

Microsatellite DNA polymorphism confirms reproductive isolation and reveals differences in population genetic structure of cryptic pipistrelle bat species

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Previous studies have indicated that the common European pipistrelle bat (*Pipistrellus pipistrellus*) comprises two cryptic species, *P. pipistrellus* and *Pipistrellus pygmaeus*, which differ in echolocation call frequency and mitochondrial DNA sequence. However, levels of divergence based on nuclear markers have not been examined, and hence the potential for male-mediated gene flow between the species cannot be discounted. Moreover, little is known about population structure and migration patterns in either species. Here, we describe the use of microsatellites to investigate nuclear DNA differentiation between, and the pattern of population genetic structure within, the two cryptic pipistrelle species. In total, 1300 individuals from 82 maternity colonies were sampled across the British Isles and Continental Europe. We show, using multivariate analyses, that colonies of the same species are generally genetically more similar to each other than to those from the other species regardless of geographical location. Our findings support the hypothesis that the species are reproductively isolated. Significant patterns of genetic isolation by distance were identified in both species, indicating that mating may occur before any long-distance autumnal migration. The presence of a sea channel does not confer higher levels of genetic differentiation among colonies over and above distance alone in either species. Differences in genetic population structure were identified between the species, with *P. pipistrellus* showing a wider range of levels of genetic differentiation among colonies and a stronger relationship between genetic and geographical distance than *P. pygmaeus*. Differences in dispersal, mating behaviour, colony size and/or postglacial colonization patterns could contribute to the differences observed. © 2007 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2007, 90, 539–550.

ADDITIONAL KEYWORDS: Chiroptera – isolation by distance – microsatellite – migration.

INTRODUCTION

The common pipistrelle, *Pipistrellus pipistrellus*, is one of Europe's most abundant and well-studied bats. Despite this, the finding that it is actually two cryptic species is relatively recent. Jones & van Parijs (1993) first reported the occurrence of two phonic types in this species, with the echolocation call frequency of most energy at either 46 kHz or 55 kHz (named 45-kHz and 55-kHz phonic types, respectively). Barratt

et al. (1997) subsequently identified two highly divergent mitochondrial DNA (mtDNA) groupings (termed clades I and II), which show high levels of sequence divergence both at the cytochrome *b* (Barratt *et al.*, 1997) and the *ND1* gene (Mayer & von Helversen, 2001a). The two mtDNA clades correspond completely to the two phonic types (46-kHz bats have the clade II haplotype, whereas 55-kHz bats are clade I) and all individuals within a single colony are of the same phonic type and mtDNA clade (Jones & van Parijs, 1993; Barratt *et al.*, 1997). Jones & Barratt (1999) proposed retaining the name *P. pipistrellus* for the

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45-kHz cryptic species, and using the next available synonym, *P. pygmaeus* for the 55-kHz cryptic species. This nomenclature has now been accepted by the International Commission on Zoological Nomenclature (2003), with *P. pipistrellus mediterraneus* recognized as a junior synonym of *P. pygmaeus*.

Identification of cryptic species within the order Chiroptera is being increasingly demonstrated as the use of molecular techniques more becomes widespread and routine (Burland & Worthington Wilmer, 2001). In the majority of cases reported, genetic differentiation among putative species has been demonstrated using mtDNA markers (Barratt *et al.*, 1997; Cardinal & Christidis, 2000; Kingston *et al.*, 2000; Mayer & von Helversen, 2001a, b; Spitzenberger, Piálek & Haring, 2001; von Helversen *et al.*, 2001). However, high levels of mtDNA differentiation cannot alone prove reproductive isolation because its matrilineal mode of inheritance means male-mediated gene flow can be missed. As recently shown in a number of bat species, sex-biased dispersal can lead to high levels of mtDNA differentiation, despite high levels of male-mediated gene flow (Castella, Reudi & Excoffier, 2001; Kerth, Mayer & Petit, 2002). Thus, although behavioural studies at mating sites have identified assortative roosting of the phonic types (Park, Altringham & Jones, 1996) and differences among the two species at a single nuclear marker have been identified in a small sample of bats (Mayer & von Helversen, 2001a), male-mediated gene flow between the cryptic pipistrelle species has yet to be unambiguously discounted.

Previous surveys of the two cryptic pipistrelle species have demonstrated a sympatric distribution across much of their European ranges (Barratt *et al.*, 1997; Mayer & von Helversen, 2001a), but little is known of their respective population structure and migration patterns. Pipistrelle bat summer colonies consist of females recruited into their natal colony (Thompson, 1992), whereas males disperse from their natal colony and establish mating territories elsewhere (Gerell & Lundberg, 1985; Gerell-Lundberg & Gerell, 1994; Park *et al.*, 1996). Strelkov (1969) classified European pipistrelle bats as migratory, although migration distances are unclear. Furthermore, whether individuals mate before migration, 'en-route', at hibernation sites, or a combination, is uncertain.

In the present study, we describe the spatial distribution of microsatellite DNA variation from pipistrelle bats sampled across the British Isles and continental Europe. We determine whether differentiation at nuclear DNA loci supports the inferences of previous studies that the two cryptic species are reproductively isolated. In addition, we investigate patterns of genetic differentiation within each cryptic species to: (1) understand more comprehensively their population structure and migration patterns and (2)

determine whether behavioural and morphological differences previously identified between the two species (Barlow & Jones, 1999) are reflected in differences in their population genetic structure.

MATERIAL AND METHODS

SAMPLING PROCEDURE AND SPECIES ASSIGNMENT

Bats were caught and sampled using 3-mm wing biopsies (Worthington Wilmer & Barratt, 1996) at a total of 82 pipistrelle maternity colonies across the British Isles, Jersey and Continental Europe (Fig. 1, Appendix 1). The species of each colony was assigned from a representative number of samples from echolocation call frequency, determined by analysis of time-expanded recordings (Barlow & Jones, 1999) and/or mitochondrial mtDNA haplotype (Barratt *et al.*, 1997).

MICROSATELLITE ISOLATION AND GENOTYPING

Six polymorphic microsatellite loci (*Ppip01–06*) were isolated and characterized from pooled DNA extracted from individuals of both species using an enrichment procedure outlined in Burland, Barratt & Racey (1998) and Hammond *et al.* (1998). Polymerase chain reaction (PCR) primer sequences, PCR conditions, number of resolved alleles, allele size ranges, and observed heterozygosities are provided in Table 1. Microsatellite genotype data were generated for 1300 individuals at between four and six loci, using the procedure described by Burland *et al.* (1998).

DATA ANALYSIS

Pairwise values of genetic distance (Nei, 1978) were calculated among the 82 colonies from the multilocus genotypes generated for all 1300 individuals with the six *Pipistrellus* microsatellite loci using the program Genetic Data Analysis, version 1.0 (Lewis & Zaykin, 2001). Relationships between colonies were then visualized using multidimensional scaling, using SPSS, version 9.0 (SPSS Inc.).

Within each species, levels of genetic differentiation among colonies were determined from the parameter F_{ST} calculated in FSTAT (Goudet, 1995). Patterns of isolation by distance were examined following the recommendations of Rousset (1997), whereby correlation is examined between $F_{ST}/(1 - F_{ST})$ and the logarithm of euclidean geographical distance between colonies in kilometres (determined from latitude and longitude co-ordinates). The significance of the relationship was tested using the Mantel procedure (Manly, 1986) executed using the program SMT, version 1.2 (E. Bonnet, unpublished software; available from http://www.geocities.com/eb_ce/mantel.html).

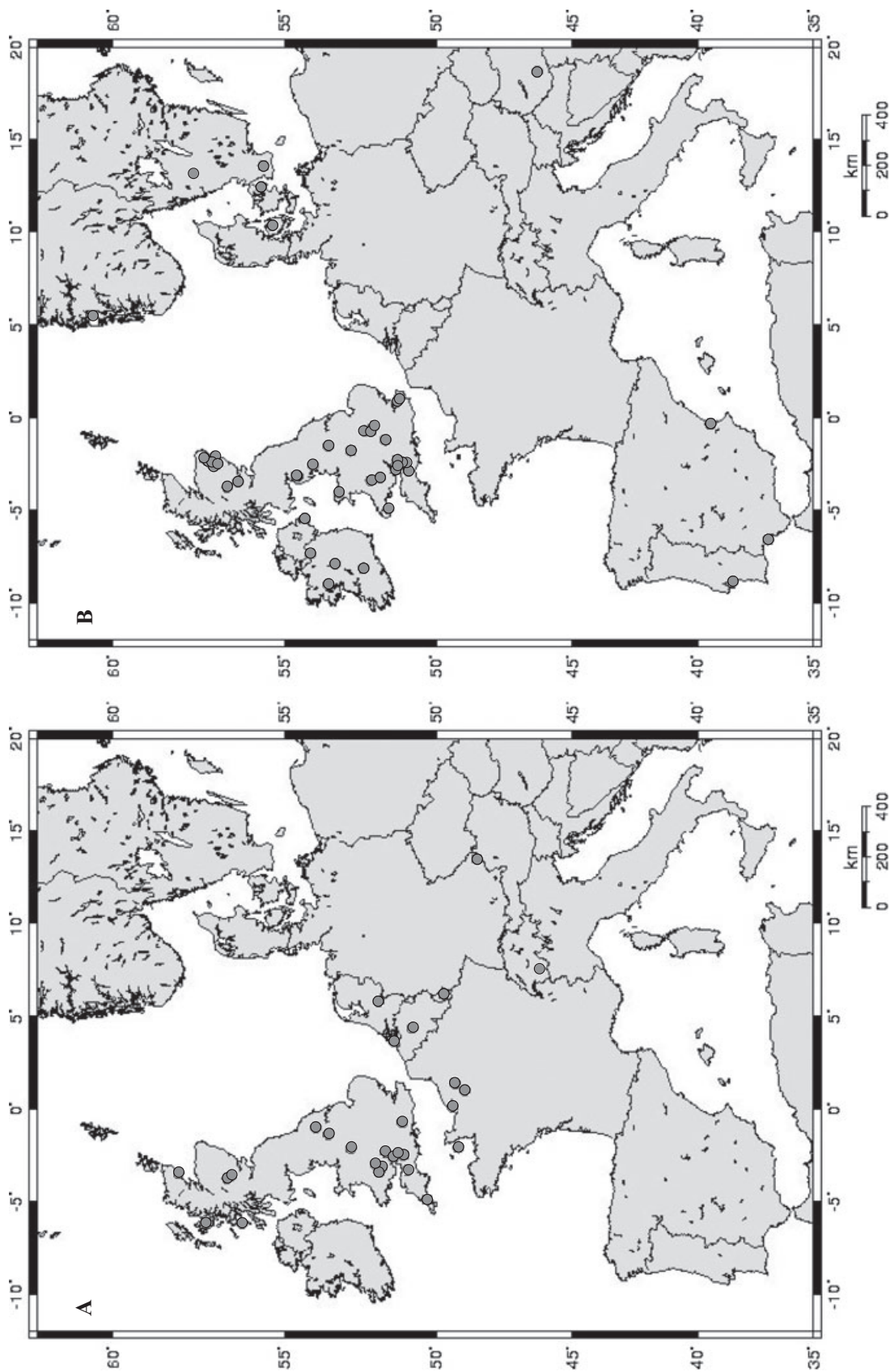


Figure 1. Distribution of (A) *Pipistrellus pipistrellus* and (B) *Pipistrellus pygmaeus* colonies sampled. The location of colonies is represented by closed circles. Colonies in close proximity are represented by a single circle. Latitude and longitude are indicated on the border of each map, with the longitude values west of zero degrees denoted as negatives. The pipistrelle species comprising each colony was determined by echolocation frequency and/or mtDNA clade (see text). Maps were prepared with Online Map creation software (http://www.aquarius-geomar.de/omc/omc_intro.html).

Table 1. Primer sequences and polymerase chain reaction conditions for microsatellite loci *Ppip01–06*

Primer name	Sequence (5'–3')	Tann (°C)	Mg ²⁺ concentration/ number of cycles	Number of alleles	Allele size range (bp)	H_{obs}	Gene diversity
<i>Ppip01-F</i>	CCCAAGGCGTAATCCCTTACC	51	1.5 mM/35	16/19	151–183/151–189	0.83/0.77	0.89/0.85
<i>Ppip01-R</i>	AAAGCGGACACAATGCGGAG						
<i>Ppip02-F</i>	ATCAGAGCCCCATTTTATC	57	1.5 mM/30	9/11	124–140/122–142	0.75/0.78	0.81/0.79
<i>Ppip02-R</i>	GTCAAATTCCTTCAACCCAC						
<i>Ppip03-F</i>	CCAAAATTTGTGAAGATGCAGAC	48	2 mM/30	10/19	189–209/107–297	0.66/0.76	0.76/0.83
<i>Ppip03-R</i>	CTCCCTCTCTCAATAATAATGAAC						
<i>Ppip04-F</i>	ATCATCTAAGAGCTGTCCCC	53	2 mM/30	15/18	205–233/201–237	0.81/0.53	0.91/0.90
<i>Ppip04-R</i>	CACCCCATGACAAAATGAAC						
<i>Ppip05-F</i>	GTTGACTCTGTTGGGAAAGGAG	48	2.5 mM/35	13/17	128–154/130–168	0.73/0.84	0.79/0.85
<i>Ppip05-R</i>	TGGGACTAAGACACAAGCCTGG						
<i>Ppip06-F</i>	TGCCCCAACCAAGTGAGCTACAG	55	2 mM/30	22/26	118–174/114–172	0.73/0.82	0.88/0.91
<i>Ppip06-R</i>	GCTCCAGTGTGACTTTTCCTCTCTC						

For each locus, the number of alleles, allele size ranges in base pairs, observed heterozygosity (H_{obs}), and gene diversity (determined using the program FSTAT) are given first for *Pipistrellus pipistrellus* followed by those for *Pipistrellus pygmaeus*. Values were determined using all the genotype data generated for each locus (for sample numbers, see Appendix 2).

Because bodies of water may represent a larger barrier to gene flow than distance alone in some bat species (Castella *et al.*, 2000; Burland & Worthington Wilmer, 2001), a partial Mantel test (Smouse, Long & Sokal, 1986) was employed to determine whether the presence of a sea channel (detailed in a further matrix as '1' = presence or '0' = absence) affects patterns of genetic differentiation among the colonies, while controlling for distance. Partial Mantel tests were performed using the program PMT version 1.1 (E. Bonnet, unpublished software; available from http://www.geocities.com/eb_ce/mantel.html). The sea channels concerned were the English Channel and the North Sea (separating colonies from the British Isles and continental Europe in both *P. pipistrellus* and *P. pygmaeus*, Fig. 1), and the Irish Sea (separating colonies between Britain and Ireland in *P. pygmaeus* only, Fig. 1).

RESULTS

Analysis of echolocation call frequency and mtDNA haplotype data revealed that a total of 32 *P. pipistrellus* and 50 *P. pygmaeus* colonies had been sampled (Appendix 1). Both species were identified in mainland Great Britain. *P. pipistrellus* samples were also obtained from France, Belgium, Luxembourg, the Netherlands, Germany, and Switzerland, whereas further *P. pygmaeus* samples came from Norway, Sweden, Denmark, Spain, Portugal, and Ireland (Fig. 1, Appendix 1).

INTER-SPECIFIC GENETIC DIFFERENTIATION

Multi-dimensional scaling of the 82 colonies, following calculation of pairwise Nei's genetic distances using six *Pipistrellus* loci, demonstrated a clear separation of colonies of each species (Fig. 2). Allele frequencies for each species are given for all loci in Appendix 2.

INTRA-SPECIFIC GENETIC DIFFERENTIATION

Levels of genetic differentiation among colonies within species were investigated at two geographical scales: (1) using colonies sampled only in mainland Great Britain and (2) across all sampled colonies. Both the mean F_{ST} and range of pairwise F_{ST} values identified were low in the two species (Table 2), although *P. pipistrellus* showed higher mean values and a wider range of pairwise F_{ST} values than *P. pygmaeus* at both geographical scales. This was most pronounced at the larger geographical scale, where both the mean and maximum F_{ST} values for *P. pipistrellus* were approximately double those for *P. pygmaeus*, despite the wider geographical sampling of *P. pygmaeus* (Fig. 1, Table 2).

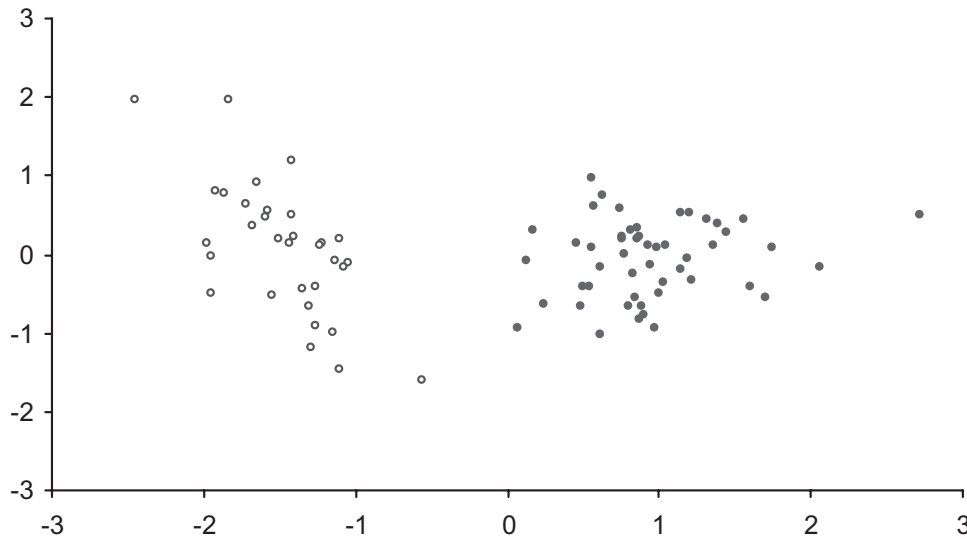


Figure 2. Multi-dimensional scaling plot of Nei's genetic distance for the 82 pipistrelle colonies. Open circles represent colonies classified as *Pipistrellus pipistrellus*, closed circles represent colonies classified as *Pipistrellus pygmaeus*. Species was assigned using echolocation and/or mitochondrial DNA.

Table 2. Mean \pm standard error (SE) F_{ST} and range of pairwise F_{ST} estimates among colonies in mainland Great Britain (GB) and among all sampled colonies (All)

	<i>Pipistrellus pipistrellus</i> (GB)	<i>Pipistrellus pygmaeus</i> (GB)	<i>Pipistrellus pipistrellus</i> (All)	<i>Pipistrellus pygmaeus</i> (All)
Number of colonies sampled	20	34	32	50
Mean \pm SE F_{ST}	0.029 \pm 0.004	0.023 \pm 0.008	0.044 \pm 0.007	0.024 \pm 0.007
Pairwise F_{ST} range	< 0.001–0.116	< 0.001–0.084	< 0.001–0.238	< 0.001–0.105
Pairwise distance range (km)	1.5–794	1.5–693	< 1–2069	< 1–2829

The range of pairwise geographical distances among colonies are also given. Removal of the colony 'Basdescharri' did not affect the mean F_{ST} value returned for *P. pipistrellus* (see text).

The distribution of pairwise distance vs. pairwise F_{ST} values are shown for both species at both geographical scales in Figure 3. Before determining whether a significant pattern of isolation by distance was present, a partial Mantel test was performed to investigate the relationship between $F_{ST}/1 - F_{ST}$ and the presence of a sea channel, while controlling for the effects of geographical distance. Although a nonsignificant correlation was observed in *P. pygmaeus* ($r = -0.06$, $P = 0.14$), this was not the case in *P. pipistrellus* ($r = 0.19$, $P < 0.05$). However, the result obtained for *P. pipistrellus* was heavily biased by a single colony sampled on the island of Jersey (colony 'Basdescharri'; Appendix 1). Of the seven apparently out-lying points on the distribution of pairwise $F_{ST}/1 - F_{ST}$ estimates (Fig. 3C), six resulted from pairwise comparisons between this colony and either British or French col-

onies. Removal of this colony resulted in a nonsignificant correlation between $F_{ST}/1 - F_{ST}$ and sea channel in *P. pipistrellus* ($r = 0.05$, $P = 0.27$), but did not affect the mean F_{ST} value (0.44).

Because the presence of a sea channel was regarded as having little or no effect over and above distance in either species, a standard Mantel test was used at both geographical scales (excluding *P. pipistrellus* colony 'Basdescharri' Jersey). Significant patterns were identified in both species at both scales (Fig. 3), although the slope of the relationship between geographical distance and genetic differentiation was significantly greater in *P. pipistrellus* than *P. pygmaeus* at both geographical scales [Students's t -test (Zar, 1996): Great Britain, $t = 11.39$, $\nu = 747$, $P < 0.001$; all sampled colonies, $t = 14.16$, $\nu = 1686$, $P < 0.001$].

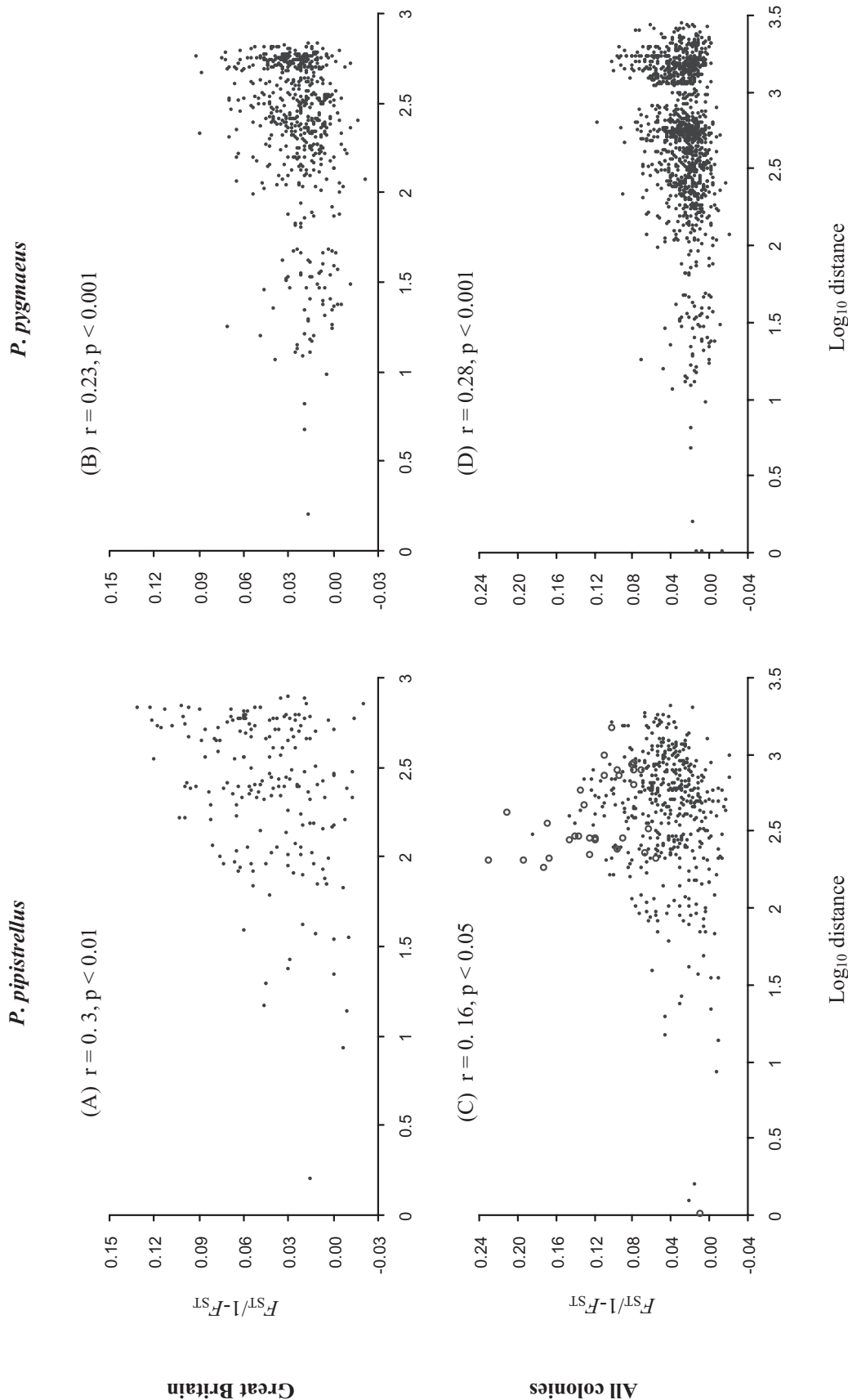


Figure 3. Plot of \log_{10} distance versus $F_{ST}/1 - F_{ST}$ for comparisons among (A) *Pipistrellus pipistrellus* colonies within Great Britain; (B) *Pipistrellus pygmaeus* colonies within Great Britain; (C) all *P. pipistrellus* colonies; (D) all *P. pygmaeus* colonies. For each, the correlation coefficient and significance of the relationship obtained using a standard Mantel test is given. Pairwise comparisons with colony Badescharri (Jersey) are shown as open circles in (C); the correlation coefficient and significance values given are for tests excluding this colony.

DISCUSSION

NUCLEAR DNA DIFFERENTIATION BETWEEN CRYPTIC PIPISTRELLE SPECIES

This comprehensive study, incorporating colonies from across the British Isles and continental Western Europe, has demonstrated that the high levels of genetic differentiation between the two cryptic pipistrelle species previously identified for mtDNA are also apparent in nuclear DNA. Across the sampled range, colonies from the same species were genetically more similar to each other than to those from the other species (Fig. 2). Thus, data from both nuclear and mtDNA markers, coupled with identification of differences in echolocation call (Jones & von Parijs, 1993), the structure of (Barlow & Jones, 1997a) and responses to (Barlow & Jones, 1997b) social calls, differences in skull morphology (Barlow, Jones & Barratt, 1997), habitat use (Vaughan, Jones & Harris, 1997), diet (Barlow, 1997), roost size, and wing morphology (Barlow & Jones, 1999), all strongly support the proposal that the two phonic types represent two reproductively isolated species.

Samples identified as *P. pipistrellus* came from central Europe and mainland Great Britain, whereas *P. pygmaeus* samples originated from northern, southern, and western Europe. Although both species are known to be sympatric over much of Europe, the distribution of species identified in the present study (Fig. 1) supports the hypothesis that *P. pygmaeus* is the dominant species around the edge of continental Europe, with *P. pipistrellus* being more abundant in central Europe (Jones & Parijs, 1993; Mayer & von Helversen, 2001a).

INTRA-SPECIFIC POPULATION GENETIC STRUCTURE IN PIPISTRELLE BATS

Previous genetic studies of pipistrelle bats across Europe (Barratt *et al.*, 1997; Mayer & von Helversen, 2001a) have provided little information on the degree of genetic structure within each cryptic species. The present study therefore represents the first assessment of population genetic structure within two of Europe's most common bat species. The patterns of nuclear genetic differentiation identified suggest that, for both species, the European population cannot be regarded as panmictic. Instead, both species displayed significant genetic isolation by distance across the sampled range.

Little is known of the migration patterns of pipistrelle bats and most available data is confounded by the fact that it was collected before identification of the two cryptic species. Strelkov (1969) reported migration distances between summer colonies and hibernacula of over 1000 km in continental Europe.

By contrast, the maximum recorded migration in Great Britain is just 69 km (Avery, 1991). Although the genetic data from the present study provide little information on winter migration distances *per se*, they do suggest that, despite the classification of *Pipistrellus* as a migratory genus (Strelkov, 1969), some distance boundaries to gene flow exist in the two cryptic pipistrelle species. Thus, if long-distance autumnal and spring migrations do occur, the majority of mating may take place prior to this. Such inferences are consistent with the formation of mating groups in the late summer (August and September) in both species (Park *et al.*, 1996), prior to the migration season.

It is informative to compare these findings to those from the migratory noctule bat (*Nyctalus noctula*) in Europe. Males of this species disperse long distances from their natal colony, setting up mating territories along females' migration routes (Petit & Mayer, 1999; Petit, Balloux & Goudet, 2001). *Nyctalus noctula* exhibits extremely low levels of colony genetic differentiation over distances of up to 4000 km (pairwise F_{ST} range < 0.001–0.03), whereas genetic isolation by distance across Europe is not supported (Petit & Mayer, 1999). Moreover, genetic isolation by distance has yet to be identified in any migratory bat species (Burland & Worthington Wilmer, 2001). It may be that winter migration distances are therefore significantly less in pipistrelles than other migratory species. However, it is also possible that differences in the timing of mating (pipistrelles before migration, other species during or after migration), or in dispersal distances of males from their natal colony, could also explain our finding of isolation by distance in the *Pipistrellus* species.

The presence of a sea channel was found to exert little or no effect over and above that of distance in either species, suggesting individuals could undergo sea crossings. This supports the study by Ahlen (1997) who found evidence for the crossing of sea channels by pipistrelle bats, explaining the capture of three *P. pipistrellus* bats on North Sea oil installations (identified using DNA analysis; F. Marshall and P. A. Racey, unpublished data). Given that rabies-type lyssaviruses occur in pipistrelles in continental Europe (Schneider & Cox, 1992), the finding that pipistrelle bats may cross sea channels suggests the potential for transmission of lyssaviruses into islands such as Britain.

The observation that sea crossings do not pose a barrier to gene flow is in contrast to a study of the mouse eared bat (*Myotis myotis*) in Spain and Morocco, which showed that genetic differentiation among populations on either side of the 14 km Gibraltar strait is at a level that these populations probably represent a different species (Castella

et al., 2000). *Myotis myotis* has a wing shape that is more adapted to high manoeuvrability than pipistrelle bats, whose wing shapes are instead more suited for longer distance flight (Norberg & Rayner, 1987).

DIFFERENCES IN POPULATION GENETIC STRUCTURE BETWEEN CRYPTIC PIPISTRELLE SPECIES

The present study has identified differences in population genetic structure between the two cryptic pipistrelle species. At both geographical scales, *P. pipistrellus* exhibited a wider range of pairwise F_{ST} values and a stronger relationship between genetic and geographical distance than *P. pygmaeus*, despite the wider geographical sampling in *P. pygmaeus* (Table 2, Fig. 3). In addition, *P. pygmaeus* displayed a consistently greater number of alleles, although heterozygosity and gene diversity were similar in both species (Table 1). The patterns observed are likely to be influenced both by historic phylogeographical and contemporary processes and, to a lesser extent, possibly by differences in mutation and inherent levels of variation in the microsatellite loci. Hulva *et al.* (2004) resolved patterns of phylogeographical structure consistent with long-term allopatry between the phonic types in the eastern and western Mediterranean, with subsequent expansion occurring within the Holocene. Differences in long-term population size and the rate and scale of recolonization will define extant levels of diversity. Moreover, contemporary differences in morphology and behaviour between the two species may also influence the patterns of genetic structure observed. Jones & van Parijs (1993) identified a lower wing aspect ratio in *P. pipistrellus*, whereas Barlow & Jones (1999) suggested that flight is slower in this species. Both these findings suggest *P. pipistrellus* may be the more sedentary species (*sensu* Entwistle, Racey & Speakman, 2000), and that the distance travelled to mating sites by females and/or male natal dispersal distances could be lower in this species. Differences could also reflect the timing of mating in the two species, or the differential growth of vaginal plugs in the two *Pipistrellus* species (Racey, 1979). In addition, *P. pipistrellus* forms significantly smaller colonies than *P. pygmaeus* (median colony counts of 76 and 203, respectively, Barlow & Jones, 1999). It is therefore possible that genetic differentiation among colonies in this species is more affected by stochastic events and genetic drift. Indeed, the Jersey colony, which appeared to be particularly differentiated from other colonies, may suggest a founder effect. Further investigation into differences in colony dynamics, mating patterns, and migration distances of these two species is now required.

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APPENDIX 1

PIPISTRELLE COLONIES INCLUDED IN THE STUDY

Country	Colony name	Latitude/ longitude	Echolocation frequency	mtDNA clade	Number of individuals <i>Ppip01-06</i>	Species assigned	
England	Barrow Gurney	51°25'N 2°41'W	53.2 ± 1.6	I	34	<i>Pipistrellus pygmaeus</i>	
	Braithwaite	54°36'N 3°11'W		I	16	<i>Pipistrellus pygmaeus</i>	
	Bretton	53°38'N 1°34'W	52.4 ± 1.7		19	<i>Pipistrellus pygmaeus</i>	
	Bromham	52°9'N 0°32'W	53.6 ± 0.6		11	<i>Pipistrellus pygmaeus</i>	
	Cambridge (Glous.)	51°44'N 2°22'W	45.0 ± 1.8		10	<i>Pipistrellus pipistrellus</i>	
	Castle Cary	51°6'N 2°31'W	53.6 ± 1.7		20	<i>Pipistrellus pygmaeus</i>	
	Conger Kent	51°21'N 0°49'E	55		12	<i>Pipistrellus pygmaeus</i>	
	Ditcheat	51°7'N 2°31'W	44.5 ± 0.9		10	<i>Pipistrellus pipistrellus</i>	
	Doveridge	52°54'N 1°49'W	52.0 ± 1.4		20	<i>Pipistrellus pygmaeus</i>	
	Earswick	54°1'N 1°3'W	44.9 ± 1.5		20	<i>Pipistrellus pipistrellus</i>	
	Frensham	51°10'N 0°46'W	45.1 ± 1.6		14	<i>Pipistrellus pipistrellus</i>	
	Grendon	52°14'N 0°43'W	57.2 ± 2.3	I	8	<i>Pipistrellus pygmaeus</i>	
	Haigh	53°37'N 1°39'W		II	9	<i>Pipistrellus pipistrellus</i>	
	Limpley Stoke	51°20'N 2°19'W	57.6 ± 1.6	I	17	<i>Pipistrellus pygmaeus</i>	
	Newton	52°5'N 3°1'W	44.3 ± 1.7		9	<i>Pipistrellus pipistrellus</i>	
	Priston	51°20'N 2°26'W	45.2 ± 1.0		8	<i>Pipistrellus pipistrellus</i>	
	Puckington	50°58'N 2°53'W	53.4 ± 1.3		10	<i>Pipistrellus pygmaeus</i>	
	Ravenstone	54°39'N 3°11'W		I	14	<i>Pipistrellus pygmaeus</i>	
	Risinghurst	51°45'N 1°13'W		I	19	<i>Pipistrellus pygmaeus</i>	
	Rushton	52°26'N 0°46'W	56.2 ± 1.7	I	11	<i>Pipistrellus pygmaeus</i>	
	Sharpham	51°10'N 2°46'W	57.0 ± 2.2	I	8	<i>Pipistrellus pygmaeus</i>	
	Stoke Bishop	51°29'N 2°38'W	46.1 ± 1.5	II	10	<i>Pipistrellus pipistrellus</i>	
	Stone	52°54'N 2°9'W	44.9 ± 1.2		11	<i>Pipistrellus pipistrellus</i>	
	Tracebridge	51°0'N 3°20'W	46.1 ± 1.5		10	<i>Pipistrellus pipistrellus</i>	
	Trendal	50°20'N 4°55'W	45.3 ± 2.1		20	<i>Pipistrellus pipistrellus</i>	
	Trenowth	50°19'N 4°55'W	45.2 ± 0.7		20	<i>Pipistrellus pipistrellus</i>	
	Ubley	51°19'N 2°40'W	54.2 ± 2.3	I	67	<i>Pipistrellus pygmaeus</i>	
	Waterham	51°18'N 0°58'E	51.9 ± 1.3		35	<i>Pipistrellus pygmaeus</i>	
	Winsley	51°21'N 2°19'W	53.4 ± 1.7		9	<i>Pipistrellus pygmaeus</i>	
	Wray	54°7'N 2°36'W	55	I	12	<i>Pipistrellus pygmaeus</i>	
	Yatton	51°24'N 2°49'W	55.6 ± 2.0	I	10	<i>Pipistrellus pygmaeus</i>	
	Scotland	Ballinluig	56°39'N 3°40'W	45		16	<i>Pipistrellus pipistrellus</i>
		Banchory	57°3'N 2°30'W	53.3 ± 1.87		24	<i>Pipistrellus pygmaeus</i>
Battleby		56°27'N 3°29'W	55		15	<i>Pipistrellus pygmaeus</i>	
Mull		56°19'N 6°14'W		II	4	<i>Pipistrellus pipistrellus</i>	
Keithfield		57°26'N 2°13'W		I	22	<i>Pipistrellus pygmaeus</i>	
Kemnay		57°14'N 2°27'W	55		24	<i>Pipistrellus pygmaeus</i>	
Killiecrankie I		56°45'N 3°46'W		I	9	<i>Pipistrellus pygmaeus</i>	
Killiecrankie II		56°45'N 3°46'W	46.7 ± 1.9		15	<i>Pipistrellus pipistrellus</i>	
Kinellar		57°12'N 2°18'W	55		15	<i>Pipistrellus pygmaeus</i>	
Latheron		58°11'N 3°30'W	45.6 ± 1.3		20	<i>Pipistrellus pipistrellus</i>	
Monymusk		57°13'N 2°31'W	55	I	24	<i>Pipistrellus pygmaeus</i>	
Pitmedden		57°7'N 2°36'W	53.2 ± 2.3	I	33	<i>Pipistrellus pygmaeus</i>	
Skye		57°24'N 6°12'W	45		10	<i>Pipistrellus pipistrellus</i>	
Aberdeen 1		57°8'N 2°10'W		I	16	<i>Pipistrellus pygmaeus</i>	
Aberdeen 2		57°20'N 2°19'W		I	21	<i>Pipistrellus pygmaeus</i>	

APPENDIX 1 *Continued*

Country	Colony name	Latitude/ longitude	Echolocation frequency	mtDNA clade	Number of individuals <i>Ppip01–06</i>	Species assigned
Wales	Beaumaris	53°16'N 4°5'W	52.6 ± 0.9		9	<i>Pipistrellus pygmaeus</i>
	Bettws	51°52'N 3°19'W	45		4	<i>Pipistrellus pipistrellus</i>
	Bwlch	51°54'N 3°14'W	45.2 ± 1.2		10	<i>Pipistrellus pipistrellus</i>
	Dale	51°43'N 5°11'W	54.1 ± 3.1		21	<i>Pipistrellus pygmaeus</i>
	Disserth	52°13'N 3°25'W		I	6	<i>Pipistrellus pygmaeus</i>
	Llangors	51°56'N 3°16'W	53.0 ± 2.0		12	<i>Pipistrellus pygmaeus</i>
	Llanspyddid	51°57'N 3°26'W	45.0 ± 1.0		10	<i>Pipistrellus pipistrellus</i>
	Orielton	51°39'N 4°57'W	53.7 ± 1.3		10	<i>Pipistrellus pygmaeus</i>
N. Ireland	Castle Ward	54°22'N 5°30'W		I	23	<i>Pipistrellus pygmaeus</i>
	Crom Estate	54°12'N 7°23'W		I	24	<i>Pipistrellus pygmaeus</i>
Eire	Athlone	53°25'N 7°56'W		I	18	<i>Pipistrellus pygmaeus</i>
	Galway	53°16'N 9°3'W		I	6	<i>Pipistrellus pygmaeus</i>
Jersey	Tipperary	52°29'N 8°10'W		I	9	<i>Pipistrellus pygmaeus</i>
	Basdescharri	49°13'N 2°7'W		II	9	<i>Pipistrellus pipistrellus</i>
Belgium	Jersey 2	49°13'N 2°7'W		II	10	<i>Pipistrellus pipistrellus</i>
	Brussels	50°50'N 4°20'E		II	24	<i>Pipistrellus pipistrellus</i>
Denmark	Copenhagen	55°45'N 12°25'E		I	24	<i>Pipistrellus pygmaeus</i>
	Odense	55°24'N 10°23'E		I	14	<i>Pipistrellus pygmaeus</i>
France	Chars	49°0'N 0°59'E		II	12	<i>Pipistrellus pipistrellus</i>
	Croisy	49°21'N 1°21'E		II	6	<i>Pipistrellus pipistrellus</i>
	Rouen 1	49°26'N 0°5'E		II	8	<i>Pipistrellus pipistrellus</i>
	Rouen 2	49°26'N 0°5'E		II	4	<i>Pipistrellus pipistrellus</i>
Germany	Passau	48°35'N 13°28'E		II	10	<i>Pipistrellus pipistrellus</i>
Holland	Doorwerth	51°59'N 5°48'E		II	28	<i>Pipistrellus pipistrellus</i>
	Ritthem	51°27'N 3°38'E		II	20	<i>Pipistrellus pipistrellus</i>
Hungary	Szekszard	46°21'N 18°41'E		I	28	<i>Pipistrellus pygmaeus</i>
Luxembourg	Sanem	49°45'N 6°10'E	45.8 ± 1.2	II	12	<i>Pipistrellus pipistrellus</i>
Norway	Askoy	60°30'N 5°9'E	54.5 ± 1.8		24	<i>Pipistrellus pygmaeus</i>
Portugal	Setubal	38°32'N 8°54'W		I	24	<i>Pipistrellus pygmaeus</i>
	Donana	37°0'N 6°38'W	50.5 ± 1.3	I	9	<i>Pipistrellus pygmaeus</i>
Spain	Valencia 1	39°28'N 0°22'W	51.4 ± 1.9		10	<i>Pipistrellus pygmaeus</i>
	Valencia 2	39°28'N 0°22'W	51.6 ± 2.4		10	<i>Pipistrellus pygmaeus</i>
	Valencia 3	39°28'N 0°22'W	51.7 ± 2.5		10	<i>Pipistrellus pygmaeus</i>
Sweden	Lund	55°40'N 13°34'E		I	23	<i>Pipistrellus pygmaeus</i>
	Ulricehamn	57°47'N 13°25'E	53.6 ± 1.8		24	<i>Pipistrellus pygmaeus</i>
Switzerland	Valais	46°8'N 7°38'E		II	24	<i>Pipistrellus pipistrellus</i>

APPENDIX 2

ALLELE FREQUENCIES OBSERVED AT EACH LOCUS.

For each locus, the frequency of each allele is given first for *Pipistrellus pipistrellus* followed by that for *Pipistrellus pygmaeus*

<i>Ppip01</i>	<i>Ppip02</i>	<i>Ppip03</i>	<i>Ppip04</i>	<i>Ppip05</i>	<i>Ppip06</i>																
151	0.005	0.021	122	0	0.001	187	0	0.001	201	0	0.001	128	0.003	0	0.003	0	0.003	114	0	0.001	0.001
153	0.032	0.008	124	0.008	0.023	189	0.001	0.001	203	0	0.005	130	0.055	0.003	0.055	0.003	0.003	118	0.008	0.009	0.009
155	0.003	0.007	126	0.028	0.026	191	0	0.036	205	0.005	0.076	132	0.016	0.004	0.016	0.004	0.006	120	0.006	0.017	0.017
157	0	0.003	128	0.069	0.156	193	0.015	0.193	207	0.017	0.027	134	0.007	0.004	0.007	0.004	0.044	122	0.044	0.035	0.035
159	0.036	0.013	130	0.088	0.306	195	0.040	0.227	209	0.026	0.024	136	0.019	0.020	0.019	0.020	0.011	124	0.011	0.053	0.053
161	0.138	0.012	132	0.267	0.247	197	0.096	0.225	211	0.031	0.062	138	0.075	0.158	0.075	0.158	0.001	126	0.001	0.052	0.052
163	0.066	0.021	134	0.276	0.151	199	0.345	0.173	213	0.051	0.105	140	0.395	0.246	0.395	0.246	0.053	128	0.053	0.073	0.073
165	0.107	0.038	136	0.111	0.054	201	0.299	0.048	215	0.086	0.099	142	0.065	0.101	0.065	0.101	0.017	130	0.017	0.097	0.097
167	0.138	0.042	138	0.125	0.024	203	0.124	0.026	217	0.074	0.161	144	0.135	0.078	0.135	0.078	0.011	132	0.011	0.134	0.134
169	0.138	0.091	140	0.028	0.012	205	0.056	0.012	219	0.048	0.137	146	0.095	0.171	0.095	0.171	0.040	134	0.040	0.154	0.154
171	0.127	0.169	142	0	0.001	207	0.020	0.015	221	0.056	0.123	148	0.128	0.109	0.128	0.109	0.060	136	0.060	0.106	0.106
173	0.139	0.260	<i>N</i>	375	900	209	0.004	0.018	223	0.116	0.103	150	0.008	0.036	0.008	0.036	0.227	138	0.227	0.087	0.087
175	0.029	0.201				211	0	0.006	225	0.132	0.043	152	0	0.033	0	0.033	0.163	140	0.163	0.051	0.051
177	0.007	0.058				213	0	0.002	227	0.105	0.017	154	0.001	0.031	0.001	0.031	0.140	142	0.140	0.043	0.043
179	0.001	0.020				215	0	0.004	229	0.140	0.010	156	0	0.003	0	0.003	0.091	144	0.091	0.020	0.020
181	0.028	0.023				217	0	0.002	231	0.095	0.005	158	0	0.001	0	0.001	0.061	146	0.061	0.012	0.012
183	0.007	0.008				219	0	0.009	233	0.017	0.002	160	0	0.001	0	0.001	0.028	148	0.028	0.006	0.006
185	0	0.002				223	0	0.001	237	0	0.001	168	0	0.001	0	0.001	0.010	150	0.010	0.007	0.007
189	0	0.001				227	0	0.001	<i>N</i>	372	877	<i>N</i>	375	897	375	897	0.008	152	0.008	0.008	0.008
<i>N</i>	374	885				<i>N</i>	375	905										154	0	0.017	0.017
																		156	0	0.012	0.012
																		158	0	0.001	0.001
																		160	0	0.001	0.001
																		162	0	0.002	0.002
																		166	0	0.001	0.001
																		168	0.001	0	0
																		170	0.001	0	0
																		172	0.001	0.001	0.001
																		174	0.017	0	0
																		<i>N</i>	361	869	869

The number of individuals (*N*) typed for each species at each locus is given below the respective frequency.