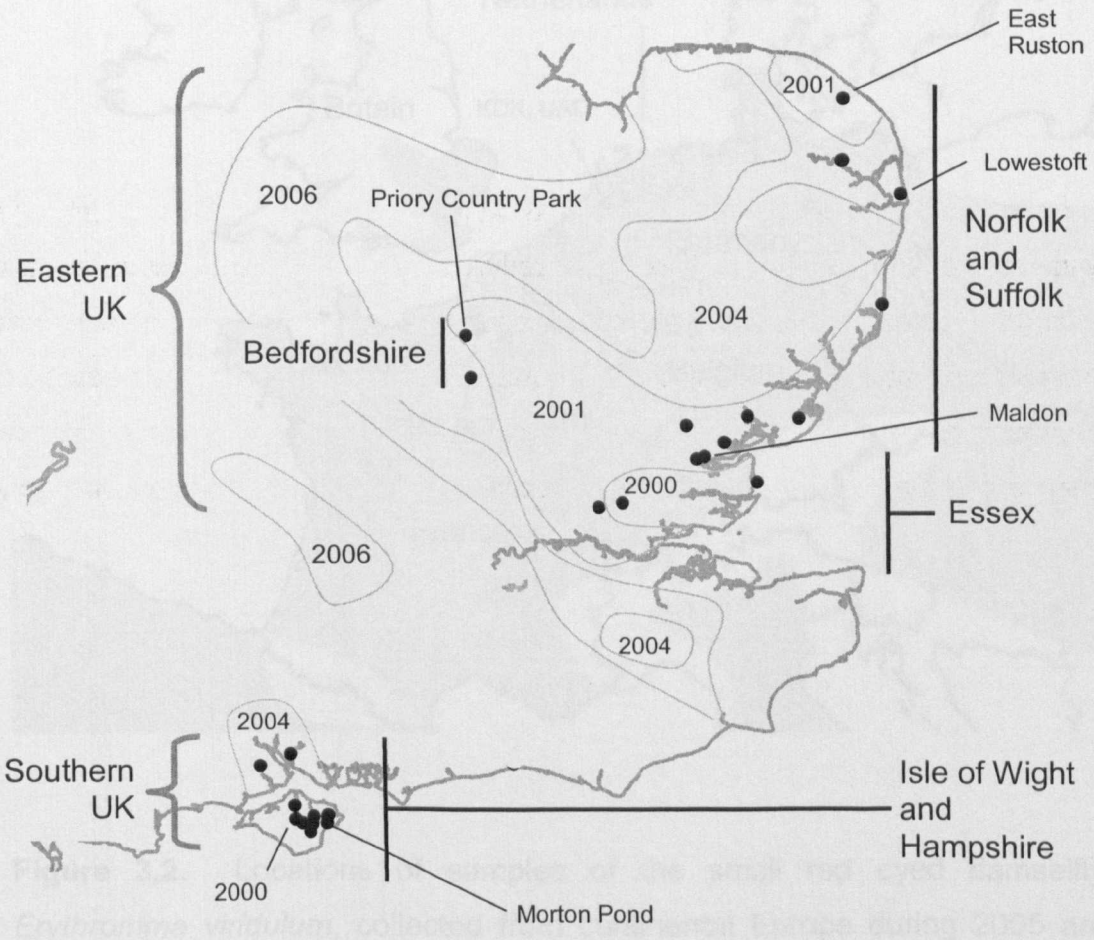


Figure 3.1. Locations of samples of the small red eyed damselfly, *Erythromma viridulum*, collected from the UK during 2002-2006. Contour lines indicate the limit of *E. viridulum*'s range in 2000, 2001, 2004 and 2006.

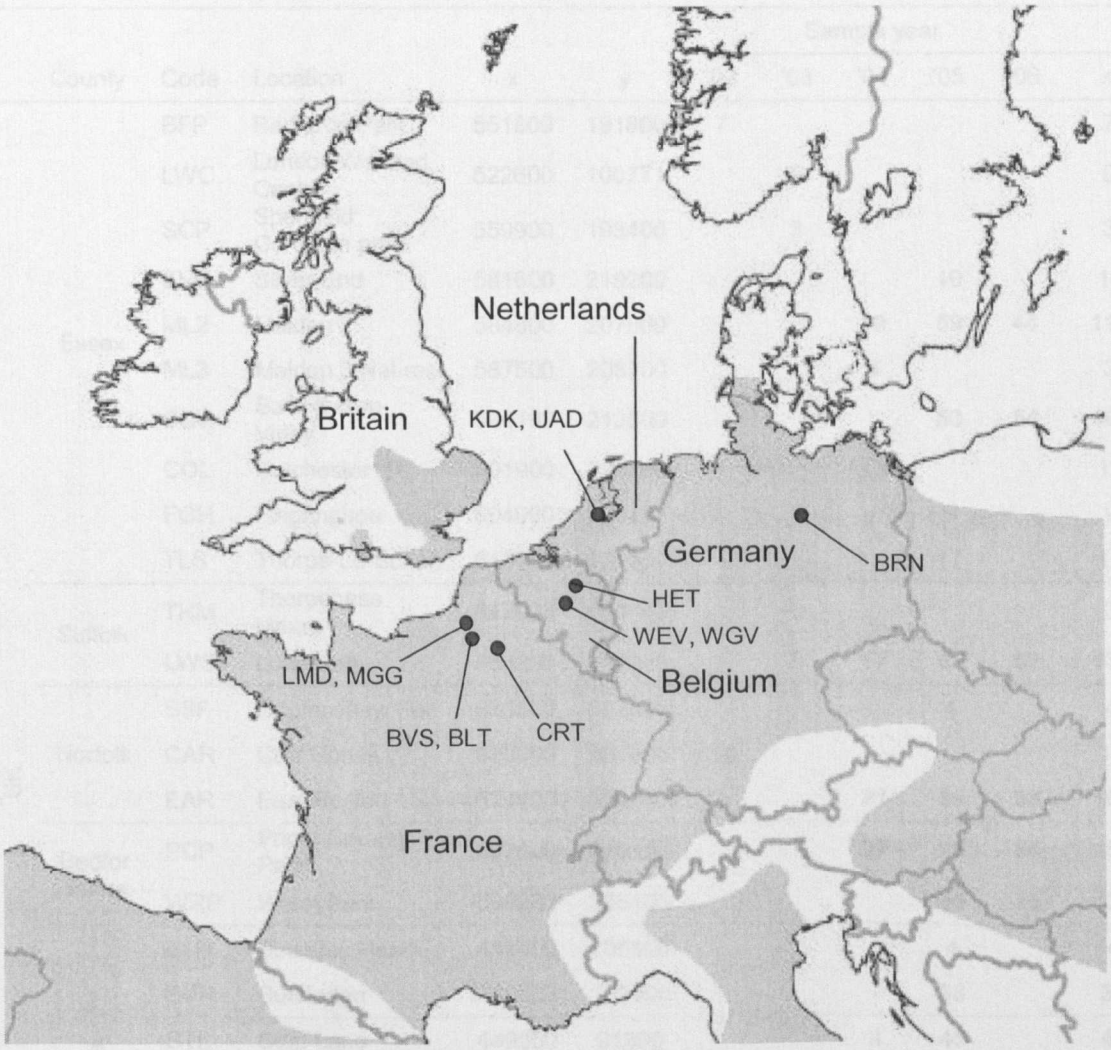


Figure 3.2. Locations of samples of the small red eyed damselfly, *Erythromma viridulum*, collected from continental Europe during 2005 and 2006: Braunschweig (BRN), Koudekerke (KDK), Alphen Ann Den Rijn (UAD), Wijvenheide Eutroof Ven (WEV), Wijvenheide Grote Vijer (WGV), Het Vinne (HET), Courtemont Varennes (CRT), Boves (BVS), Blangy-Tronville (BLT) and Le Marais de Guines (LMD) and (MGG). The shaded area shows the range of *E. viridulum* in 2006 (Adapted from Askew, 1988).

Table 3.1. Summary data on sample sizes, collection dates and spatial locations of *E. viridulum* sites used in this study. x, y values are rectangular grid coordinates converted from Ordinance Survey Landranger format.

						Sample year					
	County	Code	Location	x	y	'02	'03	'04	'05	'06	*n*
UK	Essex	BFP	Bedfords Park	551800	191800	7					7
		LWC	London Wetland Centre	522600	100771		9				9
		SCP	Shenfield Common pond	559900	193400		3				3
		SVE	Silver End	581600	219200				10		10
		ML2	Maldon 2	584800	207900			10	**59**	**44**	**113**
		ML3	Maldon 3 Nat.res.	587500	208700			3			3
		SCV	Salcott-cum-Virley	594100	213500				**53**	**54**	**107**
		COL	Colchester	601900	222300			13			13
		FGH	Fingringhoe	604900	200193				9		9
		TLS	Thorpe-Le-Soken	618900	221500				17		17
	Suffolk	THM	Thorpeness Meare	647000	259400		3				3
		LWS	Lowestoft	653300	296200		9	17	**58**	**52**	**136**
	Norfolk	SSF	Strumpshaw Fen	633500	307300				4		4
		CAR	Carr House	633900	307400	10					10
		EAR	East Ruston	634100	328200			**21**	**50**	**33**	**104**
	Bedfordshire	PCP	Priory Country Park	507600	249100		**1**	**27**	**68**	**54**	**150**
		WRP	Wrest Park	509200	235100				**46**	**75**	**121**
	Isle of Wight and Hampshire	BLH	Beaulieu Heath	438400	105100				4		4
		BUR	Bursledon	448600	109100				**38**		**38**
		STL	Stag Lane	449800	91800			4	**45**		**49**
		MAR	Marvel Farm	450200	87300	9		**31**	**37**		**77**
		SFR	Stone Farm Reservoir	452500	86000	7					7
		HOL	Hollier Farm	455200	83200	11					11
		EVC	East View Cottage	455900	85800				**33**		**33**
		PPP	Parsonage Peat Pond	456200	88200	10			**30**	**39**	**79**
		MOR	Morton Pond	460700	86100			**27**	**32**	**39**	**98**
		MRH	Marsh House	460900	88200	10			1		11
		CLP	Carpenters Lane Pond	461100	88600	6					6
						70	25	153	594	390	1,232

Table 3.1 continued. Summary data on sample sizes, collection dates and spatial locations of *E. viridulum* sites used in this study.

					Sample year		
Country	Code	Location	Latitude N	Longitude E	'05	'06	*n*
Germany	BRN	Braunschweig	52:15:51	10:31:35	**53**		**53**
Netherlands	KDK	Koudekerke	51:28:58	3:33:17		**19**	**19**
	UAD	Alphen Ann Den Rijn	52:07:43	4:39:29	**19**	**19**	**38**
Belgium	HET	Zoutleeuw, Het Vinne	50:50:00	5:06:13		10	10
	WEV	Wijvenheide Eutroof Ven	51:00:50	5:20:57		**19**	**19**
	WGV	Wijvenheide Grote Vijver	50:59:05	5:18:55		5	5
France	BLT	Blangy-Tronville	49:52:49	2:25:24		**25**	**25**
	BVS	Boves	49:50:40	2:22:59		6	6
	CRT	Courtemont Varennes	49:04:45	3:32:02		10	10
	LMD	Le Marais de Guines	50:52:45	1:52:36		3	3
	MGG	Le Marais de Guines	50:52:45	1:52:36		7	7
					72	123	195

3.2.2. DNA extraction and polymerase chain reaction

Total genomic DNA was extracted using a high salt protocol from either a single tibia (samples from 2004) or thoracic muscle (samples from 2005 and 2006). Samples were stored in 1.5 ml tubes in 100 % ethanol prior to DNA extraction. Legs were used in 2004 because their loss does not measurably affect fitness in damselflies (Fincke & Hadrys, 2001) however whole bodies were collected in 2005 to facilitate sampling. All samples were genotyped at 10 polymorphic microsatellite loci that are characterised in detail by Keat *et al.* (2005) and in Chapter 2.

Approximately 5 ng of DNA was used for a 10 µl polymerase chain reaction (PCR) containing 75 mM Tris-HCl pH 8.9, 20mM $(NH_4)_2SO_4$, 0.01 % Tween-20, 0.2 mM each dNTP, 3.0 mM $MgCl_2$, 2 pmol of each forward and reverse primer, 0.25 U *Taq* polymerase (ABgene, Epsom, UK). PCR temperature conditions were as described in Chapter 2 and by Keat *et al.* (2005). PCR products were pooled into one of two genotyping pools depending on allelic size range and the 5' fluorescent dye (either 6-FAM, NED, PET or VIC) (see also Figures 2.1a, b) with GENESCAN-500 LIZ size standard (Applied Biosystems) and separated using capillary electrophoresis through a denaturing polymer on an ABI3100 sequencer (Applied Biosystems). Allele sizes were determined using the cubic model in GENEMAPPER v. 3.0 genetic analysis software (Applied Biosystems).

3.2.3. Data analysis

3.2.3.1. General analysis of levels of genetic diversity

Microchecker software (van Oosterhout *et al.*, 2004) was used to test for the presence of null alleles and large allele drop out. Tests for significant deviation from Hardy–Weinberg Equilibrium (HWE) conditions and for linkage disequilibrium between all locus-pair combinations were carried out using the online version of GENEPOP v. 3.1d (Raymond & Rousset, 1995), making 2,000 permutations of alleles among individuals within samples.

Genetic diversity within each sample was quantified as allelic richness (A_R), expected heterozygosity (H_e), observed heterozygosity (H_o) and Wright's

(1951) inbreeding co-efficient (*f*), all calculated using FSTAT v. 2.9.3 (Goudet, 1995): A_R was standardised to 19 individuals to account for the size of the smallest sample during the permutation procedure. Mean values of genetic diversity parameter were plotted with standard errors calculated by jackknifing over loci (Sokal & Rohlf, 1995). A permutation procedure implemented by FSTAT, with 2,000 permutations of samples among groups, was used to test the significance of any differences in genetic diversity (A_R, H_e, H_o and *f*) among the following groups of three samples: (i) sites from the southern UK (Isle of Wight and Hampshire) compared with all other UK sites, (ii) sites from southern UK compared with continental European samples (Germany, Holland, Belgium and France) and (iii) populations from continental Europe and those from locations in the western UK (*i.e.* Essex, Suffolk, Norfolk and Bedfordshire).

3.2.3.2. Detection of population bottleneck

Evidence of a recent population bottleneck may be taken from the characteristic signature of a significant excess of heterozygotes from that expected under genetic equilibrium conditions, which is a consequence of a faster rate of reduction in the number of alleles than loss heterozygosity (Cornuet & Luikart, 1996, Luikart & Cornuet, 1998). I used BOTTLENECK v.1.2.02 (Piry *et al.*, 1999) software to compute an expected distribution of heterozygosities (H_e) under mutation-drift equilibrium from the allelic diversity of each sample for three different models of allelic mutation: infinite allele model (IAM), stepwise mutation model (SMM) and two-phase model (TPM) which is an intermediate to the SMM and IAM and believed to be the most appropriate model of allelic mutation for microsatellite loci (Jarne & Lagoda 1996). Both the Wilcoxon signed-rank test and a sign test were used to assess significance of whether the observed H_e is greater than that expected at equilibrium, although the latter is only robust when more than 20 loci are used (see Cornuet & Luikart, 1996 for details).

3.2.3.3. Genetic variation among successive sample years

Hierarchical analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) was employed to partition the contribution to genetic diversity arising from

spatial variation with that occurring among successive sampling periods, possibly through multiple colonisation events. To achieve statistical rigour for this analysis only populations with large sample sizes (n > 20) from the UK that were collected during 2004, 2005 and 2006 were used. Hence, the final groups were: 2004 (PCP, MAR, MOR and EAR), 2005 (PCP, WRP, ML2, SCV, BUR, EVC, MAR, MOR, PPP, STL, EAR and LWS) and 2006 (PCP, WRP, ML2, SCV, MOR, PPP, EAR and LWS). The significance of the fixation indices were tested using the permutation procedure (10,000 permutations) that is implemented by ARLEQUIN software (Schneider *et al.*, 2000).

3.2.3.4. Spatial genetic structure

Allele frequency differences among samples were tested for using the exact test employed by FSTAT v. 2.9.3 (Goudet, 1995); HWE within samples was not assumed and genotypes were permuted 2,000 times among samples. Genetic differentiation between samples was calculated using Weir & Cockerham's (1984) estimator of Wright's (1951) F_{ST} (θ in Weir and Cockerham's terminology) using FSTAT v. 2.9.3; the significance of estimates of F_{ST} from zero was assessed by making 2,000 permutations of genotypes between populations. The pattern of genetic relationships among populations (for samples sizes > 19 and data pooled over years) was summarised by multidimensional scaling analysis of the values of F_{ST} using SPSS v. 13.0 (SPSS Inc. Chicago).

Most species cannot disperse freely throughout their geographic ranges and, when genetic equilibrium conditions are attained, are therefore characterised by isolation by distance (IBD) genetic structure, where neighbouring areas are genetically more alike than distantly separated populations (Wright, 1943). The appropriate test for IBD genetic structure in a two dimensional habitat is a regression of pairwise estimates of a linearised multilocus genetic distance (F_{ST} / [1–F_{ST}]) against the corresponding natural logarithm of the geographic distance separating the populations (Rousset, 1997). Distance between samples was measured as the shortest (Euclidian) distance between sample sites. A Mantel test (2,000 permutations of population

locations among all locations) was then used to assess the significance of any relationship between genetic distance and (ln) geographic separation using GENEPOP v. 3.1c (Raymond & Rousset, 1995). I tested for IBD among (i) all pairs of UK populations, (ii) samples from the Isle of Wight and Hampshire only and (iii) between populations from Essex, Suffolk, Norfolk and Bedfordshire only. Only large samples (n > 19) were included in these analyses of IBD to limit the effect of sampling error upon estimates of F_{ST} (*e.g.* Waples, 1998).

The spatial pattern of individual genetic variation was investigated by spatial autocorrelation (Sokal & Oden, 1978), since this technique yields information about both the pattern and scale of spatial substructure and is sensitive at recovering fine-scale genetic structure (Sokal *et al.*, 1997; Peakall *et al.*, 2003; Vekemans & Hardy, 2004). For each data treatment described below, SPAGEDI v. 1.2 software (Hardy & Vekemans, 2002) was used to calculate the correlation in average kinship (F_{ij}; Loiselle *et al.*, 1995) relative to the whole data set between pairs of *E. viridulum* separated by a range of increasing spatial scales. Although the results are essentially the same with other measures more frequently used in spatial autocorrelation (such as Moran's *I*, see also Hardy & Vekemans, 1999), Loiselle *et al.*'s (1995) F_{ij} tends to be more powerful at detecting spatial genetic structure than other estimators of relatedness (Vekemans & Hardy, 2004). To avoid a bias in the correlation coefficient arising because of unequal sample sizes within each spatial category we allowed SPAGEDI v. 1.2 to assign distance categories that contained a similar number of pairwise comparisons (for further details see Hardy & Vekemans, 2002 and the manual, which is available at http://www.ulb.ac.be/sciences/ecoevol/docs/manual_SPAGeDi_1-2.pdf). The average correlogram over all loci is presented as this avoids variation in correlogram profiles based on the frequencies of individual alleles that are subject to stochastic processes (Hardy & Vekemans, 1999; Smouse & Peakall, 1999). Ninety-five percent confidence intervals for multilocus kinship coefficients at each distance class were generated from the distribution of 2,000 permutations of spatial group locations among the spatial groups. Euclidian geographical distances between individuals and population centres

were calculated from the GPS *x* and *y* coordinates. The pattern of spatial genetic structure was examined for (i) for each sample period separately (excluding 2003 where there are too few data for meaningful statistical analysis), (ii) over the whole UK data set, (iii) over the entire UK data set but restricting comparisons within years to assess the level of temporal stability to observed geographic differences and finally (iv) for males and females separately to examine whether there is sex-biased dispersal.

Population structure was assessed using the model-based clustering approach implemented by STRUCTURE v. 2.0 (see Pritchard *et al.*, 2000 for full background) that simultaneously identifies clusters (populations) and assigns individuals to populations using a Bayesian approach. Briefly, STRUCTURE models *K* populations (where *K* may be unknown) that are characterised by a set of allele frequencies at each locus. Individuals are (probabilistically) assigned to populations (or jointly if their genotypes indicate that they are admixed) on the basis of their multilocus genotypes, assuming unlinked loci and Hardy-Weinberg equilibrium conditions within populations. The actual number of distinct populations (*K*) may be estimated from the value of *K* that maximises the posterior probability of the data for a given posterior probability distribution Pr(*K*|*X*) that is calculated from the posterior distribution of Pr(*X*|*K*) (where *X* is the multilocus genotypes of sampled individuals); in STRUCTURE output, this criterion 'Ln P(D)' is calculated by computing an average of the log likelihood of the data at each step of the Monte Carlo Markov Chain (MCMC) and then half their variance is subtracted from the mean. STRUCTURE also calculates the proportion of membership of each individual in each cluster (*Q*).

In situations where there is distinct genetic structure, the true number of populations is identified by the model of *K* that returns the maximal value of Ln P(D). However, real and simulated data have demonstrated that choosing an appropriate value of *K* can be difficult, particularly for large and/or complex population structures, and under such circumstances several recommendations have been proposed to identify the best model. First, Pritchard & Wen (2003) suggest that the value of *K* at the beginning of a

'plateau' of estimates of Ln P(D) be selected, *i.e.* use the smallest value of *K* that captures the major structure of the data set. It is worth noting that rather than determine the actual number of populations this approach provides a heuristic guide to the models that are most consistent with the data set. A second method to detect the true number of clusters in an unknown sample is to use the *ad hoc* measure, ΔK, which is the second order rate of change of Ln P(D) with respect to *K* (Evanno *et al.*, 2005). ΔK is calculated from:

$$\Delta K = mean\ (|L(K+1) - 2L(K) + L(K-1)|) \,/\, sd\ [L(K)],$$

where $L(K)$ denotes Ln P(D) for a particular model run (value of *K*), *mean* is the average and *sd* is the standard deviation [of $L(K)$] across replicate runs of STRUCTURE for each putative value of *K*. Computer simulations demonstrate that the modal value of ΔK corresponds to the most pronounced partition of the data set.

Five independent runs of STRUCTURE were carried out for the total data set for $K = 1$ to $K = 19$ using the admixture model and correlated allele frequencies, since this is suggested to permits differentiation of closely related populations (Pritchard *et al.*, 2000). All model runs were based on 500,000 iterations after an initial burn-in period of 50,000 iterations, which was sufficient to ensure convergence of the MCMC. Five independent runs were made for each value of *K* to assess consistency of the results and also to calculate the second order rate of change of Ln P(D) that is described above. All genotype data were used in this analysis including those from sites where too few individuals could be collected for analyses of differentiation among populations (see Table 3.1).

Where appropriate a sequential Bonferroni correction (Rice, 1989) was applied to adjust the significance of (*k*) multiple tests.

3.3. Results

3.3.1. Genetic diversity

None of the samples showed evidence of large allele drop out. Evidence for null (non-amplifying) alleles was present at some of the locus-sample comparisons but not strongly associated with any locus in particular, indicating that positive results were chance occurrences (see Appendix 3.1). All of the probabilities of linkage disequilibrium among pairs of loci are presented in Appendix 3.2; briefly, only one locus pair demonstrated significant linkage disequilibrium over the total sample (LIST14-021 and LIST14-042, $P < 0.001$) so all loci were assumed to be unlinked. The majority of samples met expected HWE conditions with only 26 out of the 620 (~4 %) possible sample-locus combinations having a significant heterozygote deficiencies after correction for multiple testing; these deficits were not associated with any particular locus or sample (see Appendix 3.3). Therefore, all ten loci were retained for subsequent analyses.

Raw sample-locus results for measures of genetic diversity, including allelic richness (A_R), expected heterozygosity (H_e), observed heterozygosity (H_o) and Wright's (1951) inbreeding coefficient (f), are presented in Appendix 3.4. The maximum number of alleles found at any locus was eight at LIST14-002 and the least diverse was LIST14-021 which was monomorphic in many populations. LIST14-040 also showed little diversity, with seldom more than two alleles in a population. The highest value of allelic richness was found in the German sample (BRN) ($A_R = 6.595$) for LIST14-002. Values of H_o and H_e varied between 1.0 and 0.0 with the highest value of observed heterozygosity for a sample of >18 individuals was at locus LIST14-006 for the Belgian HET population ($H_o = 1.0$).

While expected heterozygosity varies little among samples, it is lowest in samples from the Isle of Wight and Hampshire, intermediate in the eastern UK and greatest in populations from continental Europe (Figure 3.3a). This pattern is evident and more pronounced for allelic richness (Figure 3.3b) but not for Wright's (1951) inbreeding coefficient, which is highly variable, and mostly positive, across the UK populations and generally close to zero for

samples from continental Europe (Figure 3.3c). Overall, therefore, *E. viridulum* shows a slight but clear trend of a reduction in genetic diversity from east to west that follows its path of invasion from continental Europe into the UK.

Permutation analyses showed that there were significant differences in A_R and H_e between populations from southern UK (Isle of Wight and Hampshire) and the rest of the mainland British populations (P = 0.027and 0.003 respectively) but not for f or F_{ST} (P = 0.399 and 0.161 respectively). There were significant differences in A_R and H_e (P < 0.001 and = 0.028 respectively) between samples from continental Europe and those from the southern UK, but not between populations from continental Europe and the eastern UK. In all instances, A_R and H_e were reduced in the Isle of Wight and Hampshire populations (Table 3.2). Of the four tests for genetic differentiation, only those between Eastern UK and Southern UK and between Southern UK and continental Europe remained significant after a sequential Bonferroni correction.

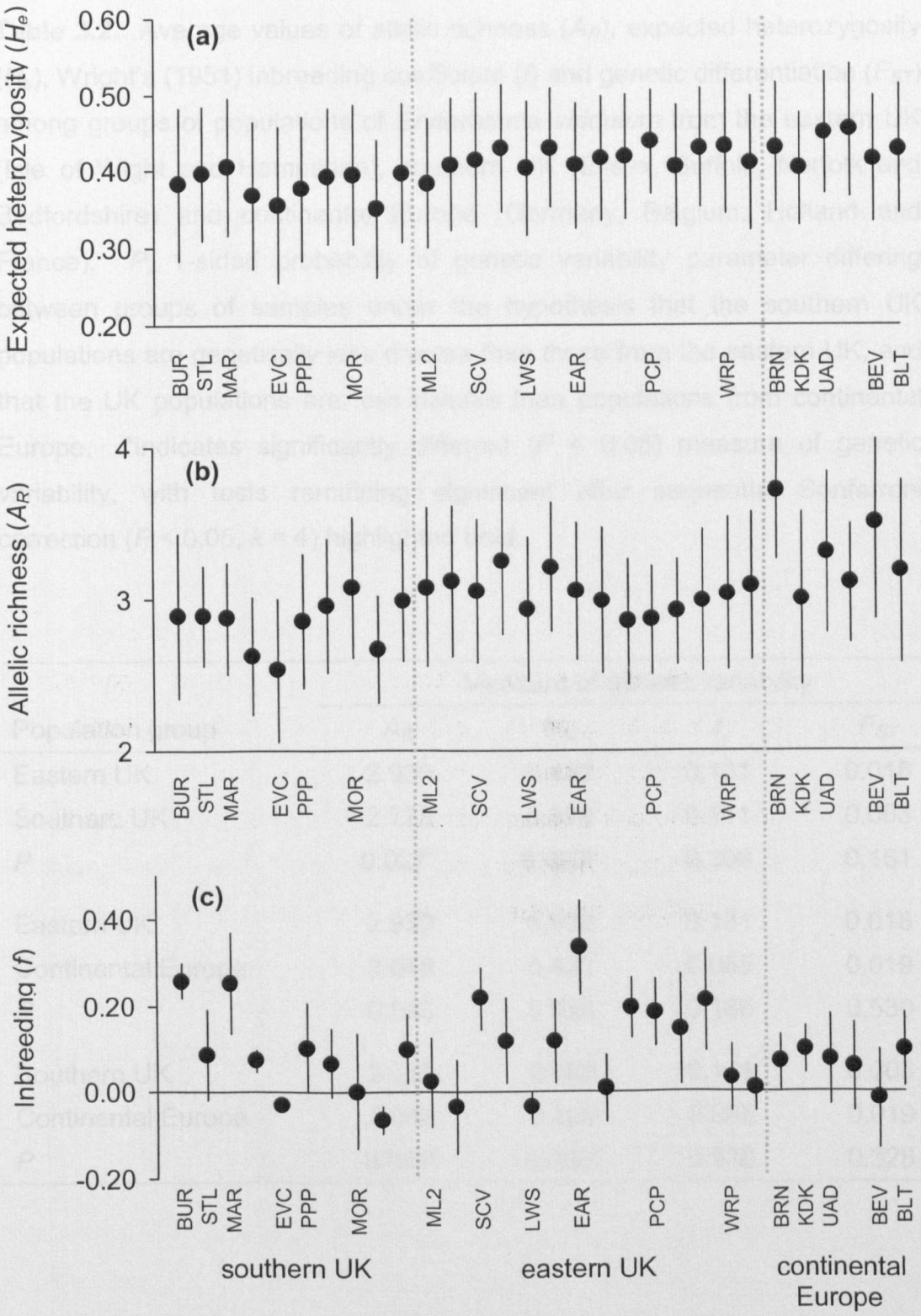


Figure 3.3. Geographic variation in genetic diversity (mean ± standard error) for populations of the small red-eyed damselfly, *Erythromma viridulum*, from northern Europe. (a) expected heterozygosity (H_e); (b) allelic richness (A_R); (c) inbreeding coefficient (f) (Wright, 1951).

Table 3.2. Average values of allelic richness (A_R), expected heterozygosity (H_e), Wright's (1951) inbreeding coefficient (f) and genetic differentiation (F_{ST}) among groups of populations of *Erythromma viridulum* from the eastern UK (Isle of Wight and Hampshire), southern UK (Essex, Suffolk, Norfolk and Bedfordshire) and continental Europe (Germany, Belgium, Holland and France). P, 1-sided probability of genetic variability parameter differing between groups of samples under the hypothesis that the southern UK populations are genetically less diverse than those from the eastern UK, and that the UK populations are less diverse than populations from continental Europe. *indicates significantly different ($P < 0.05$) measure of genetic variability, with tests remaining significant after sequential Bonferroni correction ($P < 0.05$, $k = 4$) highlighted bold.

	Measure of genetic variability			
Population group	A_R	H_e	f	F_{ST}
Eastern UK	2.930	0.432	0.131	0.018
Southern UK	2.728	0.389	0.111	0.003
P	0.027*	**0.003***	0.399	0.161
Eastern UK	2.930	0.432	0.131	0.018
Continental Europe	3.048	0.429	0.065	0.019
P	0.845	0.456	0.166	0.530
Southern UK	2.728	0.389	0.111	0.003
Continental Europe	3.048	0.429	0.065	0.019
P	**0.001***	0.028*	0.520	0.328

3.3.2. Population bottleneck

Approximately 30 % of the *E. viridulum* populations (n = 10 out of 32) showed a significant ($P < 0.05$) excess of heterozygotes that is indicative of a recent population bottleneck assuming the infinite allele model (IAM) of mutation and using the Wilcoxon sign-rank test (Table 3.3). By contrast, using the two phase (TPM) or stepwise models (SMM) of allele mutation, which are believed to be more appropriate for microsatellite loci, only four and one population(s) respectively demonstrated significant evidence for a population bottleneck. Overall, significant results included samples from the UK and continental Europe (Holland) and also throughout the sample periods (2004, 2005 and 2006), so there is no obvious spatial or temporal pattern to the distribution of populations that may have suffered a recent bottleneck. Moreover, some caution must be applied to inferring that any of these populations have demonstrate a genetic bottleneck since no significant heterozygote excesses are observed if a sequential Bonferroni correction for k = 32 multiple tests is applied.

3.3.3. Variation among temporal samples

No significant genetic differences were attributed to variation among the temporal groups of samples (P = 0.254), with just 0.2 % of the total genetic variance attributable to temporal genetic variation. Approximately 6.5 % of the total genetic variance (F_{ST} = 0.066, $P < 0.001$) was attributed to differences among spatial groups within each sample period, and the overwhelming majority of genetic variation occurred with populations (Table 3.4).

The clear lack of temporal variation among samples justifies the use of pooling samples at the same site but from different years to increase statistical power for analyses of genetic differentiation among populations.

Table 3.3. Probability values for tests for a significant heterozygote excess indicative of a population bottleneck for samples of *Erythromma viridulum* using three models of microsatellite allele mutation (IAM, infinite allele model; TPM, two phase model; SMM, stepwise model) and two methods of analysis (Sign test and Wilcoxon sign-rank test). *indicates a significant ($P < 0.05$) heterozygote excess, however no tests remain significant ($P < 0.05$) after a sequential Bonferroni correction for $k = 32$ multiple tests.

			Sign test			Wilcoxon test		
Region	Site	Year	IAM	TPM	SMM	IAM	TPM	SMM
Isle of Wight & Hampshire	EVC	2005	0.1700	0.2106	0.2196	0.0156*	0.0234*	0.2188
	MOR	2004	0.2798	0.3278	0.6050	0.3262	0.4102	0.8203
	MOR	2005	0.2732	0.3828	0.0375*	0.3672	0.6328	0.8750
	MOR	2006	0.3820	0.4872	0.2373	0.3125	0.5391	0.8623
	MAR	2004	0.3557	0.4204	0.4635	0.0967	0.2783	0.3848
	MAR	2005	0.2455	0.3197	0.3657	0.1250	0.3672	0.4551
	PPP	2005	0.2675	0.5921	0.3577	0.1504	0.5898	0.8496
	PPP	2006	0.0934	0.5717	0.3594	0.0645	0.4102	0.8496
	STL	2005	0.4058	0.5184	0.4012	0.2783	0.6523	0.7539
	BUR	2005	0.0874	0.3434	0.3662	0.0117*	0.0391	0.4063
Essex	ML2	2005	0.1701	0.0903	0.0789	0.0371*	0.7695	0.9863
	ML2	2006	0.1075	0.1760	0.1696	0.1016	0.6328	0.9863
	SCV	2005	0.2044	0.2589	0.0117*	0.0977	0.3203	0.9805
	SCV	2006	0.2889	0.3976	0.0493*	0.0645	0.5000	0.9180
East Anglia & Bedfordshire	LWS	2005	0.0410*	0.2065	0.0770	0.0059*	0.1563	0.9023
	LWS	2006	0.3971	0.5309	0.0931	0.1875	0.7217	0.9346
	EAR	2004	0.2066	0.2501	0.2054	0.0977	0.3203	0.8750
	EAR	2005	0.0242*	0.1464	0.0406*	0.0645	0.0820	0.9932
	EAR	2006	0.0476*	0.0698	0.1980	0.0195*	0.0273*	0.6289
	PCP	2004	0.0233*	0.1352	0.3654	0.0049*	0.0244*	0.6328
	PCP	2005	0.1634	0.5419	0.4898	0.0967	0.3477	0.8838
	PCP	2006	0.0156*	0.2941	0.4279	0.0068*	0.0820	0.6328
	WRP	2005	0.0504	0.2385	0.5466	0.0137*	0.1563	0.5781
	WRP	2006	0.0888	0.3065	0.1679	0.0645	0.2481	0.8984
Germany	BRN	2005	0.6065	0.1135	0.0274*	0.5000	0.9180	0.9971
Belgium	HET	2006	0.3082	0.6267	0.2976	0.1504	0.5000	0.8750
	WEV	2006	0.4507	0.0997	0.0762	0.5391	0.9033	0.9951
Holland	KDK	2006	0.3108	0.3415	0.1450	0.1504	0.4551	0.8203
	UAD	2005	0.1028	0.1188	0.4124	0.1016	0.2129	0.5449
	UAD	2006	0.0044*	0.0484*	0.2193	0.0020*	0.0039*	0.0371*
France	CRT	2006	0.1684	0.2112	0.2728	0.0977	0.2734	0.3203
	BLT	2006	0.0669	0.2627	0.2933	0.0244*	0.0820	0.7871

Table 3.4. Analysis of molecular variance (AMOVA) of samples of the small red eyed damselfly *Erythromma viridulum* from the UK collected between 2004 and 2006. Variation is quantified among three temporal groups of samples (2004, 2005 and 2006), among samples within each temporal group and within populations. Values that are significantly ($P < 0.05$) different from zero are highlighted bold.

Source of variation	*df*	SS	Variance components	% variance	Fixation index	*P*
Among temporal groups	2	31.6	0.005	0.21	0.0021	0.254
Among populations within temporal groups	21	297.2	0.139	6.55	0.0656	**<0.000**
Within populations	2066	4098.5	1.984	93.24	0.0676	**<0.000**
Total	2089	4427.3	2.128	100.00		

3.3.4. Spatial genetic structure

Overall, the level of genetic differentiation among the UK and continental European populations could be considered moderate or low given the distances among sample sites (F_{ST} = 0.038). There was little evidence for estimates of genetic differentiation being biased by values at one or few loci (*i.e.* locus-specific effects) since most loci showed similar values of average F_{ST}, varying from F_{ST} = 0.166 and 0.109 at LIST14-005 and LIST14-042 respectively down to F_{ST} = 0.003 at one locus only, LIST14-009 (Table 3.5).

Estimates of pairwise genotypic differentiation between all pairs of samples are provided in Table 3.6. A striking pattern is that significantly different (P < 0.05, k = 231) genotypic differences and values of pairwise F_{ST} were observed for all of the comparisons between populations from southern UK and those from elsewhere, but not between any of the pairs of samples within the Isle of Wight and Hampshire. Overall, average pairwise population genetic differentiation (F_{ST}) was greatest for comparisons between populations from the southern UK and those from eastern UK and continental Europe (F_{ST} = 0.066 and 0.064 respectively), intermediate for comparisons between the eastern UK (EUK) and continental Europe (CEE) (EUK-CEE F_{ST} = 0.0187; CEE-CEE F_{ST} = 0.0169; EUK-EUK F_{ST} = 0.0157) and lowest among populations from the southern UK only (F_{ST} = 0.004). Given this clear distinction in population differentiation between the three broad regions, it is not surprising that the multidimensional scaling analysis of values of pairwise F_{ST} reveals two discreet clusters: (i) a tight group of samples comprising populations from the Isle of Wight and Hampshire and (ii) a looser cluster of populations that contains all other UK populations as well as samples from continental Europe (Figure 3.4). In summary, the somewhat surprising conclusion is that greatest genetic differences occur between populations from southern UK and those from eastern UK and continental Europe, and not between the populations from UK and continental Europe.

There was a significant (y = 0.018x - 0.033, P = 0.002) relationship between genetic differentiation among UK populations (data from 2004 and 2005 combined) and the distance separating them. An apparently significant

pattern of IBD was also uncovered when sites from 2005 were considered only (data not presented), though too few samples were collected during other years for a meaningful statistical analysis of IBD among populations. In both cases of apparent IBD, however, the level of genetic differentiation remained similarly low (F_{ST} up to ~0.02) over distances between 0 km and 160 km (*i.e.* within each of the southern and eastern areas of colonisation within the UK, Figure 3.5). Thus, it is clear that the apparent statistical significance to this pattern of IBD arises because of the high differentiation among populations separated by larger geographic scales (> 160 km) rather than a more general increase in the level of population differentiation as distance increases that is characteristic of IBD (see Figure 3.5). Therefore, when samples were partitioned for analyses within regions (*i.e.* considering pairs of populations within either the eastern or southern UK only), significant IBD genetic structure was not detected for either samples from the Isle of Wight and Hampshire (y = -0.0058x + 0.0194, P = 0.885), or those samples from Essex, Suffolk, Norfolk and Bedfordshire (y = -0.0025x + 0.0200, P = 0.962).

Individual correlogram profiles of the relationship between kinship (F_{ij}) and spatial separation varied somewhat among years. For example, none of the average values of F_{ij} in the 2002 sample were significantly different from zero, and in 2006 only kinship in first distance class is significantly (P = 0.007) different (greater) from zero. By contrast, in 2004 and 2005 there is a stronger trend of declining average kinship with increasing spatial separation: F_{ij} is significantly ($P < 0.05$) greater than zero for distances of up to 100-150 km, and then negative thereafter (see Figure 3.6a). Combining the data set over all sample periods (2002-2006) to increase statistical power, also generates a significant pattern of decreasing kinship with geographical distance, with pairs of individuals up to about 100 km or less apart having values of F_{ij} significantly greater than zero, while at distances beyond approximately 160 km average kinship among pairs of individuals (relative to the sample) is significantly ($P < 0.05$) less than zero (Figure 3.6b). As with the population-based analyses of pairwise F_{ST} describe above, this decline in average kinship with distance is driven by broad genetic similarities between

E. viridulum within reach of the three broad geographic 'regions' (*i.e.* southern UK, eastern UK or continental Europe) rather than a general pattern of IBS at local scales, since within each region there is no significant trend in kinship with distance separating individuals (see Figures 3.7a-c).

There is no evidence for sex-biased dispersal in *E. viridulum* since the correlogram profiles for males and females follow the same qualitative pattern and do not differ significantly, with overlapping 95 % confidence intervals (Figure 3.8). Raw data for significance of average pairwise kinship for each distance class are presented in Appendix 3.5.

Independent runs of structure generated similar clustering solutions. Overall, values of L(K) produced by STRUCTURE analysis were similar for values of k between 1 and 6, and were highest at k = 2 and 5. The variance in values of L(K) increases with greater values of k, but is only particularly evident at k > 11 (Figure 3.9a). The mean difference between successive likelihood values of K, which corresponds to the rate of change of the likelihood function with respect to K, shows little obvious trend as values of K increase (Figure 3.9b), while the difference between successive values of $L'(K)$, which correspond to the second order rate of change of $L(K)$ with respect to K, show a gradual increase in absolute value with increasing K (Figure 3.9c). Finally, examination of ΔK from STRUCTURE output produces a clear modal value at k = 2 (Figure 3.9d) and thus strong evidence for pronounced subdivision of *E. viridulum* from UK and the northern continental European populations into 2 distinct genetic units, with the partition of the data set following geographic location; cluster 1 comprises samples from the southern UK (Isle of Wight and Hampshire) while cluster 2 consists of individuals from the rest of mainland UK and also all of the samples from continental Europe (Germany, Holland, Belgium and France). Although there is clear evidence for two discreet clusters, a substantial proportion if individuals are inferred to have mixed ancestry, whereby there is an approximately equal probability of membership to either cluster (Figure 3.10 and Table 3.7).

Average membership coefficients hypothetical cluster 1 stand out as being generally high (> 0.6) for most of the southern UK populations (with the exception of two small populations). Average membership coefficients for the eastern UK and continental European samples are mostly high for cluster 2, however a greater percentage of populations, in particular three of the French samples, have roughly equal probabilities of membership to either simulated cluster (Table 3.7). This pattern of inferred mixed ancestry likely represents high gene flow and the concomitant weak spatial genetic structure and non-equilibrium conditions, but could also arise through some broad-scale clinal genetic variation or IBD spatial genetic structure.

Table 3.5. Individual locus *F*-statistics for populations of *Erythromma viridulum* from the UK and continental Europe.

	F_{IT}		F_{ST}		F_{IS}	
	Mean	SE	Mean	SE	Mean	SE
LIST14-002	0.064	0.018	0.028	0.010	0.037	0.020
LIST14-005	0.810	0.064	0.166	0.050	0.769	0.060
LIST14-006	0.066	0.027	0.015	0.007	0.052	0.026
LIST14-014	0.199	0.051	0.069	0.019	0.140	0.059
LIST14-019	0.058	0.015	0.003	0.004	0.055	0.015
LIST14-021	0.795	0.072	0.007	0.005	0.794	0.073
LIST14-025	0.090	0.028	0.019	0.008	0.073	0.031
LIST14-035	0.092	0.025	0.015	0.005	0.078	0.025
LIST14-040	0.139	0.069	0.015	0.005	0.126	0.072
LIST14-042	0.184	0.071	0.109	0.036	0.084	0.066
All Loci	0.141	0.047	0.038	0.013	0.107	0.040

Table 3.6. Pairwise estimates of genotypic differentiation (above diagonal) and F_{ST} (below diagonal) between samples of *Erythromma viridulum* from the UK and continental Europe; *indicates significant genetic difference between population pairs ($P < 0.05$); pairwise probability values that remain significant ($P < 0.05$, $k = 153$) after correction for multiple tests highlighted bold.

	Essex, Suffolk, Norfolk and Bedfordshire							Isle of Wight and Hampshire						Continental Europe				
	ML	SCV	LWC	LWS	EAR	PCP	WRP	BUR	STL	MAR	EVC	PPP	MOR	BRN	KDK	UAD	WEV	BLT
ML		0.2007	0.0029*	0.0010*	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	0.0510	**0.0003***	0.0026*	0.1863
SCV	0.0041*		0.0121*	0.0516	**0.0003***	**0.0003***	0.0624	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	0.0007*	0.1033	**0.0003***	0.0029*	0.8131
LWC	0.0036	0.0030		0.0016*	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	0.0232*	**0.0003***	0.0010*	0.2219
LWS	0.0043*	0.0009	0.0134*		**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	0.0007*	0.0356*	**0.0003***	0.0095*	0.1775
EAR	**0.0233***	**0.0198***	0.0228*	**0.0132***		**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	0.0078*	**0.0003***	**0.0003***	0.0062*
PCP	**0.0366***	**0.0302***	0.0325*	**0.0235***	0.0038*		**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***
WRP	0.0118*	0.0036*	0.0117*	**0.0084***	**0.0255***	**0.0337***		**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***	0.0013*	**0.0003***	0.1422
BUR	**0.0498***	**0.0554***	**0.0617***	**0.0485***	**0.0588***	**0.0804***	**0.0699***		0.0049*	0.0007*	0.0085	0.0092*	0.0043*	**0.0003***	**0.0003***	**0.0003***	0.0007*	**0.0003***
STL	**0.0538***	**0.0543***	**0.0613***	**0.0455***	**0.0533***	**0.0722***	**0.0675***	-0.0049		0.4366	0.1039	0.3118	0.5578	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***
MAR	**0.0684***	**0.0639***	**0.0813***	**0.0533***	**0.0659***	**0.0838***	**0.0681***	0.0058	-0.0010		0.2709	0.0088*	0.3418	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***
EVC	**0.0600***	**0.0632***	**0.0799***	**0.0487***	**0.0656***	**0.0770***	**0.0715***	0.0075	0.0055	0.0036		0.0673	0.1850	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***
PPP	**0.0657***	**0.0642***	**0.0723***	**0.0545***	**0.0668***	**0.0888***	**0.0797***	0.0007	-0.0023	0.0049	0.0140*		0.3797	**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***
MOR	**0.0668***	**0.0671***	**0.0754***	**0.0568***	**0.0693***	**0.0867***	**0.0739***	0.0034	-0.0022	-0.0004	0.0063	0.0030		**0.0003***	**0.0003***	**0.0003***	**0.0003***	**0.0003***
BRN	0.0138*	0.0096*	0.0204*	0.0089*	**0.0260***	**0.0282***	**0.0199***	**0.0447***	**0.0419***	**0.0515***	**0.0368***	**0.0556***	**0.0557***		0.0118*	**0.0003***	0.0010*	0.0317*
KDK	0.0122*	0.0131	0.0147	0.0122*	0.0278*	0.0445*	**0.0426***	**0.0549***	**0.0565***	**0.0823***	**0.0765***	**0.0573***	**0.0790***	0.0181*		**0.0003***	0.7157	0.3389
UAD	**0.0161***	0.0069	0.0113	0.0109*	0.0128*	0.0232*	0.0083*	**0.0617***	**0.0549***	**0.0637***	**0.0681***	**0.0683***	**0.0651***	0.0213*	0.0292*		**0.0003***	0.0768
WEV	0.0171*	0.0188*	0.0317*	0.0131*	0.0331*	0.0481*	**0.0377***	**0.0486***	**0.0533***	**0.0658***	**0.0703***	**0.0519***	**0.0657***	0.0362*	0.0002	0.0323*		0.2392
BLT	0.0068	-0.0028	-0.0034	0.0064	0.0207*	0.0289*	0.0086	**0.0743***	**0.0690***	**0.0839***	**0.0891***	**0.0769***	**0.0841***	0.0165*	0.0023	0.0031	0.0095	

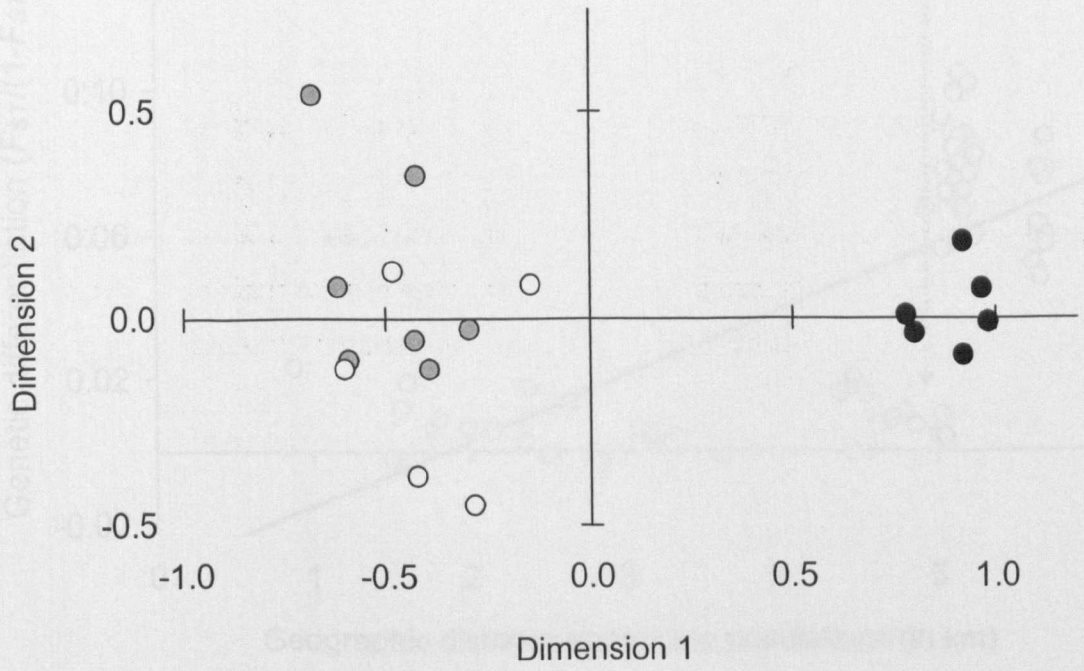


Figure 3.4. Multidimensional scaling plot of values of pairwise F_{ST} between samples of the small red-eyed damselfly, *Erythromma viridulum*, from (i) southern UK (Isle of Wight and Hampshire) (black), (ii) eastern UK (Essex, Suffolk, Norfolk and Bedfordshire) (grey) and (iii) continental Europe (Germany, Belgium, Netherlands and France) (white).

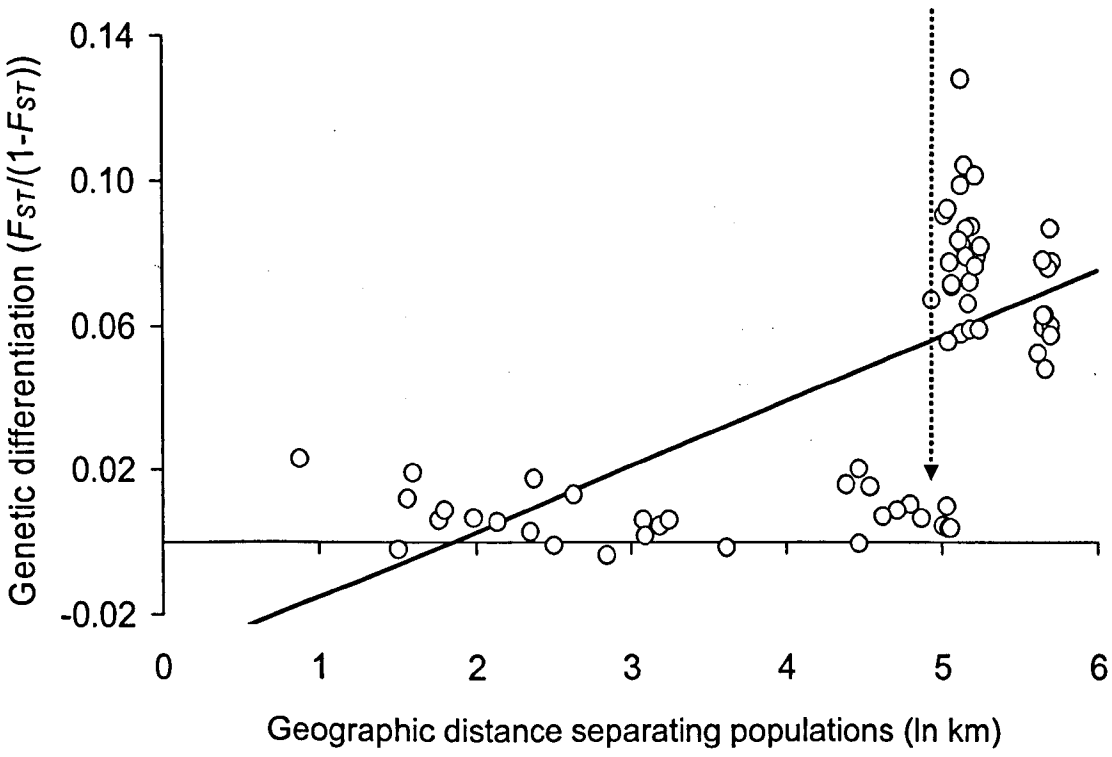
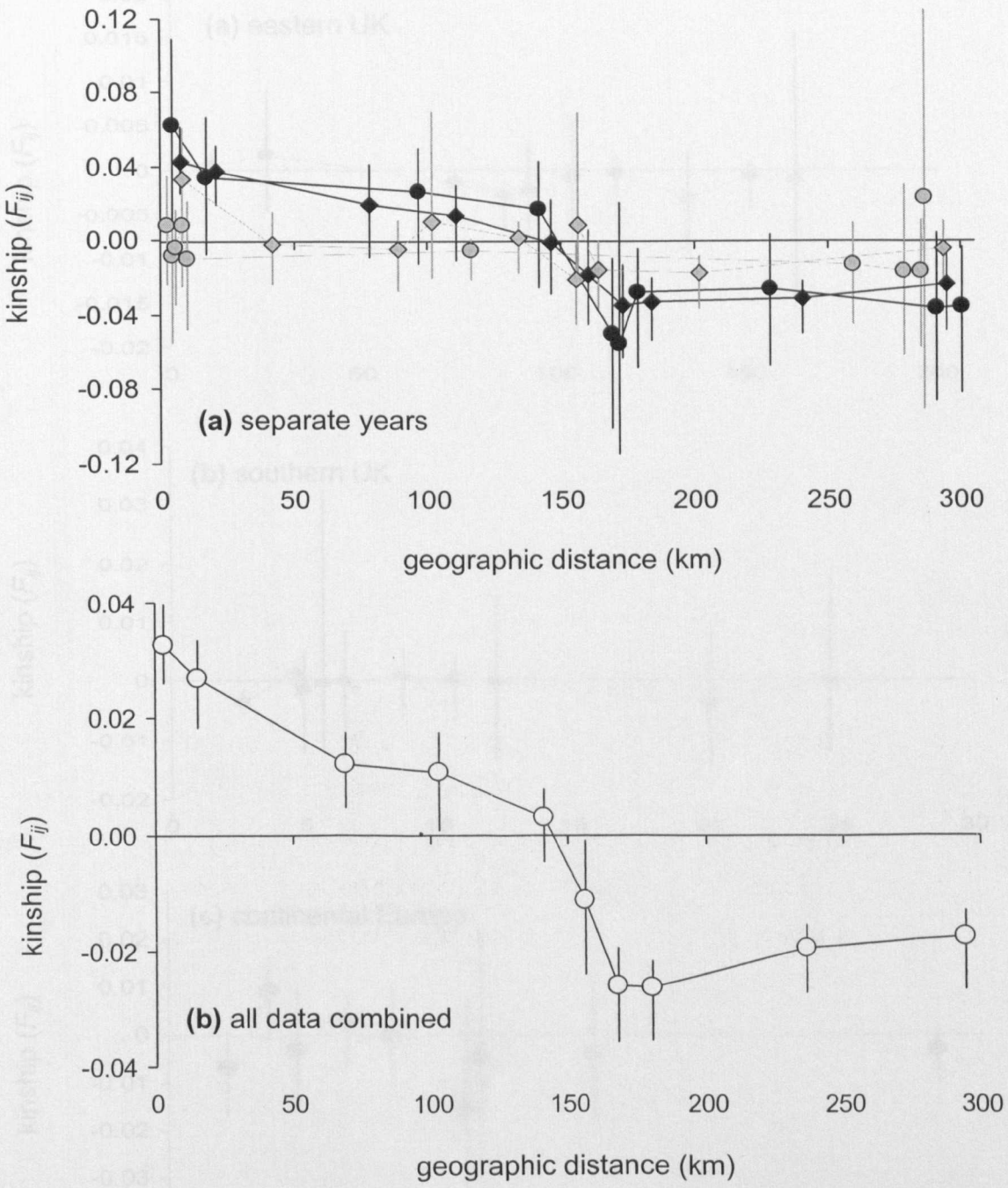
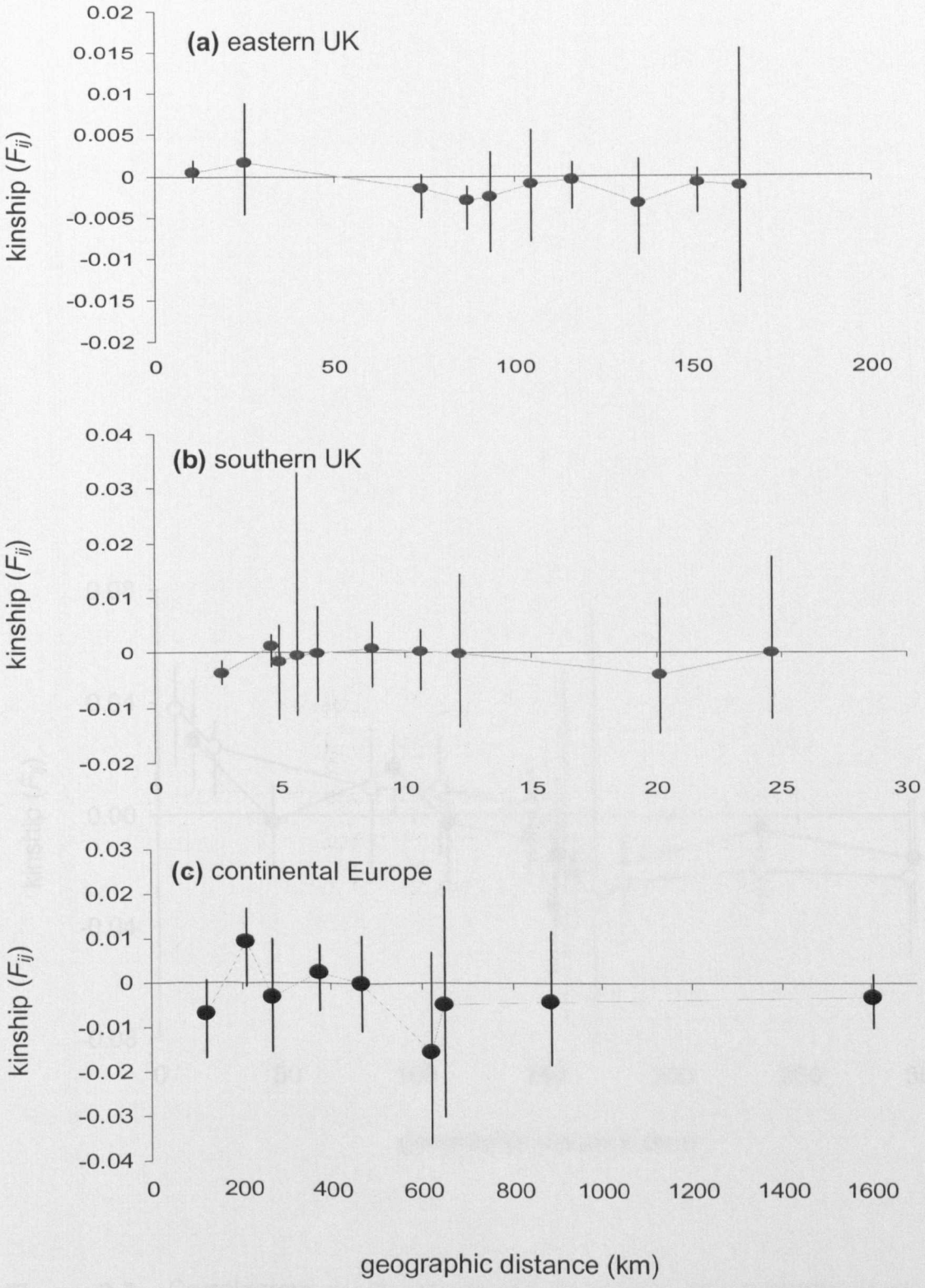


Figure 3.5. Relationship between the level of genetic differentiation [F_{ST} / (1-F_{ST})] and geographic separation for pairs of populations of the small red-eyed damselfly *Erythromma viridulum* from the UK in 2004 and 2005. The arrow highlights the spatial scale where there is a significant break in genetic structure.



Figures 3.6a, b. Correlogram profiles of the variation in kinship (F_{ij}) (Loiselle *et al.* 1995) as a function of the average distance separating pairs of *Erythromma viridulum* from locations in the UK. Confidence intervals are twice the standard error, obtained by jackknifing over 10 microsatellite loci. (a) individual profiles for samples collected during 2002 (grey circles), 2004 (black circles), 2005 (black diamonds) or 2006 (grey diamonds); (b) variation in F_{ij} over all years combined.



Figures 3.7a, b, c. Spatial variation in average kinship (F_{ij}) (Loiselle *et al.* 1995) among pairs of *Erythromma viridulum* from (a) eastern UK (Essex, Bedfordshire, Suffolk, Norfolk), (b) southern UK (Isle of Wight, Hampshire) and (c) continental Europe (Germany, Belgium, Holland, France).

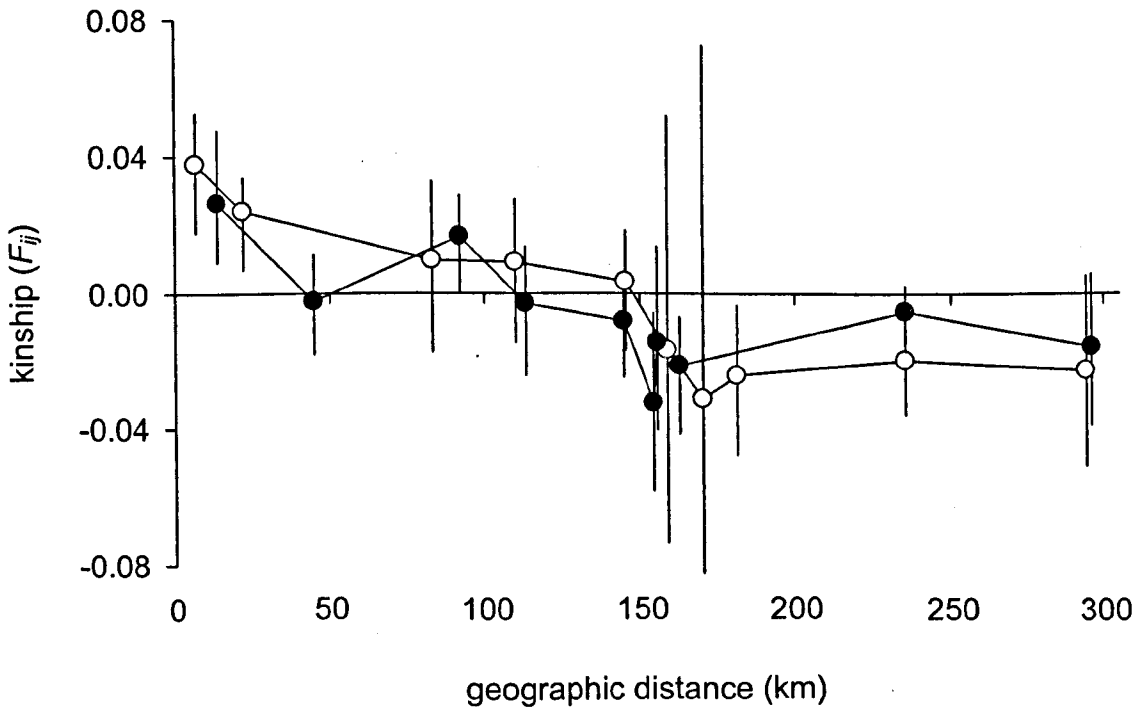
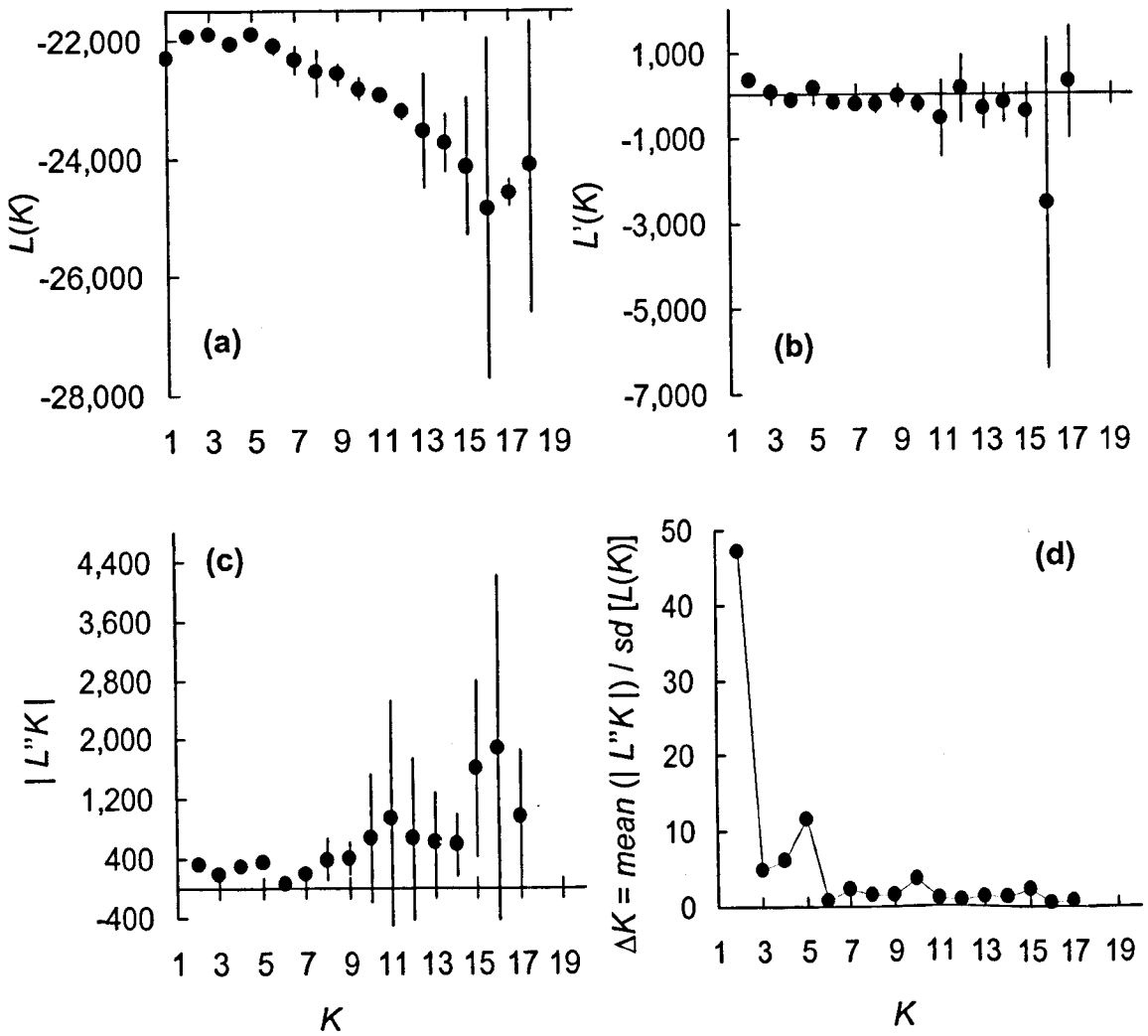


Figure 3.8. Correlogram profile of variation in kinship (F_{ij}) (Loiselle *et al.* 1995) as a function of the average distance separating pairs of male (open) and female (black) *Erythromma viridulum* that were caught in the UK between 2002-2006. Confidence intervals are twice the standard error, obtained by jackknifing over 10 microsatellite loci.



Figures 3.9a, b, c, d. Description of the four steps for the graphical method that allows detection of the true number of groups (K) in a sample. (a) Mean L(K) (±95 % CI) over 5 independent runs for each K value. (b) Rate of change of the likelihood distribution (mean ± SD) calculated as $L'(K) = L(K) - L(K - 1)$. (c) Absolute values of the second order rate of change of the likelihood distribution (mean ± SD) calculated according to Evanno *et al.*'s (2005) formula: $|L''(K)| = |L'(K + 1) - L'(K)|$. (d) ΔK calculated as $\Delta K = m|L''(K)|/s[L(K)]$. The modal value of this distribution represents either the true value of K or the uppermost level of genetic structure.

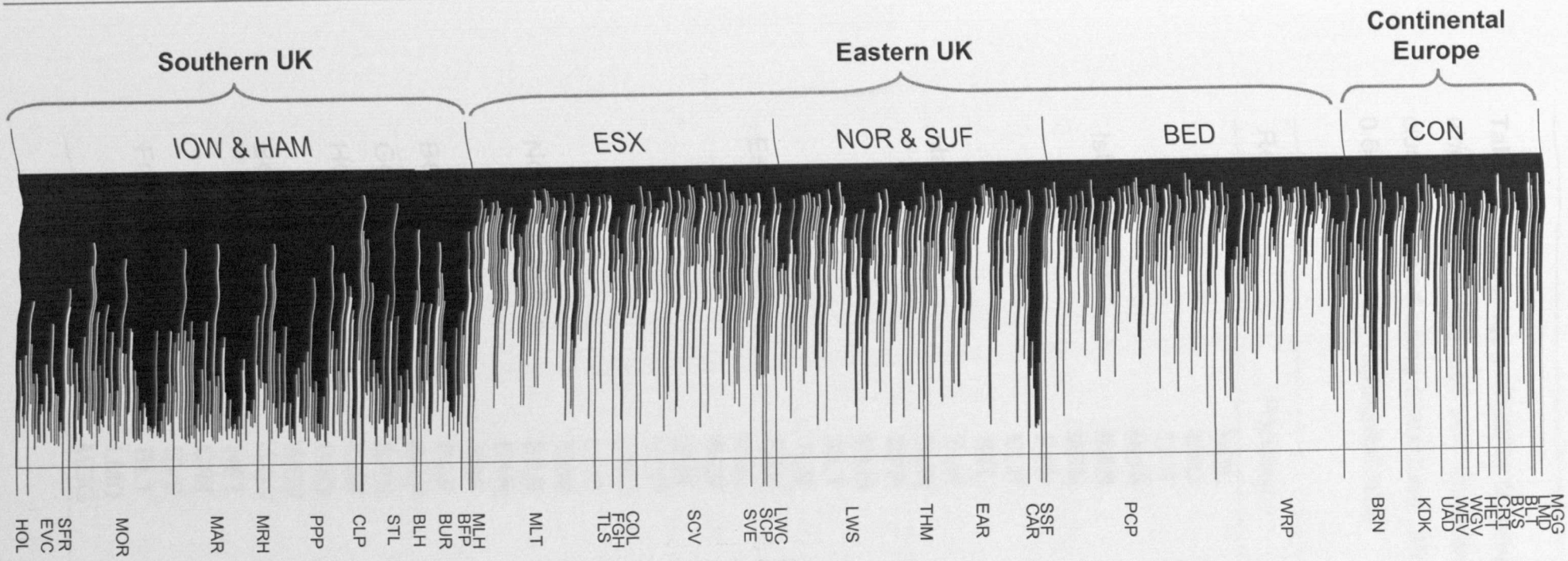


Figure 3.10. Probabilities of individual membership to clusters in a two cluster simulation. Each bar represents one individual and the proportion of the bar that is black or white represents the proportion of assignment to cluster one or two respectively. Data from all sampling years (2002-2006) have been combined. Broad geographic regions are coded Isle of Wight (IOW), Hampshire (HAM), Essex (ESX), Norfolk (NOR), Suffolk (SUF), Bedfordshire (BED) and continental Europe (CON).

Table 3.7. Average probability of membership for populations of *Erythromma viridulum* from the UK and continental Europe to one of two simulated clusters derived using STRUCTURE. Populations with average membership > 0.6 to a cluster are highlighted bold.

Region	Population	Probability of Assignment		*n*
		Cluster 1	Cluster 2	
Isle of Wight	**HOL**	**0.790**	0.210	11
	EVC	**0.798**	0.202	33
	SFR	0.579	0.421	7
	MOR	**0.785**	0.215	98
	MAR	**0.792**	0.208	77
	MRH	**0.683**	0.317	11
	PPP	**0.756**	0.244	79
	CLP	**0.695**	0.305	6
	STL	**0.757**	0.243	49
Hampshire	BLH	0.503	0.497	4
	BUR	**0.707**	0.293	38
Essex	**BFP**	**0.629**	0.371	7
	MLH	0.299	**0.701**	4
	MLT	0.375	**0.625**	112
	TLS	0.302	**0.698**	17
	FGH	0.378	**0.622**	9
	COL	0.390	**0.610**	13
	SCV	0.376	**0.624**	107
	SVE	0.289	**0.711**	10
Norfolk & Suffolk	SCP	0.547	0.453	3
	LWC	0.484	0.516	9
	LWS	0.404	0.596	136
	THM	0.180	**0.820**	3
	EAR	0.363	**0.637**	104
	CAR	**0.823**	0.177	10
	SSF	0.366	**0.634**	4
Bedfordshire	**PCP**	0.316	**0.684**	150
	WRP	0.329	**0.671**	121
Germany	BRN	0.479	0.521	53
Holland	**KDK**	0.353	**0.647**	19
	UAD	0.332	**0.668**	38
Belgium	WEV	0.423	0.577	19
	WGV	0.488	0.512	5
	HET	0.262	**0.738**	10
	CRT	0.253	**0.747**	10
France	BVS	0.597	0.403	6
	BLT	0.365	**0.635**	25
	LMD	0.584	0.416	3
	MGG	0.450	0.550	7

3.4. Discussion

In this chapter I used 10 microsatellite loci to characterise patterns of genetic diversity and spatial genetic structure in 1,322 *E. viridulum* that were collected between 2002 and 2006 from 28 populations that had recently colonised the UK, and 195 individuals from 11 populations from Germany, Belgium, Holland and France that represent putative source populations, or populations close to source of invasion. Genetic data were used to explore the level of population structure, loss of variability during colonisation and to compare UK samples with those from continental Europe.

3.4.1. Patterns of genetic variability

Rapid range expansion is expected to produce genetically homogenous populations because factors that create genetic structure, such as genetic drift and local adaptation are counteracted by high migration rates and recent founding of populations (Slatkin, 1993). Theory predicts that only very low levels of migration between populations are needed to prevent genetic differentiation (Wright 1931; Slatkin, 1985; Mills & Allendorf, 1996). In models where range expansion occurs by means of long distance migrants, known as leptokurtic dispersal, increasingly large patches of homozygosity are produced in comparison to the fragmented patchwork of high and low frequency areas produced in the other forms of dispersal (Ibrahim *et al.*, 1996). Furthermore, founder effects are well documented to reduce genetic variability and therefore structure, in newly established populations (Tsutsui *et al.*, 2000).

The only other genetic study of a zygopteran using microsatellites was on the endangered species, *Coenagrion mercuriale*, which in Britain exists in fragmented patches with little dispersal occurring between populations (Watts *et al.*, 2004a, 2005). Levels of genetic diversity were found to be similar but slightly higher in *C. mercuriale* to those in *E. viridulum*, despite their contrasting distributions and dispersal ability. A_R was higher in *C. mercuriale* than *E. viridulum* (3.46 and 3.00 respectively) and *f* was lower (0.040 and 0.100 respectively). This demonstrates that similar low levels of genetic

diversity can be generated by different processes and that founder effects have not prevented or slowed the spread of *E. viridulum* in the British Isles.

3.4.2. Population bottleneck

Genetic bottlenecks caused by founder effects are expected to produce populations with reduced genetic variability when compared with their source populations (Tsutsui *at al.*, 2000). I found significant differences in the variability among the populations of *E. viridulum* in terms of their allelic richness and expected heterozygosity (see Table 3.2). These differences were indicative of a loss of genetic variability during colonisation of the British Isles, with the Isle of Wight and Hampshire populations showing more loss than those from the rest of mainland Britain when compared with continental samples. This evidence suggests effective population sizes have differed between the regions studied at some time.

3.4.3. Spatial genetic structure

The main purpose of this study was to investigate the population genetic structure of *E. viridulum* in the British Isles and relate this to potential source populations in continental Europe. From the correlogram profiles it is apparent that spatial clustering of similar genotypes, *i.e.* some IBD-like genetic structure, is evident only at relatively large scales – i.e. within 100-160 km. The combined 2002-2006 correlogram shows positive kinship coefficients (significantly greater than zero) observed for groups of samples up to 150 km. No small-scale pattern of IBD could be found when (i) the Isle of Wight and Hampshire (ii) sites from mainland Britain or (iii) continental European samples were considered. This confirms that the *E. viridulum* in the British Isles represents two populations originating from discrete invasion events; one on the Isle of Wight which subsequently spread into Hampshire and the other centred on the east coast of southern UK.

I conclude that large scale patters of IBD in the British Isles were generated by differences between the Isle of Wight and Hampshire populations and those from the rest of mainland Britain, indicative of two discrete invasions. This result is consistent with the results from STRUCTURE indicating the

highest value of ΔK, which is the second order rate of change of Ln P(D) with respect to K, was found when K = 2, meaning the best model was two clusters, or populations. When individuals were probabilistically assigned to these clusters (or jointly if their genotypes indicate that they are admixed) by STRUCTURE, a loose pattern emerged of genetic differentiation between the Isle of Wight and Hampshire populations and those from mainland British sites. Assignment to these populations was rarely definitive as most individuals were admixed though tending to have a greater proportion of probability assigned to their geographic origin. Samples from continental Europe tended to cluster with those from Essex and East Anglia rather than the Isle of Wight, implying their source population. As such, the source for the Isle of Wight population remains unknown and it is likely it was from an area further west of my sample range in northern Europe, for example from an area close to the Cap de la Hague in northern France. Alternatively, the allele frequencies may have been highly skewed as the result of an extreme founder effect, although as discussed above no evidence for a bottleneck was detected.

There was some temporal variation in the strength of the large scale pattern of IBD-like genetic structure with correlograms from 2002 and 2006 showing a weaker pattern of decreasing kinship (F_{ij}) and higher variances than those from 2004 and 2005, most likely a result of smaller sample size in those years. The point at which positive kinships switch to negative remained constant at around 150 km over the different years of sampling. The reasons behind this variation are unclear at present, possibly representing variation in the level of immigration or an artefact of sampling.

Genetic evidence did not implicate sex-biased dispersal occurring in *E. viridulum*, with males and females having a similar pattern of decreasing relatedness with geographic distance over all years combined (Figure 3.8), though sample size for females was small (n = 170). Capture-mark-recapture studies of damselflies have found a general pattern of higher likelihood of recapture at least once for males than for females at both natal

sites and breeding sites, attributed to higher female-biased dispersal (reviewed by Bierinckx *et al.*, 2006).

No significant pattern of genetic isolation by distance within broad areas (*i.e.* populations on the Isle of Wight and Hampshire, mainland Britain or continental Europe) was detected. This may seem surprising considering the mainland Britain samples span an area of 160 km and the continental European samples extend over 1,600 km. There are a number of factors describing to the range expansion of *E. viridulum* that are likely to have contributed to the observed allelic homogeneity. Movement between water bodies by odonates, particularly zygopterans, is generally regarded as uncommon during their mature adult lives (Fincke, 1982; Utzeri *et al.*, 1984; Banks & Thompson, 1985; Michiels & Dhondt, 1991) however *E. viridulum* has expanded its range in the British Isles by an average of 31.68 km per year (source: British dragonfly recording network, courtesy of S. Cham) indicating routine long-distance dispersal (indeed, since *E. viridulum* colonised the UK from continental Europe, it must be capable of dispersing over longer distance than this). A pattern is emerging of *E. viridulum* migration events taking place towards the end of the flying season, when population densities are greatest, increasing the viability of new colonies (Cham, 2002). There is some evidence that *E. viridulum* may be migrating by night (Jones, 2004) potentially utilising low level winds to cover large distances as with other insect species including odonates (Gatehouse, 1997). Similarly, large-scale seasonal migrations by night have been documented in China for the dragonfly, *Pantala flavescens*. Radar observations indicated this species regularly makes journeys of 150-400 km in a single flight at altitudes of up to 1,000 km over open sea (Feng *et al.*, 2006). *Ischnura hastata* has also been documented to travel large distances over sea after it was caught in nets mounted on an aeroplane at an altitude of 300m, which has allowed it to colonise remote islands (Dunkle, 1989)

Other factors that may have contributed to the weak spatial genetic structure in *E. viridulum* in the British Isles and on the continent are the relatively short time since these populations were founded and the repeated invasions of the

British Isles. The first record of *E. viridulum* in the British Isles was in 1999 at three localities in Essex (Dewick & Gerussi, 2000) and in the following years sites were added along the coast between north-Norfolk and the Isle of Wight in addition to significant inland movement (Cham 2001, 2002, 2003, 2004). Furthermore, as this species is predominantly semivoltine in the British Isles (see Chapter 5) it means there has been limited scope for local adaptation or genetic drift to generate local genetic differentiation. The rate at which *E viridulum* has spread in the British Isles implies either founding populations were of sufficient numbers to rapidly breed and spread, or that further reinforcements from the continent were a contributing factor. Indeed, repeated invasions of the British Isles by *E. viridulum* have been documented at coastal stations with waves of individuals observed coming inland from the sea (Cham, 2002; Bowman, 2004; Parr, 2004, 2005) which may have ameliorated potential genetic founder effects typically associated with biological invasions (Goodisman, 2001).

There was a lack of genetic isolation by distance in the continental populations despite samples being taken over a range of over 1,600 km in northern Europe. It might be expected that the pattern of genetic isolation by distance would be stronger in the native range compared to the introduced range in the British Isles, as has been the case in studies of other invasive insects (Tsutsui & Case, 1994; Gomulski *et al.*, 1998; Malacrida *et al.*, 1998; Gasperi *et al.*, 2002; Kourti, 2002). However, the area sampled in northern Europe had itself only been significantly colonised as recently as the 1970s in the Netherlands (Ketelaar, 2002) and in the 1990s in Belgium (De Kniff *et al.*, 2001). This range expansion was rapid and may account for the lack of genetic structuring for the same reasons as the lack of small-scale structure in the British Isles.

No general conclusions have emerged in comparative studies into the population genetic structure of zygopteran species. Most published population genetic studies of zygopterans have reported only low or non significant genetic differences at small distances (Geenen *et al.* 2000; Wong *et al.*, 2003; De Block, 2005). Significant differentiation was found between

populations separated by similar scales to those examined in this study (Andrés *et al.* 2000, 2002; Svensson, 2004). The only damselfly study to have found small scale genetic isolation by distance was on *Coenagrion mercuriale* in the Itchen valley, where a pattern of IBD emerged over just 1,000 m (Watts *et al.*, 2004a). The primary factors generating of this small scale pattern of IBD was very limited dispersal, in contrast to *E. viridulum* and habitat fragmentation. *C. mercuriale* is a weak flier and in a capture-mark-recapture experiment, 78 % of adults moved less than 50 m from where they were first captured over their lifetime (Watts *et al.*, 2004a).

As this is the first population genetic study of damselfly undergoing range expansion, comparisons can only be drawn with other invasive species or those undergoing range expansion. The most informative studies are those which look at genetic variation in both the introduced and native range of a species. In Argentine ants, the pattern of genetic isolation by distance among nests and colonies in the native range resembled a population at equilibrium under a stepping-stone model of population structure in contrast to introduced populations which showed no pattern of genetic isolation by distance (Tsutsui & Case, 1994). Isolation by distance has been observed between populations of the medfly *Ceratitis capitata* in Africa (the native area) and in the Mediterranean basin, where it was introduced and first recorded in 1842 (Gomulski *et al.*, 1998; Malacrida *et al.*, 1998; Gasperi *et al.*, 2002; Kourti, 2002). In a study of the introduced wasp *Vespula germanica*, in Australia a significant pattern of genetic isolation by distance was only found when a larger spatial scale was investigated either because of limited dispersal or multiple introductions (Goodisman *et al.*, 2001). In 1935 the cane toad, *Bufo marinus*, was introduced to Australia where it spread rapidly. Subsequent genetic studies showed no pattern of isolation by distance at local scales (Leblois *et al.*, 2000) though founder effects and discontinuities in the colonisation process may have generated the apparently significant relationship between genetic and geographic distance reported in previous studies (Easteal, 1985), similar to the results described above for *E. viridulum*.

These studies together with this investigation provide an insight into the population genetics of species undergoing range expansion and human mediated invasions. They emphasise that dispersal ability is a critical factor in determining spatial genetic structure. In addition a clear understanding of the history of an invasion is important so that patterns of IBD are correctly attributed to discontinuities in the invasion process rather than local adaptation or genetic drift. In the case of *E. viridulum*, questions remain about the origin of the Isle of Wight and Hampshire populations and how voltinism and effective population sizes influence the spatial variation in allele frequencies.

3.4.4. Summary

I found a lack of genetic structure even at regional spatial scales, which were attributed to high levels of gene flow, brought about by high migration rate and multiple colonisation events by large numbers of individuals. An exception to this lack of structure was the differentiation between the southern and eastern UK populations ascribed to different putative source populations. Data strongly implied that eastern UK populations were founded by migrants from the area of continental Europe that was sampled in this investigation. However the source of the southern UK populations remains unknown and is likely to be either outside the range sampled in this investigation or the result of an extreme founder effect. The southern UK showed a greater loss of genetic variability than eastern UK populations when compared with continental European populations. Overall we found that migration rate and source populations were the critical factors in determining the current population genetic structure of *E. viridulum* in the British Isles.

Chapter 4

Temporal genetic variation at recently colonised populations of the small red-eyed damselfly *Erythromma viridulum* (Charpentier, 1840).

4.1. Introduction

Genetic surveys that explore temporal changes in genetic structure are valuable because they provide insights into how patterns of spatial genetic structure develop and are maintained (de Barro *et al.*, 1995). Typically, temporal genetic variation is assumed to be minimal and putative key determinants of population genetic structure such as dispersal ability or landscape are the foci of study for a wide range of taxa, including odonates (see discussion and references in Chapter 3). However, it is essential to recognise that, in addition to dispersal ability *per se*, genetic structure is determined by other demographic and behavioural factors, such as natal site philopatry (Avise, 1994), mating system (Jarne & Städler, 1995; Fournier *et al.*, 2002; Lenoir *et al.*, 2007), historical patterns of gene flow (Liebherr & Zimmerman, 1998; Leblois *et al.*, 2000), effective population size (Adkison, 1995, Lehmann *et al.*, 1998, Adams *et al.*, 2007; Watts *et al.*, 2007b) and developmental polymorphism (Robinson *et al.*, 1992; Schultheis *et al.*, 2002; Charmantier *et al.*, 2006). This chapter principally deals with the latter two properties.

Often, species' life histories have an invariant component, such as a specific larval type, that leaves a characteristic signature on the pattern of spatial genetic structure (reviewed by Avise, 1986; Bohonak, 1999). Many insects, however, combine developmental polymorphism with facultative expression of particular stages during their life cycle; for example, species may use or bypassing a diapause stage to avoid or exploit certain environmental conditions respectively (*e.g.* Solbreck, 1979; Bradford & Roff, 1995; Hunter & McNeil, 1997; Hockham *et al.*, 2001). Odonates have an inherently complex terrestrial-aquatic life-cycle, and many species are able to vary their

development time in response to certain environmental conditions, such as latitude (Parr, 1970; Norling, 1984; Corbet, 1999; Johansson, 2003; Corbet *et al.*, 2006), perceived time constraint (Johansson, 1999), food stress (de Block, 2004; Mikolajewski *et al.*, 2007), predation risk (Brodin, 2006) and larval density (Banks & Thompson, 1987). While many odonates regulate development with a diapause, this can be averted by appropriate treatment and therefore it is regarded as a facultative rather than obligate condition (reviewed by Corbet 1999; Corbet *et al.*, 2006).

Developmental polymorphism can lead to variation in the number of generations that may be completed within a given period. Corbet (1999; Corbet *et al.*, 2006) reviewed levels of voltinism (the number of generations completed in a year) in odonates: univoltinism, *i.e.* completing one generation per year, predominates in temperate species, followed by semivoltinism and partivoltinism (one generation every two or three years, respectively), bivoltinism (two generations per year) and then other levels of multivoltinism. For some species, such as many species within the Lestidae, voltinism is fixed at one generation per year, however, for many species the level of voltinism can vary with latitude (*i.e.* correlating with photoperiod and/or temperature) (Parr, 1970; Norling, 1984; Johansson, 2003; reviewed by Corbet, 1999; Corbet *et al.*, 2006). Thus, odonate generation time can vary intraspecifically according to the perceived environmental conditions.

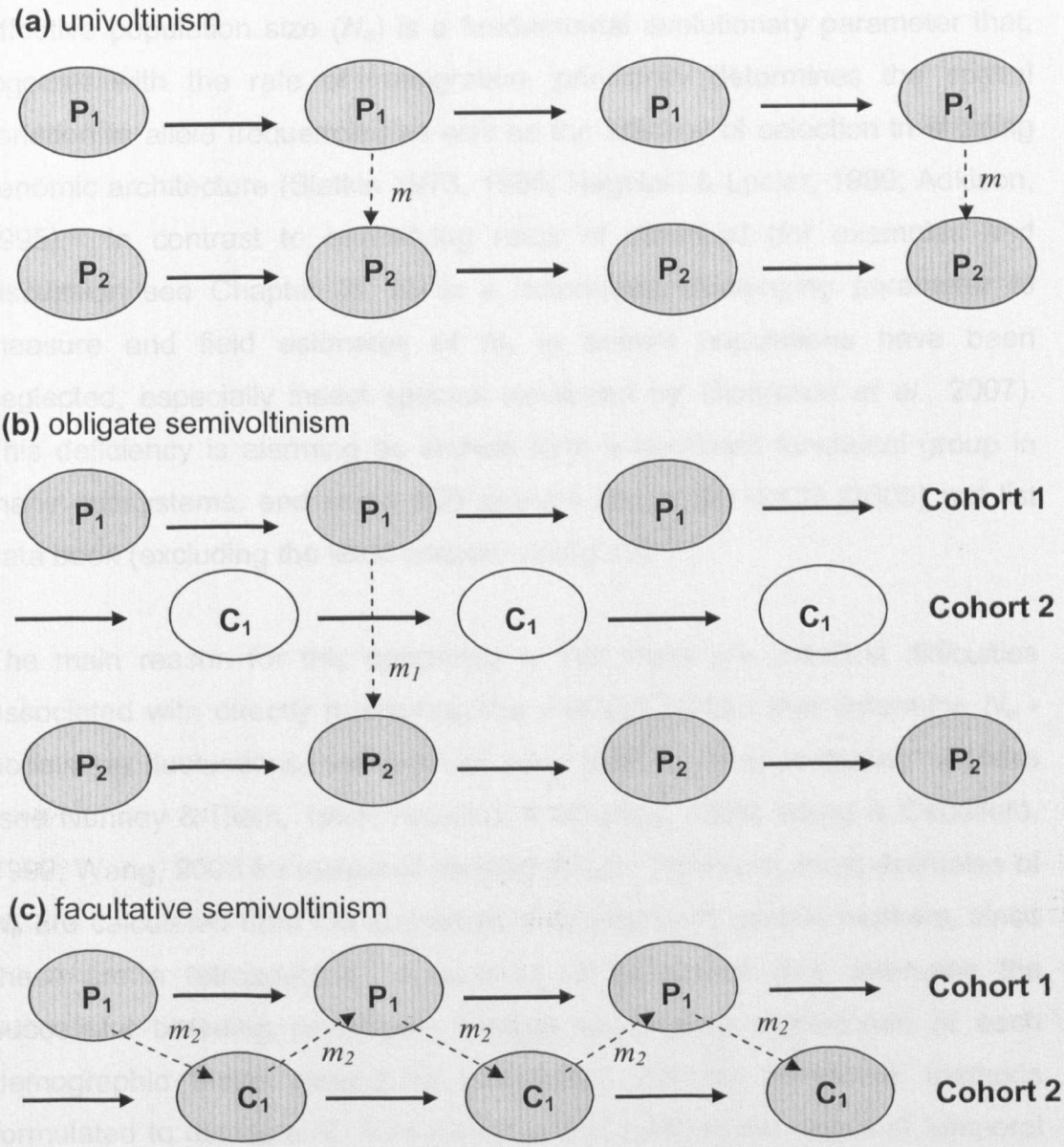
Temporal genetic divergence in univoltine species will reflect patterns of immigration, selection and divergence due to genetic drift (described in more detail below). Thus, when populations have attained migration-drift equilibrium conditions samples from different generations could be used to characterise population genetic parameters without a severe bias. By contrast, in an obligate semivoltine species, where larval development time is fixed at two years, any particular breeding site will comprise two distinct cohorts that are effectively reproductively isolated (Figure 4.1b), and thus be expected to diverge through the action of genetic drift, response to selection and mutation; indeed, in some insects, reproductive isolation among discrete cohorts is sufficient to generate distinct races (*e.g.* Coates *et al.*, 2004).

Clearly, estimates of spatial genetic structure may not reflect actual patterns of gene flow when sampling is confounded by a mixture of distinct cohorts. Where larval developmental time is not fixed, however, there is potential for genetic 'leakage' between cohorts whereby individuals from both cohorts emerging in any breeding season (Hughes, 1999; Schultheis *et al.*, 2002; Watts *et al.*, 2005). In a predominantly semivoltine species, even just a small number of individuals taking one or three years to complete development, should provide sufficient gene flow between cohorts to mitigate the effects of genetic drift and selection (Figure 4.1c).

E. viridulum is predominantly semivoltine in the British Isles, though under favourable conditions some individuals may complete a generation within one year, a subject which is covered in Chapter 5. This species is known to be bivoltine in Greece (Galletti & Pavesi, 1983) and therefore is assumed to be univoltine throughout central Europe. Developmental plasticity and a mixture of semivoltine and univoltine life histories in the British Isles would prevent separate cohorts of *E. viridulum* from becoming reproductively isolated. Accordingly, low levels of inter-cohort differentiation are expected.

Irrespective of divergence among cohorts, genetic differences will accumulate between successive generations through the evolutionary forces of selection, migration, mutation and genetic drift. The concept of N_e was introduced by Wright (1931) to predict the genetic properties of a finite population that meets the assumptions of random mating, constant population size and non-overlapping generations (the Wright-Fisher model) (Fisher, 1930; Wright, 1931). In the absence of significant migration, selection or mutation, the allele frequencies of any population will fluctuate among generations due to random sampling of gametes during reproduction, with the amount of this genetic drift inversely proportional to the effective population size (N_e). Natural populations seldom conform to the Wright-Fisher model and various factors reduce the number of reproductively successful individuals (*i.e.* N_e) below that of the total adult census (N) (reviewed by Frankham, 1995; Frankham *et al.*, 2002). This has widely-recognised evolutionary consequences because it is N_e and not N that

determines levels of inbreeding and the rate of loss of genetic diversity, which are important correlates of population persistence (Saccheri *et al.*, 1998; Spielman *et al.*, 2004) and future evolutionary potential (Franklin, 1980).



Figures 4.1a, b, c. Potential evolutionary consequences of voltinism among successive generations (solid arrows) of populations (open or grey ovals) connected, or not, by gene flow (*m*, dashed arrows). (a) 2 spatially separated populations (P_1 and P_2) of a univoltine species remain genetically similar (both grey) through intermittent migration events (*m*); (b) as above but for an obligate semivoltine species with 2 cohorts (P_1 and C_1) shown at one site. Individuals from P_1 and C_1 genetically diverge because they always emerge during different years and do not interbreed. (c) facultative semivoltine species with some individuals taking either one or three years to develop and therefore recruiting to a different cohort; gene flow between cohorts reduces rate of genetic divergence.

Effective population size (N_e) is a fundamental evolutionary parameter that, together with the rate of immigration, principally determines the spatial variation in allele frequencies as well as the efficacy of selection in shaping genomic architecture (Slatkin 1973, 1985; Nagylaki & Lucier, 1980; Adkison, 1995). In contrast to quantifying rates of dispersal (for examples and discussion see Chapter 3), N_e is a notoriously challenging parameter to measure and field estimates of N_e in animal populations have been neglected, especially insect species (reviewed by Thompson *et al.*, 2007). This deficiency is alarming as insects form a dominant functional group in many ecosystems, and some 800 species are in the IUCN (2006) red list data book (excluding the least concern category).

The main reason for this deficiency is that there are manifest difficulties associated with directly measuring the principal factors that determine N_e - population fluctuations, uneven sex ratio and variance in mating success (see Nunney & Elam, 1994; Falconer & MacKay, 1996; Wang & Caballero, 1999; Wang, 2005 for review of methodology). Therefore, most estimates of N_e are calculated from the population frequencies of genetic markers, since these are a retrospective measure of all processes that determine the successful breeding population, though the relative significance of each demographic factor cannot be quantified. Of the numerous methods formulated to estimate N_e from genetic data, such as the extent of temporal variation in allele frequencies (Nei & Tajima, 1981; Waples, 1989 and others), linkage disequilibrium (Hill, 1981) or heterozygote excess (Pudovkin *et al.*, 1996), the former is believed to have the greatest precision and is the most frequently employed.

For species existing as a dynamic network of partially connected patches (captured by the metapopulation paradigm, see Hanski, 2003 for review) one issue in particular may complicate estimation of N_e: the original genetic methods developed to calculate N_e assume that populations are closed (*i.e.* they experience no immigration) is often invalid – certainly for a highly mobile species such as *E. viridulum* that is rapidly expanding its range. Migration alters the gene frequencies of a population and this will bias estimation of N_e

if not taken into account. For this reason, new techniques have been derived that are able to jointly estimate N_e and immigration rates (m) (Vitalis & Couvet, 2001; Wang & Whitlock, 2003) and there are now several examples of the application of these techniques to characterise vertebrate populations (Wilson *et al.*, 2004; Jehle *et al.*, 2005), and, more recently, in odonates (Watts *et al.*, 2007b).

In this Chapter I (1) examine the level of genetic differentiation variation among cohorts, and (2) quantify N_e of three populations of the invading small red-eyed damselfly *Erythromma viridulum*.

4.2. Materials and Methods

4.2.1 Study sites

Samples used for analyses of temporal variation are provided in Table 4.1.

Briefly, adult *E. viridulum* samples, collected from 9 sites during the summers of 2004, 2005 and 2006, were used to quantify the level of genetic differentiation between cohorts. The final groups of samples examined as cohorts were: cohort 1 (MAR from 2004, and PCP, WRP, ML2, SCV, MOR, PPP, EAR and LWS from 2006) and cohort 2 (PCP, WRP, ML2, SCV, MAR, MOR, PPP, EAR and LWS all from 2005).

Samples were taken from successive generations (2004 and 2006) at three sites to estimate N_e, with genotype data from a range of potential source populations pooled into a single source population used to estimate immigration rates (see 4.2.4 below). Grid references and a map of locations for these sites are given in Chapter 3, in Table 3.1 and Figure 3.1 respectively.

4.2.2 DNA Extraction and Polymerase Chain Reaction

Microsatellite loci and methods used for DNA extraction and genotyping are provided in Chapter 3.2.2.

4.2.3 Genetic differentiation among cohorts

Basic genetic analyses, genetic diversity, linkage among loci, agreement with expected Hardy-Weinberg equilibrium conditions and level of differentiation between pairs of populations, are provided in Chapter 3 and, therefore, are not presented here because the main aim of this chapter is to determine the pattern of temporal variation in genetic structure. The permutation procedure implemented by FSTAT was used (2,000 permutations of samples among groups) to test the significance of any differences in genetic diversity (A_R, H_e, H_o and f) between samples from different alternate-year cohorts.

Principal component analysis (PCA) aims to explain multivariate data by a few linear combinations of the original variables while still retaining nearly as much of the total variation between samples (Johnson & Wichern, 1992). A plot of the sample scores (eigenvectors) of significant principal components offers a convenient representation of the overall variation in data as long as the principal components still account for a significant amount of the total between-sample variation. Rather than attempt to describe the spatial pattern of individual allele frequencies (56 variables) I reduced the multiallelic variation between *E. viridulum* samples to two-dimensions by a PCA of the sample allele frequencies using PCA-GEN version 1.2.1 (Goudet, 1999). The significance of each principal component was assessed from 5,000 randomisations of genotypes.

Next, hierarchical analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) was used to separate the contribution to genetic diversity arising from spatial variation with that occurring among cohorts. The significance of the fixation indices were tested using the permutation procedure (10,000 permutations) that is implemented by ARLEQUIN software (Schneider *et al.*, 2000).

SPAGEDI v. 1.2 (Hardy & Vekemans, 2002) was used to calculate the correlation in average kinship (F_{ij}; Loiselle *et al.*, 1995) relative to the whole data set between pairs of *E. viridulum* separated by various spatial scales. To avoid a bias in the correlation coefficient arising because of unequal

sample sizes within each spatial category I allowed SPAGEDI v. 1.2 to assign ten distance categories that contained a similar number of pairwise comparisons (for further details see Hardy & Vekemans, 2002). Ninety-five percent confidence intervals for F_{ij} at each distance class were generated from the distribution of 2,000 permutations of spatial group locations among the spatial groups. Euclidian geographical distances between samples were calculated from the GPS *x* and *y* coordinates. Correlograms were generated for (1) odd- and even-year cohorts separately and (2) by restricting comparisons between cohorts to determine whether there were any substantial differences in pattern of spatial structure between cohorts.

Finally, the strength of any isolation by distance (IBD) was examined for both cohorts by a regression of estimates of genetic differentiation among pairs of samples, defined by $F_{ST}/(1-F_{ST})$, against the corresponding distances (ln km) separating them (Rousset, 1997); values of $F_{ST}/(1-F_{ST})$ were calculated using SPAGEDI v. 1.2 software.

4.2.4 Effective population size

Temporal methods to estimate N_e are based on the premise that allele frequencies drift apart more rapidly in smaller populations than in larger ones, and hence the magnitude of fluctuations in allele frequencies is directly related to N_e. Recent statistical improvements on the original temporal methods overcome the restrictive assumption of no immigration into populations (*e.g.* see Berthier *et al.*, 2002; Wang & Whitlock, 2003), though a recent comparison of Waples' (1989) original temporal method with the moment and maximum-likelihood (ML) estimators derived by Wang & Whitlock (2003) found relatively little difference in estimates of N_e between various methods (Watts *et al.*, 2007b). I calculated N_e using Waples' (1989) original method as it is still widely used, even though it does assume that the population is isolated. NeESTIMATOR ver. 1.3 (Peel *et al.*, 2004) was used to calculate N_e and ninety-five percent confidence intervals using methods provided by Waples (1989).

Better estimates of N_e should be obtained using more realistic estimators, since *E. viridulum* is highly mobile and its populations likely experience substantial immigration. Therefore, I employed the latter Wang & Whitlock's moment and maximum-likelihood (ML) estimators that jointly estimate N_e and an immigration rate (m), using MNE ver. 2.3 software (Wang & Whitlock, 2003). These methods estimate N_e and m of a focal population under the assumption that immigrants are provided by an infinitely large source, but are robust to deviations from this model and may be applied to a source comprising one or more finite subpopulations. For this analysis, the focal populations consisted of (1) Bedford, PCP, (2) Isle of Wight, MOR and (3) Norfolk, EAR, with the pooled genotypes of *E. viridulum* samples from (1) East Anglia and Bedfordshire or (2) Hampshire/Isle of White (excluding those of the focal population) used as appropriate source populations (see Table 4.1). Finally, I used MNE to calculate N_e assuming that the sites are isolated and experience no immigration. Ninety-five percent confidence intervals are calculated for the ML method (see Wang & Whitlock, 2003 for details).

Table 4.1. Summary of population used for (1) cohort analysis (highlighted bold) and (2) the focal populations (underlined) and the samples pooled to generate potential source populations to estimate effective population size (N_e). N_e was estimated using Wang & Whitlock's (2003) moment and maximum-likelihood estimators. *N* indicates sample sizes. Full details of sample locations are provided in Table 3.1 and Figure 3.1 (Chapter 3).

			N			
County	Code	Location	'04	'05	'06	*n*
Essex	SVE	Silver End		11		11
	ML2	**Maldon 2**	10	**59**	**44**	113
	SCV	**Salcott-cum-Virley**		**53**	**54**	107
	FGH	Fingringhoe		9		9
	TLS	Thorpe-Le-Soken		17		17
Suffolk	**LWS**	**Lowestoft**	17	**58**	**52**	127
Norfolk	**EAR**	**East Ruston**	21	**50**	**33**	104
Bedfordshire	**PCP**	**Priory Country Park**	27	**68**	**54**	149
	WRP	**Wrest Park**		**46**	**75**	121
Isle of Wight and Hampshire	BLH	Beaulieu Heath		4		4
	BUR	Bursledon		38		38
	STL	Stag Lane	4	45		49
	MAR	**Marvel Farm**	**31**	**37**		68
	EVC	East View Cottage		33		33
	PPP	**Parsonage Peat Pond**		**30**	**39**	69
	MOR	**Morton Pond**	27	**32**	**39**	98

4.3. Results

4.3.1 Genetic variation between cohorts

While some measures of genetic diversity (*e.g.* allelic richness and expected heterozygosity) were slightly greater in the even-year cohort, there were no significant differences between odd- and even-year cohorts in any measure of genetic diversity (A_R = 3.13, 3.24, P = 0.273; H_e = 0.398, 0.415, P = 0.075; f = 0.061, 0.105, P = 0.321 for odd, even years respectively) or overall level of population differentiation (F_{ST} = 0.038, 0.041, P = 0.850 for odd, even years respectively).

Only the first principal component axis accounts for a significant amount of the genetic variation among samples (F_{ST} = 0.0274, P = 0.0002, 59 % of the total inertia) (Table 4.2). Nonetheless, the second axis accounts for a substantial (approximately 14 %) amount of the variation in allele frequency differences among samples, so the overall variation is visualised using a two-dimensional scatterplot of population sample scores (eigenvectors) that account for nearly three-quarters of the global F_{ST} (=0.0462) among samples. Differences in the first two principal component scores reveal three distinct clusters (Figure 4.2). As expected given the results from Chapter 3, the two largest clusters are separated on the basis of geography – that is, samples from southern (Isle of Wight and Hampshire) and eastern England (Suffolk, Norfolk and Bedfordshire) – rather than between cohorts. Despite this strong effect of sample location, two populations from eastern England, PCP and EAR in 2006, form a small, third cluster that fall outside the main group of samples from southern England.

AMOVA clearly demonstrates that the level of genetic differentiation among cohorts is non-significant (P > 0.05), with more than 95 % of the variation occurring between individuals within samples. A small (~4 %) but significant (P < 0.001) amount of differentiation was present among samples within each cohort (Table 4.3).

Table 4.2. Summary statistics for principal component analysis of allele frequency variation between samples of the small red-eyed damselfly *Erythromma viridulum* from the UK.

Principal component axis	Per axis inertia (eigenvalue)	Percentage inertia	Cumulative percentage	F_{ST}	P
1	0.1155	59.41	59.41	**0.0270**	**0.0002**
2	0.0264	13.56	72.97	0.0063	0.9972
3	0.0138	7.07	80.04	0.0033	
4	0.0112	5.78	85.82	0.0027	
5	0.0065	3.34	89.16	0.0015	
6	0.0056	2.88	92.04	0.0013	
7	0.0045	2.30	94.35	0.0011	
8	0.0024	1.22	95.56	0.0006	
9	0.0023	1.16	96.73	0.0005	
10	0.0018	0.92	97.65	0.0004	
11	0.0014	0.70	98.35	0.0003	
12	0.0010	0.52	98.86	0.0002	
13	0.0008	0.42	99.28	0.0002	
14	0.0005	0.28	99.56	0.0001	
15	0.0005	0.23	99.79	0.0001	
16	0.0003	0.13	99.92	0.0001	

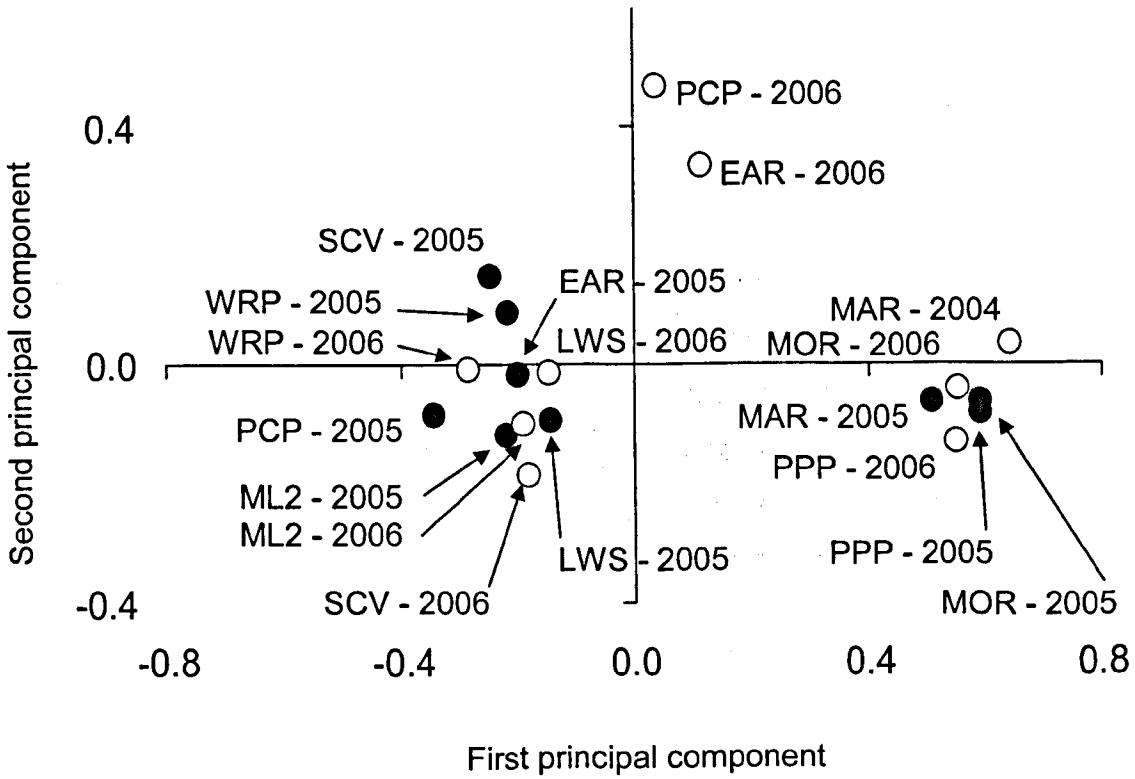


Figure 4.2. Scatterplot of the first and second principal component scores (based on variation in allele frequency) for samples of the small red-eyed damselfly *Erythromma viridulum* within the UK representing odd- (black) and even- (white) year cohorts. Only the first principal component axis accounts for statistically significant ($P < 0.001$) variation in genetic differences between samples, but together both axes represent ~73 % of the total variation in allele frequencies (see Table 4.2 for full details).

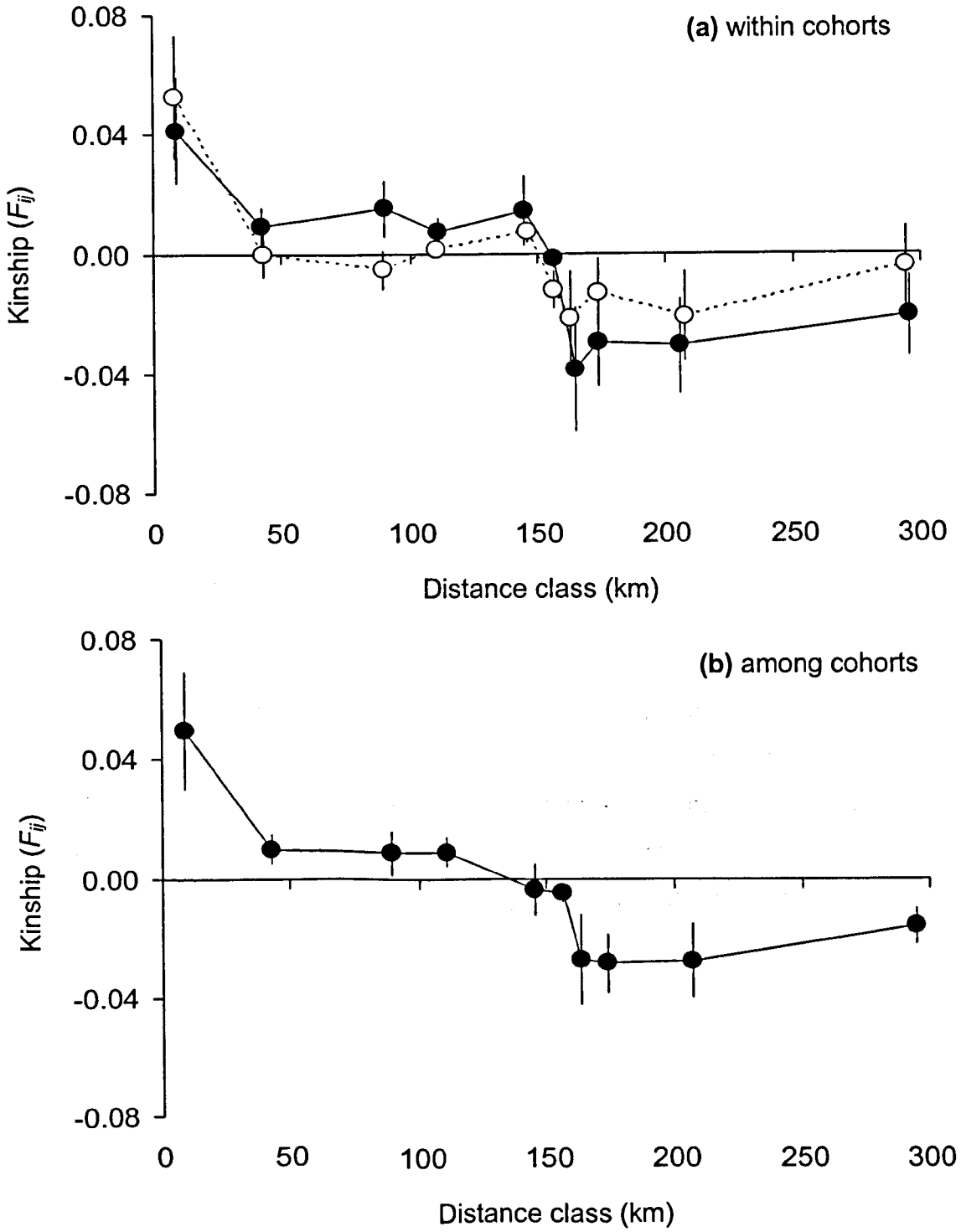
Table 4.3. Analysis of molecular variance (AMOVA) of samples of the small red eyed damselfly *Erythromma viridulum* from the UK collected between 2004 and 2006. Variation is quantified among two potentially discrete cohorts (odd and even years from 2004, 2005 and 2006), among samples within each cohort and within populations.

Source of variation	*df*	SS	Variance components	% variance	Fixation index	*P*
Among cohorts	1	5.64	-0.00569	-0.27	-0.00274	0.664
Among populations within temporal groups	16	158	0.08371	4.03	0.04016	**<0.000**
Within populations	1690	3382	2.00099	96.25	0.03753	**<0.000**
Total	1707	3649	2.148	100.00		

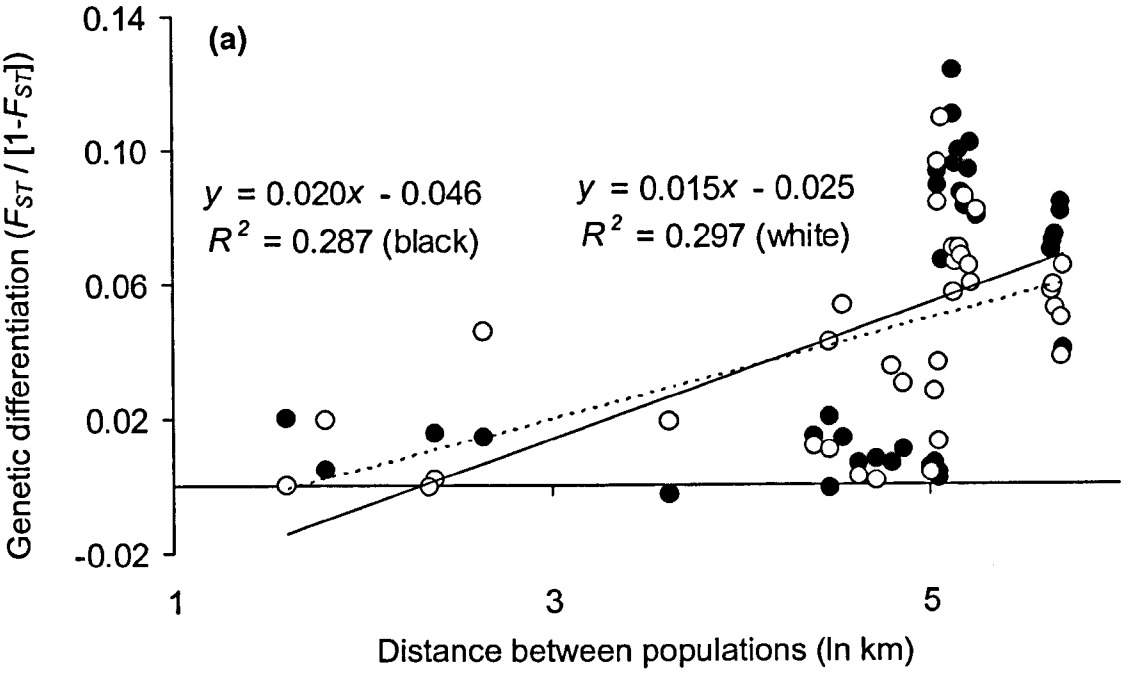
Correlogram profiles for both cohorts show a similar pattern of decreasing kinship (F_{ij}) with increasing geographical separation (Figure 4.3a). Despite this, there is no evidence for a general pattern of isolation by distance among these samples (see also Chapter 3) as the decline in kinship with distance is not linear. Rather, for both cohorts there is a sharp decline in kinship between the first and second distance class (at approximately 50 km). Thereafter, average kinship varies little, being low but positive for the odd year cohort and effectively zero for the even-year cohort, over the next four distance classes until at roughly 150 km average F_{ij} drops sharply to become negative. The same spatial variation in average kinship is observed when pairwise estimates are restricted to comparisons between cohorts (Figure 4.3b) indicating that thee spatial pattern of population structure is broadly similar over both cohorts.

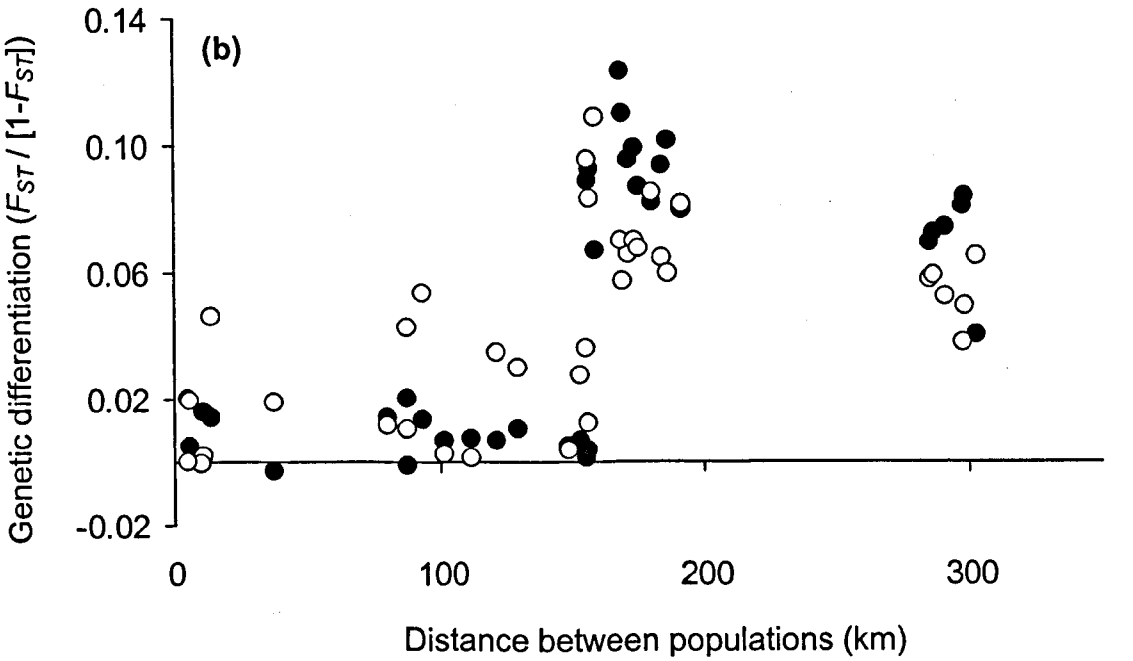
Pairwise population differentiation shows a general increase in both the average values of $F_{ST}/(1-F_{ST})$ and the variance of these estimates with increasing spatial scale; the overall relationship between differentiation and distance is similar for both cohorts, odd-year cohort, $y = 0.02x - 0.046$, $R^2 = 0.287$ and even-year cohort, $y = 0.015x - 0.025$, $R^2 = 0.297$ (regression of $F_{ST}/(1-F_{ST})$ against ln distance), with distance accounting for a relatively small amount of the variation (<30 %) in genetic differentiation among populations. Indeed, within each cohort, values of $F_{ST}/(1-F_{ST})$ actually remain relatively low over short distances (< 150 km), are greatest, both in value and variance, at moderate distances (150-200 km) and intermediate in value with low variance among the most distantly (~ 300 km) separated pairs of samples (Figures 4.4a, b). Moreover, it is notable that there is some variation to this pattern between cohorts, with the even-year cohort having greater values of $F_{ST}/(1-F_{ST})$ at smaller distances than do many pairs of samples from within the odd-year cohort (Figure 4.4b).

An almost identical pattern of genetic structure ($y = 0.019x - 0.044$, $R^2 = 0.341$, for regression of $F_{ST}/(1-F_{ST})$ against ln distance) to that described above is obtained when pairwise comparisons are restricted to samples from different cohorts (see Figures 4.5a, b).

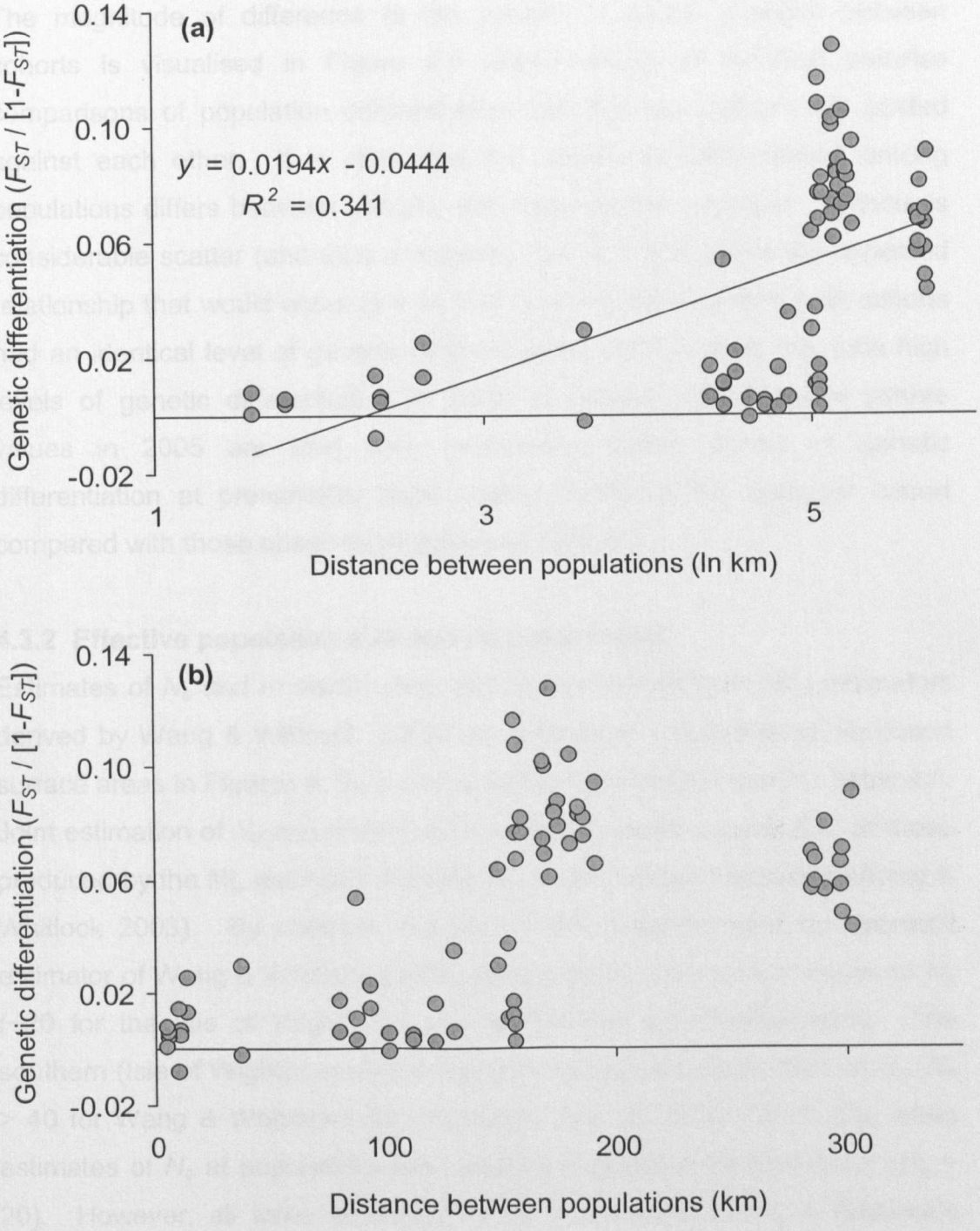


Figures 4.3a, b. Spatial variation in average (± SE) kinship (F_{ij}) among pairs of small red-eye damselflies, *Erythromma viridulum*, collected between 2004 and 2006 from populations in the UK. (a) estimates of F_{ij} restricted to pairs of individuals within distinct 2005 (black) and 2004, 2006 (open) cohorts; (b) estimates of F_{ij} restricted to pairs of individuals from separate cohorts only.





Figures 4.4a, b. Comparison of the pattern of pairwise genetic differentiation (F_{ST} / [1-F_{ST}]) and spatial separation for pairs of populations of odd- (black) and even- (white) year cohorts of the small red-eyed damselfly *Erythromma viridulum* collected from the UK between 2004 and 2006. Values of pairwise differentiation plotted on (a) a log scale as appropriate for two-dimensional array of populations and (b) a linear scale for simple visualisation.



Figures 4.5a, b. Comparison of the pattern of pairwise genetic differentiation (F_{ST} / [1-F_{ST}]) and spatial separation for pairs of populations from different odd- and even-year cohorts of the small red-eyed damselfly *Erythromma viridulum* collected from the UK between 2004 and 2006. Values of pairwise differentiation plotted on (a) a log scale as appropriate for two-dimensional array of populations and (b) a linear scale for simple visualisation.

The magnitude of difference to the pattern of spatial variation between cohorts is visualised in Figure 4.6 where values of identical pairwise comparisons of population differentiation from the two cohorts are plotted against each other. It is clear that the pattern of differentiation among populations differs between cohorts, with two features apparent: (1) there is considerable scatter (and thus a relatively low R^2 = 0.61) from the expected relationship that would occur ($y = x$) if all pairs of samples from both cohorts had an identical level of genetic differentiation, and (2) there are quite high levels of genetic differentiation in 2006 at smaller spatial scales (where values in 2005 are low) and, conversely, higher values of genetic differentiation at presumably large spatial scales in the odd-year cohort compared with those observed in even-year cohort.

4.3.2 Effective population size and migration rates

Estimates of N_e and m made using the maximum-likelihood (ML) estimators derived by Wang & Whitlock (2003) are presented graphically as likelihood surface areas in Figures 4.7a, b and c, and for all methods used in Table 4.4. Joint estimation of N_e and m produced generally similar values of N_e to those produced by the ML estimator assuming a single, closed population (Wang & Whitlock 2003). By contrast, Waples' (1989) estimator and the moment estimator of Wang & Whitlock (2003) always produced very low values of N_e (~20 for the Isle of Wight and <10 for Norfolk and Bedfordshire). The southern (Isle of Wight) population had the highest estimated value of N_e (N_e > 40 for Wang & Whitlock's ML estimator) and the widest 95 % CIs, while estimates of N_e at populations from eastern England were both lower (N_e ~ 20). However, all three estimates of N_e made using Wang & Whitlock's (2003) ML estimator had overlapping 95% CIs (Table 4.4).

Similar to the trend described above for N_e, there was a contrast in estimated immigration rate (m) between the southern and eastern populations (Figure 4.7c), which had low (m = 0.008) and high (m = 0.43 and 0.85) estimated immigration rates respectively; these values of m have non-overlapping 95% CIs (Table 4.4).

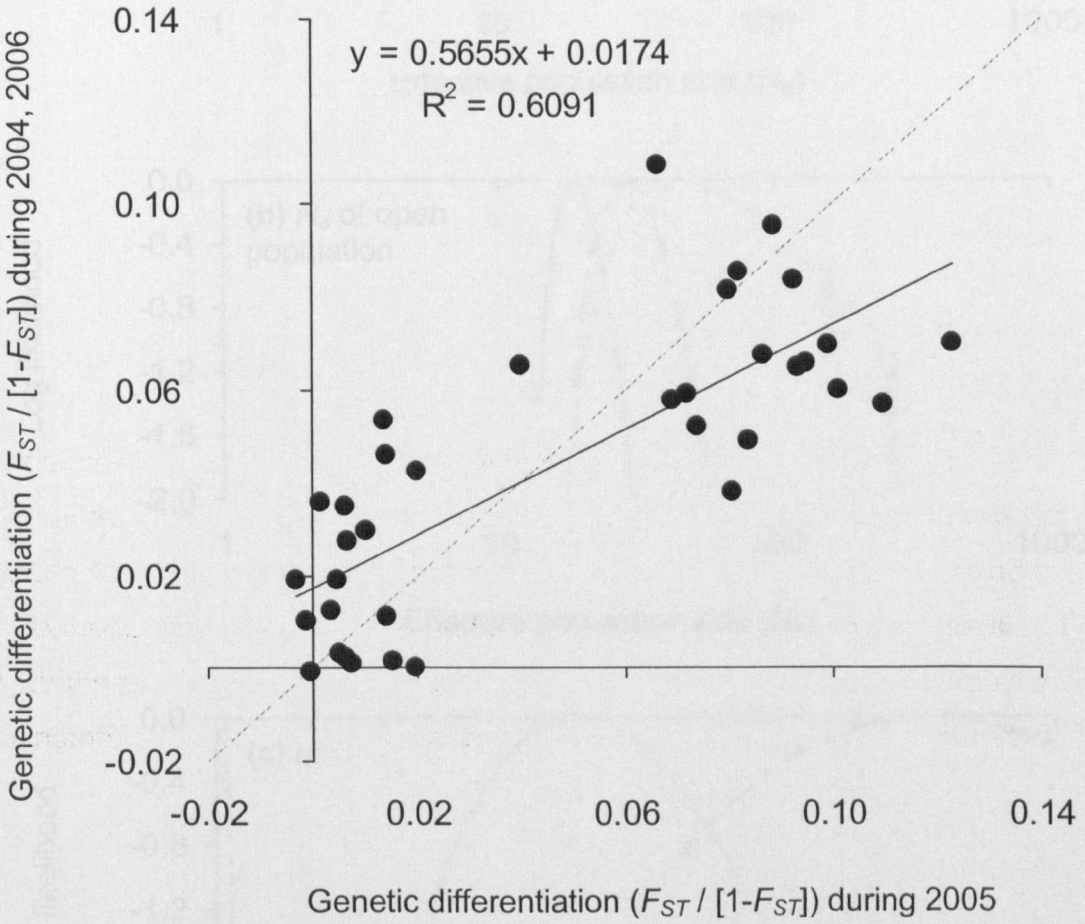
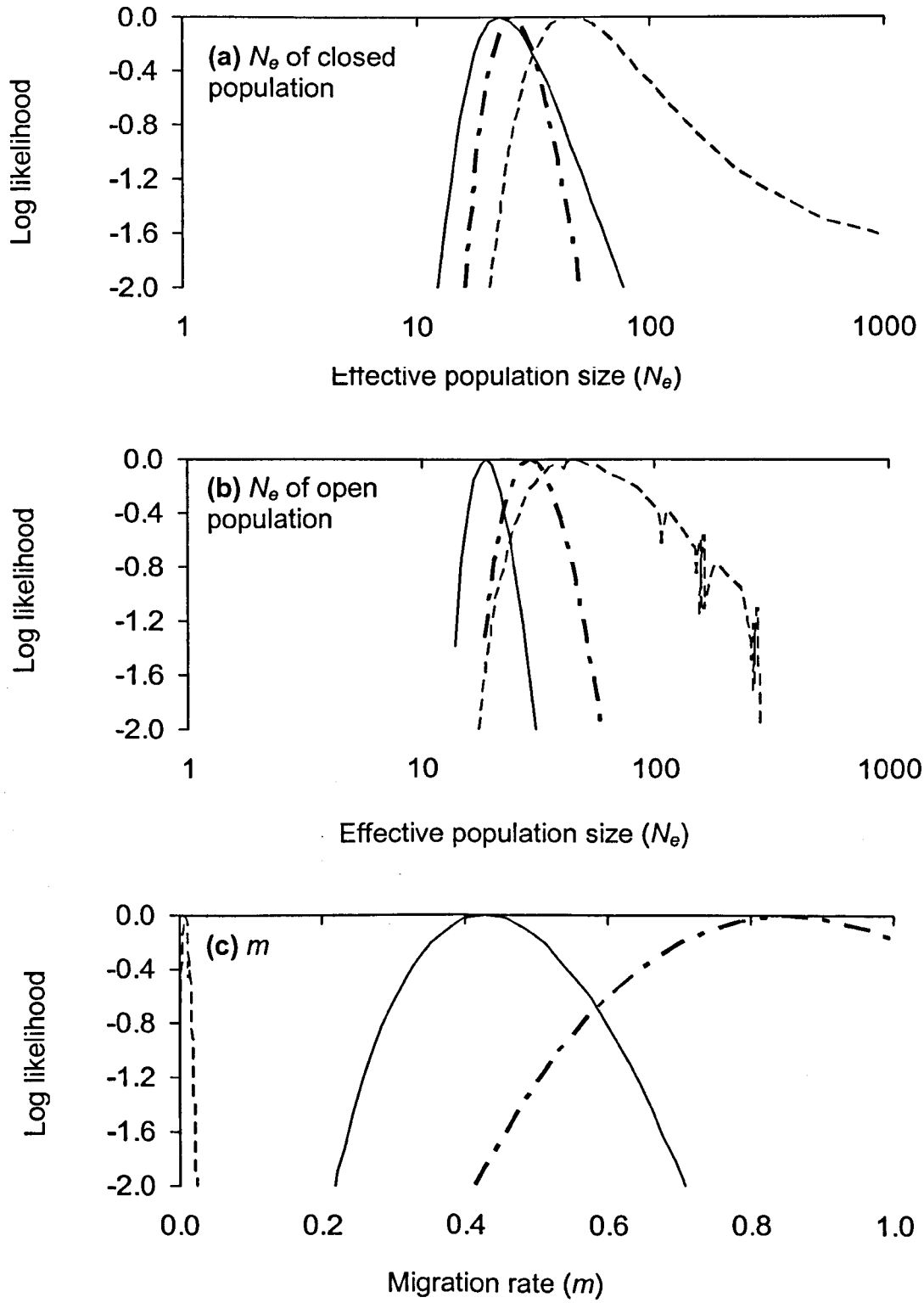


Figure 4.6. Contrast to the level of genetic differentiation (F_{ST} / [1-F_{ST}]) observed among identical pairwise comparisons of populations of the small red-eyed damselfly *Erythromma viridulum* from odd- (2005) and even- (2004, 2006) year cohorts. Dashed grey line indicates the relationship ($y = x$) that would occur if the pattern of pairwise differentiation among samples is identical for both cohorts; solid black line indicates the actual relationship between pairwise differentiation and spatial separation for identical pairwise comparisons.



Figures 4.7a, b, c. Likelihood surfaces for maximum-likelihood estimation of N_e and migration rate (m) for populations of the small red-eyed damselfly from Bedfordshire (solid line), Norfolk (solid-dashed line) and Isle of Wight (dashed line). (a) N_e estimated assuming that the populations and experience no immigration, (b) N_e estimated jointly with (c) migration rate.

Table 4.4. Effective population size (N_e) and migration rates (m) estimated using the moment and maximum-likelihood (ML) estimators derived by Wang & Whitlock (2003) and Waples' (1989) temporal method.

			Maximum-Likelihood			Moment
Method	Population			-95% CI	+95% CI	
Wang & Whitlock (closed)	Isle of Wight	N_e	45.40	20.80	>5,000	18.25
	Norfolk		22.46	12.33	77.55	8.21
	Bedfordshire		25.26	16.00	50.27	5.35
Wang & Whitlock (open)	Isle of Wight	N_e	43.80	18.63	275.77	22.72
		m	0.008	<0.001	0.022	0.015
	Norfolk	N_e	28.94	16.71	58.76	4.64
		m	0.852	0.413	1.000	0.290
	Bedfordshire	N_e	18.81	13.05	30.54	9.60
		m	0.427	0.217	0.708	0.967
Waples (closed)	Isle of Wight	N_e	20.2	6.2	248.6	
	Norfolk		8.6	3.3	25.6	
	Bedfordshire		4.0	1.8	7.8	

4.4. Discussion

The aim of this investigation was to investigate temporal differences in genetic structure and also to determine effective population sizes and migration rates of a highly mobile damselfly species. The main findings of this study were: (1) there was generally little difference to the overall pattern of spatial genetic structuring between cohorts, although (2) the cohorts at some populations did genetically differ (3) and the level of genetic differentiation among populations was slightly weaker in even-year cohort; in addition (4) N_e was estimated to be very low for all populations and (5) m was several orders of magnitude lower in the southern England population than in populations from eastern England.

4.4.1 Genetic differentiation among cohorts

Overall there were apparently few major differences to the spatial pattern of genetic structure between the different cohorts, both at an individual- (*cf.* Figures 4.3a and b) and population level (*cf.* Figures 4.4a, b and 4.5a, b); indeed AMOVA uncovered no significant genetic differences between alternate year-class cohorts (Table 4.2). These results are consistent with the overall lack of significant temporal structure identified in Chapter 3 (see Table 3.4). Therefore, at a broad level, analysing spatial genetic structure of *E. viridulum* samples using samples from a mixture of cohorts would appear to have little impact on the overall analysis of spatial genetic structure. Nonetheless, the pattern of differentiation between pairs of samples is not identical across cohorts (Figure 4.6), indicating that there are subtle genetic differences between cohorts that can be identified using other methods. Thus, we are faced with a picture of subtle genetic differences between cohorts underlying broad genetic homogeneity; the former points at restricted gene flow between cohorts (*i.e.* a tendency for most, if not all, *E. viridulum* in the UK to be semivoltine) while the latter is indicative of developmental plasticity that facilitates inter-cohort migration (*i.e.* a mixture of voltinism strategies). Untangling the relative importance of these factors is not yet possible as the observed pattern of genetic divergence between cohorts continues to be driven by the highly dynamic nature of gene flow for *E. viridulum* in the British Isles, insufficient time required to reach genetic equilibrium conditions and

the ongoing changes in voltinism as this species responds to new environmental conditions (see discussion in Chapter 5).

High dispersal rates and repeated immigration from continental Europe (over the past eight years (Ketelaar, 2002; Cham, 2003; Parr, 2005) had lead to numerous, independent colonisation events. The stochastic nature of the colonisation process raises the possibility that sites contain individuals that originated from several different continental sites, either directly of via other colonisation routes in England, particularly during different years. Where this occurs there will be some genetic differentiation between cohorts, however strong genetic differences are not uncovered as generally weak genetic differentiation was identified between many of the study sites, including those from continental Europe and eastern England (see Chapter 3).

One exception to this is the strong differentiation at two of the eastern English sites – (PCP and EAR, Figure 4.2). One explanation could be that these populations experienced a different wave of immigration in 2006 compared with migrants from 2005. Certainly, this view is reinforced by records of the range expansion of *E. viridulum* in 2005 and 2006. There was comparatively little range expansion in the British Isles in 2005, however 2006 saw significant northerly and westerly movement with new colonies appearing over 120 km from previously known sites (Parr, 2006), which may account for changes in spatial genetic distribution between years. Of course, other effects such as sampling families or strong drift cannot be excluded at this time.

Very little gene flow, roughly one (effective) migrant every other generation (Spieth, 1974; Kimura & Ohta 1971; Lewontin 1974), is required to mitigate the effects of genetic drift and prevent population divergence in the absence of strong selection. It is unclear what selective pressures could drive genetic divergence between cohorts, so these data imply that *E. viridulum* is an obligatory semivoltine species in the UK, or at least very nearly so, or that reproduction between individuals from different cohorts has been unsuccessful/not taken place. Data from Chapter 5, where a study of larval

growth showed that *E. viridulum* is predominantly semivoltine in the British Isles, supports this idea. Obligate levels of voltinism appear to be rare in odonates (Corbet, 1999; Corbet *et al.*, 2006), so the most plausible explanation for divergence is that there has been insufficient time for the cohorts to reach migration-drift equilibrium conditions; indeed, sampling of cohorts in 2004-2006 represents a maximum 3 generation period after the initial colonisation. The corollary is that genetic differences between cohorts are not due to divergence arising from ongoing reproductive isolation as a consequence of an obligate semivoltine life-history, but more likely because of temporal variations in migration rate and discontinuities in the invasion process. At present, it appears that the pattern of differentiation between cohorts doe not depend on the level of voltinism, however further work to characterise future levels of genetic divergence or convergence between cohorts would be extremely interesting and assess the impact of developmental plasticity.

The results for cohort differentiation of *E. viridulum* contrast strongly with the stable pattern of temporal genetic structure found in the highly sedentary endangered damselfly *Coenagrion mercuriale* (P.C. Watts, unpublished data), which is also at the northern edge of its range and semivoltine in the UK (Purse & Thompson, 2002). At one site in Dorset, no significant genetic differences were detected between successive cohorts of *C. mercuriale* (Watts *et al.*, 2005), similar to the AMOVA results presented in this Chapter. Further analyses of nine *C. mercuriale* populations, however revealed a striking, identical pattern of differentiation among pairs of population in both cohorts (regression of relationship between values of pairwise F_{ST} for odd and even-year cohorts $y \approx x$, $R^2 = 0.985$, P.C. Watts, unpublished data) that could only have arisen if there is inter-cohort gene flow since this species is liable to substantial genetic differentiation in the absence of dispersal (Watts *et al.*, 2004a, 2005, 2006, 2007b). The contrast reflects the longer period of establishment of *C. mercuriale* populations that will be closer, or have attained, genetic equilibrium conditions.

More generally, only a handful of studies have quantified genetic divergence between distinct cohorts of insect species. For example, non-significant genetic differentiation among successive cohorts that has been interpreted as evidence for variation in larval developmental time (Schultheis *et al.*, 2002, stonefly *Peltoperla tarteri*) and an annual rather then biennial life cycle (Vila & Björklund, 2004). Similar results to the previous examples may arise through lack of evolutionary time for cohorts to diverge (Marçon *et al.*, 1999; Bourget *et al.*, 2000, European corn borer *Ostrinia nublialis*) or insufficient marker resolution (Kankare *et al.*, 2002, noctuid moth *Xestia tecta*). Subsequent work uncovered divergence among cohorts of *O. nublialis* at one study site but not another (Coates *et al.*, 2004), while significant genetic differences between cohorts of the spruce sawfly *Cephalcia arvensis* are possibly indicative of incipient speciation (Battisti *et al.*, 2000). Beyond demonstrating the range of possible evolutionary outcomes of possessing distinct cohorts, it is hard to generalise further about the causes and consequences of life-history plasticity because these studies were restricted to only one or two sites. Together, the results of these odonate studies emphasise the importance of quantifying life-history traits and levels of gene flow of a species before making conclusions about spatial genetic structure.

4.4.2 Effective population size

The estimates of N_e for all populations of *E. viridulum* were very low (<46, Table 4.4), irrespective of the method used, or whether the population was assumed to be open or closed. Interestingly, the Isle of Wight population had a larger N_e than those from eastern England (Table 4.4 and Figure 4.7a, b), which is expected as it has been established for the longest time and is likely either reflection of large N, or at least a greater number of successful breeders, possibly because of more favourable conditions (see discussion below). Few studies have quantified N_e in aquatic insects to make broad comparisons (see Thompson *et al.*, 2007 for review), with the notable exception of Watts *et al.*'s (2007b) recent study of *C. mercuriale*. Estimates of N_e in this odonate were also quite low (~40 - 400) but generally higher than in *E. viridulum* which is surprising as *C. mercuriale* mainly exists as fragmented, small populations in the UK. If these estimates of N_e for *E.*

viridulum are correct they raise questions about the long-term persistence of *E. viridulum* populations in the UK (in the absence of continued immigration), since it has been suggested that N_e greater than 50 or 500 is required for to prevent immediate detrimental effects of inbreeding and long-term evolutionary potential respectively (Simberloff, 1988; Mack *et al.*, 2000; Frankham *et al.*, 2002; Allendorf & Luikart, 2007). Accordingly, it is somewhat counterintuitive that *E. viridulum* maintains apparently healthy breeding populations in the UK (Cham, 2004) and is even expanding its range at a rapid pace. Indeed, in other studies of successful invasive species, levels of N_e have also been found to be highly variable (Holland, 2001; Tsutsui *et al.*, 2001; Estoup *et al.*, 2004), indicating that factors other than N_e may be more critical in determining invasive success, at least in the short-term; for example, Lande (1988) emphasised the importance of demographic, rather than genetic, factors in ensuring population survival.

No accurate data exists on the total population sizes (N) of *E. viridulum* in England, however, some sites on the Isle of Wight are documented to have hundreds of adults (Cham, 2002) and both Priory County Park and Wrest Park contained hundreds, if not thousands of individuals, at peak densities (S. Keat, pers. obs.). Thus, it is clear that total numbers of adults (N) at these sites far exceed the estimates of N_e and thus the ratio of N_e/N will be very low, almost certainly much lower than values observed for *C. mercuriale* (which varies between 0.006 and 0.42). Since the N_e/N ratios for *C. mercuriale* were lower than most values reported in a review of 17 studies of insects, with the exception of the seaweed fly *Coelopa frigida* (N_e/N = 0.0047 and 0.0009) (Frankham, 1995), *E. viridulum* potentially has lowest reported values of N_e/N for any insect so far studied. N_e/N ratios less than one are typical of animal populations, largely because of fluctuations in population size, but also through variance in mating success and uneven sex ratios (see Frankham, 1995; Frankham *et al.*, 2002; Allendorf & Luikart 2007 for reviews). Further work to confirm the low N_e, quantify adult population sizes and then determine the factors that drive this apparently low N_e/N ratio would be extremely interesting.

It is probable that variance in mating success is a principal factor in generating the low N_e to N ratios for *E. viridulum*. Failure of eggs to hatch in sub-optimal environments has been suggested as a reason for disappearance of *E. viridulum* from British sites where in previous years they have been observed ovipositing (Cham, 2003). Studies have suggested *E. viridulum* is eurytolerant but breeding success is dependant on environmental factors such as water phosphate levels and the species of aquatic macrophytes present (Sternberg & Buchwald, 1999; Ketelaar, 2002). Although speculative, it is likely that low values of N_e can be partially assigned to high reproductive success of a very few migrants at sites where environmental conditions were (partially) favourable. Highly variable survivorship of offspring has been documented in a neotropical damselfly, where typically less than half of parents realised any fitness from their reproductive investment (Fincke & Hadrys, 2001).

Genetic estimates of N_e encompass all factors affecting reproductive success and therefore should be more accurate than studies based on demographic parameters, which typically can only quantify one or two factors at a time and rarely determine the actual reproductive success of individuals. As *E. viridulum* is rapidly expanding its range in the UK it may attempt to breed at sub optimal sites that are encountered during the colonisation process. Breeding at these sites may therefore be either very limited or not occur; if the former, then the amount of genetic drift occurring between successive generations may be confounded with genetic signal from recent immigration, and given the latter then the genetic divergence between 'generations' is not due to small N_e, but simply reflects separate invasion events. While I used an estimator that was derived specifically to account for potential confounding effects of migration (Wang & Whitlock, 2003), it is known to perform poorly when populations are weakly differentiated (Hauser *et al.*, 2002; Richards & Leberg, 1996; Luikart *et al.*, 1998; Watts *et al.*, 2007b). Further work is required to partition the contribution of drift and migration, and especially to establish whether successful breeding occurs. Chapter 5 documents successful breeding at Priory Country Park (also recorded by Cham, 2004), Maldon and Salcott-cum-Virley, however the breeding status at

other sites remains unknown. A clear source of imprecision for this and many studies is the small sampling interval (here, a single generation), such that there is a large sampling effect relative to the amount of drift; an intermediate, rather than large or small, number of generations between sampling has been suggested to provide the most accurate estimates (Wang & Caballero, 1999). Clearly, further studies are essential before any general conclusions can be made about whether low ratios of N_e/N calculated using genetic methods are typical for damselflies.

There are a variety of techniques to calculate N_e, however in this investigation Waples' (1989) original method was compared with Wang & Whitlock's moment and maximum-likelihood (ML) methods. Waples' (1989) method assumes that the population is closed to migration, a condition which is not met in this study, whereas Wang & Whitlock's method incorporates the role of migration. Nonetheless, Waples' (1989) method produced consistently lower values, consistent with results presented by Watts *et al.*, (2007b).

4.4.3 Migration rate

Discussion on migration rates is tentative as Wang & Whitlock's (2003) estimator can produce inflated estimates of m when populations are poorly differentiated, as appears to be typical of many *E. viridulum* populations (Chapter 3). Nonetheless, a clear contrast in immigration rate was observed between the Isle of Wight and the eastern UK populations (Table 4.4, Figure 4.7c). Migration rate was found to be low for the Isle of Wight, which may be explained by the relative geographic isolation of this population. The Isle of Wight is an island off the coast of the British Isles, the major source of immigrants is currently most likely to come from continental Europe, which is at least 160 km away over the English Channel. Distributional records fro the British Dragonfly Recording Network indicate that the Isle of Wight and Hampshire population is currently isolated from the Eastern UK population, though if the rate of expansion, averaging 31.68 km per year (Chapter 1.3.3.), continues at this rate they will converge within 1-2 years. So

presently the likelihood of migrants being exchanged between the Isle of Wight and Eastern UK populations remains low.

The Bedfordshire population showed higher levels of migration than the other two populations. Since its arrival in the British Isles in 1999, *E. viridulum* has expanded its range inland in a predominantly north-westerly direction with the largest expansions originating from north-Essex and Norfolk. It was first recorded in Bedfordshire in 2003, where a large population became established at Priory Country Park (Cham, 2004). Since then range expansion has continued further in a north westerly direction and Bedfordshire currently lies close to the centre of the inland expansion, giving it a high likelihood of regularly receiving immigrants from the surrounding network of *E. viridulum* populations.

A number of studies have attempted to quantify migration rates in odonates using capture-mark-recapture methods (*e.g.* Conrad *et al.* 1999, 2002; Angelibert & Giani, 2003; Watts *et al.*, 2004a, 2007; Rouquette & Thompson, 2007). The general relationship between the probability of dispersal and the distance dispersed has been found to be a negative exponential, though this relationship varies strongly depending on species. Watts *et al.* (2007a) is currently the only study to compare ecological and genetic methods of calculating migration rates and they found that ecological methods tended to underestimate migration. In their study, genetic methods estimates of migration in *C. mercuriale* varied from 0.006 up to 0.758, which were far too large to be realistic this sedentary species and reinforce the need for caution when interpreting the data presented in Table 4.4. Indeed, while N_e and m calculated by genetic means can provide valuable insights into underlying population processes, specific values must be interpreted with caution as they can be severely biased by departure from underlying genetic model assumptions (see Whitlock & McCauley, 1999 for review); particularly relevant for *E. viridulum* is the certain departure from genetic equilibrium conditions that leaves a characteristic signature of low levels of genetic differentiation at large spatial scales (Slatkin, 1993; see *e.g.* Figures 4. 4a, b and 4.5a, b). Obvious discrepancies between genetic and ecological

estimates of these processes leave room for either improvements in the models used, or empirical exploration of unquantified factors affecting these processes.

In conclusion we found limited differences between cohorts and assigning these small differences to particular process remains somewhat speculative. Estimates of N_e were generally low and likely to have been affected by the overall low levels of genetic differentiation between UK populations. Low levels of *m* for the southern UK population compared to the Eastern UK were attributed to the geographic isolation and the current lack of connectivity between these populations. It is likely that the same factors that generated a general lack of local spatial genetic structure (described in Chapter 3) operated to drive the absence of significant temporal variation presented in this chapter; these factors are: highly dynamic and high levels of gene flow in UK *E. viridulum* populations, repeated immigrations from continental Europe bringing about discontinuities in the invasion process and the lack of time required to reach genetic equilibrium conditions.

Chapter 5

Larval growth and voltinism in recently-founded populations of the small red-eyed damselfly *Erythromma viridulum* (Charpentier, 1840).

5.1. Introduction

Insects in seasonal environments often have alternative developmental pathways allowing a flexible allocation of time to growth in favourable parts of the season and dormancy in unfavourable parts (Danks, 1994). Odonates are thought to have tropical origins, though have evolved life history strategies that allow them to cope with temperate climates by having cold-tolerant stages in their life cycle (Corbet, 1999). In temperate zones odonates cope with seasonal temperature fluctuations typically by overwintering in a cold-resistant larval stage, or in a state of obligate or facultative diapause at the egg stage (Corbet, 1980, 1999; Norling, 1984). There are also examples of species, such as some within the genus *Sympecma*, which survive overwinter in a cold tolerant adult stage (Corbet, 1962). The period of developmental slowing that occurs during winter increases life cycle length and often results in one, two or multi-year life histories.

The term voltinism refers to the number of generations that are completed within a year. A population completing one generation in a year is described as univoltine, two generations a year as bivoltine. If a generation requires two years to be completed it is referred to as semivoltine and more than two years as partivoltine. Obligate univoltine species, such as many within the genera *Lestes* and *Sympetrum*, have a set life cycle of one year are characterised by diapause in the egg stage and rapid larval growth during the spring (Corbet, 1999). Most odonate species however overwinter in the larval stage, where a wide range of instars are cold tolerant. This strategy gives greater flexibility to the life cycle and allows variation in adult phenology in response to the environment. Knowledge of voltinism is required to

understand how life cycles have become appropriate to environments in different regions and how seasonal regulation is achieved (Corbet, 1999).

The time spent in the aquatic larval stage of development varies depending on species and in some cases physical and biotic factors. Factors that have been documented to correlate with changes in voltinism in odonates include; presence of insectivorous fish (Macan, 1966), larval density (Banks & Thompson, 1987; Martin *et al.*, 1991), water temperature (Short *et al.*, 1987; Pritchard *et al.*, 1991; Thielen, 1992), altitude (Rowe, 1987; Norling, 1984) and latitude (Parr, 1970; Naraoka, 1976; Norling, 1984; Cordero-Rivera, 1988; Johansson, 2003). Most studies of voltinism in European Odonata have been in northern-temperate latitudes and have revealed a relationship between voltinism and latitude (*e.g.* Norling, 1984; Corbet, 1999; Corbet *et al.*, 2006). This occurs because at higher latitudes the period of the year when growth can occur becomes shorter and thus developmental time becomes longer. Generalisations about latitudinal patterns of voltinism are sometimes confounded by exceptions such as the obligate univoltine species of *Lestes* which are found north of the Arctic Circle in Canada (Cannings *et al.*, 1991).

Erythromma viridulum has recently expanded its range in Europe (Ketelaar, 2002) and in 1999 was the first recorded example of a migrant damselfly establishing colonies in the British Isles (Dewick & Gerussi, 2000). Since its initial arrival at three sites in Essex in 1999, there have been repeated immigrations from continental Europe and it has rapidly expanded its range in the British Isles as far north and west as East Yorkshire and Devon respectively and centred on the Thames valley (source: British Dragonfly Recording Network and the British Dragonfly Society website http://www.britishdragonflysoc.org.uk) (see Figure 5.1). The range expansion in the British Isles is described in greater detail in section 1.3.2. Information on the life history of this species is sparse. It is noted to be bivoltine toward the southern margin of its range in Greece (Galetti & Pavesi, 1983) and therefore assumed to be univoltine at higher latitudes. The flight period of this species extends from the beginning of June until the beginning of

September, peaking in mid August (source: British Dragonfly Recording Network, courtesy G. French). Thus *E. viridulum*'s pattern of emergence would seem typical of a "summer species" (Corbet, 1954, 1964) that does not overwinter in the last larval instar and shows an asynchronous emergence later in the summer.

The aim of this chapter is to presents quantify the pattern of the larval growth in *E. viridulum* and identify the pattern of voltinism at its north-west range margin.

5.2. Methods

5.2.1. Study sites

The study sites used to quantify larval growth in the UK were: Salcott-Cum-Virley and Maldon (both in Essex), Priory Country Park, Bedfordshire and a small sample collected from Little Grays, Kent (for locations see Figure 5.1). The pond at Salcott-Cum-Virley was small, shallow and contained large amounts of the aquatic macrophyte *Myriophyllum spicatum* and no fish. The Maldon site was medium sized, had abundant aquatic macrophytes including *Ceratophyllum demersum*, *Nymphaea alba* and *Lemna* spp., indicating probable eutrophic conditions, and a population of carp (*Cyprinus carpio*) and dace (*Leuciscus leuciscus*). The Finger Lake at Priory Country Park was the largest and deepest body of water and as with the other sites was dominated by aquatic macrophytes such as *M. spicatum* and also *Nuphar lutea*. The Finger Lakes at Priory Country Park are used for commercial coarse fishing and support a population of carp (*C. carpio*), rudd (*Scardinius erythrophthalmus*), dace (*L. leuciscus*) and tench (*Tinca tinca*). The site at Little Grays was a disused waterway, supporting similar macrophyte species to the other sites.

During the peak season at the end of July and beginning of August, *E. viridulum* was the dominant odonate species at all sites. However, notably *Erythromma najas* was present at all sites, along with other common UK zygopteran species such as *Coenagrion puella* and *Ischnura elegans*.

5.2.2 Sample collection, identification and measurement

Samples were collected on 20 August, 2005 and 6 June, 2006 from Maldon and on 6 June, 5 July and 9 August, 2006 at Salcott-Cum-Virley and Priory Country Park. Two individuals were also caught at Little Grays on 21 May in an attempt to assess the size of overwintering larvae. Larvae were collected using a square mouthed net with a 1 mm mesh size. The most effective technique was to draw the net through aquatic macrophytes near the water surface, then deposit the contents into a white plastic sorting tray, where larvae were identified and removed by hand. Larvae were stored in a drum containing pond water so they could be taken to the laboratory alive to be positively identified and measured.

Larvae were identified initially by their unpigmented, rounded lamellae (Brook, 2003) and definitively by the presence of setae on the ventral surface of the posterior margin of the first abdominal segment, but absence of setae on the mastesternum (Carchini, 1983). The head width (maximum distance across the compound eyes) and body length of larvae were measured to the nearest 0.1 mm using a light microscope and eyepiece micrometer.

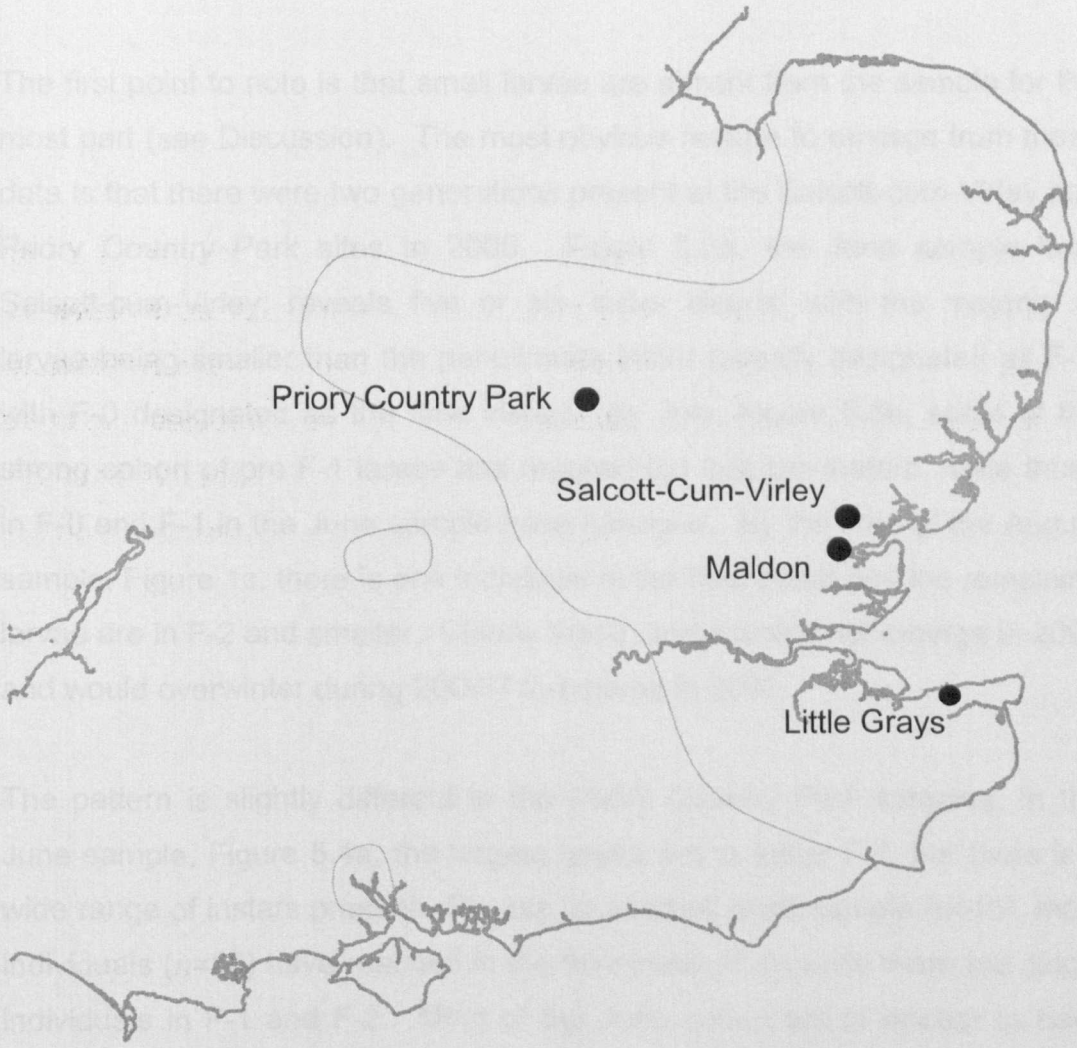


Figure 5.1. Map of the southern UK showing sites where *E. viridulum* larvae were collected and the 2006 distribution of this species, indicated by the contour lines.

5.3. Results

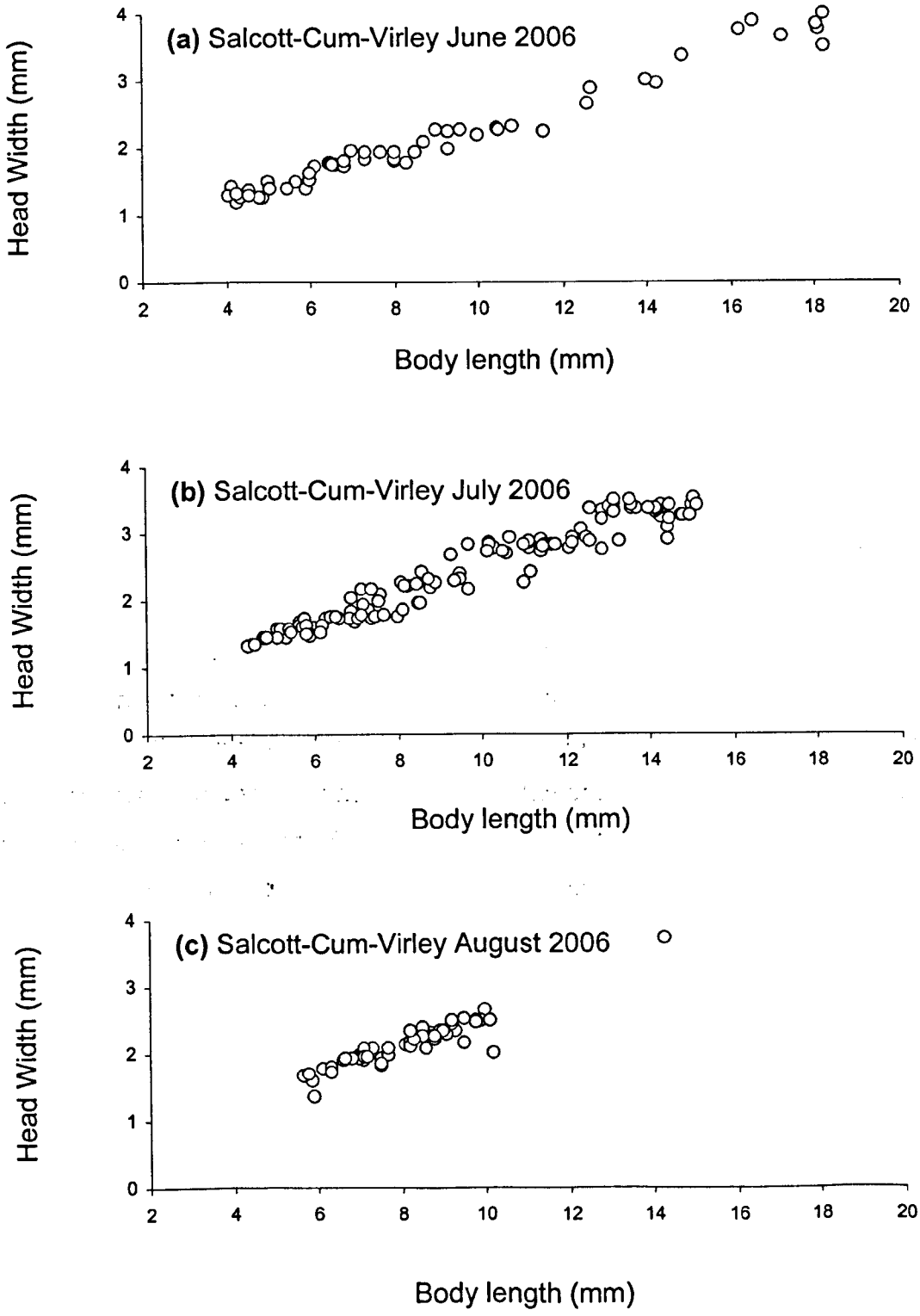
The raw data in the form of plots of body length against head width for the three main study sites, Salcott-Cum-Virley, Priory Country Park and Maldon, together with a mini-plot showing two individuals collected from Little Grays in Kent, are shown in Figures 5.3-5.6. Figures 5.3a-c show the data from Salcott-cum-Virley for the three sampling visits in June, July and August 2006 respectively; Figures 5.4a-c show the equivalent data for Priory Country Park and Figures 5.5a and 5.5b shows the data for Maldon in August 2005, together with a June sample from 2006. Figure 5.6 shows head width and body length for two individuals collected from Little Grays in May 2006.

The first point to note is that small larvae are absent from the sample for the most part (see Discussion). The most obvious feature to emerge from these data is that there were two generations present at the Salcott-cum-Virley and Priory Country Park sites in 2006. Figure 5.3a, the June sample from Salcott-cum-Virley, reveals five or six 'instar clouds' with the majority of larvae being smaller than the penultimate instar (usually designated as F-1, with F-0 designated as the final instar). By July, Figure 5.3b, some of the strong cohort of pre F-1 larvae has reached the last two instars, while those in F-0 and F-1 in the June sample have emerged. By the time of the August sample, Figure 1c, there is one individual in the final instar and the remaining larvae are in F-2 and smaller. Clearly these larvae would not emerge in 2006 and would overwinter during 2006/7 to emerge in 2007.

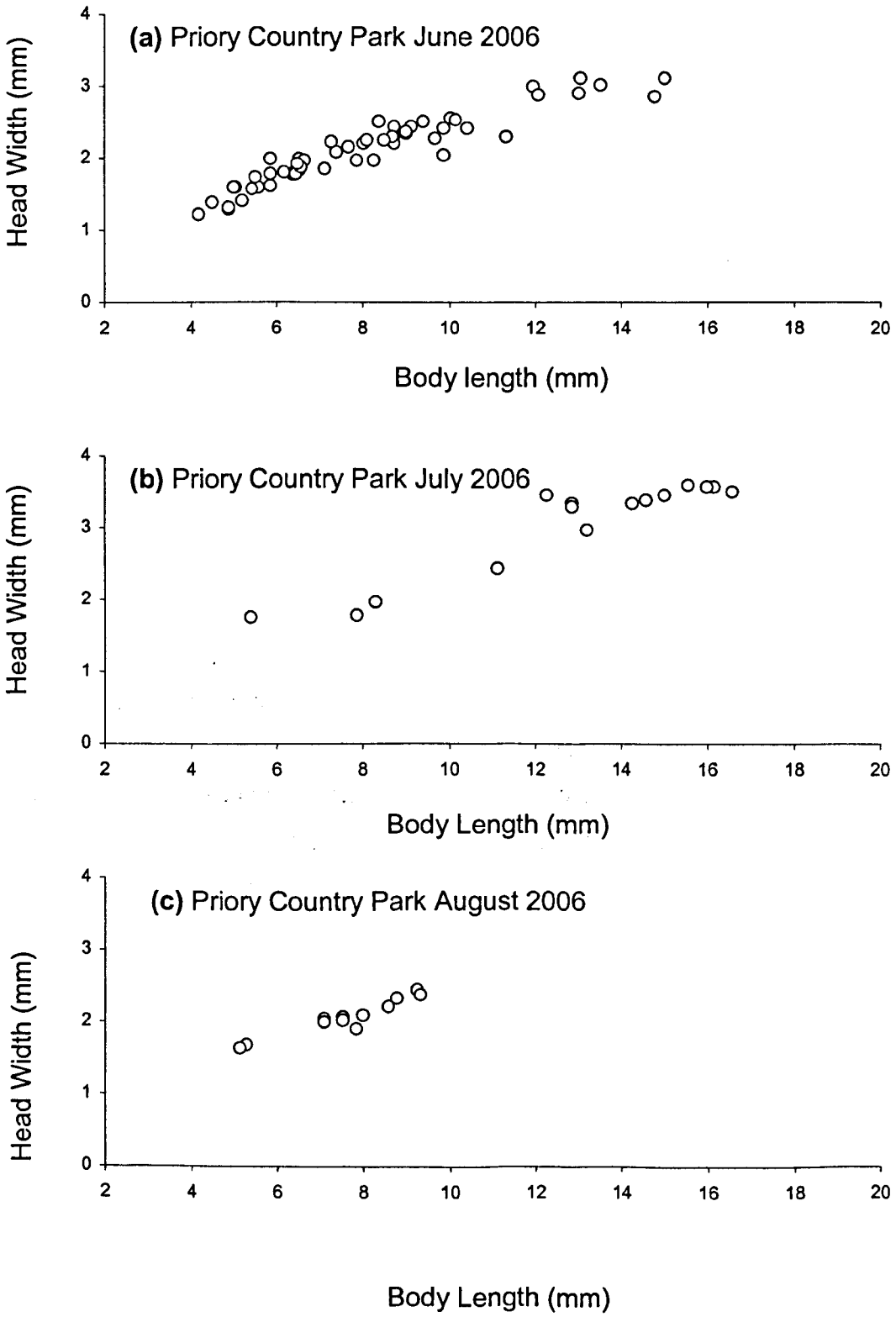
The pattern is slightly different in the Priory Country Park samples. In the June sample, Figure 5.4a, the largest larvae are in instar F-1, but there is a wide range of instars present. By July, in a rather small sample (n=15), most individuals (n=10) have reached in the final instar (F-0) while there are single individuals in F-1 and F-2. Most of the June cohort would appear to have emerged by July. In August the picture at Priory Country Park resembles that at Salcott-cum-Virley with only larvae destined to overwinter present in the sample.

The situation at Maldon is confounded by the samples being collected in different years. In late August 2005, there were final instar larvae present (unlike Priory Country Park and Salcott-cum-Virley (except for 1 individual) in 2006). However, most larvae would have over-wintered. The June sample contains a wide range of instars and includes larvae that would probably have emerged in 2006 and just coming in to the sample, some that probably would not have emerged. There is little robust information to be gained from the two individuals collected at Little Grays in Kent on 21 May 2006 other than to note that they were probably from two different cohorts.

A third feature of the data is that the sizes of individuals in particular instars differ depending upon which cohort they are in. This is best illustrated by looking in more detail at the most extensive data, the Salcott-cum-Virley samples. Figures 5.7a-c show the distribution of head widths of the June, July and August samples from Salcott-cum-Virley. In June and July the groups with the largest head widths would each have emerged in 2006. This was clear from the length of their wing buds (S. Keat, pers. obs.). However the mean head widths of these two groups differed significantly (June 3.71±0.20, July 3.35±0.08 SD; t = 4.96, P = 0.002). The June sample presumably involved a cohort whose eggs hatched in 2004 whereas the July sample involved a cohort whose eggs hatched in 2005. At Priory Country Park (Figures 5.8a-c), there appears to be just one cohort emerging in 2006, whose eggs were laid in 2005. The mean size of the final instar at Priory Country Park in July 2006 does not differ significantly from that of the final instar of the Salcott-cum-Virley sample of July 2006 (Priory Country Park 3.44±0.11, Salcott-cum-Virley 3.35±0.08 SD; t = 2.18, $P > 0.05$). At Maldon the situation is less clear because sample sizes are smaller and because the two samples were collected in different years. The final instar larvae present in the August sample were intermediate in size between the Salcott-cum-Virley June and July samples and were not significantly different from the larger (June) sample (3.61±0.03 SD; $P > 0.05$).



Figures 5.3a-c. Relationship between head width and body length (mm) for larvae of *Erythromma viridulum* collected from Salcott-cum-Virley, Essex in 2006 on (a) 6 June, (b) 5 July and (c) 9 August.



Figures 5.4a-c. Relationship between head width and body length (mm) for larvae of *Erythromma viridulum* collected from Priory Country Park, Bedfordshire in 2006 on (a) 6 June, (b) 5 July and (c) 9 August.

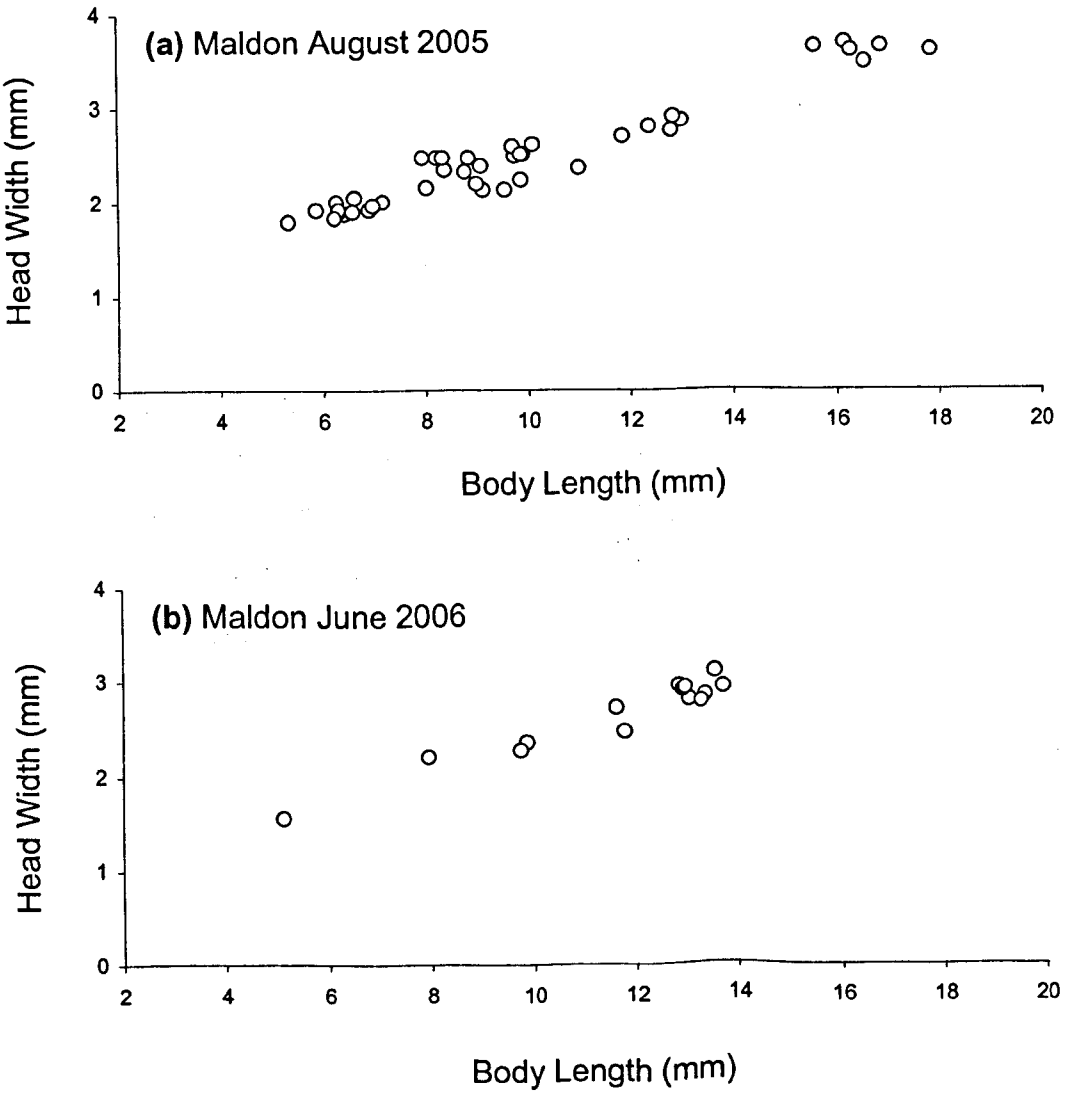


Figure 5.5. The relationship between head width and body length (mm) for larvae of *Erythromma viridulum* collected from Maldon, Essex on (a) 20 August 2005 and (b) 6 June, 2006.

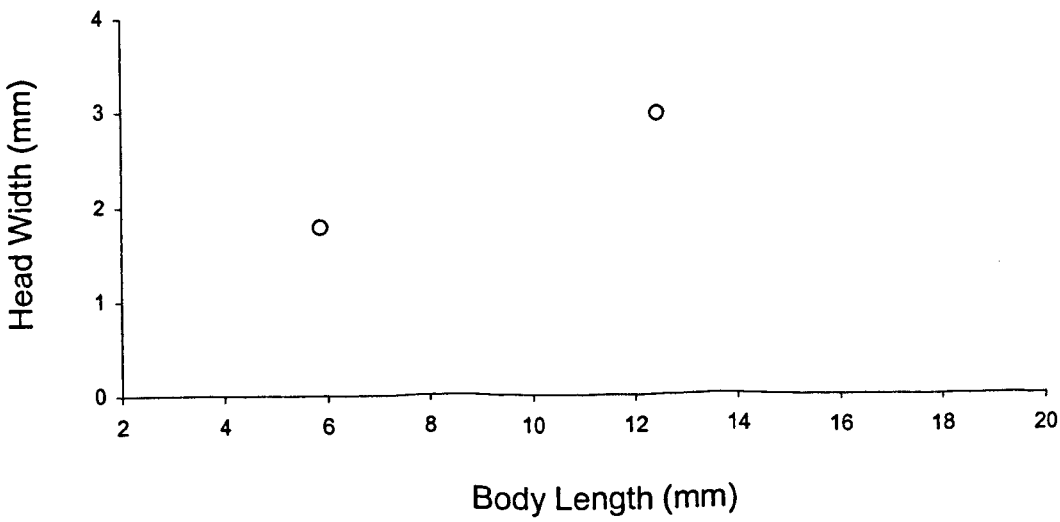
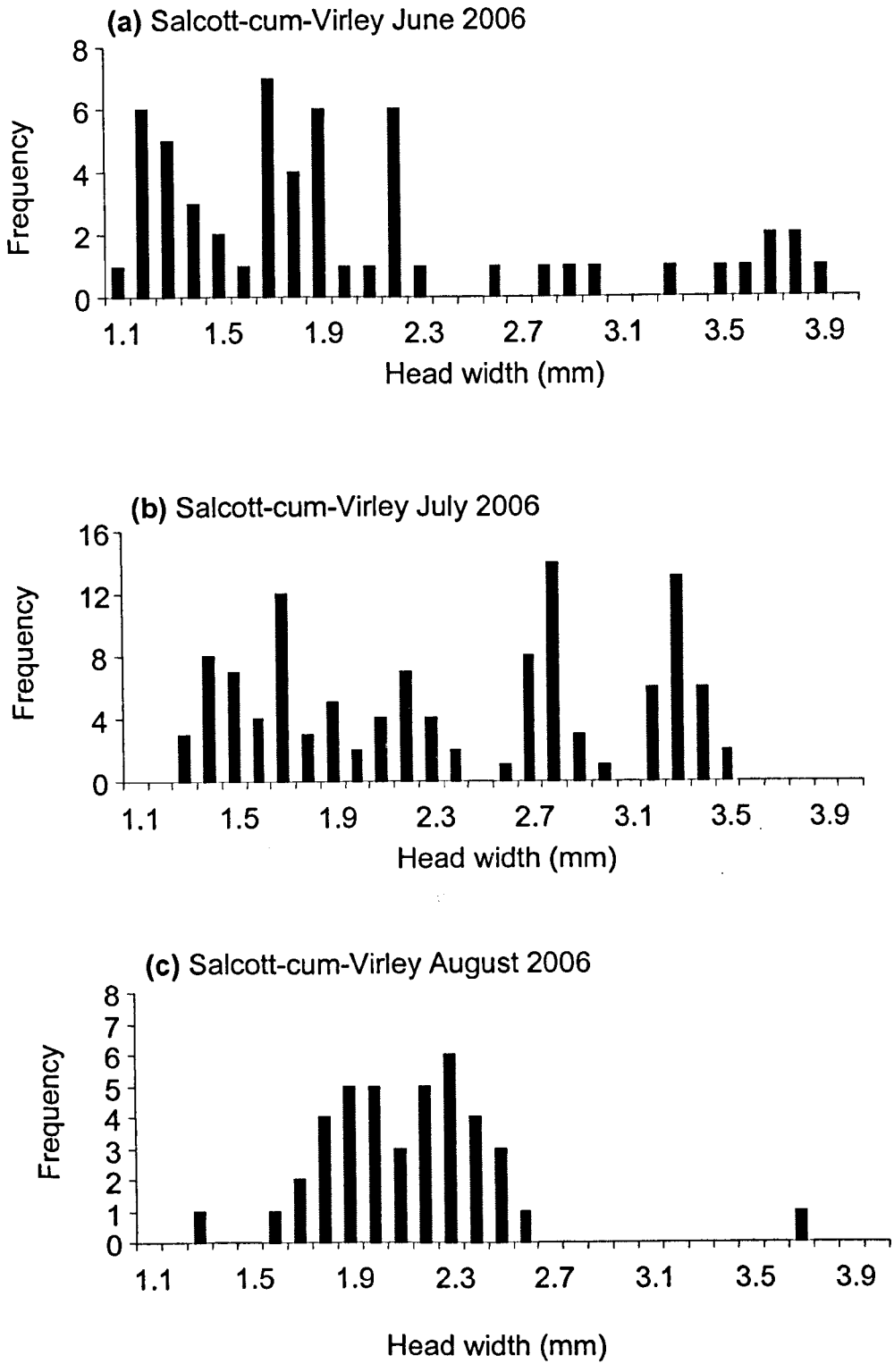
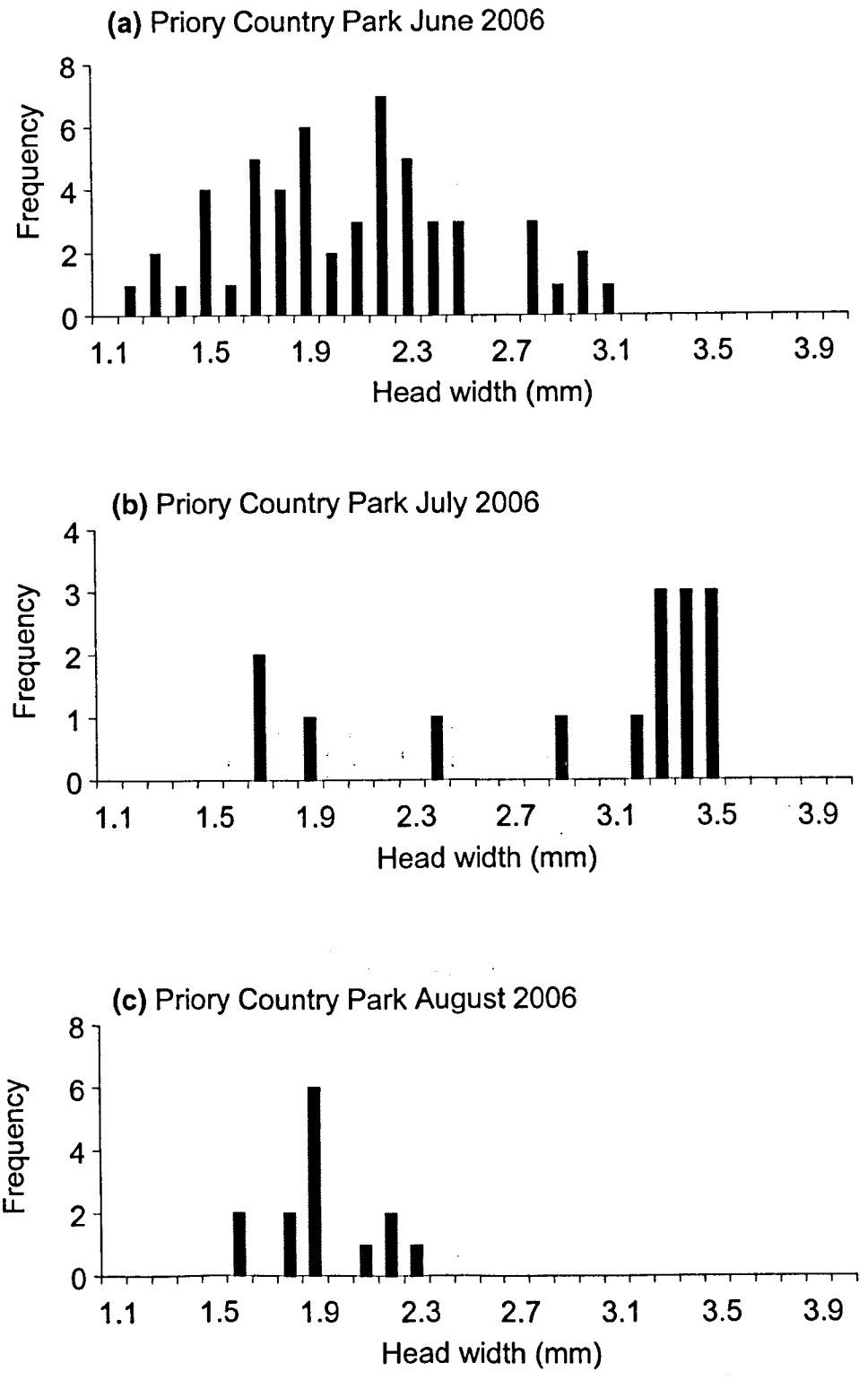


Figure 5.6. The relationship between head width and body (mm) length for larvae of *Erythromma viridulum* collected from Little Grays, Kent on 21 May 2006.



Figures 5.7a-c. The distribution of head widths in larval samples of *Erythromma viridulum* from Salcott-cum-Virley in (a) June, (b) July and (c) August, 2006.



Figures 5.8a-c. The distribution of head widths in larval samples of *Erythromma viridulum* from Priory Country Park in a) June, b) July and c) August 2006.

5.4. Discussion

Small larvae are missing from the data for a number of reasons. The most significant reason is that sampling was undertaken with a 1 mm mesh net so that larvae with head widths less than 1 mm would not be expected to be well-represented in the samples. Second, larvae were sorted in the field and small larvae are more difficult to find in trays containing vegetation and sediment. They are more difficult for the obvious reason that they are small, but also because they are highly thigmotactic, and are thus reluctant to leave small particles of sediment or pieces of vegetation to which they are attached. Some small larvae do remain in the net following sampling (attached to vegetation) but the cost-benefit balance of trying to find them is not favourable and in any case small zygopteran larvae are notoriously difficult to identify. All published larval keys concentrate on final instar larvae (including, for example, Gardner's key, reproduced with some modifications in Hammond [1983]) and some of the main distinguishing features are not well developed in earlier instars.

Although it was only possible to sample three sites it is clear that the larval growth and development of *Erythromma viridulum* has not yet developed in to a consistent pattern in the UK. At Priory Country Park near Bedford, a site at which *E. viridulum* has been recorded since 2001, the population produced one peak of emergence with a second cohort following behind that was unlikely to emerge in 2006. In other words the population at this site was entirely semi-voltine in 2006. At Salcott-cum-Virley, a site from which *E. viridulum* has also been recorded since 2001, it is likely that two different cohorts emerged, indicating that the population was composed of a predominantly semivoltine cohort, the first one to emerge, and a univoltine cohort. There was strong evidence that the cohort of larvae still present in the August sample was another semi-voltine cohort. It is highly unlikely that most of it would have emerged in 2006. At Maldon the population appears (albeit from much reduced samples) to be semivoltine.

The voltinism trends for *E. viridulum* are not at present well defined because this species is clearly still expanding and receiving migrants from continental

Europe. Those individuals that reproduce at their natal site, where there is high environmental quality may be tending towards univoltinism, but the majority, especially those that disperse long distances, later in the season, may be semi-voltine. In situations where there are natal populations and dispersers, there may be a mixture of univoltinism and semivoltinism. This is not surprising given that immigration at coastal sites has been recorded late in the season (Parr, 2004, 2005), leading to semi-voltinism initially. *E. viridulum* is thought to be univoltine on the continent and is likely to follow the same trend as some other coenagrionids in the UK, by tending towards semi-voltinism in the most northerly parts of their ranges (*e.g. Coenagrion puella*, shows a mixture of univoltine and semi-voltine life cycles around Merseyside, depending on pond quality (Banks & Thompson, 1987), but is entirely semi-voltine at higher latitudes in Scotland (D.J. Thompson, pers. comm.). Certainly almost any combination of life cycle patterns is possible once *E. viridulum* populations become more stable and widespread in the British Isles.

Though this study indicated a predominantly semi-voltinism life cycle for *E. viridulum* in the British Isles, the pattern of flight period is not what might be expected if this were the case. Prior to emergence, semi-voltine species typically overwinter in late instars and show synchronous emergence in the beginning of the summer, unlike *E. viridulum*, which predominantly emerges late in the summer (*cf.* Figure 1.10)). Also, if there was a mixture of univoltine and semivoltine individuals, two peaks of adult emergence might be expected. Current data does not suggest that this is the case (Figure 1.10), though further site specific information on emergence time would be required to make firmer conclusions. It is tempting to hypothesise that being a thermophilic, holomediterranean species, *E. viridulum* might respond to ambient temperature as well as, or instead of, using photoperiod as a cue for emergence, termed "unregulated development" by Corbet (1999). There is anecdotal support for this idea in that final instar individuals were present at Salcott-Cum-Virley in June, though no adults were observed (pers.obs.), though it is possible that these final instar larvae were on the verge of emergence.

It is interesting to note that the semivoltine cohort at Salcott-cum-Virley were larger in the final instar than the apparently univoltine cohort. It is possible that there is variation in life history strategy here and animals that miss the emergence window of univoltinism interpolate an extra instar in their second spring making them larger at the time of emergence. If this is the case, it is likely that larger individuals in the UK are those that show the greatest dispersal tendency, as was found in the Netherlands (Wasscher, 1987).

Latitudinal variation in voltinism has been documented in many invasive and range expanding insects (Glover *et al.*, 1991; Ozaki & Ohbayashi, 2001; Bryant *et al.*, 2002; Musolin, 2007). Life history plasticity is often cited as an important factor in the success of an invasive species (Sakai *et al.*, 2001). It is therefore of some interest to consider to what degree the changes in voltinism might have facilitated the recent range expansion of *E. viridulum* into higher latitudes in northern Europe. The questions raised could be best addressed with a study of voltinism in *E. viridulum*'s recently colonised range in northern Europe, compared with the historical core range in central Europe, where it assumed to be univoltine. Indeed before the cause of range expansion of *E. viridulum* is assigned to climate change the effect of life cycle changes should also be considered.

In conclusion it was found that *E. viridulum* is predominantly semivoltine in the British Isles, though under favourable conditions may a complete a generation within one year. As the British population has only recently become established and is still receiving immigration from continental Europe, the patterns of voltinism are not yet well defined. As such it is difficult to make firm conclusions, though the current mixture of life cycles implies that a gradient of voltinism may eventually form within the British Isles dependent on latitude and environmental quality. This study also emphasises the need to consider life history changes when making predictions about distributional changes in response to global climate change.

Chapter 6

Discussion and conclusions

6.1. Introduction

Non-native species can alter ecosystem processes, act as vectors of diseases, and diminish biological diversity (Vitousek *et al.*, 1996; de Wit *et al.*, 2001; Walker, 2006), as well as having significant economic impacts (Pimentel *et al.*, 2000). The threat posed by non-native species is expected to increase as a result of global climate change. Temperatures in the UK and Europe are predicted to rise by 3 to 5°C in the next 50 years (Houghton *et al.*, 2001; Schiermeier, 2004; IPCC, 2007), which will bring about rapid distributional changes in highly mobile species. Climate change has been linked to poleward shifts in range across wide a range of taxa (Allen & Breshears, 1998; Parmesan, 1999; Thomas & Lennon, 1999; Hickling *et al.*, 2006; Mieszkowska *et al.*, 2006; Parmesan, 2006). Global temperature rises are also predicted increase the threat posed by non-native species as they capitalise on the resultant ecological perturbations (Dukes & Mooney, 1999; Walther *et al.*, 2002). Though the processes of human mediated introductions and range expansions may differ in mechanism, similar themes pervade both and so literature from both areas can be considered relevant.

Odonates are thought to be warm adapted species with tropical origins (Corbet, 1999) and are therefore likely to be highly sensitive to environmental temperature changes in temperate latitudes. There is increasing evidence that odonate range margins are shifting northwards, apparently in response to climate change (Ott, 1996; Aoki 1997; Hickling *et al.*, 2005). There has been an increase in the abundance of non-breeding migratory odonates in the British Isles that cannot be wholly attributed to increased recording effort (Brooks, 2001). Non-migratory British odonates have shifted their range margin northward over the last 40 years (Hickling *et al.*, 2005). Northward

range expansion has meant that successful colonisation of the British Isles by formerly continental European odonates has been documented for two species in the last 8 years; the southern emerald damselfly *Lestes barbarus* (Fabricius, 1798) (Nobes, 2003) and the small red-eyed damselfly *Erythromma viridulum* (Charpentier, 1840) (Cham, 2002).

6.2. Rate and range of expansion

E. viridulum has expanded its range in a north westerly direction at remarkable rate over the last 30 years (Ketelaar, 2002). Over this time it has colonised an area from northern France to Denmark, utilising the Rhine valley as an arterial colonisation route. In 1999 *E. viridulum* became the first recorded example of a migrant damselfly to colonise the British Isles (Dewick & Gerussi, 2000). Since then it has expanded its range in the UK by and average of 31.68km per year (source: British Dragonfly Recording Network, courtesy G. French).

The westerly expansion from the Isle of Wight population occurred at a much slower rate than the expansion on the mainland British Isles, taking at least 4 years to spread into nearby Hampshire. This may have been because of lower population densities on the Isle of Wight leading to fewer migrants, which is supported by estimates of migration rate (*m*) several orders of magnitude lower than those for the mainland UK populations (see Table 4.4). Also because the Isle of Wight is an island, the 3 km barrier of sea between it and the mainland might have restricted expansion. In the 3 years since its arrival in Hampshire *E. viridulum* has made little recorded expansion into the surrounding areas.

The current range of *E. viridulum* in the UK extends as far north as Hull in East Yorkshire and it seems probably that the range expansion will continue further northwards still. The northernmost limit at which *E. viridulum* has been recorded in continental Europe is scattered colonies from the northern tip of Denmark (source: Birds and Wildlife in Denmark website, found at http://www.fugleognatur.dk/english/) at a latitude roughly equal to Aberdeen in Scotland. Whether *E. viridulum* will successfully breed and switch to a

three year life cycle at these northern range limits remains to be seen. It seems probable that all suitable sites in southern England will over time become occupied by *E. viridulum*, however areas connected by water courses will become colonised first followed by the surrounding areas. Barriers of unsuitable habitat, such as mountainous regions, are likely to inhibit range expansion initially, but regular long distance dispersal events mean they are likely to be overcome with time.

6.3. Genetic diversity

The rapid range expansion and the colonisation of an island such as the UK by *E. viridulum* would be predicted to have profound impacts on its population genetic structure. Populations at northern range limits have often expanded into large areas of suitable territory. This leading edge expansion would probably be by long-distance dispersers that set up colonies and rapidly expand to fill the area before others arrive. This would be repeated many times over a long colonizing route, and these founding events would lead to loss of alleles and homozygosity (Hewitt, 1993, 1999; Slatkin, 1993). This prediction was confirmed by the low levels of genetic diversity found in all UK and European populations of *E. viridulum*. To fully confirm that this was the case, samples would be needed from the centre of the range of *E. viridulum* in central and southern Europe. The southern UK population, including the Isle of Wight and Hampshire, was shown to have lower levels of genetic diversity than both the eastern UK population and continental European populations (Table 3.2). This was thought to be the result of a smaller founding population and fewer immigration events.

A striking result of this investigation was the low levels of diversity in *E. viridulum* microsatellites compared with those described for other odonates (see Chapter 2). Microsatellites isolated from *E. viridulum* were found to have short stretches of uninterrupted repeat units and low levels of expected heterozygosity (H_e) compared with all six odonate species for which reasonable numbers of loci have been characterised (Figures 2.5a, b). These differences were interpreted as real rather than an artefact of sampling effort and are thought to represent a signature of recent rapid range

expansion. *E. viridulum* also showed a slight but clear trend of reduced genetic diversity from east to west following its invasion path from continental Europe into the UK (Figures 3.3a-c). *E. viridulum* does not seem to have suffered any deleterious effects of low genetic diversity and maintains healthy, expanding breeding populations in the UK.

6.4. Population genetic structure

Rapid range expansion is expected to produce genetically homogenous populations because factors that create genetic structure, such as genetic drift and local adaptation are counteracted by high migration rates and recent founding of populations (Slatkin, 1993). Results from Chapter 3 showed overall levels of genetic differentiation from the UK and Europe were moderate or low given the distances between sample sites (F_{ST} = 0.038). No significant pattern of genetic isolation by distance within more local areas (*i.e.* populations from (1) the Isle of Wight and Hampshire, (2) East Anglia or continental Europe) was detected. This lack of genetic structure concurs with Slatkin's (1993) model where the time since a population radiates, or in this case is founded, is proportional to the degree of isolation by distance structure that will be observed. The other factor contributing to the observed genetic homogeneity was high gene flow brought about by high rates of dispersal and multiple waves of immigration, described in Chapter 1.3.3.

An apparent pattern of genetic isolation by distance was detected over a broader spatial scale when the whole of the UK was analysed (see Chapter 3.3.4). This effect was generated by differences between the Isle of Wight and Hampshire population and the eastern UK population. After its arrival in 1999, the main colonisation by *E. viridulum* occurred in north Essex and headed in a north westerly direction running along the Thames valley. Other significant invasions were documented along the south coast between Kent and Norfolk including a colonisation of the Isle of Wight that is currently still isolated from eastern UK populations. The Isle of Wight population likely gave rise to a population in nearby Hampshire in 2004, which was confirmed by analyses in STRUCTURE which grouped the Hampshire and Isle of Wight populations together (see Table 3.7 and Figure 3.10). The genetic

differentiation of the Isle of Wight is indicative of a different source population to that of eastern UK, or the result of an extreme founder event, though no significant evidence for a bottleneck was found. Figure 6.1 shows possible French source regions for the Isle of Wight and Hampshire population outside of the range sampled. Possible sources could have come from the coast of Brittany and Lower Normandy, though population densities in this area are relatively low (DJ Thompson, pers. com.). There is some support for the idea that genetic differentiation of the Isle of Wight and Hampshire population may have been the result of an extreme founder event because of the differences in allelic frequencies. The allelic frequencies from Isle of Wight and Hampshire population were mostly reductions in diversity compared with samples from other areas (S. Keat unpublished data). Further work using mtDNA markers might help to resolve the source of the Isle of Wight population, though sequencing this in *E. viridulum* has so far proved difficult.

6.5. Life cycle

It was revealed that *E. viridulum* is predominantly semivoltine in the UK, though some individuals may complete development in one year under favourable conditions (see section 5.3). A strictly semivoltine life cycle might be expected to result in reproductive isolation between cohorts causing genetic differentiation through the action of genetic drift. Overall there were apparently few major differences to the spatial pattern of genetic structure between the different cohorts, both at an individual- (*cf.* Figures 4.3a and b) and population level (*cf.* Figures 4.4a, b and 4.5a, b). Nonetheless, the pattern of differentiation between pairs of samples is not identical across cohorts (Figure 4.6), indicating there were subtle genetic differences between some of the cohorts. Although this could have been the signature of temporal genetic differentiation, it was more likely to be a reflection of discontinuities in the invasion process caused by different waves of immigration moving across the UK. Also, if cohorts split and only a few individuals completed a generation within one year, as was suggested by Chapter 5, this would mitigate the effects of reproductive isolation between cohorts (Spieth, 1974; Kimura & Ohta, 1971; Lewontin, 1974).

Extended developmental time is a common tactic amongst odonates allowing them to extend their range further north where the summer growth period is shorter, resulting in a gradient of voltinism with latitude (Parr, 1970; Naraoka, 1976; Norling, 1984; Cordero-Rivera, 1988; Johansson, 2003). It is possible that a switch to a semivoltine life cycle has been important in facilitating the range expansion of *E. viridulum* across Europe. If this were the case with *E. viridulum* it would be expected that it exhibits an at least partially semivoltine life cycle in the parts of northern Europe where it has expanded it range in recent years. No data exist on voltinism in northern Europe for this species so quantifying this is a priority for understanding the factors that have stimulated its range expansion.

The apparent paradox still exists that *E. viridulum* has been found to be semivoltine in the UK but its phenology is typical of a univoltine "summer species" (Corbet, 1999). In Chapter 5 it was hypothesised that this may be because *E. viridulum* is a thermophillic species (Sternberg & Buchwald, 1999) and responds to temperature as well as, or instead of, photoperiod as a cue for emergence. I have successfully maintained *E. viridulum* larvae in the laboratory to the emergence stage, so an experimental manipulation of temperature and photoperiod would seem the best way to answer this question. Individuals were kept individually in mesh cages within a large tray, to avoid cannibalism of smaller instars. Water was changed regularly using RO water from the laboratory and larvae were fed *Daphnia pulex*.

6.6. Migratory behaviour

As well as changes in life cycle, it is possible that the recent expansion of *E. viridulum* has been mediated by behavioural changes, in particular those that result in long-distance dispersal. The most important of these is the tendency to disperse when population densities become high, thereby increasing the likelihood of successful colonisation (Corbet, 1999). There is some evidence that these migration events are taking place at night (Jones, 2004), potentially utilising low level winds to cover large distances. These behaviours have been critical in the rate of range expansion shown by *E.*

viridulum and its ability to cross the English Channel to reach the UK.

6.7. Expansion in 2006-2007

The range expansion that occurred in 2006 was particularly notable because of the large numbers of new colonies that were discovered some distance from the core population (see Figure 1.8). Unusually in this year the expansion appeared to spread predominantly in a south-westerly, rather than north-westerly direction. It might have been that after spreading the length of the Thames valley, *E. viridulum* was forced to expand in other directions by means of long-distance, rather than stepping stone dispersal along water courses. Long-distance migration oriented by water courses, rather than wind borne dispersal has been documented in other species such as *Libellula quadrimaculata*, *Lindenia tetraphylla* and *Selysiothemis nigra* (Corbet, 1999). The new colonies in 2006 on the coast at Lower Bruckland, Devon, Weymouth, Somerset and Hull, East Yorkshire seem most likely to be the result of fresh immigration from the continent. If this is the case, rather than the result of expansion within the UK, these sites are the result of the longest migration over water yet recorded for *E. viridulum*. Figure 6.1 shows that the coast of Lower Normandy is the closest to the Isle of Wight, Somerset and Devon populations and is still over 100km away.

As records for 2007 of the distribution of *E. viridulum* are incomplete, latest "Hot News" from the British Dragonfly Society website is reported. Most notably *E. viridulum* was again reported from the new sites from 2006 in Lower Bruckland, Devon and Hull, East Yorkshire. Daily counts at these sites were higher than the previous year with 84 individuals at Hull and over 30 individuals at Lower Bruckland. This implies either fresh immigration, a univoltine life cycle or failure to record the presence of this species in previous years. *E. viridulum* was recorded at a new site in Somerset at Westhay Moor Nature Reserve, near Glastonbury. This site was closer to the expansion from central England toward Swindon and Bath that occurred in 2006 than the coastal sites in Somerset from that year. Other significant records from 2007 came from Strandford in the Vale, Oxfordshire and further records from Hampshire.

6.8. Community level effects

The community level effects of non-native species are well documented including causing reductions in biodiversity, altering ecosystem processes and reducing niche space of native species (Vitousek *et al.*, 1996; de Wit *et al.*, 2001; Woodward & Hildrew, 2001; Walker, 2006). The high densities of *E. viridulum* documented at some UK sites are likely to have important ecological impacts. Though this species has particular habitat preferences it seems to be relatively eurytolerant, though breeding success may be variable depending on environmental quality. Therefore the impact of *E. viridulum* is likely to be greatest at sites where environmental conditions are favourable for breeding. This is dependent on the presence of aquatic macrophytes such as *Ceratophyllum demersum* (Rigid Hornwort) and *Myriophyllum spicatum* (Spiked Water-milfoil) and in terms of water chemistry, *E. viridulum* is typically found at eutrophic ponds with high phosphate levels. Competition with congeneric species is likely to be most intense in the larval stage, where it has been shown that at high densities competition for food can occur (Corbet, 1999). The odonate species most likely to be affected by the arrival of *E. viridulum* are those that share a similar niche space, such as *E. najas*.

6.9. Other range expanding odonate species

The range expansion of *E. viridulum* raises the question of whether other odonate species will change their distributions in a similar manner. It is surprising that other species of non-breeding migratory odonates found in the British Isles have not established long term breeding populations, with the exception of *L. barbarus* (Nobes, 2003). *Sympetrum fonscolombii* is usually found in south-west England following strong prevailing winds which carry individuals from their breeding grounds in south-west Europe. There are currently several sites in England which have produced successive generations of the species (Brooks, 2001). Other species of migrant dragonfly that were reported as becoming more common included *Anax parthenope* (recorded in 1996 and 1997), *Sympetrum pedemontanum* (1995), *Crocothemis erythraea* (1995) and *Anax junius* (1998) (Brooks, 2001).

In Belgium three species were documented to have significantly increased their distributions in the period 1950-2000; *Aeshna mixta*, *Crocothemis erythraea* and *E. viridulum*. In common with *E. viridulum*, *C. erythraea* is a southern species that has expanded its range northwards in France, Germany, the Netherlands since the mid-1970s (Ott, 1996; De Kniff, 2001). This species was recorded for the first time in 1995 close to the southernmost tip of the British mainland (Jones, 1996). As such it will be interesting to see how far the expansion of *C. erythraea* will proceed. Other Mediterranean and tropical species that have expanded their ranges in recent decades include *Aeshna affinis*, *Hemianax ephippiger* and *Orthetrum brunneum* (Ott, 1996)

6.10. Conclusion

In the face of increasing global change the threat posed by range expansions and biological invasions is likely to increase (Dukes & Mooney, 1999; Walther *et al.*, 2002). Understanding the processes that underlie biological invasions is critical in making predictions about their future impacts. Greater understanding will help future management of biological invasions, including mitigating the effects of invasion and predicting potential invaders. Over the course of this thesis important insights into the causes and effects of the range expansion of *E. viridulum* in Europe and the UK have been revealed. Source populations from continental Europe have been identified and the population genetic structure within the British Isles has been quantified. The rapid rate of range expansion and recent arrival in the UK has been shown to have a dramatic effect on patterns of population genetic structure. The rapidity of the expansion of the British population has meant that genetic homogeneity has prevailed, with the exception of the differentiation between the southern and eastern UK populations. It has also demonstrated that low levels of genetic diversity have apparently not prevented the rapid expansion and success of this species. Links have been drawn between temporal changes in population genetic structure and the information gained about life history patterns in the UK. This study has also emphasised the importance of understanding the distributional history of a species before a population genetic study is carried out. Finally, this investigation has allowed predictions

to be made about the future of this species including suggestions about its ecological impact.

6.11. List of major conclusions

(1) The range expansion of *E, viridulum* into northern Europe has occurred a rapid rate over the last 30 years

(2) *E. viridulum* is relatively eurytolerant but has specific habitat requirements for breeding success, the most important of which is the presence of aquatic macrophytes at the water surface

(3) *E. viridulum* shows extremely low levels of genetic diversity for the microsatellite loci characterised compared with other odonates, which is thought to represent the signature of rapid range expansion

(4) There was a lack of genetic structure even at regional spatial scales, which were attributed to high levels of gene flow, brought about by high migration rate and multiple colonisation events by large numbers of individuals.

(5) There was differentiation between the southern and eastern UK populations ascribed to different putative source populations with eastern populations originating from within the range sampled in northern Europe

(6) There was generally little difference to the overall pattern of spatial genetic structuring between cohorts, although cohorts at some populations did genetically differ

(7) Estimates of N_e and m were considered low and likely to have been affected by the overall low levels of genetic differentiation between UK populations

(8) *E. viridulum* is predominantly semivoltine in the British Isles, though under favourable conditions may a complete a generation within one year

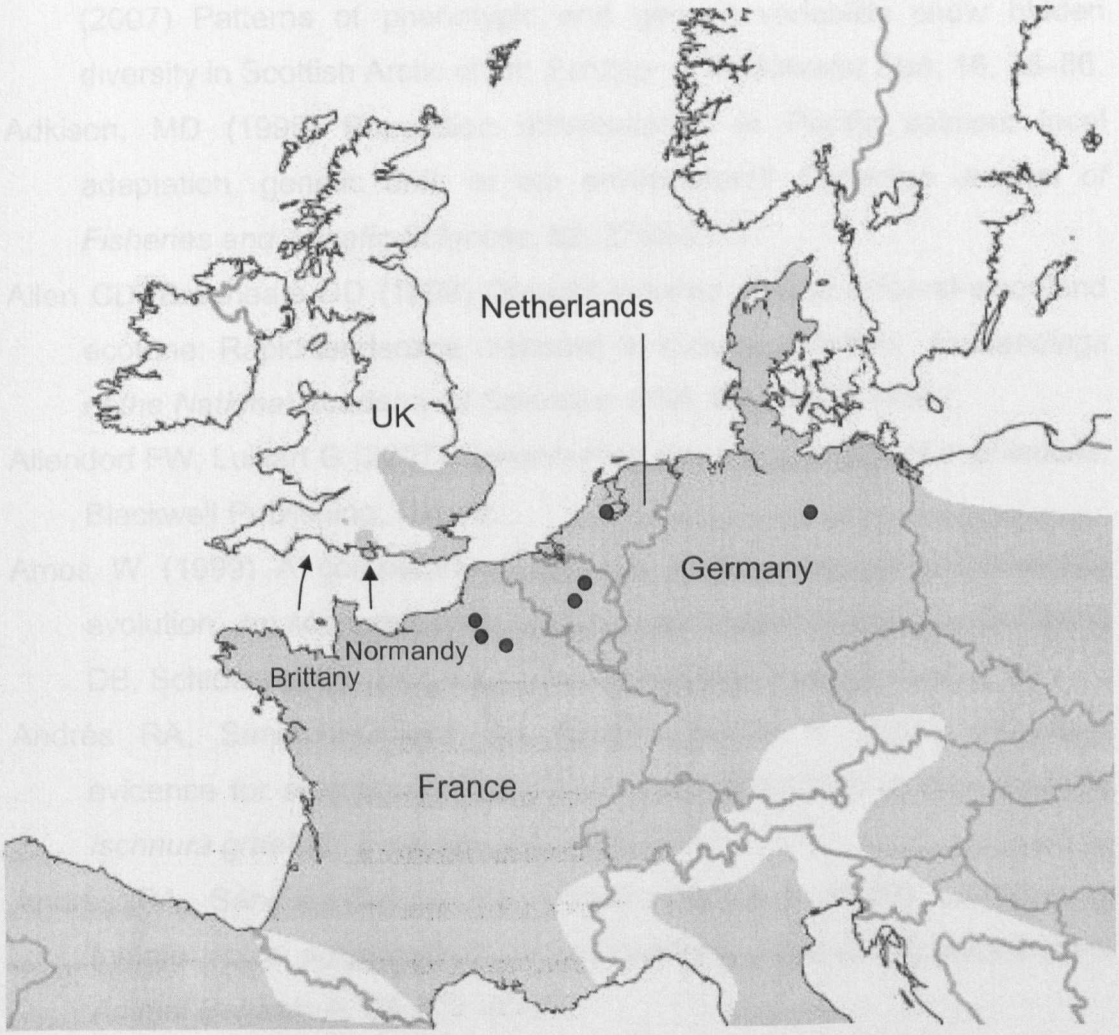


Figure 6.1 The distribution of *E. viridulum* showing sites where samples were collected from (black dots) and potential sources for the Isle of Wight and Hampshire population from Brittany and Lower Normandy outside the sampled range (redrawn from Askew, 1988)

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Appendix 2.1. Primer note describing ten polymorphic microsatellite loci isolated from the small red-eyed damselfly *Erythromma viridulum*.

Molecular Ecology Notes (2005) 5, 788–790 doi: 10.1111/j.1471-8286.2005.01063.x

PRIMER NOTE

Ten microsatellite loci for the small red-eyed damselfly *Erythromma viridulum* (Charpentier)

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Abstract

The small red-eyed damselfly, *Erythromma viridulum* (Charpentier), is the first recorded example of a migrant damselfly establishing colonies in the British Isles. To examine the population genetic structure of *E. viridulum*, a partial genomic library enriched for CA microsatellite loci was constructed. Of the 42 loci tested, 19 amplified spurious bands and 13 were monomorphic, leaving 10 polymorphic loci that resolved distinct alleles within the expected size range. The number of alleles ranged between two (LIST14-021, LIST14-40) and eight (LIST14-002). Observed and expected heterozygosities varied between 0.000–0.698 and 0.045–0.688, respectively.

Keywords: *Erythromma viridulum*, microsatellite, Odonata, range expansion, small red-eyed damselfly

Received 01 April 2005; revision accepted 28 April 2005

In recent years, there has been much debate on the effects that global temperature increase may have on species distributions. In particular, the northward range expansion of many European species has been associated with regional warming (Parmesan *et al.* 1999). The small red-eyed damselfly, *Erythromma viridulum* (Charpentier), has seen significant north-westerly range expansion in Europe in the last 30 years (Ketelaar 2002) and is the first recorded example of a migrant damselfly establishing colonies in the British Isles (Dewick & Gerussi 2000). Its range in the British Isles currently extends from Norfolk to the Isle of Wight and as far inland as Rugby in Warwickshire. *Erythromma viridulum* is now a common species in the core of its British range, in Essex, and looks set to further continue its spread across the British Isles (Cham 2004). Rapid range expansion is expected to produce populations with reduced genetic variability because the series of founder events that occur lead to loss of alleles and increased homozygosity. The reduction in allelic diversity and hetrozygosity is expected to be proportional to the severity of the population bottleneck (Hewitt 1999). As such *E. viridulum* represents a model with which to investigate the effects of rapid range expansion on genetic variability and to test the central–peripheral theory of population genetic differentiation.

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Genomic DNA was isolated from the body tissue of two adults for the construction of the microsatellite library and from hind tibia when testing for DNA polymorphisms; DNA was extracted using the high salt protocol of Sunnucks & Hales (1996), but with reduced volumes when leg samples were used. Microsatellite loci were isolated in *E. viridulum* using an enrichment technique described in detail by Bloor *et al.* (2001).

Microsatellite alleles were amplified by polymerase chain reaction (PCR) in a 10-μL reaction volume using ReddyMix PCR mix (ABgene) on a Dyad DNA Engine (MJ Research Inc). PCR conditions were 95 °C for 1 min, followed by 5 cycles at 95 °C for 30 s, T_a °C for 45 s and 72 °C for 45 s, then 25 cycles at 95 °C for 30 s, T_a °C for 30 s and 72 °C for 55 s and finally, 72 °C for 10 min, where T_a is the annealing temperature (Table 1). Each reaction contained 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, 0.2 mM each dNTP, 3.0 mM $MgCl_2$, 5–50 ng template DNA, 10 pmol each primer and 0.25 U *Taq* polymerase (ABgene). Forward primers were labelled with either 6-FAM, NED, PET or VIC fluorescent dyes (Applied Biosystems). PCR products were pooled with a 500 bp (LIZ) size standard (Applied Biosystems) and separated by capillary

Appendix 2.1 continued. Primer note describing ten polymorphic microsatellite loci isolated from the small red-eyed damselfly *Erythromma viridulum*.

Table 1 Levels of variability at 10 polymorphic microsatellite loci in adult small red-eyed damselflies (*Erythromma viridulum*) from Yarbridge, Isle of Wight ($n = 23$) and East Ruston, Norfolk ($n = 20$), both in UK. Dye, 5′ fluorescent label (Applied Biosystems); T_a, annealing temperature (°C); N_a, number of alleles observed; H_O, observed heterozygosity; H_E, expected heterozygosity

Locus GenBank Accession no.	Primer sequence (5′–3′)	Repeat motif	Dye	T_a	Size range (bp)	N_a	H_O H_E
LIST14-002	F: TTTCGTCTCAAGGACCGC	$(AC)_5(AC)_4(AC)_{10}$	NED	45	147–186	8	0.512*
BV448262	R: GAGAGTGGTTTTTGCTTCG						0.687
LIST14-005	F: CCATACCCATGGATAATAGC	$(AC)_8$	6-FAM	55	206–220	3	0.000*
BV448263	R: AAGCTTCGGATCATTTTCGG						0.126
LIST14-006	F: GGCTCAGTCTCACATTTCC	$(AC)_{10}$	6-FAM	55	297–303	5	0.488
BV448264	R: CAAAGGACCTATCCCAACG						0.605
LIST14-014	F: CTTAACCCACCTCACGACCACC	$(AC)_7$	PET	55	194–198	3	0.419
BV448265	R: TTCGCGCCCATTCTCACTCTCG						0.408
LIST14-019	F: ACGTCCTCATGCTGCATTCGC	$(GT)_{10}$	6-FAM	55	168–172	3	0.442
BV448266	R: CGACTCCTGGTTATGTCCTCC						0.424
LIST14-021	F: GGAGATAAGCGAGGATGAGG	$(GT)_2(GT)_5$	PET	48	196–200	2	0.000
BV448267	R: ACCCCACTTTTAGGAGGTCC						0.045
LIST14-025	F: ATCTCACCCCATCTTGTGC	$(TG)_5(GT)_3$	NED	48	246–256	6	0.419
BV448268	R: GTTCTCGGAAAACTGACCG						0.688
LIST14-035	F: GTGTTGTCTGCTGAATGGC	$(GT)_9$	VIC	55	333–343	5	0.698
BV448269	R: TACAGGGAAGAGGACTACC						0.662
LIST14-040	F: TATGCGACAAGTTAGCCG	$(GCT)_4(GCT)_2$	VIC	45	384–386	2	0.000
BV448270	R: ATCCCAAGGTTACAACGC						0.045
LIST14-042	F: CAGCGGTATCAAACTTCG	$(GT)_9$	VIC	50	223–230	3	0.163
BV448271	R: AAAAGGCGAGAAATCCCG						0.225

*indicates significant departure ($P < 0.05$) from expected (within site) Hardy–Weinberg equilibrium conditions

electrophoresis through a denaturing acrylamide gel on an ABI 3100 automated sequencer (Applied Biosystems).

Levels of genetic diversity were assessed from 43 adult damselflies collected from Yarbridge ($n = 23$) on the Isle of Wight and East Ruston in Norfolk ($n = 20$), both in UK. GENEPOP (Raymond & Rousset 1995) was used to calculate basic measures of genetic diversity (over all samples), the significance of any deviations from expected Hardy–Weinberg conditions (within each site) and also for linkage disequilibrium between all pairs of loci.

Of the 42 microsatellite sequences that we were able to design primers around, 19 loci amplified spurious bands and 13 loci were monomorphic, leaving 10 polymorphic loci that resolved distinct alleles within the expected size range. The number of alleles ranged between two (LIST14-021, LIST14-40) and eight (LIST14-002) (Table 1); observed and expected heterozygosities varied between 0.000 and 0.698 and 0.045–0.688, respectively (Table 1). LIST14-005 proved to be monomorphic in the Isle of Wight population but alternate alleles were found in the Norfolk population. Two loci (LIST14-002 and LIST14-005) showed significant ($P < 0.05$) deviations from expected Hardy–Weinberg conditions, although all heterozygote deficits were non-significant ($P > 0.05$) after correction for multiple testing (Rice 1989). Only two of the 45 locus comparisons showed significant ($P < 0.05$) linkage disequilibrium (LIST14-002 to LIST14-019, $P = 0.022$; LIST14-002 to LIST14-014, $P = 0.024$) but given the large number of tests involved, these loci are probably not significantly linked, although an analysis based on further samples is required to confirm this.

Acknowledgements

This work was funded by a NERC Studentship NER/S/A/2003/11297. We also would like to thank Steve Cham for his help in finding sites.

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Appendix 2.2. Raw sequence data for microsatellite-containing regions of the genome of *E. viridulum* for which primers could be designed. Primer binding sites are underlined and microsatellite motifs are highlighted bold. Values of N within the data indicate sequencing was ambiguous at that base.

LIST14-001
TTAGTGGCACACACTGCGTGAAAAA**ACACACACACACACAC**AAAAACTATTGCAAAAAATGTTAAGCA
AAAAAACTGTCAGTTGTAAGATTTGCTGTATCCTCAAAGAGC

LIST14-002
TTTCGTCTCAAGGACCGCTCAGACTCCTAATGTTGTCCTCAGACGCTAANTCACTTCCNTACTCCCAC
CACTTCAAGTCTCCCCACCCCCCAAAGTCGCACAATA**ACACACACACACCAACACACAC**GAGCTAGCACA
CTCGCCGA**ACACACACACACACACACACAC**AGACGAAGCAAAAACCACTCTC

LIST14-003
TCTCCCACATCGCTTTCCTCCCCAATTCTTTCATTCCTCCCCAATTCTTTCATTCCTCCTCCTTCCTC
ACTCCCTCTCTGTCCTCTCCCACATCTCAAAGGCATCCTTCCCAACCTTCTCCCTCAATAATCAACGT
CACC**CACAACACAACACAC**ACACTACTCCACACACAACATTTCTCCAGCCTGTTCCTCTGCCCTAAATC
CGCACTTATGCGTGTNACATTTCTCCTCTAGTTAAAAGCTGTCCCATAGCGCATTGGGATAGCGTAC

LIST14-004
TCTTACAGAGCTTTCCAGCTTATTTATCTCTTGAGTGAAATTATTTTTATTCA**GTTGTTGTTGTTGTT**
GCTTCAATTTGCAATTCATTCAGAAAATAAGAGTTTATTGTTGGAACCGAGTATCAAGAAC

LIST14-005
CCATACCCATGGATAATAGCAAAAACTATCTGCACTTATAGTCATTTGACACAGATATTGAATTTCCA
ACTCTAAATGGACCAATCAATTTTTCCTTATATTTCAGTTTCGAAAGTTTGGCCTCCNCACTT**ACACA**
CACACACACACAAGTACTGAAAAGGGAAAAATAATTATACTACTAGCCTCATCACTTAAGTATCCCGA
AAATGATCCGAAGCTT

LIST14-006
GGCTCAGTCTCACATTTCCATGGGCCGGGGATAATGAGCGTGCCGGATGGTCCAGTGTGGGAAGGGTG
CGTGTATACATATTATTTTATAACCATGAAAAGGGTTCTCTCTGCAGGAATATTT**ACACACACACACA**
CACACACGTGCATATAAAAAGGAGTAGGGGTGCAGTCGTATCCCGGGGGTTGGGTAATAAAATAAAAT
GAAGGGTAAAAGATGTTTATGGGATGAGATAAAAAAAACGTAATGAGGGAGGAAATATTGGAGGGCCA
TGAGAAACTCGTTGGGATAGGTCCTTTG

LIST14-007
AAAACAGGCAAGCAATCTGGTG**GTGTGTGTGTGTGTGTGTGT**GAGGTGCGGGTGGCTTGGTGACGGTT
TGGGTGGGTGGGGTTTTAAAAAGGGTTCCACCCTCCCCGGCCCTTCAGCTCCCCTCTCATTGGTTCAT
CTCCGTAGGCAAAGGGGTTGAAAAGGGGTTGTTTTTTTAGGCTGTGGGAGGGGTCCTGACTGGCTACT
GGGAAAGGGGTTGGTTCCGCGAGGGTACAGCAACAAAAAAAAACGAGAGAAAAGAAGGGGGAGATGGT
GTTTGGGAA

LIST14-008
CGAGTGATATTTCGATGTTAGGTTTAGTGATTCATGATGTATGAATGCGAA**GTGTGGTGTGTGTGTT**A
TTCTTTCCTCCCCCCATTAGTCCCTTTCCCGATATGCCGTGACTCGCCGGAGGACGGAATTCCCCAAG
ACAGTGGCCTTCCCCCCCCCCCCAAAGTGAATAATGGCTCGCTTTATGCAGCGGCGAAGCGGTCAAAG
GTATGCAAGTGAGCTTCCCACCCCCCAAAATGGACCCCCTACATTCCCCTTTCCCCGTCCCCGCCAGT
GGAGGGGCTAACATTTAAATAGCCGCGACAACCCAGGTTTTGGGTTGGTTTTCTATTGTTCCCGATTG
ATGCCGGAAGTACGGACGAGAGAGGCAGTACGCGAAGTGGCAAGACAGAGGACGAGACTAGAAGGGCA
GAGGAGCTGGAGCTGGANAGTGTCGGGAAGGANAGGCTGAAAGGACTTAANAGATTGAACGGAAAAAA
GTCGTCGCCGCA

LIST14-009
GAGGTGGACAACTGACAACGAAACTATTATCCAACCACATACAA**ACACACACACAC**CGCGGGAGAAAA
AGGTGCGCACGGNAAAGAGGAGGCTAAGGTNGTGGGGATTCCCGAAGCAAGAATGGGAGGGAGAACGG
GGCCAAAAGGGGGATGACGGGGTGGAGCCCACCGGTTAGGCGGGCGAAGCGACGTAGTAGAA

LIST14-010
TGATTGATACATACAGCACCGCAGTTTG**TGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTG**
TGTGTGTGTGTGTGTGTGTGTGCAGTACACACATTAGAATTAGAAATNTAGAAATTGTANGAGGCATT
CACTACGACCTGCGGTAGTGTCAGACTTT

LIST14-011
ACACCTTTTGTATCAGCCGCCTGTGTCTCTGGGCTAATCGATCAACATTCACTTTATTCGATAGTTTC
AGGCTTCACTTTGTGAAAATCCATTCTAATAACTGAAAGATATGGTTTTTTTTCAACTATTTT**CACAC**
ACACACACACACACCGGTCGACTTGAATAAATAGTGTGAAAAAAGTACCATATCTTTCAGTTATTATT
TACTTTATTTTCTGCTTCGTATAGTTTCGTACCGGGAGGTACGAAAATATCCGTTGGGTTAAAGGCTG
GCGCCGTCCAGGACAGCCCTCTGTTCGCGGCAATT

LIST14-012A
GCCCTCTAATGCTTTTGCTGGA**GTGTGTGTGTT**CTTTGTGGCGATGGTTGGGTGCGAGTGGTGACTGA
T

LIST14-012B
AATAGGCGAGGACTTCCCGGGACTCATGAGCGACCAGGAGAGTGAGGACGAAG**AGGAGGAGGAGG**AAG
AGGAGGGTGAGTACTGCACGCCTTCCTTTTTGACTGCAGTGGCTGGAGTGATTGG

LIST14-013A
AATTTCATTTGCCGACCGAACGAACACAATCGCAATCTCACACACATACACACACGCAGGAGGGGAGA
GACGGAGACTTGAAAAACAAAAGCAAAACCGCGGGCGGACTCACACGTACACACACACACACACAAAA
CTTCTTTTTCCCGATTGGTTATTCGATCCGAAGCTTGGGGTCTCTGGCCAAT

LIST14-013B
AATTTCATTTGCCGACCGAACGAACACAATCGCAATCT**CACACACATACACACAC**GCAGGAGGGGAGA
GACGGAGACTTGAAAAACAA

LIST14-014A
CAAGCCCAATAGCAGTCGGCACCCTTCCACGCATTT**CCTTTTTTCTCTC**TTGCTCGCGCAATTCCTGCC
ATTTGAAGTGTACGGGATGAGGAGCGAAAGGGGAGGAGGAGTTTTCGGAGTGGACTAGTAGGGAAACT
TGAGGTGGGGAG
AAGCAAAACCGCGGGCGGACTCACACGTACACAC

LIST14-014B
CTTAACCCACCTCACGACCACCACATA**CACACACACACACA**TTCTAACCCTTTTCCTTCGCAGNACNC
TCGCGGCTAACAGGCAGCAGCGGAGATGGAGAGAGAGGAANTTGAATAAAACGGCCAGGAGTCCACCG
ATTCCCGCCGGCGAACACAATTTTCTGTGGCCTCCCGCGCGCGCGAGAGTGAGAATGGGCGCGAA

LIST14-015
CCGCTCACTGACTGACAAGCTGGGTCGGCTCCTTCCTTCACCGCTGATTG**GTCGTC**GTTG**GTCGTCGT**
CGGCCGTAGCAGAAAACTACAGGTAGAAGTCGCTAGTTCCTTACTTTCGCTTATATTATCCGGTGAGA
ATCGCCTTTGAAC

LIST14-016
CATCTGGACCTGCTCTGTCGAGCTTATTCTCCCGGCNATTTTTAGCC**TGTGTGTGTGT**CTTT**GTGTGT**
GTGTGTTGTGTGAGTGTACGAGTGATCAACNNNNCNNNNAAGNNNNTNNTCCTTGAATGGACGTTTTT
TCAATNNGTNCANTTCATNACCAACNTNTTNNTAAGACACCNNNAAAGTNNGTNNTGGCATAAGTTNC
AANAAATNNNNNATTCCGTTCTTCCGCGAATTTCTTA

LIST14-017A
ACTGTGTGCTGGTGTTCCCGCGAGCGGCGCTGGCGTCGCGTCACGGCAG**GAGAGAGAGAGAGAGAGAG**
TGAGTGTGT**GAGAGAGAGAGA**GC**GAGAGAAGAGA**AA**GAGAGAGAGAGGAGAGA**GGAATTTGTCGACGT
GGCAGCGGAATGCGCCGCCGCCATATTCCCTCGCCGTCTCCTTATCGGCGGTCACGTGACAGCGGCGG
AGACACAACACGACA

LIST14-017B
ATCCCCCAGACAGTGGAAGCCT**CAACAACAA**CAT**CAACAACAACAA**CAGCAGCACGGGAAGAGAGAGA
GGGAGGGGGGGAGAGAGAGAGCGGGNTTCGCCCCCAATCCGCGCAAGGGCGCCTCCTCNNCCCGTGCC
GCGGACCTTCTCCCGCCGTTTCCTTTCGCAATCGA

LIST14-018
AGCTGTGAGCATTTTACCTAGGGCTCAT**ACACACACACACACACACACA**CATAATTTACATTTCTCTT
GAACCTTTCTAGGGTTTCCGATCACTGGGCTCGCGCTTTAAATTCGGTGTCCATAGGGATCCGAAGCT
TTGGGGTCTCTGGCCAA

LIST14-019
ACGTCCTCATGCTGCATTCGCCAGCGCAACGGATTGGGGAAAAAACGCAGAAAAAACGTCGTGGGTTG
GCACGCCGAGCCGCGATTCGTTTT**TTTCTTGGCCGAGAGCCGACGAGGAGGACAGACGAGAAGTGTGT
GTGTGTGTAGTGT**ATGACAGGAGGACATAACCAGGAGTCG

LIST14-020
TGGATTGGCTGAGGAACGGAATATTTGCAAAATTCTGCGACTAATGTTCCCTTGCACCGTTGGCACAT
TCTAGTGGAACCGT**CACACACACACACACACA**AACAGTGCAGAATCCCTCAAACCCTT

LIST14-021
GGAGATAAGCGAGGATGAGGAGAGGATAGCACGGAGCGGGGAGGGAGATATGAGGAGTGGACGGGGTC
ACGGATGGTTGGAGACTTATGCATGANCGAGTG**GTGTGGGTGTGTGTGT**CTGGGTGCGGGGATTAAAC
GTTAAAGGGTGTGGGGGGGTACGTGTTTTGAATGAGGACCTCCTAAAAGTGGGGT

LIST14-022
TAAAAGTGGGGTTTGAGGGGGAGAGGAGGGAGGAGAATTCTGGAGGAGGGAAGAAA**GAGGAGGAG**AGC
TGAGGCGGACGAACGCGCAGGGTTTTGCTCCCAGGCTCTTTTCATTTCCACCTCACCCAACCCTTCCC
CTAACTCCCACTCTACTCACTCCTCACTCCATACCCATACTCCCTCCTCTCTCACTCCCTCCCCCGCC
AGGGAAGAAGCCAAATT

LIST14-023
AAAAGGTCTCCAATGGCCGTAACGTGGGGGGTACCGTCCAAGTACAAGACGAGACACTGTGTGGTATA
CTGGAACGGGAGGATTCACAGCAGTGACCAACCCCAGCCTCTCAACCGGAAACCCTTCACAATCACAC
GCTAAACACGAAGGAAACTTTGCAAGGGTTGAAGGGGCCTCGCTAAGTTTTCTCCGGTTTAGATACCA
CT**CACACACACACACACA**CTAGAGACTTCGTCGAGAGCGGACGTCTTTTGCTGAGCTCCTCGCCGTTG
CTGCTTCGTGTGTT

LIST14-024
TGTAAACAGACCAGTCGGGTCGCAGAGTCAAAACGTGAGCAGCTGTAAGCAGCACAGCAACAACAGCA
AACAGCCAAGGAGGAAGG**CAACAACAACAACAACAA**AGACTGCTAAAATTATGGCAGGTCTCTGCGCA
CACCTTCTTGGAATG

LIST14-025
ATCTCACCCCATCTTGTGCAGTCTTAAACCGACGGAGATAAAAATGCCGGGAATCCTAACCCTCCCTC
TGGAAAGAAGAAAAAGTCTTTGCCGTCGTATTCATGGAAAGGGGATGGTCGGCCTTTGGAGACTGGCT
TTT**TGTGTGTGTG**CGTGTGTGGAAGGACCTTTTTCACCCATTTCCCCCATCCACCTTTCGACTCCCTT
CCCAGCGTCCTTTGCACCCCAGCCGGTCAGTTTTCCGAGAAC

LIST14-026
AAGCGTCACAGAAACCACGCCTTAACACACCAAAACAACGAAAACAAGACCAGCAATTGACANCNNGN
AATCGAGCAGCGAGTTCACGGACCCAGCCGAGTATCGCCCATGAACGTGAACGTTTCCCAGAATGCCC
CACGGCGCTGAGAGCTGGATAGACTTTCGACGAG**CACACACACA**CTTGAAGAGAATAGGGAAGGAAGG
AGACTTTGGTAAGAAGCTTCGCAGCATCGGGAGTTGCACCACACAGACAGACTGAGTTAATGGGAGCC
CAAAATGGACTCTGGTGG

LIST14-027
ACACGCTAAACACGAAGGAAACTTTGCAAGGGTTGAAGGGGCCTCGCTAAGTTTTCTCCGGTTTAGAT
ACCACT**CACACACACACACACACA**CTAGAGACTTCGTCGAGAGCGGACGTCTTTTGCTGAGCTCCTCGCC
GTTGCTGCTTCGTGTGTTCTT

LIST14-028
TTTCAGCATAACAGAGTCGGTCAAATATCAGGACAAGGTTTTTTGTGCCATTAAAGTTATGGAAAATA
ACCGGTTTTTTTTCGTCATCCCTGTATTCATATGATTTTCCATCGTCCAAGTACAGCATACCAGAGGC
TTCATTCTGCAAAGATTGAATGCAATGAAAACCCAACAAGGCC**CACACACACACACACA**CGTTTTCCA
ACAATTAAACAGAGATTTATTGTGTATAGTAATATATTGACAAATTCACTCACCTTGGCATCAAGAGC
AATGACTAAGGTGAATGG

LIST14-029
TTGTCTCTCAGCACGGTTTTCCCTTTCAAATCCAAGTTTCTCTTTCTGAATAACCTGGAAAATGCTCA
GTTTTAGAGCAATGTCCGTTCCCATTCCATACCCATGGATAATAGCAAAAACTATCTGCACTTATAGT
CATTTGACACAGATATTGAATTTCCAACTCTAATGGACCAATTTA**CACACACACACACACACA**AGTACTG
AAAAGGGAAAAATAATTATACTACTAGCCTCATCACTTAAGTATCCCGAAAATGATCCGAAGCTTG

LIST14-030
AAATGATGACTTCCCAGGGCCTTCGTAATGTTTGCTAAAATGACGGGAAGCTACCAAGTAGGAAGGAA
GAGAGGCGTGGTGGAAATACA**TGTGTGTGTGTGTGTGTGTG**TCTGTGGTGCCTCAATTTATCTGCCCT
CCAATCAGCCAAATAATTTTCGCTTTCTAGCGTCGATCCTAAAATTTTCACAGTTATTGCTCATCAAT
AAAAGCCGCG

LIST14-031
TGACTTTTCCCTGTTCACCCCCTCGCCGCGGTGAGGCTGCAGTTCGGATTTGAGGGGGAGGGAAGGGT
TGGGGGAGTGGGAATTTCTAGGAAAAGGTAGGTTAGTGTTAATAGTTCTACTCTCAACTAA**CTACTAC**
TACCAGTA**CTACTACTACTACTA**CCACTCGCTCAGTCAAACCAAATC

LIST14-032
GTGTGTGTGATTGAGACCTACGTGGAAATTTGTTCGTAAAACAAATCCCCAAGGTGCGTGCGTGTGAN
CGTGANTTTTCCAGTTNATTTTTTTGGTCTTTTCTTGGGGTGAGTGGGTCACCGTCGTAAGCGAAAGC
TTCACAAACC**TGTGTGTGTGTGTGTGTGTG**ATTGAGACCTACGCGGAATTTGTTCCTCAGAACAAATC
TGCTAGGTGAGTGAGTGAGCCTGCGTGATTGTTTTTCATAGTGTTTGAGCCTTAACCTATGACGTACA
GCCACTAAAACAAACTTAAAGGGAAAAATAACCACACCAATTATACAATATGGATATATCATGAAAAC
ATTCTCTAATCATAAAGTCATCGACCTCAGCACCATTTA

LIST14-033
TTCCCATCAATCAAGCCCACGGGAGCGTCATGCTGCCTAGAAAGGCTGAGGTTCCGAAGAGGGCTTCC
CTTTTATACCCTTAATGATTTTTTCTTATCATCCTGAGAGGCAAAGTTTTTTTCCTTTACTTCGACGG
GATTAATTTTGAGAACCGCCTCCATATCATTTATTTTTCTTGACGTCACTTTTAATCGACAACTCCAA
GAA**CACACACACACACA**AGCACAGGTGAATCTCTTTTTCCCTCACAAGCCCTTCTCATCACCCGACTA
CCCCGCAGTACCCATCTTAACCCCGAAAGTTCCCCGAGGATGCAGAGAATA

LIST14-034
AAAGGTGGTGTGGGTTAGGACAGGTAACNCCGGCGGGGGGGGGGGAGGGAAAAACTTTCCCCTTCGTC
GAGACTCGCTTT**GTGTGTGTGTGTGTGTGTG**TCGTAAGGCCTT**CTCTCTCTCTCTC**TTTCTTTCTCCCTCTC
TCTCTTTCTCTTCGTCTCGTCGTTCAAGCAGTCCGGCCGAGTCAGTCCATCGCGTGCTGGTTTCGCGT
CGACAAGTCAGGTCTTCCGGGAGTGGTGCTCTGGTGGCTACTGCCGACCCTAAGTGGATCCCAAATGT
TTTCCGCATATTCGGAATGCGGTCTTATCAG

LIST14-035
GTGTTGTCTGCTGAATGGCGGAAGGTGTGTGGAGGGATTGAAGGGTGGAGGGGGGTTGGAGGAGGAGA
TGATGGGGTGGTGTGAGGCCGCCTGGTTATTCCCCGGTCGAGCGGAGTGAGGGACTGGGGAGGGGAAA
GACTGGGCGGTTCGGAACAATCTTTCTCAGTGAGATGGATATCGTGCTGGGTGTGGGGTGGGAGGAAT
ACATGAATGTGTAATCGGTGCTTAACTTACAACCTGATACGTGCCTGGACGCG**GTGTGTGTGTGTGTG**
TGTGCTTTTGCGTGTGAATGCATGTGTATTTGTGCCCTAAGGTAGGTAGTCCTCTTCCCTGTA

LIST14-036
TGAAATCGCTTGCGTTGCCCTCGTGCGATTACAAAAATCCTCCTCGTCGGGTGGAGAAGGGTCGCCTT
GGCAACTACACT**TGTGTGTGTGTGTTTGTGTGTGTGTGTG**GGTGGGTGGGTGGGTGGTCCCCAAATTA
TGTTACGGACTGAAGCAGGAGAAAAATTGGCCCAACCCTACCCTTCATT

LIST14-037
GACCCAATCACACTAAGGAACAGGCAATTTATGAACTAGAAAAATCACGCTCACACGCACGCACCTCG
CGGATTTGCTCTTACGAACAAATTTCCACGTAGGTCTCAAT**CACACACACACACACACACA**GGTTTGT
GAAGCTTTCGCTTACGACAGT

LIST14-038
CATCGGGTGGACATTTTGGTATTATTCGAGGAGACTCCGGANG**TGTGTGTGTGTGTGT**TGGAGGAATT
GATGTGGGGTGGGGAGGGGTGAAGGTGAGGGGGTGACCCACAAACCCAGGGGAACCCTCCGAAAAGTG
T

LIST14-039
TTTGTCGTGAGAGACTCGGCCGGAGTTCGGAGGGGGGGTAAGGAGTGGAGGAGGACGCCGTGAAGTAC
ATGCTACAAGGGGGACTTCCTGCTGAGGAGGGAGTGGCCTTCANGAATCATCCCTTC**CACACACACAC**
ACACACACACCATCAACCAGCCGCCCCTCTGCGCCCATAGATCCGAAGCTTG

LIST14-040
TATGCGACAAGTTAGCCGTCTGCTGTGGACTCATGCCCGCCATGGGCTGCTGACTATGGTTCATGTAC
GCTGAGGACTGATTCGCCGCCTGAGCTTGTTGTTGCTGAGCCGCGGCCATCATTTGCTGCTGTTGTTG
ATTCGCCAGCTGTTGTTGGTATTGTT**GCTGCTGCTGCTGCT**GTT**GCTGCT**GTTGTTGTTGGTACTGCTGTT
GCTGCTGTTGTTGGTATTGCTGCTGTTGGTACTGTTGCTGCTGCTGTAACTGCTGGCTCTGCTGGTAT
TGCTGGGGTTGTTGGTACTGCGAGGCCGCCGCCGCCACCTTGTTCTGCATGCCCGCTTCGTTGTCTGC
GGCCGATATACTCCCCCTCGCCGTC**GCGTTGTAACCTTGGGAT**

LIST14-041
TCTCACCGCAAAATAACGGCGAAAATCCTCCTAGAACAGACAGAAAGGCAGCCAAAGGTGAAGCACAG
GAGAGCACGTCCTCACTCGATGACTGTCA**GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT**GAGTGAGGG
AGTGAGTGTCAAGGCTTGCCAGTTGAGAGGCTGTATT

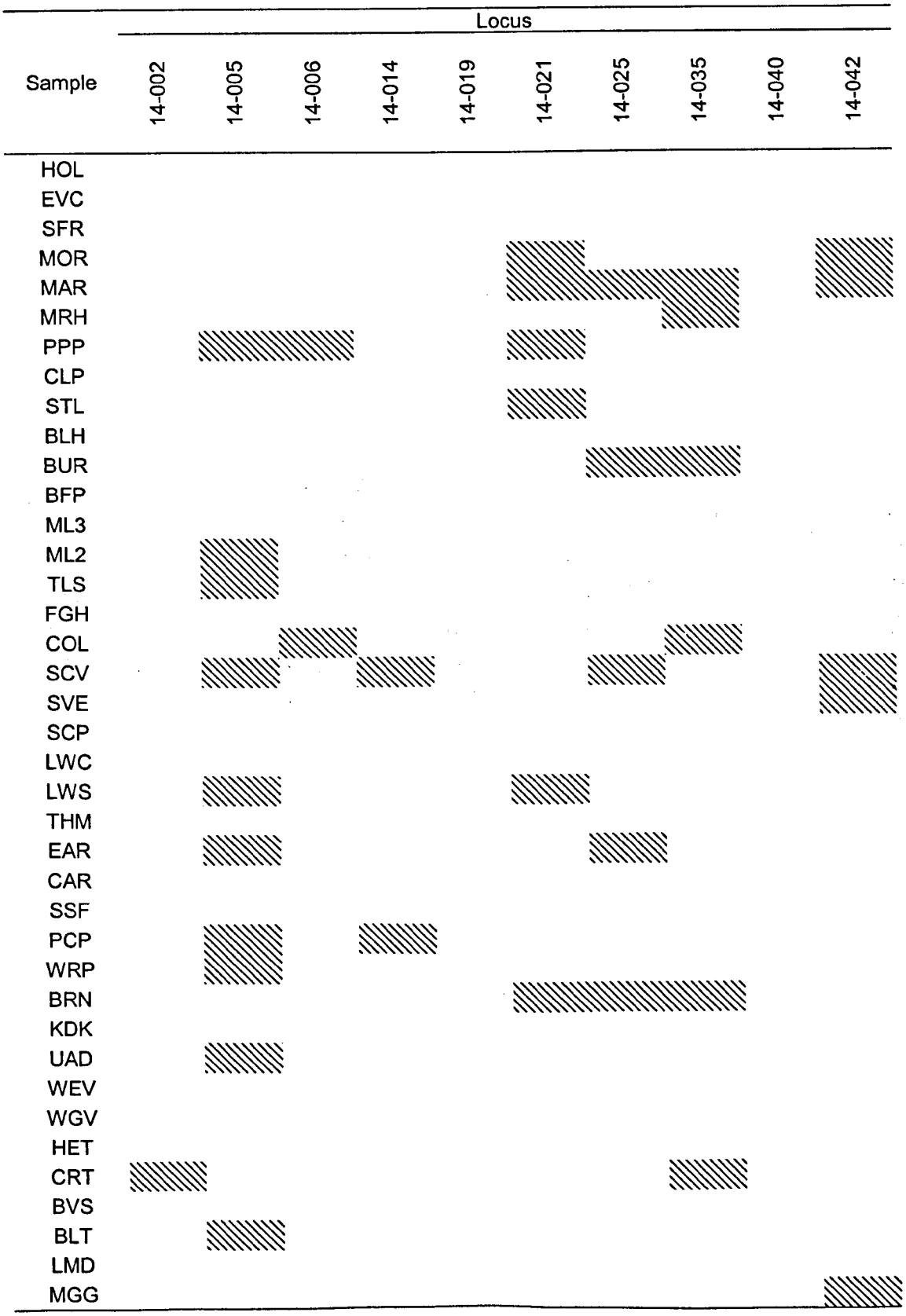
LIST14-042
CAGCGGTATCAAACTTCGATCAAGCCATTGATGCTGGTTTTAAACGGCTTTATAAACG**GTGTGTGTGT**
GTGTGTGTGGAAGATACGTGCTCGTCACGCGACTTTCGATTCTTTTTCTTTTCGCTCTCCTTAATTTT
TTTTCTTTTTGGTAATCTAGACACGCANTCGGATAAAGCTCAGGCTGGCGCTTTTCTTTTATGTCGAC
TTGACGGGATTTCTCGCCTTTT

LIST14-043
AAATGGTTTCCGAGGTCATGGAGTAAAACCTCCATGGAATGA**GTGTGTGTGTGTGTGT**CGAAACGTCG
GCTGAATGGTTATAGAGACCCGGTGTACCCCCTGAAAACCTACTGAGGCTCCAGATTCAGGATTTTG

Appendix 2.3. Primer sequences, summary characteristics and PCR conditions for 13 microsatellite loci isolated from *Erythromma viridulum* that proved to be monomorphic in the British population; $MgCl_2$, optimal magnesium chloride concentration; T_a, annealing temperature.

Locus	Primer sequence (5' -> 3')	Core motif	$MgCl_2$ (mM)	T_a (°C)
LIST14-004	F: TCTTACAGAGCTTTCCAGC	$(GTT)_5$	1.5	56
	R: GTTCTTGATACTCGGTTCC			
LIST14-012	F: GCCCTCTAATGCTTTTGCTGG	$(GT)_5$	3.0	55
	R: ATCAGCCACCACTGCCACCC			
LIST14-013	F: AATTTCATTTGCCGAAACG	$(CA)_4(AC)_4$	3.0	55
	R: ATTGGCCAGAGACCCCAAGC			
LIST14-015	F: CCGCTCACTGACTGACAAGC	$(GCT)_2(GCT)_3$	3.0	55
	R: GTTCAAAGGCGATTCTCACCG			
LIST14-022	F: TAAAAGTGGGGTTTGAGGGG	$(GAG)_3$	3.0	57
	R: AATTTGGCTTCTTCCCTGGC			
LIST14-023	F: AAAAGGTCTCCAATGGCCG	$(CA)_8$	3.0	53
	R: AACACACGAAGCAGCAACG			
LIST14-024	F: TGTAAACAGACCAGTCGGG	$(CAA)_6$	3.0	55
	R: CATTCCAAGAAGGTGTGCG			
LIST14-026	F: AAGCGTCACAGAAACCACG	$(CA)_5$	1.5	50
	R: CCACCAGAGTCCATTTTGG			
LIST14-027	F: ACACGCTAAACACGAAGG	$(CA)_8$	1.5	51
	R: AAGAACACACGAAGCAGC			
LIST14-031	F: TGACTTTTCCCTGTTCACC	$(CTA)_3(CTA)_5$	1.5	50
	R: GATTTGGTTTGACTGAGCG			
LIST14-032	F: GTGTGTGTGATTGAGACC	$(TG)_{10}$	3.0	44
	R: TAAATGGTGCTGAGGTCG			
LIST14-033	F: TTCCCATCAATCAAGCCC	$(CA)_7$	3.0	55
	R: TATTCTCTGCATCCTCGG			
LIST14-034	F: AAAGGTGGTGTGGGTTAGG	$(TC)_6$	3.0	45
	R: CTGATAAGACCGCATTCCG			

Appendix 3.1. Summary of test for null alleles at microsatellite loci; shaded boxes highlight significant ($P < 0.05$) tests.



Appendix 3.2. Probability of linkage disequilibrium among pairs of loci (data pooled over all populations) calculated using GENEPOP (Raymond & Rousset, 1995). χ^2, chi-squared value; *df*, degrees of freedom; *P*, significance level. *indicates significant ($P < 0.05$) linkage disequilibrium, with tests that remain significant after correction for $k = 45$ multiple tests highlighted bold.

Locus pair		χ^2	*df*	*P*
LIST14-002	LIST14-005	38.771	44	0.695
LIST14-002	LIST14-006	84.858	66	0.059
LIST14-005	LIST14-006	29.071	46	0.976
LIST14-002	LIST14-014	77.563	66	0.156
LIST14-005	LIST14-014	33.238	44	0.882
LIST14-006	LIST14-014	57.147	76	0.948
LIST14-002	LIST14-019	42.884	68	0.993
LIST14-005	LIST14-019	56.204	42	0.070
LIST14-006	LIST14-019	66.017	74	0.734
LIST14-014	LIST14-019	49.502	76	0.992
LIST14-002	LIST14-021	16.263	18	0.574
LIST14-005	LIST14-021	5.376	10	0.865
LIST14-006	LIST14-021	17.774	18	0.471
LIST14-014	LIST14-021	14.443	16	0.566
LIST14-019	LIST14-021	13.828	18	0.740
LIST14-002	LIST14-025	65.562	68	0.561
LIST14-005	LIST14-025	24.616	44	0.992
LIST14-006	LIST14-025	58.778	76	0.928
LIST14-014	LIST14-025	63.195	78	0.888
LIST14-019	LIST14-025	64.448	76	0.825
LIST14-021	LIST14-025	23.641	16	0.098
LIST14-002	LIST14-035	51.717	70	0.950
LIST14-005	LIST14-035	43.518	44	0.492
LIST14-006	LIST14-035	68.617	74	0.655
LIST14-014	LIST14-035	40.057	76	1.000
LIST14-019	LIST14-035	44.607	74	0.997
LIST14-021	LIST14-035	15.051	16	0.521
LIST14-025	LIST14-035	55.610	76	0.962
LIST14-002	LIST14-040	11.222	36	1.000
LIST14-005	LIST14-040	6.009	22	1.000
LIST14-006	LIST14-040	27.395	36	0.848
LIST14-014	LIST14-040	18.429	34	0.986
LIST14-019	LIST14-040	29.439	36	0.772
LIST14-021	LIST14-040	4.331	14	0.993
LIST14-025	LIST14-040	12.693	34	1.000
LIST14-035	LIST14-040	26.278	38	0.924
LIST14-002	LIST14-042	50.962	56	0.666
LIST14-005	LIST14-042	15.202	38	1.000
LIST14-006	LIST14-042	44.080	56	0.876
LIST14-014	LIST14-042	51.444	62	0.828
LIST14-019	LIST14-042	49.044	56	0.733
LIST14-021	LIST14-042	42.648	12	**0.000***
LIST14-025	LIST14-042	50.485	60	0.804
LIST14-035	LIST14-042	64.063	62	0.404
LIST14-040	LIST14-042	4.109	28	1.000

Appendix 3.3. Deviations from expected Hardy-Weinberg equilibrium conditions. *indicates significant deviation ($P < 0.05$), with those tests remaining significant after correction for multiple testing ($k = 62$) highlighted bold.

Population	Year	LIST14-002	LIST14-005	LIST14-006	LIST14-0014	LIST14-019	LIST14-021	LIST14-025	LIST14-035	LIST14-040	LIST14-042
HOL	2002	0.3875	-	0.9505	0.7027	0.7749	-	0.2117	0.5134	-	-
	2005	-	-	0.6012	0.1983	0.5977	-	1.0000	1.0000	-	-
EVC	2005	0.5554	-	0.7700	0.5814	0.3164	-	0.5937	0.6192	-	-
SFR	2002	0.2175	-	0.9622	1.0000	0.2743	-	0.7389	0.0961	-	-
MOR	2004	0.5885	-	0.6706	*0.0350	1.0000	*0.0012	0.6318	0.9867	-	***0.0008**
	2005	0.5107	-	0.5535	0.8540	0.9800	-	0.6489	0.3552	1.0000	-
	2006	0.5087	*0.0023	0.1086	0.1529	0.1409	-	0.5633	0.3375	0.0794	-
MAR	2002	0.9906	-	0.5681	0.5354	1.0000	-	0.3793	*0.0023	-	-
	2004	*0.0053	0.1008	0.2605	0.5655	0.2939	*0.0009	*0.0187	0.0819	-	***0.0007**
MAR	2005	0.4956	-	0.7382	0.3234	0.1911	-	0.1157	0.2195	-	1.0000
MRH	2002	0.8379	-	1.0000	0.4933	0.582	-	0.3493	*0.0013	-	-
	2005	-	-	-	-	-	-	-	-	-	-
PPP	2002	0.1906	-	0.47	0.6993	0.8526	-	0.3775	*0.0243	-	-
	2005	0.5548	-	0.1113	0.761	*0.016	*0.0166	0.7413	0.1628	1.0000	-
	2006	0.1041	*0.0024	0.1178	*0.0477	0.9913	1.0000	0.7403	0.6144	-	1.0000
CLP	2002	1.0000	-	0.1687	0.2703	0.514	-	1.0000	0.1463	-	0.0914
STL	2004	0.3445	0.598	0.7714	1.0000	0.7731	-	1.0000	0.2576	-	-
	2005	0.7729	***0.0002**	0.3473	0.4994	0.7648	*0.0017	*0.0427	0.4017	1.0000	1.0000
BLH	2005	0.7851	-	0.1979	1.0000	1.0000	-	0.4227	0.7685	-	-
BUR	2005	0.3164	-	***0.0004**	0.5003	0.1209	-	*0.0018	***0.0001**	0.1317	-
BFP	2003	0.5475	-	0.2287	1.0000	0.2941	-	0.8496	0.1284	-	0.1055
ML3	2004	0.1988	-	0.6004	-	-	-	-	-	-	-
ML2	2004	0.8165	1.0000	0.0853	0.4878	0.9708	-	0.2732	0.1241	-	0.4712
	2005	0.1040	***0.0002**	0.8371	0.8293	0.3900	-	0.9034	0.1716	-	0.7259
	2006	0.0574	***0.0004**	0.9110	0.1820	0.7563	-	0.9336	0.9427	-	0.8416
TLS	2005	0.8622	*0.0033	0.5008	0.0657	1.0000	-	0.6120	0.5369	-	0.2227
FGH	2005	0.9162	-	0.1044	*0.0307	1.0000	-	0.1843	0.7216	-	0.3392
COL	2004	0.0508	*0.0414	*0.018	0.7939	0.3607	-	0.0607	*0.0073	-	1.0000
SCV	2005	0.9791	0.0578	0.0711	***0.0000**	0.2296	-	***0.0001**	0.2637	-	*0.0103
	2006	0.4239	***0.0000**	0.1619	0.2145	0.4031	-	0.297	0.4504	0.0562	0.5692
SVE	2005	0.7584	1.0000	0.8311	0.7075	1.0000	-	0.8058	0.8769	-	*0.0011
SCP	2003	-	-	0.5944	0.5984	1.0000	-	0.5976	1.0000	-	-
LWC	2002	-	-	-	-	-	-	-	-	-	-
	2003	0.6889	0.0694	0.701	0.5913	0.3864	-	1.0000	0.0507	-	0.1527
LWS	2003	0.7327	0.1095	0.5815	0.1803	0.8575	-	*0.0172	0.4043	-	1.0000
	2004	*0.0180	-	0.4622	0.968	0.1986	**0.0002**	0.1377	0.3433	-	0.0653
	2005	0.5759	1.0000	0.8129	0.9953	0.3459	-	0.8804	0.7816	-	*0.0066
	2006	0.3172	***0.0000**	0.2038	0.0503	0.5169	-	0.285	0.2203	-	0.6819

Appendix 3.3 continued. Deviations from expected Hardy-Weinberg equilibrium conditions. *indicates significant deviation ($P < 0.05$), with those tests remaining significant after correction for multiple testing ($k = 62$) highlighted bold.

Population	Year	LIST14-002	LIST14-005	LIST14-006	LIST14-0014	LIST14-019	LIST14-021	LIST14-025	LIST14-035	LIST14-040	LIST14-042
THM	2003	0.6021	1.0000	1.0000	1.0000	0.5995	-	-	1.0000	-	1.0000
	2004	***0.0000**	***0.0000**	***0.0003**	1.0000	0.2943	-	***0.0005**	*0.0057	-	0.5511
EAR	2005	0.6383	***0.0003**	0.2860	0.9709	0.5558	-	0.5983	0.7319	-	0.4547
	2006	0.5009	*0.0029	0.6868	*0.0330	0.8353	-	***0.0008**	*0.0165	-	0.1060
CAR	2002	0.7731	-	0.6482	1.0000	0.8689	-	0.9785	*0.0180	-	-
SSF	2005	1.0000	-	1.0000	0.7746	0.4284	-	1.0000	1.0000	-	0.0863
PCP	2003	-	-	-	-	-	-	-	-	-	-
	2004	***0.0003**	0.2492	*0.0348	0.0894	0.5234	-	0.9460	***0.0002**	1.0000	0.6407
	2005	*0.0238	***0.0000**	0.2157	0.2931	0.9112	*0.0072	0.1422	*0.0124	1.0000	*0.0284
	2006	0.7491	***0.0000**	0.9911	*0.0082	*0.0097	-	*0.0012	0.2956	1.0000	0.6218
WRP	2005	0.8576	***0.0001**	0.1313	0.4753	0.757	-	0.0629	0.5669	-	0.8797
	2006	0.7770	0.0660	*0.0026	0.6132	0.0843	-	0.3604	0.5534	1.0000	0.9975
BRN	2005	0.5717	***0.0000**	0.0895	*0.0013	0.4685	-	0.7245	0.3099	1.0000	0.3474
KDK	2006	0.4481	*0.0020	*0.0072	*0.0010	*0.0029	-	*0.0087	*0.0033	-	***0.0000**
UAD	2005	0.4404	***0.0001**	0.4328	0.9534	0.6267	-	0.0670	0.2291	-	0.6075
	2006	0.2034	***0.0002**	0.8535	0.8727	0.3485	-	0.3792	0.8239	-	0.9595
WEV	2006	0.3352	-	0.9763	0.9222	0.5518	*0.0260	0.6579	*0.0395	-	1.0000
WGV	2006	0.8746	0.1119	0.8773	1.0000	0.3335	-	1.0000	0.0790	-	-
HET	2006	0.9811	0.1580	0.9525	1.0000	0.6601	-	0.5360	0.7715	-	0.6917
CRT	2006	*0.0068	0.0542	0.0876	1.0000	0.4785	-	0.4014	*0.0046	-	0.0711
BVS	2006	1.0000	-	0.7590	0.7561	1.0000	-	0.9307	0.8874	-	1.0000
BLT	2006	0.8721	*0.0011	0.2366	0.3134	0.3581	-	0.8072	0.2312	-	0.6482
LMD	2006	0.6009	-	0.5950	-	0.2007	-	0.5988	1.0000	-	-
MGG	2006	0.4816	0.0797	1.0000	1.0000	1.0000	-	1.0000	1.0000	-	0.2120

Appendix 3.4. Basic measures of genetic diversity (*n*, sample size; N_a, number of alleles; A_R, allelic richness; H_o, observed heterozygosity; H_e, expected heterozygosity; *f* Wright's (1951) inbreeding coefficient) in samples of *Erythromma viridulum* from the UK and continental Europe.

Site	Year		Locus										All Loci
			002	005	006	014	019	021	025	035	040	042	
HOL	2002	*n*	8	8	8	8	8	8	8	8	8	5	7.70
		N_a	4	1	4	2	2	1	4	6	1	1	2.60
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.50	0.00	0.88	0.50	0.50	0.00	0.50	0.75	0.00	0.00	0.36
		H_e	0.58	0.00	0.69	0.53	0.50	0.00	0.69	0.78	0.00	0.00	0.38
		f	0.138	-	-0.289	0.067	0	-	0.291	0.045	-	-	0.04
	2005	*n*	1	3	3	3	3	3	3	3	3	3	2.80
		N_a	2	1	3	2	2	1	2	3	1	2	1.90
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	1.00	0.00	0.67	0.00	0.33	0.00	0.67	1.00	0.00	0.33	0.40
		H_e	1.00	0.00	0.73	0.53	0.60	0.00	0.53	0.73	0.00	0.33	0.45
		f	-	-	0.111	1	0.5	-	-0.333	-0.5	-	0	0.13
EVC	2005	*n*	32	33	31	33	33	31	33	33	32	33	32.40
		N_a	5	1	4	3	3	1	4	4	1	1	2.70
		A_R	5	1	4	3	3	1	4	4	1	1	2.56
		H_o	0.78	0.00	0.48	0.52	0.42	0.00	0.67	0.79	0.00	0.00	0.37
		H_e	0.74	0.00	0.45	0.51	0.47	0.00	0.66	0.73	0.00	0.00	0.36
		f	-0.052	-	-0.077	-0.013	0.101	-	-0.015	-0.083	-	-	-0.02
SFR	2002	*n*	7	7	7	6	6	7	7	6	7	6	6.60
		N_a	3	1	3	2	2	1	3	3	1	2	2.10
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.57	0.00	0.86	0.33	0.17	0.00	0.71	0.17	0.00	0.17	0.30
		H_e	0.69	0.00	0.65	0.30	0.41	0.00	0.67	0.44	0.00	0.17	0.33
		f	0.186	-	-0.358	-0.111	0.615	-	-0.071	0.643	-	0	0.13
MOR	2004	*n*	27	26	27	27	26	27	27	27	26	27	26.70
		N_a	6	1	4	3	2	2	5	5	2	3	3.30
		A_R	5.926	1	3.963	3	2	2	4.962	4.962	2	2.963	3.077
		H_o	0.70	0.00	0.63	0.48	0.46	0.00	0.74	0.85	0.04	0.04	0.39
		H_e	0.70	0.00	0.62	0.52	0.36	0.14	0.71	0.70	0.04	0.17	0.40
		f	-0.005	-	-0.018	0.079	-0.282	1	-0.052	-0.23	0	0.79	0.14
	2005	*n*	32	31	32	32	31	31	32	32	32	32	31.70
		N_a	5	2	4	4	3	1	3	3	3	2	3.00
		A_R	4.967	1.839	3.812	3.625	2.839	1	3	3	2.625	1.813	2.852
		H_o	0.63	0.03	0.56	0.56	0.58	0.00	0.69	0.59	0.06	0.03	0.37
		H_e	0.61	0.03	0.55	0.49	0.45	0.00	0.65	0.64	0.06	0.03	0.35
		f	-0.027	0	-0.025	-0.153	-0.284	-	-0.057	0.069	-0.008	0	-0.05
	2006	*n*	39	39	39	34	39	39	39	38	38	39	38.30
		N_a	6	2	4	3	3	2	3	5	3	2	3.30
		A_R	5.656	1.997	3.955	2.765	2.966	1.667	3	4.684	2.942	1.667	3.130
		H_o	0.67	0.03	0.59	0.38	0.44	0.03	0.69	0.63	0.11	0.03	0.36
		H_e	0.72	0.12	0.57	0.47	0.54	0.03	0.67	0.68	0.15	0.03	0.40
		f	0.08	0.791	-0.044	0.195	0.189	0	-0.036	0.072	0.304	0	0.16

Appendix 3.4 continued.

MAR	2002	*n*	9	9	9	9	9	7	9	8	9	9	8.70
		N_a	5	1	3	3	3	1	3	3	2	1	2.50
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.89	0.00	0.56	0.44	0.78	0.00	0.67	0.13	0.11	0.00	0.36
		H_e	0.69	0.00	0.58	0.50	0.54	0.00	0.69	0.69	0.11	0.00	0.38
		f	-0.306	-	0.048	0.123	-0.474	-	0.03	0.829	0	-	0.04
	2004	*n*	30	30	31	31	31	31	30	31	31	31	30.70
		N_a	5	2	4	2	3	2	4	4	2	2	3.00
		A_R	5	2	4	2	3	2	4	4	2	2	2.90
		H_o	0.53	0.07	0.52	0.35	0.45	0.00	0.50	0.65	0.03	0.03	0.31
		H_e	0.70	0.13	0.62	0.37	0.51	0.12	0.69	0.75	0.03	0.12	0.40
		f	0.337	0.477	0.166	0.049	0.122	1	0.275	0.138	0	1	0.36
	2005	*n*	37	36	37	37	36	37	36	36	37	37	36.60
		N_a	5	2	3	3	3	1	3	4	2	2	2.80
		A_R	5	2	3	3	3	1	3	4	2	2	2.64
		H_o	0.76	0.03	0.54	0.46	0.42	0.00	0.56	0.58	0.03	0.05	0.34
		H_e	0.74	0.03	0.50	0.51	0.50	0.00	0.66	0.67	0.03	0.05	0.37
		f	-0.021	0	-0.074	0.104	0.167	-	0.156	0.127	0	-0.014	0.05
MRH	2002	*n*	10	10	10	10	9	10	10	10	9	9	9.70
		N_a	4	1	4	3	2	1	3	4	1	2	2.50
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.90	0.00	0.60	0.50	0.44	0.00	0.60	0.20	0.00	0.11	0.34
		H_e	0.75	0.00	0.50	0.54	0.52	0.00	0.69	0.71	0.00	0.11	0.38
		f	-0.209	-	-0.213	0.082	0.158	-	0.143	0.727	-	0	0.10
	2005	*n*	1	1	1	1	1	1	1	1	1	1	1.00
		N_a	2	1	1	2	1	1	2	1	1	1	1.30
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.30
		H_e	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.30
		f	-	-	-	-	-	-	-	-	-	-	-
PPP	2002	*n*	10	10	10	10	10	10	10	10	10	10	10.00
		N_a	4	1	3	3	2	1	3	4	2	2	-
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.81	0.00	0.68	0.50	0.52	0.00	0.60	0.80	0.11	0.60	0.46
		H_e	0.72	0.00	0.52	0.54	0.42	0.00	0.65	0.76	0.11	0.57	0.43
		f	0.286	-	0.107	-0.069	-0.111	-	0.079	0.467	0	0	0.09
	2005	*n*	30	30	29	30	28	29	28	29	28	30	29.10
		N_a	6	2	3	3	3	2	4	5	2	1	3.10
		A_R	5	2	4	3	3	2	4	5	2	2	3.02
		H_o	0.50	0.03	0.38	0.35	0.24	0.00	0.53	0.49	0.05	0.00	0.26
		H_e	0.50	0.03	0.44	0.33	0.40	0.05	0.51	0.54	0.05	0.00	0.28
		f	-0.004	0	0.129	-0.07	0.414	1	-0.029	0.099	-0.018	-	0.17
	2006	*n*	39	39	39	39	39	39	39	39	39	39	39.00
		N_a	6	2	4	3	3	2	5	5	1	2	3.30
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.62	0.08	0.44	0.36	0.62	0.05	0.72	0.72	0.00	0.08	0.37
		H_e	0.66	0.21	0.58	0.50	0.48	0.05	0.68	0.70	0.00	0.07	0.39
		f	0.075	0.631	0.248	0.291	-0.3	-	-0.058	-0.032	-	-0.027	0.09
CLP	2002	*n*	6	6	6	6	6	6	5	6	5	6	5.80
		N_a	4	1	4	3	2	1	3	3	1	2	2.40
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	1.00	0.00	0.50	0.33	0.33	0.00	0.80	0.33	0.00	0.00	0.33
		H_e	0.74	0.00	0.65	0.53	0.48	0.00	0.62	0.62	0.00	0.30	0.40
		f	-0.395	-	0.25	0.394	0.333	-	-0.333	0.487	-	1	0.25

Appendix 3.4 continued.

STL	2004	*n*	3	3	4	4	4	4	4	4	4	4	3.80
		N_a	4	2	2	2	2	1	3	3	2	1	2.20
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.67	0.33	0.50	0.50	0.50	0.00	1.00	0.50	0.25	0.00	0.43
		H_e	0.87	0.60	0.57	0.43	0.57	0.00	0.68	0.71	0.25	0.00	0.47
		f	0.273	0.5	0.143	-0.2	0.143	-	-0.6	0.333	0	-	0.07
	2005	*n*	44	43	45	45	44	45	45	44	45	45	44.50
		N_a	6	3	3	3	3	2	3	4	3	3	3.30
		A_R	5	3	3	2	3	2	3	4	2	2	2.93
		H_o	0.73	0.02	0.53	0.47	0.52	0.02	0.53	0.66	0.04	0.09	0.36
		H_e	0.71	0.11	0.59	0.48	0.48	0.11	0.66	0.68	0.04	0.09	0.40
		f	-0.022	0.795	0.102	0.023	-0.081	0.792	0.198	0.029	-0.006	-0.026	0.18
BLH	2005	*n*	4	4	4	4	4	4	4	4	4	4	4.00
		N_a	5	1	4	3	3	1	3	4	1	1	2.60
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.75	0.00	0.50	0.75	0.75	0.00	0.50	0.75	0.00	0.00	0.40
		H_e	0.79	0.00	0.75	0.61	0.61	0.00	0.61	0.75	0.00	0.00	0.41
		f	0.053	-	0.368	-0.286	-0.286	-	0.2	0	-	-	0.01
BUR	2005	*n*	38	38	37	36	37	38	38	35	38	38	37.30
		N_a	7	1	4	3	3	1	4	5	2	1	3.10
		A_R	6	1	4	3	3	1	4	5	2	1	2.92
		H_o	0.63	0.00	0.46	0.47	0.38	0.00	0.45	0.37	0.08	0.00	0.28
		H_e	0.69	0.00	0.63	0.49	0.49	0.00	0.68	0.71	0.12	0.00	0.38
		f	0.087	-	0.274	0.03	0.225	-	0.345	0.483	0.369	-	0.26
BFP	2003	*n*	7	7	7	7	7	7	7	7	5	7	6.80
		N_a	5	1	4	2	2	1	2	5	1	2	2.50
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.71	0.00	0.57	0.43	0.29	0.00	0.57	0.57	0.00	0.14	0.33
		H_e	0.76	0.00	0.76	0.36	0.53	0.00	0.53	0.82	0.00	0.49	0.43
		f	0.063	-	0.262	-0.2	0.478	-	-0.091	0.324	-	0.727	0.22
ML3	2004	*n*	3	3	3	3	3	3	3	3	3	3	3.00
		N_a	3	1	2	2	2	1	2	2	1	2	1.80
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.33	0.00	0.33	0.33	0.33	0.00	0.33	0.33	0.00	0.33	0.23
		H_e	0.73	0.00	0.60	0.33	0.33	0.00	0.33	0.33	0.00	0.33	0.30
		f	0.6	-	0.5	0	0	-	0	0	-	0	0.16
MLT	2004	*n*	10	10	8	10	10	10	9	10	10	10	9.70
		N_a	4	3	3	3	2	1	4	3	1	3	2.70
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.80	0.40	0.50	0.40	0.70	0.00	0.56	0.40	0.00	0.40	0.42
		H_e	0.68	0.35	0.57	0.47	0.52	0.00	0.71	0.61	0.00	0.42	0.43
		f	-0.18	-0.143	0.125	0.153	-0.37	-	0.231	0.357	-	0.04	0.03
	2005	*n*	59	59	59	59	59	59	59	59	59	58	58.90
		N_a	7	2	5	3	2	1	5	7	1	4	3.70
		A_R	6	2	4	3	2	1	4	6	1	3	3.14
		H_o											-
		H_e	0.69	0.07	0.58	0.49	0.42	0.00	0.54	0.63	0.00	0.41	0.38
		f	0.147	1	-0.081	-0.097	0.074	-	-0.121	0.114	-	-0.047	0.12
	2006	*n*	44	42	43	43	44	44	44	44	44	44	43.60
		N_a	5	2	6	4	2	2	4	7	1	4	3.70
		A_R	4	2	5	3	2	1	4	6	1	3	3.18
		H_o	0.64	0.07	0.72	0.40	0.43	0.02	0.75	0.75	0.00	0.50	0.43
		H_e	0.68	0.09	0.64	0.47	0.41	0.02	0.64	0.68	0.00	0.45	0.41
		f	0.065	1	-0.132	0.156	-0.05	0	-0.18	-0.105	-	-0.114	0.07

Appendix 3.4 continued.

TLS	2005	*n*	17	17	17	13	17	17	17	17	16	17	16.50
		N_a	4	2	4	3	2	1	4	5	2	2	2.90
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.76	0.00	0.59	0.23	0.35	0.00	0.71	0.65	0.06	0.35	0.37
		H_e	0.68	0.21	0.61	0.44	0.30	0.00	0.67	0.67	0.06	0.50	0.41
		f	-0.134	1	0.045	0.486	-0.185	-	-0.058	0.041	0	0.299	0.17
FGH	2005	*n*	9	9	9	9	9	9	9	8	9	9	8.90
		N_a	5	1	3	2	2	1	4	4	2	2	2.60
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.89	0.00	0.44	0.11	0.44	0.00	0.44	0.75	0.11	0.11	0.33
		H_e	0.77	0.00	0.62	0.50	0.37	0.00	0.63	0.73	0.11	0.37	0.41
		f	-0.164	-	0.297	0.789	-0.231	-	0.312	-0.037	0	0.407	0.17
COL	2004	*n*	12	13	13	13	13	13	11	13	13	13	12.70
		N_a	5	3	4	3	2	1	4	5	1	2	3.00
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.67	0.08	0.38	0.54	0.38	0.00	0.55	0.38	0.00	0.54	0.35
		H_e	0.76	0.22	0.65	0.50	0.51	0.00	0.66	0.70	0.00	0.41	0.44
		f	0.133	0.657	0.417	-0.091	0.25	-	0.184	0.464	-	-0.333	0.21
SCV	2005	*n*	53	53	52	48	53	53	53	51	46	53	51.50
		N_a	8	3	4	4	2	1	5	6	1	4	3.80
		A_R	5	2	4	3	2	1	4	5	1	3	3.12
		H_o	0.75	0.06	0.52	0.29	0.38	0.00	0.43	0.65	0.00	0.17	0.33
		H_e	0.65	0.09	0.57	0.52	0.44	0.00	0.63	0.70	0.00	0.53	0.41
		f	-0.157	0.383	0.092	0.446	0.143	-	0.315	0.07	-	0.682	0.25
	2006	*n*	54	54	53	54	54	54	53	52	53	54	53.50
		N_a	6	2	5	4	2	1	6	6	2	4	3.80
		A_R	5	2	4	3	2	1	5	5	2	3	3.31
		H_o	0.69	0.00	0.49	0.41	0.44	0.00	0.57	0.73	0.04	0.43	0.38
		H_e	0.70	0.17	0.61	0.47	0.48	0.00	0.63	0.75	0.07	0.42	0.43
		f	0.019	1	0.192	0.126	0.074	-	0.106	0.022	0.488	-0.006	0.22
SVE	2005	*n*	10	10	10	8	10	10	10	10	8	10	9.60
		N_a	4	2	3	3	2	1	3	4	1	2	2.50
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.80	0.20	0.70	0.63	0.20	0.00	0.70	0.90	0.00	0.00	0.41
		H_e	0.70	0.19	0.61	0.59	0.19	0.00	0.59	0.72	0.00	0.53	0.41
		f	-0.152	-0.059	-0.156	-0.061	-0.059	-	-0.189	-0.266	-	1	0.01
SCP	2003	*n*	3	3	3	3	3	3	3	3	3	2	2.90
		N_a	2	1	3	2	3	1	2	3	2	2	2.10
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.33	0.00	0.67	0.33	0.67	0.00	0.33	1.00	0.33	0.50	0.42
		H_e	0.33	0.00	0.73	0.60	0.60	0.00	0.60	0.73	0.33	0.50	0.44
		f	0	-	0.111	0.5	-0.143	-	0.5	-0.5	0	0	0.06
LWC	2002	*n*	1	1	0	1	1	1	1	1	1	1	7.60
		N_a	1	1	-	2	1	1	1	2	1	1	2.60
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.00	0.00	-	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.22
		H_e	0.00	0.00	-	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.22
		f	-	-	-	-	-	-	-	-	-	-	-
	2003	*n*	8	8	8	8	8	8	8	8	6	6	0.90
		N_a	4	2	3	2	3	1	3	5	1	2	1.22
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.88	0.00	0.63	0.38	0.38	0.00	0.63	0.50	0.00	0.17	0.35
		H_e	0.74	0.23	0.59	0.46	0.49	0.00	0.49	0.76	0.00	0.53	0.43
		f	-0.195	1	-0.061	0.192	0.25	-	-0.296	0.356	-	0.706	0.24

Appendix 3.4 continued.

LWS	2003	*n*	9	9	9	9	9	8	9	9	9	9	8.90
		N_a	4	3	2	4	2	1	5	3	1	2	2.70
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.67	0.67	0.44	0.33	0.56	0.00	0.33	0.67	0.00	0.44	0.41
		H_e	0.63	0.60	0.52	0.47	0.50	0.00	0.67	0.70	0.00	0.37	0.45
		f	-0.067	-0.116	0.158	0.304	-0.111	-	0.515	0.05	-	-0.231	0.06
	2004	*n*	14	17	17	16	17	17	17	17	17	17	16.60
		N_a	5	2	3	2	2	2	5	5	2	5	3.30
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.57	0.06	0.53	0.63	0.35	0.00	0.53	0.59	0.06	0.47	0.38
		H_e	0.71	0.06	0.55	0.48	0.51	0.37	0.66	0.66	0.06	0.70	0.48
		f	0.203	0	0.046	-0.304	0.319	1	0.2	0.114	0	0.332	0.19
	2005	*n*	58	58	58	58	58	57	58	58	58	58	57.90
		N_a	6	2	4	3	2	1	5	6	1	4	3.40
		A_R	5	2	4	3	2	1	4	5	1	3	2.98
		H_o	0.67	0.07	0.59	0.60	0.45	0.00	0.67	0.74	0.00	0.38	0.42
		H_e	0.68	0.07	0.54	0.47	0.49	0.00	0.64	0.70	0.00	0.45	0.40
		f	0.006	-0.027	-0.09	-0.297	0.085	-	-0.054	-0.052	-	0.161	-0.03
	2006	*n*	52	52	52	52	51	52	51	52	52	51	51.70
		N_a	7	3	4	3	2	2	6	5	2	5	3.90
		A_R	5	3	4	3	2	1	5	5	1	4	3.27
		H_o	0.63	0.02	0.60	0.35	0.45	0.02	0.61	0.62	0.02	0.49	0.38
		H_e	0.62	0.29	0.58	0.52	0.47	0.02	0.65	0.68	0.02	0.45	0.43
		f	-0.018	0.934	-0.029	0.331	0.034	0	0.072	0.095	0	-0.081	0.13
THM	2003	*n*	3	3	3	3	3	3	3	3	3	3	3.00
		N_a	3	2	2	2	2	1	2	3	1	2	2.00
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.67	1.00	0.67	0.67	0.33	0.00	0.33	0.67	0.00	1.00	0.53
		H_e	0.73	0.60	0.53	0.53	0.60	0.00	0.33	0.60	0.00	0.60	0.45
		f	-0.333	-0.333	0.5	-	0	-	-	-1	-	0.111	-0.17
EAR	2004	*n*	21	21	21	21	21	21	21	21	21	21	21.00
		N_a	4	3	4	2	3	1	5	5	1	3	3.10
		A_R	4	3	4	2	3	1	5	5	1	3	3.10
		H_o	0.24	0.00	0.29	0.43	0.43	0.00	0.52	0.48	0.00	0.33	0.27
		H_e	0.66	0.26	0.59	0.34	0.50	0.00	0.68	0.65	0.00	0.35	0.40
		f	0.642	1	0.521	-0.25	0.147	-	0.235	0.277	-	0.06	0.33
	2005	N	50	47	50	50	50	50	50	49	50	50	49.60
		A	5	2	4	3	3	1	6	7	2	3	3.60
		Ar	4	2	3	3	2	1	5	6	1	2	3.04
		Ho	0.68	0.06	0.48	0.56	0.50	0.00	0.64	0.76	0.02	0.42	0.41
		He	0.62	0.21	0.53	0.46	0.50	0.00	0.66	0.71	0.02	0.44	0.42
		Fis	-0.092	0.697	0.09	-0.207	0.007	-	0.033	-0.064	0	0.042	0.06
	2006	*n*	33	31	33	33	32	33	32	33	33	33	32.60
		N_a	3	3	4	3	2	1	5	5	1	3	3.00
		A_R	3	3	4	3	2	1	5	5	1	3	2.88
		H_o	0.58	0.10	0.61	0.21	0.53	0.00	0.44	0.55	0.00	0.36	0.34
		H_e	0.61	0.21	0.59	0.48	0.48	0.00	0.68	0.65	0.00	0.47	0.42
		f	0.065	0.541	-0.033	0.56	-0.1	-	0.358	0.158	-	0.231	0.22
CAR	2002	*n*	10	10	10	10	10	10	10	10	10	9	9.90
		N_a	4	1	3	2	2	1	4	4	1	1	2.30
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.70	0.00	0.60	0.40	0.60	0.00	0.90	0.40	0.00	0.00	0.36
		H_e	0.66	0.00	0.59	0.34	0.53	0.00	0.73	0.73	0.00	0.00	0.36
		f	-0.068	-	-0.009	-0.2	-0.149	-	-0.256	0.463	-	-	-0.04

Appendix 3.4 continued.

SSF	2005	*n*	4	4	3	4	4	4	4	4	4	4	3.90
		N_a	4	1	3	2	2	1	3	3	1	2	2.20
		A_R	-	-	-	-	-	-	-	-	-	-	
		H_o	0.75	0.00	1.00	0.50	0.25	0.00	1.00	1.00	0.00	0.00	0.45
		H_e	0.64	0.00	0.73	0.57	0.54	0.00	0.68	0.75	0.00	0.57	0.45
		f	-0.2	-	-0.5	0.143	0.571	-	-0.6	-0.412	-	1	0.00
PCP	2003	*n*	1	1	1	1	1	1	1	1	1	1	1.00
		N_a	2	1	1	2	1	1	1	2	1	1	1.30
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		H_e	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		f	-	-	-	-	-	-	-	-	-	-	-
	2004	*n*	27	27	27	26	27	27	24	26	27	27	26.50
		N_a	4	2	4	2	3	1	3	5	2	4	3.00
		A_R	4	2	4	2	3	1	3	5	2	4	2.90
		H_o	0.37	0.41	0.44	0.23	0.44	0.00	0.67	0.31	0.15	0.56	0.36
		H_e	0.61	0.51	0.59	0.36	0.45	0.00	0.53	0.65	0.14	0.52	0.44
		f	0.3946	0.2033	0.2563	0.3671	0.0142	-	-	0.5338	-	-	0.15
	2005	*n*	68	67	68	68	67	68	68	68	67	68	67.70
		N_a	4	2	4	2	4	2	4	6	3	3	3.40
		A_R	4	2	4	2	3	2	4	5	2	3	2.96
		H_o	0.56	0.00	0.43	0.29	0.54	0.00	0.59	0.60	0.12	0.35	0.35
		H_e	0.66	0.11	0.48	0.33	0.47	0.03	0.65	0.73	0.11	0.49	0.41
		f	0.1522	1	0.1122	0.1079	-0.147	1	0.0911	0.1737	-	0.2839	0.27
	2006	*n*	54	54	54	54	54	54	53	54	54	54	53.90
		N_a	6	2	4	3	2	1	6	6	2	2	3.40
		A_R	5	2	4	3	2	1	5	5	2	2	3.03
		H_o	0.67	0.00	0.57	0.15	0.31	0.00	0.68	0.07	0.50	0.59	0.35
		H_e	0.64	0.25	0.48	0.47	0.48	0.00	0.72	0.07	0.50	0.55	0.42
		f	-	1	-	0.6846	0.3513	-	0.3569	0.0522	-	-	0.24
WRP	2005	*n*	46	46	46	46	46	46	46	45	46	45	45.80
		N_a	7	3	4	3	3	1	5	6	1	2	3.50
		A_R	6	2	4	3	2	1	4	5	1	2	3.08
		H_o	0.70	0.04	0.54	0.50	0.54	0.00	0.54	0.78	0.00	0.53	0.42
		H_e	0.64	0.16	0.65	0.52	0.50	0.00	0.63	0.75	0.00	0.48	0.43
		f	-	0.7341	0.1645	0.0345	-	-	0.1336	-	-	-	0.09
	2006	*n*	75	74	74	75	75	74	75	74	75	75	74.60
		N_a	6	2	5	3	2	1	6	5	2	4	3.60
		A_R	5	2	5	3	2	1	5	5	1	3	3.14
		H_o	0.63	0.04	0.58	0.47	0.36	0.00	0.68	0.66	0.03	0.63	0.41
		H_e	0.59	0.07	0.61	0.46	0.44	0.00	0.70	0.71	0.03	0.50	0.41
		f	-	0.3848	0.0488	-	0.1881	-	0.0223	0.0687	-	-0.25	0.04
BRN	2005	*n*	52	53	52	52	53	51	53	52	53	53	52.40
		N_a	8	5	6	3	4	1	7	6	3	4	4.70
		A_R	7	4	5	3	3	1	5	5	2	4	3.81
		H_o	0.75	0.09	0.48	0.35	0.40	0.00	0.70	0.77	0.04	0.43	0.40
		H_e	0.75	0.23	0.56	0.48	0.41	0.00	0.62	0.76	0.04	0.46	0.43
		f	-0.005	0.588	0.143	0.283	0.025	-	-0.118	-0.016	-0.005	0.06	0.11
KDK	2006	*n*	19	19	19	19	19	19	19	19	19	19	19.00
		N_a	4	2	3	3	2	2	4	5	1	4	3.00
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.58	0.21	0.53	0.32	0.32	0.05	0.53	0.63	0.00	0.47	0.36
		H_e	0.62	0.27	0.52	0.48	0.44	0.05	0.50	0.73	0.00	0.40	0.40
		f	0.062	0.234	-0.008	0.351	0.294	0	-0.056	0.134	-	-0.191	0.09

Appendix 3.4 continued.

UAD	2005	*n*	19	19	19	19	19	19	19	19	19	19	19.00
		N_a	7	2	5	2	2	1	5	5	2	2	3.30
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.63	0.00	0.63	0.63	0.47	0.00	0.63	0.68	0.05	0.42	0.42
		H_e	0.64	0.34	0.66	0.50	0.49	0.00	0.69	0.67	0.05	0.44	0.45
		f	0.007	1	0.048	-0.271	0.036	-	0.081	-0.022	0	0.053	0.10
	2006	*n*	19	19	19	19	19	19	19	19	19	19	19.00
		N_a	6	2	5	2	2	1	5	5	1	2	3.10
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.58	0.05	0.79	0.47	0.32	0.00	0.68	0.79	0.00	0.58	0.43
		H_e	0.71	0.42	0.70	0.42	0.40	0.00	0.73	0.67	0.00	0.46	0.45
		f	0.187	0.878	-0.132	-0.125	0.212	-	0.07	-0.182	-	-0.261	0.08
WEV	2006	*n*	19	19	19	19	19	19	19	19	19	19	19.00
		N_a	4	2	5	3	3	2	5	6	2	3	3.50
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.63	0.05	1.00	0.68	0.58	0.00	0.95	0.68	0.05	0.47	0.51
		H_e	0.69	0.15	0.76	0.58	0.67	0.10	0.86	0.88	0.05	0.41	0.52
		f	0.0831	0.6567	-	-	0.1395	1	-	0.2274	0	-	0.13
WGV	2006	*n*	5	5	5	5	5	5	5	5	5	5	5.00
		N_a	2	2	3	2	2	1	3	4	1	2	2.20
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		H_e	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		f	-0.091	1	-0.28	-0.143	0.6	-	-0.391	0.484	-	0	0.15
HET	2006	*n*	10	10	10	10	10	10	10	10	10	10	10.00
		N_a	5	2	5	2	3	2	5	5	1	3	3.30
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.90	0.10	0.90	0.40	0.50	0.10	0.60	0.80	0.00	0.60	0.49
		H_e	0.69	0.27	0.75	0.34	0.51	0.10	0.65	0.76	0.00	0.57	0.47
		f	-	0.64	-0.209	-0.2	0.0217	0	0.0847	-	-	-	-0.01
CRT	2006	*n*	10	10	10	10	10	9	10	10	10	10	9.90
		N_a	4	2	3	3	2	1	3	5	1	2	2.60
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.30	0.00	0.30	0.30	0.30	0.00	0.50	0.40	0.00	0.20	0.23
		H_e	0.70	0.19	0.56	0.28	0.39	0.00	0.58	0.75	0.00	0.53	0.40
		f	0.585	1	0.481	-0.08	0.25	-	0.151	0.482	-	0.633	0.44
BVS	2006	*n*	6	6	6	6	6	6	6	6	6	6	6.00
		N_a	4	1	3	3	2	2	3	3	2	2	2.50
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	1.00	0.00	0.67	0.67	0.67	0.17	0.83	0.83	0.17	0.33	0.53
		H_e	0.76	0.00	0.62	0.62	0.48	0.17	0.68	0.68	0.17	0.30	0.45
		f	-0.364	-	-0.081	-0.081	-0.429	0	-0.25	-0.25	0	-0.111	-0.17
BLT	2006	*n*	25	24	25	25	25	25	25	25	25	24	24.80
		N_a	6	2	4	2	2	1	5	7	2	3	3.40
		A_R	5	3	4	3	2	1	4	6	2	2	3.24
		H_o	0.76	0.04	0.48	0.28	0.36	0.00	0.72	0.68	0.04	0.49	0.39
		H_e	0.69	0.25	0.58	0.35	0.43	0.00	0.67	0.77	0.04	0.50	0.43
		f	-0.103	0.839	0.171	0.204	0.163	-	-0.075	0.114	0	-0.013	0.14
LMD	2006	*n*	3	3	3	3	3	3	3	3	3	3	3.00
		N_a	3	1	3	2	2	1	3	3	1	2	2.10
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.67	0.00	0.67	0.33	0.00	0.00	0.67	1.00	0.00	0.33	0.37
		H_e	0.73	0.00	0.73	0.33	0.53	0.00	0.73	0.73	0.00	0.33	0.41
		f	0.111	-	0.111	0	1	-	0.111	-0.5	-	0	0.12

Appendix 3.4 continued.

MGG	2006	*n*	7	7	7	7	7	7	7	7	7	7	7.00
		N_a	4	2	3	3	3	1	3	6	1	2	2.80
		A_R	-	-	-	-	-	-	-	-	-	-	-
		H_o	0.71	0.00	1.00	0.43	0.86	0.00	0.71	0.86	0.00	0.00	0.46
		H_e	0.75	0.26	0.67	0.38	0.58	0.00	0.56	0.80	0.00	0.44	0.45
		f	0.048	1	-0.556	-0.125	-0.532	-	-0.304	-0.075	-	1	0.06

Appendix 3.5. Values of kinship (F_{ij}) (Loiselle *et al.*, 1995) for pairs of *E. viridiulum* separated by mean distance (km) categories. -95 %CI, lower 95 % confidence interval; +95 % CI, upper 95 % CI; *P* (o<e), probability that the observed value of F_{ij} is less than zero; *P* (o>e) probability that the observed value of F_{ij} is greater than zero.

2002	Distance	2.56	4.52	5.77	8.11	10.26	116.77	259.70	279.11	285.05	286.70
	F_{ij}	0.0082	*-0.0081*	*-0.0043*	0.0081	*-0.0099*	-0.0052	*-0.0134*	*-0.0170*	*-0.0172*	0.0232
	-95%CI	-0.0237	-0.0542	-0.0338	-0.0247	-0.0478	-0.0210	-0.0451	-0.0616	-0.0569	-0.0905
	+95%CI	0.0028	-0.0075	-0.0131	0.0003	-0.0164	-0.0153	-0.0225	-0.0148	-0.0289	0.0475
	P(o<e)	0.4998	0.6727	0.2089	0.3843	0.7711	0.4553	0.8591	0.4223	0.1074	0.9440
	P(o>e)	0.5007	0.3298	0.7926	0.6162	0.2304	0.5452	0.1429	0.5802	0.8956	0.0890
2004	Distance	4.20	17.40	97.01	141.78	169.27	171.68	178.66	228.36	290.98	300.76
	F_{ij}	**0.0627**	0.0341	0.0263	0.0168	*-0.0506*	*-0.0556*	*-0.0279*	*-0.0260*	*-0.0365*	*-0.0361*
	-95%CI	0.0111	-0.0073	-0.0122	-0.0256	-0.1012	-0.1148	-0.0684	-0.0676	-0.0865	-0.0822
	+95%CI	0.0576	0.0253	0.0113	0.0002	-0.0604	-0.0394	-0.0450	-0.0388	-0.0458	-0.0499
	P(o<e)	0.9985	0.9820	0.9895	0.8951	0.0285	0.0640	0.1254	0.1759	0.1404	0.0940
	P(o>e)	0.0065	0.0185	0.0110	0.1054	0.9760	0.9735	0.8756	0.8246	0.8641	0.9090
2005	Distance	7.42	20.70	78.35	110.74	146.21	160.78	173.47	184.17	240.80	294.54
	F_{ij}	**0.0422**	**0.0376**	0.0195	0.0130	*-0.0013*	*-0.0188*	*-0.0349*	*-0.0333*	*-0.0312*	*-0.0237*
	-95%CI	0.0252	0.0192	-0.0087	-0.0111	-0.0284	-0.0448	-0.0631	-0.0537	-0.0505	-0.0482
	+95%CI	0.0448	0.0325	0.0129	0.0057	-0.0056	-0.0207	-0.0419	-0.0402	-0.0412	-0.0316
	P(o<e)	0.9980	1.0000	0.9665	0.9590	0.5472	0.0990	0.0060	0.0010	0.0025	0.0280
	P(o>e)	0.0025	0.0000	0.0340	0.0415	0.4533	0.9015	0.9945	0.9995	0.9980	0.9725
2006	Distance	8.35	42.23	89.42	101.71	134.28	155.99	156.52	164.14	202.14	293.26
	F_{ij}	**0.0332**	*-0.0021*	*-0.0048*	0.0096	*0.0017*	*-0.0212*	0.0081	*-0.0156*	*-0.0176*	*-0.0051*
	-95%CI	0.0087	-0.0238	-0.0270	-0.0201	-0.0176	-0.0452	-0.0216	-0.0369	-0.0366	-0.0272
	+95%CI	0.0364	-0.0069	-0.0116	0.0399	-0.0096	-0.0251	0.0384	-0.0216	-0.0282	-0.0119
	P(o<e)	0.9765	0.2699	0.7301	0.3943	0.7526	0.0505	0.8186	0.1719	0.3893	0.1534
	P(o>e)	0.0270	0.7311	0.2719	0.6397	0.2479	0.9540	0.2169	0.8306	0.6112	0.8486
Males	Distance	7.10	22.58	83.72	110.27	146.04	159.00	170.94	181.26	235.29	293.90
	F_{ij}	0.0373	0.0236	0.0092	0.0089	0.0032	-0.0172	-0.0310	-0.0244	-0.0196	-0.0226
	-95%CI	-0.0168	-0.0156	-0.0166	-0.0212	-0.0167	-0.0256	-0.0258	-0.0203	-0.0168	-0.0229
	+95%CI	0.0212	0.0138	0.0122	0.0168	0.0106	0.0264	0.0284	0.0145	0.0077	0.0214
	P(o<e)	0.9995	0.9980	0.9500	0.9215	0.8406	0.1259	0.0060	0.0065	0.0130	0.0270
	P(o>e)	0.0010	0.0025	0.0505	0.0790	0.1599	0.8746	0.9945	0.9940	0.9875	0.9735
females	Distance	14.28	45.47	92.18	113.31	145.27	154.87	156.09	162.90	235.64	295.93
	F_{ij}	0.0259	-0.0027	0.0165	-0.0031	-0.0085	-0.0326	-0.0147	-0.0215	-0.0057	-0.0160
	-95%CI	-0.0199	-0.0169	-0.0266	-0.0233	-0.0203	-0.0564	-0.0715	-0.0235	-0.0160	-0.0280
	+95%CI	0.0152	0.0098	0.0229	0.0184	0.0148	0.0692	0.1036	0.0207	0.0088	0.0277
	P(o<e)	0.9960	0.5807	0.9500	0.5227	0.2984	0.1384	0.3403	0.0370	0.4018	0.1354
	P(o>e)	0.0045	0.4198	0.0505	0.4778	0.7021	0.8621	0.6602	0.9635	0.5987	0.8651