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Diversity, genetic structure and evidence of outcrossing in British populations of the rock fern *Adiantum capillus-veneris* using microsatellites

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Abstract

Microsatellites were isolated and a marker system was developed in the fern *Adiantum capillus-veneris*. Polymorphic markers were then used to study the genetic diversity and structure of populations within the UK and Ireland where this species grows at the northern edge of its range, requiring a specific rock habitat and limited to a few scattered populations. Three dinucleotide loci detected a high level of diversity (23 alleles and 28 multilocus genotypes) across the UK and Ireland, with nearly all variation partitioned among rather than within populations. Of 17 populations represented by multiple samples, all except four were monomorphic. Heterozygosity was detected in three populations, all within Glamorgan, Wales (UK), showing evidence of outcrossing. We make inferences on the factors determining the observed levels and patterns of genetic variation and the possible evolutionary history of the populations.

Keywords: *Adiantum capillus-veneris*, fern, microsatellite, population structure

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Introduction

Adiantum capillus-veneris, the Maidenhair Fern, is a homosporous pteridophyte of wide global range achieving greatest abundance in the Mediterranean, which reaches its northern limit in the UK and Ireland (Jalas & Suominen 1972). The sexual life cycle of *A. capillus-veneris* is typical of homosporous pteridophytes, involving two free-living life forms or generations, the gametophyte, which is haploid, and the sporophyte, which is diploid. The sporophyte (2n) produces spores via meiosis which develop into gametophytes (n). Gametophytes then form gametes via mitosis. Fusion of egg and sperm results in a zygote, which develops into the next sporophyte generation. In homosporous pteridophytes zygotes may arise as a result of three potential fertilization events (Lloyd 1974; Klekowski 1979): (i) intra-gametophytic selfing: the fusion of sperm and egg from the same gametophyte resulting in a homozygote; (ii) inter-gametophytic selfing: the fusion of sperm and egg from different gametophytes derived from spores from the same parental sporophyte; and (iii) outcrossing: the fusion of sperm and egg from gametophytes derived from spores of different sporophytes. The sporophyte generation, as in all pteridophytes, is the dominant 'leafy' generation and is well known in this species due to its horticultural appeal. In contrast, the gametophyte generation is a minute structure, often near microscopic and rarely seen. This species also has the ability to spread vegetatively by means of a creeping, branching rhizome in the sporophyte generation.

In contrast to its more widespread distribution in Europe (Jalas & Suominen 1972) *A. capillus-veneris* is nationally scarce in the UK and Ireland where it only occurs in crevices or on tufa deposits of damp limestone rock and is limited to a few scattered coastal localities (Stewart *et al*. 1994). This species has also naturalized in a number of localities around the UK and Ireland on man-made substrates, such as the mortar in walls and railway bridges. The levels of genetic diversity, the genetic structure of the natural populations, and the breeding systems operating to maintain them are unknown. Nor is anything known of the origins of the

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naturalized populations, which may have arisen through spread from natural spore sources or from non-native cultivated stock. The popularity of this species amongst horticulturists has led to its widespread cultivation in the past and collection from natural sites within the British Isles (Page 1982).

Compared to seed plants, relatively few studies of levels and distribution of genetic variation within and between populations have been published in pteridophytes, and those have mostly investigated woodland species, with few studies of rock/xeric pteridophytes. In seed plants factors considered to be important in determining genetic structure in populations include mating system, gene flow, selection pressure, mutation, genetic drift, intraspecific phylogeography, evolutionary history and life history attributes, and physical features of the habitat itself (Stebbins 1957; Levin & Kerster 1974; Jain 1976; Brown 1979; Loveless & Hamrick 1984; Hamrick & Godt 1989). In pteridophytes, the determinants of genetic structure and their relative importance are still little understood.

The mating system has been reported to be an important determinant of the genetic structure of plant populations (Stebbins 1957; Brown 1979; Loveless & Hamrick 1984; Hamrick & Godt 1989). Despite their potential for inbreeding, the majority of pteridophyte species studied so far have been reported to be outcrossing, e.g. *Pellaea andromedifolia* (Gastony & Gottlieb 1985) and *Pteridium aquilinum* (Wolf *et al*. 1988). A smaller proportion of species are primarily inbreeding, e.g. *Botrychium dissectum* (McCauley *et al*. 1985) and *B. virginianum* (Soltis & Soltis 1986), and only a few species have shown evidence of a mixed mating system, e.g. *Dryopteris expansa* (Soltis & Soltis 1987a); *Blechnum spicant* (Soltis & Soltis 1988) and *Hemionitis palmata* (Ranker 1992).

Studies of seed plants have led to the generalization that outcrossing species maintain most genetic variability within rather than among populations, whereas inbreeding species partition most variability among populations (Brown 1979; Hamrick *et al*. 1979; Gottlieb 1981). This hypothesis has been upheld in some pteridophyte populations, e.g. populations of *Blechnum spicant* (Soltis & Soltis 1988) and *Polystichum otomasui* (Maki & Asada 1998), but not in others, e.g. populations of *Equisetum arvense* and *E. hyemale* (Korpelainen & Kolkkala 1996), *Sadleria pallida* and *S. cyatheoides* (Ranker *et al*. 1996) and *H. palmata* (Ranker 1992), which show high interpopulation divergence and an outcrossing mating system. In these populations, factors other than mating system have been suggested to be important in determining genetic structure. The high level of interpopulation divergence in *E. arvense* and *E. hyemale* was explained in terms of the life history of *Equisetum*, i.e. the inefficiency of spore germination and gametophyte reproduction in non-colonizing situations (Korpelainen & Kolkkala 1996). In *H. palmata* it was accounted for by the ability of this species to inbreed and by founder effects (Ranker 1992), and in *S. pallida* and *S. cyatheoides* it was attributed to spatial heterogeneity of habitat (Ranker *et al*. 1996).

Another generalization based on data from seed plants predicts that in outcrossing populations genotypes are randomly distributed whereas inbreeding populations exhibit genetic structure, i.e. nonrandom distribution of genotypes in space (Loveless & Hamrick 1984). Again this hypothesis has been shown to hold for some pteridophytes, e.g. *P. munitum* (Soltis & Soltis 1987b) and *Blechnum spicant* (Soltis & Soltis 1988). However, the rock fern *Cheilanthes gracillima* has shown both a highly outcrossing mating system and substantial genetic structuring within populations (Soltis *et al*. 1989). Such structuring was attributed to patchiness of suitable habitat as well as availability of 'safe sites' for germination (Cousens *et al*. 1988) which may further limit exploitable habitat in established populations.

In the light of their results for *C. gracillima* Soltis *et al*. (1989) hypothesized that populations of xeric or rock dwelling pteridophytes may be more likely to exhibit genetic structuring than pteridophytes of mesic environments due to patchiness of suitable habitats. More than a decade has passed since their study, yet only a handful of population studies have examined rock-dwelling pteridophytes (e.g. Holderegger & Schneller 1994; Schneller & Holderegger 1996; Vogel *et al*. 1999; Suter *et al*. 2000). Interestingly, all investigations to date have shown evidence of genetic structuring, either within or between populations, in support of the Soltis *et al*. (1989) hypothesis.

Apart from a few recent studies using random amplified polymorphic DNA (RAPD) markers (e.g. Korpelainen 1996; Schneller *et al*. 1998) and restriction fragment length polymorphisms (RFLPs) of amplified fragments of the chloroplast *trn*L_{UAA} intron (Ji *et al*. 1994; Rumsey *et al*. 1996, 1998) virtually all population genetic studies in pteridophytes have used allozymes. This technique has been invaluable in advancing our understanding of pteridophyte population genetics (reviewed by Werth 1989), however, it has the disadvantage that it does not always detect sufficient variation within a species to enable study of genetic variation at the population level (Murphy *et al*. 1996 and F.J. Rumsey unpublished data).

Microsatellite markers offer an alternative to allozymes for pteridophyte population studies. They have the advantage that their interpretation is comparatively straightforward (Bruford & Wayne 1993) and their greater sensitivity compared to allozymes in detecting polymorphism has been well documented (e.g. Chase *et al*. 1996; Degen *et al*. 1999). Microsatellites have been isolated and markers developed in a range of plant taxa including angiosperms (e.g. Dayanandan *et al*. 1999; Reusch *et al*. 1999; Rossetto *et al*. 1999), gymnosperms (Echt *et al*. 1996; Pfeiffer *et al*. 1997) and algae (Luo *et al*. 1999), however, to date there are no published reports of microsatellite markers in pteridophytes.

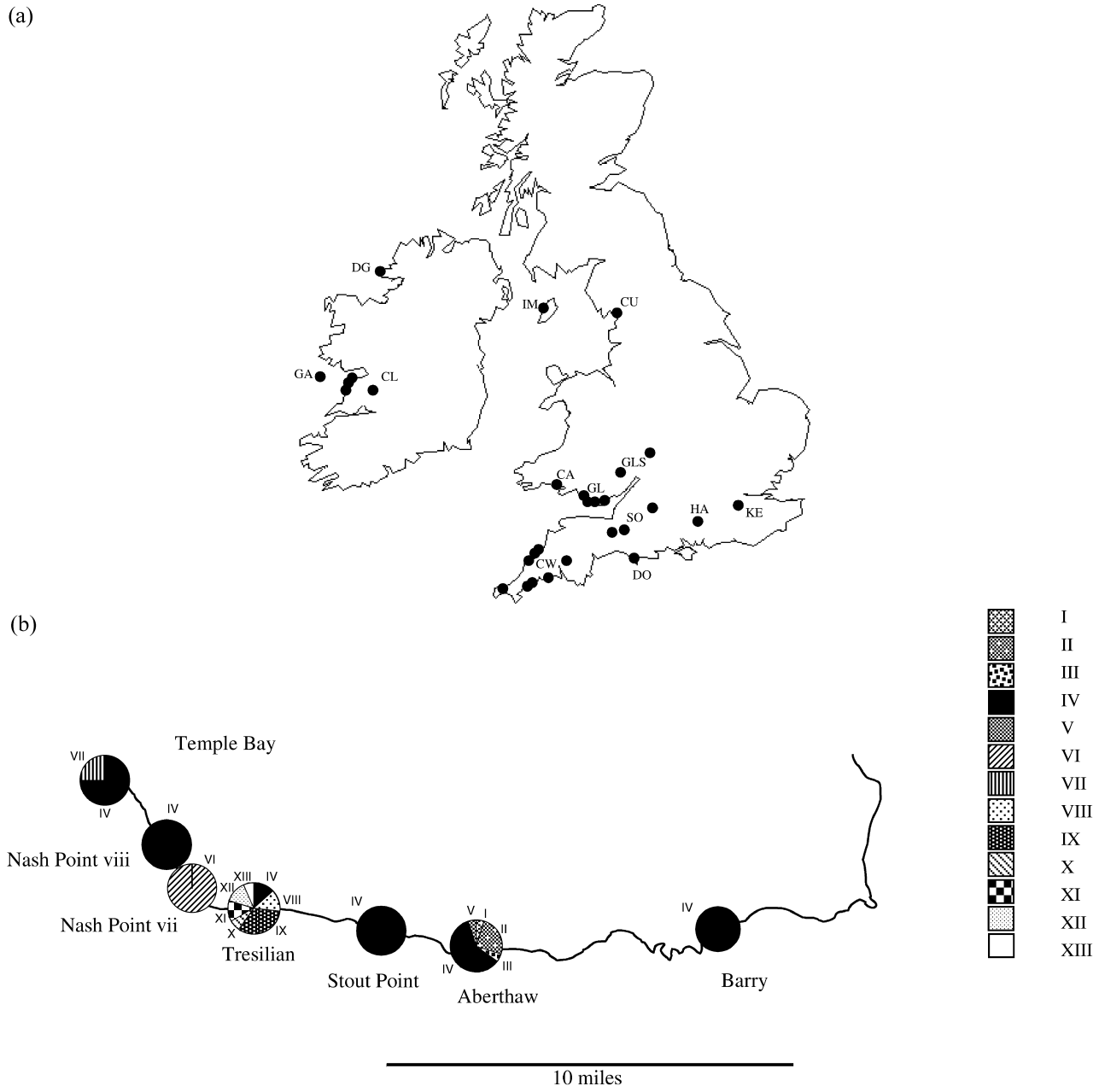

Fig. 1 (a) Location of populations sampled in the British Isles. Letters refer to counties which are given in full in Table 1. (b) Populations sampled on the Glamorgan coast (GL). Pie charts indicate the prevalence of the 13 different haplotypes identified by microsatellite analysis of three loci (further details in Tables 1 and 5).

In this paper we characterize microsatellites in the genome of the Maidenhair Fern (*A. capillus-veneris*) and develop a highly informative marker system. We then report on the application of this marker system to characterize genetic diversity and structure in sporophyte populations of *A. capillus-veneris* in the UK and Ireland. We make inferences on mating system and other factors determining the observed levels and patterns of genetic variation.

Materials and methods

Populations were defined as discrete, more or less continuous stands of sporophytes, separated from one another by a physical barrier, discontinuity of habitat, or geographical distance.

Locations of UK and Irish populations are shown in Fig. 1. Codes, counties and numbers sampled per population are given in Table 1. Natural populations were

Table 1 Populations of *Adiantum capillus-veneris* assayed and numbers of multilocus genotypes detected

Population and code	County, country and code (in parenthesis)	Status	*n*	No. of MLGs	Codes of MLGs detected
UK/Irish populations					
Aberthaw (Aber)	Glamorgan, UK (GL)	Natural	20	5	I; II; III; IV; V
Aran IG (IG)	Aran Is. Galway, Ireland (GA)	Natural	1	1	XVIII
Aran IT (IT)	Aran Is. Galway, Ireland (GA)	Natural	4	2	XX; XV
Arnside (Arn)	Cumbria, UK (CU)	Natural	1	1	XIV
Barry (Barry)	Glamorgan, UK (GL)	Natural	11	1	IV
Boscastle (Bosc)	Cornwall, UK (CW)	Natural	5	1	XVI
Burren B5 (B5)	Clare, Ireland (CL)	Natural	1	1	XV
Burren B8 (B8)	Clare, Ireland (CL)	Natural	3	1	XV
Burren FB1 (FB1)	Clare, Ireland (CL)	Natural	1	1	XVII
Burren FB6 (FB6)	Clare, Ireland (CL)	Natural	1	1	XV
E. Portland (Eport)	Dorset, UK (DO)	Natural	1	1	XI
Isle of Man 1 (IoM1)	Isle of Man, UK (IM)	Natural	1	1	XIX
Isle of Man 2 (IoM2)	Isle of Man, UK (IM)	Natural	1	1	XIX
Lantic Bay (Lant)	Cornwall, UK (CW)	Natural	16	1	XI
Llanstephan (Llan)	Carmarthenshire, UK (CA)	Natural	11	1	XI
Malin Beg (Malin)	Donegal, Ireland (DG)	Natural	2	1	XXI
Meathop (Mea)	Cumbria, UK (CU)	Natural	1	1	XIV
Mullaghmore (Mull)	Clare, Ireland (CL)	Natural	1	1	XXII
Nash Point vii (Nvii)	Glamorgan, UK (GL)	Natural	3	1	VI
Nash Point viii (Nviii)	Glamorgan, UK (GL)	Natural	1	1	IV
Poulsallagh 1 (Poul1)	Clare, Ireland (CL)	Natural	1	1	XV
Poulsallagh 2 (Poul2)	Clare, Ireland (CL)	Natural	1	1	XXIII
Poulsallagh 3 (Poul3)	Clare, Ireland (CL)	Natural	1	1	XXIII
Poulsallagh 4 (Poul4)	Clare, Ireland (CL)	Natural	1	1	XV
Stout Point (Stout)	Glamorgan, UK (GL)	Natural	8	1	IV
Temple Bay (Temp)	Glamorgan, UK (GL)	Natural	4	2	IV; VII
Tintagel (Tint)	Cornwall, UK (CW)	Natural	3	1	XIX
Tresilian (Tres)	Glamorgan, UK (GL)	Natural	15	7	VIII; IX; X; XI; XII; XIII; IV
Aldershot (Ald)	Hampshire, UK (HA)	Naturalized	1	1	XXIV
Batheaston (Bath)	Somerset, UK (SO)	Naturalized	1	1	XIV
Chard (Chd)	Somerset, UK (SO)	Naturalized	1	1	XXV
Coleford (Cole)	Gloucester, UK (GLS)	Naturalized	1	1	XXVI
Cotehele B (CoteB)	Cornwall, UK (CW)	Naturalized	1	1	XI
Cotehele D (CoteD)	Cornwall, UK (CW)	Naturalized	1	1	XI
Farningham (Farn)	Kent, UK (KE)	Naturalized	1	1	XI
Illminster (Ill)	Somerset, UK (SO)	Naturalized	1	1	XXVII
Ledbury (Ledb)	Gloucester, UK (GLS)	Naturalized	1	1	XI
Penzance (Penz)	Cornwall, UK (CW)	Naturalized	1	1	XI
RMPenzance 1 (RMPZ1)	Cornwall, UK (CW)	Naturalized	1	1	XI
RMPenzance 2 (RMPZ2)	Cornwall, UK (CW)	Naturalized	1	1	XI
RMPenzance 3 (RMPZ3)	Cornwall, UK (CW)	Naturalized	1	1	XI
St Mawes (StMaw)	Cornwall, UK (CW)	Naturalized	3	1	XI
Trelissick (Treli)	Cornwall, UK (CW)	Naturalized	3	1	XXVIII
W. Portland (WPort)	Dorset, UK (DO)	Naturalized	2	1	XI
Wadebridge (Wade)	Cornwall, UK (CW)	Naturalized	3	1	XI
All UK/Irish populations	—	—	144	28	—
Non UK/Irish populations					
France D63 (D63)	France (FR)	Natural	1	1	XXIX
France GdN (GdN)	France (FR)	Natural	1	1	XXX
Salerno (Sal)	Italy (IT)	Natural	1	1	XXXIV
Spain MdM (Mdm)	Spain (SP)	Natural	1	1	XXXII
Azores RdC (RdC)	Azores (AZ)	Naturalized	1	1	XXXIII
Giardini Publico (Gia)	Sicily (SI)	Naturalized	1	1	XXXI
San Clemente (San)	Italy (IT)	Naturalized	1	1	XXXV
All non-UK/Irish populations	—	—	7	7	—
All populations	—	—	151	35	—

n refers to the number of samples analysed per population. No. of MLGs refers to the number of multilocus genotypes detected per population. MLGs referred to by codes are given in Appendix I.

sampled as extensively as possible by removal of fresh green sporophyte fronds from up to 20 individuals per population. In total, the population analysis (151 samples) comprised 28 natural populations, 17 naturalized populations and a small sample from seven non-UK/Irish populations for a wider scale geographical comparison. All samples were collected directly from sporophytes growing *in situ* with the exception of four which were taken from sporophytes raised in cultivation from spore collections (i.e. Meathop, E. Portland, Illminster and Ledbury). Sample sizes varied widely, from one to 20, with many populations only represented by one or two individuals. In some populations this was due to very small population size. In others our small sample was due to inaccessibility due to populations growing high up on vertical cliff faces. Representatives of naturalized populations and a range of non-UK/Irish populations (one to three samples per population) were also included in the analysis for comparison. Due to the ability of this species to spread vegetatively by rhizome it was not always possible to differentiate whether 'individual' sporophyte plants were unique products of sexual events, clonal fragments, or multiclonal patches which appeared to be single individuals.

Fresh green sporophyte material was collected from a cultivated plant of Glamorgan, UK (population: Tresilian) origin for construction of the genomic library.

DNA extraction

Genomic DNA was extracted from frozen or dried sporophyte tissue (≈ 0.1 g) following a CTAB protocol adapted specifically for pteridophyte material (Dempster *et al*. 1999). For the genomic library, a CTAB extraction protocol was slightly modified from Doyle & Doyle (1987), followed by purification by caesium chloride gradients.

Library construction

A microsatellite-enriched genomic library was constructed following Edwards *et al*. (1996). The following oligonucleotides were used to enrich for DNA fragments containing microsatellites: $[GA]_{15}$, $[GT]_{15}$, $[AT]_{15}$, $[GC]_{15}$, $[CAA]_{10}$, $[CATA]_{10}$, $[ATT]_{10}$, $[GATA]_{10}$, $[GCC]_{10}$ and $[ATAG]_{10}$. Enriched DNA was digested with 1 unit of *Mlu*I and ligated into a modified pUC19 vector (pJV1) containing a *Mlu*I compatible *Bss*HI site. Plasmids were transformed into *Escherichia coli* DH10B™ (Life Technologies).

Screening, sequencing and primer design

Sequencing was performed following the manufacturer's recommendations using the ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction kit on the ABI 377 automated sequencer, using both forward and reverse M13 primers. Specific primers flanking microsatellite repeats were designed using Primerselect (DNASTAR Ltd).

Polymerase chain reaction optimization of primers and polymorphism testing

Polymerase chain reaction (PCR) conditions were optimized as follows: 15 min at 95 °C; 28–35 cycles of 45 s at 94 °C, 45 s at annealing temperature (48–59 °C) and 45 s at 72 °C; followed by 72 °C for 5 min. All reactions used a 'hot-start' *Taq* polymerase, Hotstar *Taq* DNA Polymerase (Qiagen Ltd). Standard reaction conditions after optimization were 50 ng of each primer; 1 × Qiagen *Taq* polymerase buffer (containing Tris-HCl, KCl $(NH_4)_2SO_4$, $MgCl_2$); 0.2 mM dNTPs; 0.625 U *Taq* polymerase; 2–3 mM $MgCl_2$ and ≈ 2–5 ng DNA in a 25 μL reaction volume.

PCR products were visualized on 1.5% agarose followed by 1.5% Nusieve 3 : 1 (Flowgen) high-resolution agarose. Primer sets revealing polymorphism were selected for subsequent population analyses. All PCR reactions were performed using an OmniGene Thermal Cycler (Hybaid), a GeneAmp PCR System 2400 (Perkin Elmer) or an MJ-Research PTC-200 Peltier Thermal Cycler.

Resolving simple sequence repeat (SSR) polymorphism

Primers were labelled with ABI fluorescent dyes (FAM or HEX) and PCR products were visualized and sized by automated detection during gel electrophoresis [4.2% (w/v) denaturing polyacrylamide] using an ABI 377 automated sequencer. At least one previously amplified and sized DNA sample was included in every PCR reaction and gel run to check allele sizing. Where allelic differences of 2 bp or less were observed, samples were re-amplified and run again to confirm sizing. All heterozygote samples were verified by re-amplification. GENESCAN™ Tamra 350 was used as an internal size standard for all analyses. All gels were run using GENESCAN™ 2.0 software and sample files were imported into GENOTYPER™ 2.1 for further analysis.

Data analysis

Basic statistics including allele frequencies and number of alleles detected per locus, number of multilocus genotypes (MLGs), and observed and expected heterozygosity per locus were calculated for each population and global values across all samples using GENEPOP version 3.1d (Raymond & Rousset 1995) and POPASSIGN version 3.8 (S. Funk, Institute of Zoology, London). Genotypic disequilibria and inbreeding coefficients (F_{IS}) were estimated using GENEPOP, and significance was tested using Fisher's exact tests. Multilocus genotypes in heterozygous populations were

further examined, and potential scenarios were investigated which could explain the distribution of MLGs in these populations. Allele sharing distances (ASD, e.g. Bowcock *et al*. 1994) were calculated using POPASSIGN for all individuals pairwise in the data set, as a measure of the genetic distance between MLGs. An unrooted phenogram of allele sharing pairwise genetic distances was constructed using neighbour joining (Saitou & Nei 1987) as implemented in PHYLIP (Felsenstein 1993). Genetic distance measures based on allele sizes were not calculated due to the irregular allele size distribution at all three loci. Estimates of gene flow (*Nm*) and the rate of intragametophytic selfing (Holsinger 1987) were not attempted due to small population sizes in some cases.

Results

Identification and characterization of SSRs and results of the marker development process

Of 41 random clones sequenced, 38 contained microsatellites. All microsatellites isolated were unique and all contained dinucleotide repeats. Repeat length ranged from five to 49 repeats.

According to the categories defined by Weber (1990) 24 of the 38 microsatellites isolated were perfect (uninterrupted) dinucleotide repeats, five were imperfect (interrupted) dinucleotide repeats and nine were compound repeats. Only one tri- and one tetranucleotide repeat were isolated and both adjoined a dinucleotide repeat forming a compound SSR. The most frequent motif was $[GT]_n$ occurring in 31 out of 38 microsatellites (82%), followed by $[GA]_n$ occurring in 13 out of 38 (34%).

Of the 38 positive clones, primer sets could be designed for 13. Of these, three (23%) produced a multiple band pattern on high-resolution agarose, four (31%) produced an indistinct band, one produced no signal for genomic DNA, and one produced a monomorphic signal.

Four primer sets (KA, KH, KJ and KK), amplified single, polymorphic bands on high-resolution agarose and were selected for analysis. Primers were initially tested on a sample of six individuals from Cornwall (St Mawes), Sicily, Dorset (Portland), Glamorgan (Stout Point), Glamorgan (Aberthaw) and the Azores. Only loci amplified using three primer sets, KH, KJ and KK (Table 2), gave clear unambiguous signals of the expected product size on the ABI377 and these three primer sets were used in the following population analysis.

Results of the population analysis

For the three loci analysed, 23 alleles and 28 MLGs were detected within the UK and Irish samples, and 27 alleles and 35 MLGs were detected when including non-UK/Irish

Table 2 Oligonucleotide primers used in the population analyses: sequences and optimized PCR conditions, numbers of alleles detected, observed and expected heterozygosity across all samples (per locus and mean values across loci) and allele size range for Wales, rest of British Isles (BI) and non-British Isles samples

Locus	Primer sequence (5′–3′)	Product size range (bp)	T_a (°C)	$MgCl_2$ concn (mM)	No. PCR cycles	N_{ALL}	H_E	H_O	Allele size range (Wales)	Allele size range (rest of BI)	Allele size range (non-BI)
KH	GTGTTCGTTTGCGTATATGTATTT AGGGGATTCCACTGTTCTTG	141–183	52	3	33	9	0.73	0.06	152–183	141–159	141–169
KJ	AAAACCAGCTATCTTTTGAACTGT ATAACAATGGCCCGACCCTTAG	123–177	50	3	33	13	0.73	0.03	130–161	130–177	128–159
KK	AGGCAAAGGGGAGGGAGGGATAGT TTTTTGCAGTTTTGTTGTCACC	143–161	52	3	33	5	0.70	0.07	143–159	143–159	155–161
Mean	—	—	—	—	—	9	0.72	0.05	—	—	—

T_a = optimized annealing temperature; H_E = expected heterozygosity; H_O = observed heterozygosity, N_{ALL} = total number of alleles detected.

Table 3 Numbers of alleles detected for each locus, observed and expected heterozygosity, Inbreeding coefficients (F_{IS}, Weir & Cockerham *et al*. 1984) and results of Hardy–Weinberg exact tests per locus for the four polymorphic populations

Locus	No. of alleles/locus	H_E	H_O	F_{IS}
Aberthaw (UK: GL)				
KH	3	0.37	0.30	0.19
KJ	1	—	—	—
KK	2	0.30	0.35	−0.19
Aran IT, Clare (Ireland: CL)				
KH	1	—	—	—
KJ	1	—	—	—
KK	2	0.43	0	1
Temple Bay (UK: GL)				
KH	2	0.33	0.33	0
KJ	2	0.33	0.33	0
KK	2	0.33	0.33	0
Tresilian (UK: GL)				
KH	3	0.63	0.13	0.79*
KJ	3	0.6	0.27	0.56*
KK	2	0.2	0.37	0.47

H_E = expected heterozygosity; H_O = observed heterozygosity; F_{IS}, inbreeding coefficient.
*indicates significant deviation from Hardy–Weinberg at $P < 0.05$ level.

collections (Table 1 and Appendix I). All three loci were highly polymorphic (Table 2), with alleles detected ranging from five to 13 across all samples, resulting in a mean of nine alleles per locus. However, of the 17 UK/Irish populations analysed which were represented by multiple samples, all except four were monomorphic (Table 1). These multiple sample populations varied in size from two to four samples (10 populations), 5–10 samples (two populations) and 11–20 samples (five populations), and the four polymorphic populations comprised four, four, 15 and 20 samples, respectively. Expected heterozygosities, calculated globally across all samples (Table 2) were very high for all three loci, resulting in a mean of 0.72. In contrast, observed heterozygosities were close to zero for all loci, with a mean of 0.05. Observed and expected heterozygosities per locus calculated for the four nonmonomorphic populations (Table 3) show that heterozygotes were detected in only three populations, all within Glamorgan, Wales, UK (i.e. Aberthaw, Tresilian and Temple Bay). In all three populations virtually all heterozygous samples showed heterozygosity at more than one locus (Table 5).

Inbreeding coefficients and results of the Hardy–Weinberg exact tests (Table 3) for the four nonmonomorphic populations show two significant deviations from Hardy–Weinberg equilibrium (HWE), both for loci within the same population (Tresilian, Glamorgan, UK). Both were due to high positive F_{IS} values, indicating heterozygote deficiencies. The other two polymorphic Glamorgan populations, Aberthaw and Temple Bay, were in HWE for all loci although the results for the Temple Bay population could be due to sampling ($n = 4$). The absence of heterozygosity in the Aran IT population (Aran Islands, Clare, Ireland) resulted in a high inbreeding coefficient ($F_{IS} = 1.0$) for the polymorphic KK locus. Exact tests for linkage disequilibrium (Table 4) were significant for all three locus pairs within the Tresilian population, and for the KH/KK locus pair within the Aberthaw population.

Table 4 Results of exact tests for linkage disequilibrium; *P* values

Population	KH/KJ loci	KH/KK loci	KJ/KK loci
Aberthaw (UK:GL)	—	0.0048*	—
Temple Bay (UK:GL)	0.2509	0.2519	0.2500
Tresilian (UK:GL)	0.0015*	0.0136*	0.0008*

*indicates significant genotypic linkage disequilibrium.

Detailed examination of the MLGs in each of the three heterozygous populations (Glamorgan, UK) revealed the following.

Aberthaw. This population comprises five MLGs, two of which are relatively common and the commonest of which (IV) is the sole homozygous MLG present. The remainder all contain at least one heterozygous locus. Although the KJ locus is fixed for allele 161 in this population, KH contains three alleles and KK contains two alleles, with the majority of MLGs being heterozygous for this locus.

Temple Bay. This population has a much simpler structure with MLG IV again predominating, but with the second MLG (VII) showing unequivocal evidence for outcrossing, with all three loci being heterozygous and sharing one allele with MLG IV.

Tresilian. The Tresilian population is the most complex of these populations, comprising seven MLGs, four of which (VIII, IX, XI and IV) are homozygous. This population is also the most variable, with three, three and two alleles present at loci KH, KJ and KK, respectively. As with Aberthaw and Temple Bay, the heterozygous MLGs are rarer than the homozygous (4 : 11). Furthermore, the heterozygous MLGs in Tresilian only comprise combinations of the homozygous MLGs present.

The neighbour-joining phenogram of ASD values among samples (Fig. 2) shows a pattern typically seen when using highly variable markers in populations which are highly structured through genetic drift, selection, or inbreeding, i.e. terminal branches are generally much longer than internal branches. The statistical support for this topology could not be assessed rigorously due to the nature of the data set, but the topology of the phenogram suggests the existence of a cluster grouping only Glamorgan samples, of

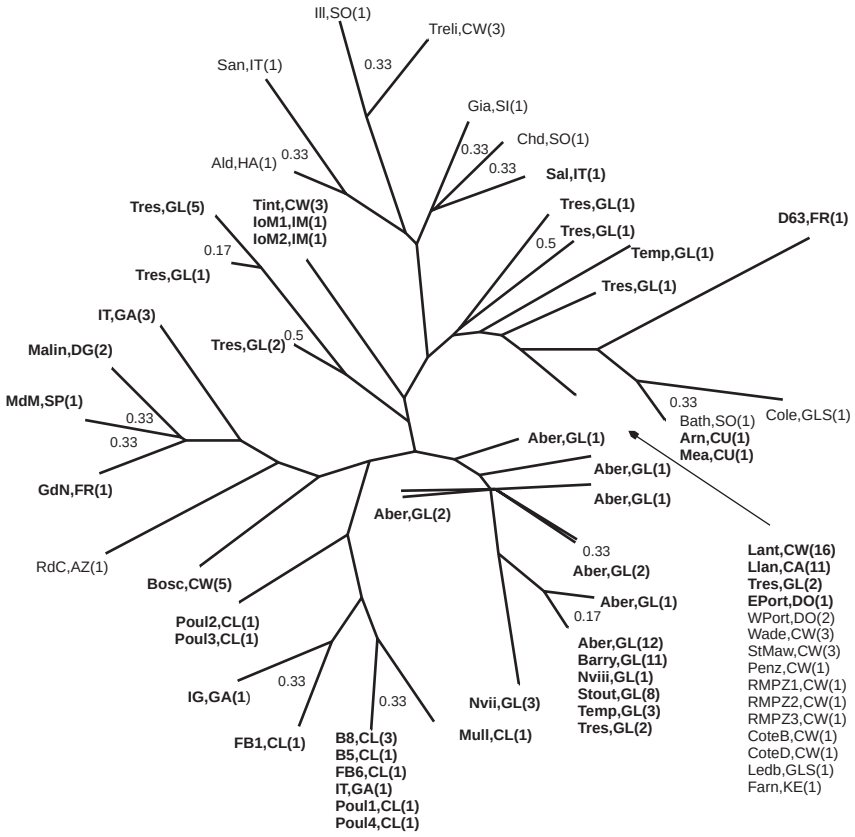

Fig. 2 Unrooted phenogram of allele sharing pairwise genetic distances constructed using the neighbour joining method. Allele sharing distances are shown for terminal branches. Abbreviations refer to populations and letters refer to counties. Both are given in full in Table 1. Numbers in brackets after populations and county codes refer to the number of samples in each population possessing the MLG represented by that terminal branch. Natural populations are in bold; naturalized populations are not in bold.

a cluster grouping samples from Ireland (Clare, Galway) and of a large cluster grouping some Glamorgan samples, Cumbria, additional sites in southwest Wales and southwest England. The three native Cornwall populations (Boscastle, Lantic Bay and Tintagel) appear highly differentiated, and an unexpected result is the clustering of the Tintagel population in North Cornwall with the populations on the Isle of Man. Some of the naturalized UK/Irish populations are shown to group with non-UK/Irish samples [i.e. Aldershot (Hampshire); Trelissick (Cornwall); Illminster and Chard (Somerset)], although the majority group with natural UK/Irish samples. The seven non-UK/Irish samples do not group together but each one occupies an unshared position on a separate branch of the phenogram.

Discussion

Genetic diversity, population genetic structure and mating system in Adiantum capillus-veneris

Our results show high levels of genetic diversity in *Adiantum capillus-veneris* overall across UK and Irish samples, the evidence being the total number of alleles (23), multilocus genotypes (28), and mean number of alleles detected per locus (Mean N_{ALL}: 9). Global expected heterozygosities are also high, with a mean H_E across all loci of 0.72. Comparisons with these results are, however, problematic for pteridophytes since there are no microsatellite data available, and although our values appear high empirically, higher levels of diversity have been detected in seed plants using microsatellites, e.g. a mean N_{ALL} of 21, and mean global H_E of 0.74, were obtained for *Pinus contorta* var *latifolia*, lodgepole pine, based on analysis of 120 samples from four populations using five SSR loci (Thomas *et al*. 1999).

In contrast to the very high global expected heterozygosities estimated for *A. capillus-veneris*, observed heterozygosities are close to zero for all loci. This discrepancy can be explained in terms of the partitioning of variation within and between populations. Of the 17 populations analysed which were represented by multiple samples, all except four are monomorphic, i.e. virtually all genetic variation in the *A. capillus-veneris* populations sampled is partitioned between rather than within populations. We recognize the possibility that sample size may be confounding this result but the nature of our data set is such that statistical analyses to test its effect would not be meaningful. Importantly, other populations with relatively large sample sizes [e.g. Barry (n = 11), Lantic Bay (n = 16) and Llanstephan (n = 11)] were monomorphic. Although outcrossing has been shown by the presence of heterozygotes in three populations, no inferences on mating system can be made for the monomorphic populations.

Thus, it is not possible to test the hypotheses from flowering plant studies which predict partitioning of variation on the basis of predominant mating system (Brown 1979; Hamrick *et al*. 1979; Gottlieb 1981).

The detection of multiple heterozygotes in three populations within Glamorgan (Tresilian, Aberthaw and Temple Bay) provides direct evidence of outcrossing. The high positive F_{IS} values observed at two loci within the Tresilian population may be due to a number of factors including an inbreeding mating system (either intragametophytic or intergametophytic selfing), genetic drift, selection, genetic structure within populations, allelic dropout or the presence of null alleles. Allelic dropout and the presence of null alleles would be expected to be locus specific and possibly detectable in other heterozygous populations. Selection for homozygosity would seem unlikely, even in the presence of strong fine-scale adaptation, and genetic drift should reduce heterozygosity at all loci, which was not the case. The more likely explanation of the heterozygote deficiency is inbreeding via intragametophytic or intergametophytic selfing and localized genetic substructuring within the population.

The significant linkage disequilibrium detected for all three locus pairs within Tresilian, and for the KH/KK locus pair within Aberthaw could be a consequence of admixture or linkage. Although linkage cannot be ruled out it is unlikely given the large number of chromosomes in *A. capillus-veneris* ($n = 30$; Manton 1950) and the small number of loci analysed. A more likely explanation is that these populations exhibit localized genetic structure, comprising a number of incompletely mixed subpopulations, leading to non-random association between alleles in the genotypes sampled.

Both the significant linkage disequilibrium values and the HWE deficiencies could be a result of spatial genetic structuring within populations. Such structuring could have arisen due to temporal factors, e.g. alleles introduced through independent spore arrivals over time may not have been present for a sufficient length of time to allow mixing. Alternatively, genetic structuring may arise as a result of physical patchiness of the habitat itself reducing effective gene flow within what we considered to be a population. Effective gene flow may be further reduced where suitable habitat patches have become fully occupied by the first plants to establish. Such limited gene flow could result in inbreeding, whether by intra- or intergametophytic selfing. Both Tresilian and Aberthaw are typical of natural populations of *A. capillus-veneris* in the UK in that suitable habitat is patchily distributed, even on a very local scale, with suitable moist tufa substrate interspersed with less suitable substrate such as bare rock patches. We therefore propose the latter scenario as the more likely explanation for these results. It is also possible that genetic structuring may have arisen in part due to uneven spread of rhizomatous clones, although the extent of this is unknown.

Partitioning of genetic variation among rather than within populations has been observed in other rock-dwelling pteridophytes, including, *Sadleria pallida* and *S. cyatheoides* (Ranker *et al*. 1996), *Asplenium septentrionale* (Holderegger & Schneller 1994), *Asplenium ruta-muraria* (Schneller & Holderegger 1996) and *Asplenium csikii* (Vogel *et al*. 1999), and patchiness of suitable habitat has been proposed as a major factor determining such genetic structure (Holderegger & Schneller 1994; Ranker *et al*. 1996; Schneller & Holderegger 1996; Vogel *et al*. 1999). Gene flow into existing populations may also be limited by suitable niches being fully occupied by previously established plants, e.g. Vogel *et al*. (1999). These factors may account for the partitioning of variation among, rather than within, populations of *A. capillus-veneris* in the UK and Ireland.

Natural populations in the UK and Ireland could have two origins. They may be relicts of a once more widespread and continuous distribution, but there is no evidence that suitable habitat was any more widespread in the past than it is today, or alternatively populations may have been founded by long distance spore dispersal followed by intragametophytic selfing.

The latter scenario has been proposed to account for observed genetic structuring between populations of *Asplenium septentrionale* (Holderegger & Schneller 1994), *Asplenium ruta-muraria* (Schneller & Holderegger 1996) and *Asplenium trichomanes* ssp. *quadrivalens* (Suter *et al*. 2000). These populations are also limited to a specific rock habitat which occurs discontinuously, and most genetic variation was detected among rather than within populations. Monomorphic populations were suggested to have arisen through independent single spore arrivals, and genetic variation detected within some populations was attributed to the arrival of two or more independent spore dispersal events over time. Intra-population variability in *Asplenium ruta-muraria* was shown to be related to age of population, with recently established populations on man-made substrates showing no polymorphism and longer established populations showing variability (Schneller & Holderegger 1996).

Long-distance, single-spore founding events followed by intragametophytic selfing may also explain the observed variation in *A. capillus-veneris* in the UK and Ireland. Support for this hypothesis is provided by the monomorphism in the majority of natural populations with multiple samples, despite the high level of diversity detected overall. Further evidence is provided by the observation that intragametophytic selfing does occur in gametophyte isolates of *A. capillus-veneris* raised in cultivation, resulting in the production of healthy sporophytes (K.V.P. unpublished data). Our data have also shown heterozygosity and evidence of outcrossing in three populations within Glamorgan, UK. We therefore infer a mixed mating system in these populations similar to that reported for *Hemionitis palmata* (Adiantaceae; Ranker 1992).

Table 5 Multi-locus genotypes (MLGs) detected within Glamorgan populations

KH locus	KJ locus	KK locus	MLG code number	*n*
Aberthaw				
152 181	161 161	143 143	I	1
152 183	161 161	143 155	II	5
183 183	161 161	143 155	III	1
183 183	161 161	143 143	IV	12
152 152	161 161	143 155	V	1
Barry				
183 183	161 161	143 143	IV	11
Nash Point vii				
183 183	148 148	143 143	VI	3
Nash Point viii				
183 183	161 161	143 143	IV	1
Stout Point				
183 183	161 161	143 143	IV	8
Temple Bay				
183 183	161 161	143 143	IV	3
152 183	130 161	143 159	VII	1
Tresilian				
152 152	159 159	143 143	VIII	2
179 179	159 159	143 143	IX	5
152 152	130 161	143 159	X	1
152 152	130 130	159 159	XI	2
152 179	130 159	143 159	XII	2
179 179	130 159	143 143	XIII	1
183 183	161 161	143 143	IV	2

n refers to the number of samples per population which possess each MLG.
MLGs referred to by codes are given in Appendix I.

The variability detected in four populations could be attributed to multiple spore arrivals over time or mutation. A detailed examination of the MLGs and their frequencies (Table 5) enables a more detailed interpretation of their origins.

Four out of five MLGs at Aberthaw are heterozygous, although the one homozygous MLG (IV) predominates, a MLG common to six of the seven Glamorgan populations sampled. Two loci are polymorphic, and at the KK locus, all heterozygote MLGs share at least one allele with IV, and could therefore descend from this MLG. However, two MLGs (I and V) do not share an allele with IV at the other locus (KH). The possibility that MLG I arose as a result of mutation from MLG IV is unlikely given that MLG I is a heterozygote for two different alleles (152 and 181) at the KH locus, which are different to those found in MLG IV (183/183). Although a 2-bp slippage-induced mutation is a possible source of the 181 allele, the occurrence of a second mutation, resulting in a 31-bp deletion to generate a 152-bp allele, does not seem plausible. Mutation is also an unlikely explanation for MLG V given the large disparity in allele size at the KH locus and the presence of the 152/152 genotype at KH, which is also present in Tresilian, Glamorgan, UK (MLG XI). Therefore, the descendants of at least one additional founding MLG appear to be present in this population, with the haplotype 152–161–155, potentially explaining MLG combinations II, III and V (most parsimoniously, but not necessarily, at F_1, F_2, or F_1 backcross stage).

In Temple Bay, one homozygous MLG (IV) is present with one heterozygous MLG (VII). Here MLG VII could not have arisen by mutation given the heterozygosity at the three independent loci and the fact that allele sizes differ by 31, 31 and 16 bp, respectively, for loci KH, KJ and KK. The most plausible explanation is that a MLG IV has outcrossed with minimally a second, unsampled MLG, the most likely combination for which would be 152–130–159.

Finally, in Tresilian, seven MLGs are present, four of which are homozygous. These homozygous MLGs are highly divergent, with differing alleles at two or more loci and/or with large allele size differences. Thus, the possibility that these MLGs were generated through mutation from a shared common origin is extremely unlikely. The three heterozygous MLGs appear to have arisen through outcrossing since they all comprise potential combinations of the homozygous MLGs present, possibly at F_1, F_2, or F_1 backcross stage.

The genetic variation detected within Glamorgan populations was unexpected and has illustrated the potential importance of these populations in terms of maintaining the diversity of *A. capillus-veneris* in the UK. Many alleles are shared between populations within the local area of Glamorgan (Table 5, Fig. 1b) suggesting gene flow between locally established populations, although this could not be measured meaningfully given the small sample sizes and the presence of linkage disequilibrium and inbreeding within several of these populations. Population size within Glamorgan is consistently large compared to natural populations in other UK regions (K.V.P. unpublished data), which may result in greater overall spore production and potential for gene flow. Longevity of populations is also a potentially important factor and can be verified by early records, e.g. the first record of *A. capillus-veneris* from the Glamorgan coast dates back to 1698 (Hyde & Wade 1962). The proximity of these populations may also be important in enabling gene flow since they occur at intervals of only a few miles (Fig. 1b), and are interspersed by other populations growing too high on the cliffs to be accessible.

The genetic distance data provide some insights into the origins and relationships among the UK and Irish natural populations. Many samples from populations which are close geographically cluster together on the phenogram (Fig. 2), although others do not, e.g. the unexpected clustering of Tintagel in North Cornwall with the Isle of Man populations. The distance data also provide interesting

insights into the origins of naturalized populations around UK and Ireland. We can infer that some populations have probably originated from local natural spore sources. For example the naturalized West Portland population clusters with the natural population at East Portland, and many of the naturalized Cornwall populations (Wadebridge, St Mawes, Cotehele, and all Penzance and RMPenzance samples) group with the extensive natural population at Lantic Bay on the south Cornwall coast. Other naturalized populations group only with non-native samples: Aldershot (Hampshire), Illminster (Somerset), Trelissick (Cornwall) and Chard (Somerset), suggest a possible cultivated origin from non-native stock. However, origins of some populations remain less clear, e.g. the naturalized populations of Batheaston (Somerset) and Coleford (Gloucester) cluster with the natural populations in Cumbria. Each of the seven non-UK/Irish samples analysed occupies a unique terminal branch on the phenogram, showing no clear evidence of shared origins with UK/Irish samples.

The results of our study have highlighted the applicability of microsatellites to the study of pteridophyte populations and have provided insights into levels of diversity, genetic structure and mating systems in *A. capillus-veneris* in the UK and Ireland even given the limitations of sample size. The pattern and extent of diversity detected in *A. capillus-veneris* by the three microsatellite loci might be considered surprising given the limited, edge of range distribution of this species in the UK and Ireland and its restriction to a narrow ecological niche (e.g. see Vogel *et al*. 1999). Although most variation is partitioned between rather than within populations the variability and heterozygosity detected within three populations within Glamorgan was also unexpected and has revealed the importance of these populations in terms of UK biodiversity. However, it is important to recognize that the distribution of *A. capillus-veneris* in the UK and Ireland (growing on the northern edge of its range), precludes generalizations emerging from this study regarding diversity, genetic structure and breeding systems in other parts of its global range. Further study across its global range would be required before any such broad generalizations could be made.

However, on the basis of our results, we predict that microsatellites will be an invaluable tool in the future for assessing levels and patterns of genetic variation in pteridophyte populations, and in particular in the study of rare species where microsatellite data may facilitate more informed conservation policy.

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Appendix I

Unique multilocus genotypes (MLGs) detected

MLG code	KH locus	KJ locus	KK locus
I	152 181	161 161	143 143
II	152 183	161 161	143 155
III	183 183	161 161	143 155
IV	183 183	161 161	143 143
V	152 152	161 161	143 155
VI	183 183	148 148	143 143
VII	152 183	130 161	143 159
VIII	152 152	159 159	143 143
IX	179 179	159 159	143 143
X	152 152	130 161	143 159
XI	152 152	130 130	159 159
XII	152 179	130 159	143 159
XIII	179 179	130 159	143 143
XIV	152 152	177 177	159 159
XV	157 157	159 159	157 157
XVI	159 159	161 161	155 155
XVII	155 155	159 159	157 157
XVIII	155 155	157 157	157 157
XIX	157 157	130 130	143 143
XX	157 157	159 159	155 155
XX1	141 141	155 155	155 155
XXII	159 159	159 159	157 157
XXIII	157 157	161 161	157 157
XXIV	152 152	173 173	161 161
XXV	152 152	175 175	161 161
XXVI	141 141	177 177	159 159
XXVII	155 155	123 123	161 161
XXVIII	159 159	123 123	161 161
XXIX	169 169	153 153	159 159
XXX	141 141	159 159	155 155
XXXI	152 152	177 177	161 161
XXXII	141 141	128 128	155 155
XXXIII	181 181	141 141	155 155
XXXIV	152 152	128 128	161 161
XXXV	157 157	173 173	161 161