Development and application of 14 microsatellite markers in the burying beetle *Nicrophorus vespilloides* reveals population genetic differentiation at local spatial scales

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Burying beetles (genus *Nicrophorus*) are relatively rare among insects in providing sophisticated parental care. Consequently, they have become model species in research analysing social evolution, the evolution of parental care and mating systems. We used the recently published *N. vespilloides* genome and transcriptome to develop microsatellite markers. Specifically, we developed 14 polymorphic markers with 5 to 13 alleles per locus and used them to investigate levels of genetic differentiation in four south Cambridgeshire (UK) populations *of N. vespilloides*, separated by 21 km at most. The markers revealed significant genetic structuring among populations (global F_{ST} = 0.023) with all but one of the pairwise comparisons among populations being significant. The single exception was the comparison between the two closest populations, which are approximately 2.5 km apart. In general, the microsatellite markers showed lower observed heterozygosity than expected. We infer that there is limited dispersal between populations and potentially also some inbreeding within them and suggest that this may be due to habitat fragmentation. We discuss these results in the context of recent laboratory experiments on inbreeding and beetle flight.

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5   SUMMARY

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7   parental care. Consequently, they have become model species in research analysing social

8   evolution, the evolution of parental care and mating systems. We used the recently published *N.*

9   *vespilloides* genome and transcriptome to develop microsatellite markers. Specifically, we

10   developed 14 polymorphic markers with 5 to 13 alleles per locus and used them to investigate

11   levels of genetic differentiation in four south Cambridgeshire (UK) populations *of N.*

12   *vespilloides*, separated by 21 km at most. The markers revealed significant genetic structuring

13   among populations (global F_{ST}= 0.023) with all but one of the pairwise comparisons among

14   populations being significant. The single exception was the comparison between the two closest

15   populations, which are approximately 2.5 km apart. In general, the microsatellite markers

16   showed lower observed heterozygosity than expected. We infer that there is limited dispersal

17   between populations and potentially also some inbreeding within them and suggest that this may

18   be due to habitat fragmentation. We discuss these results in the context of recent laboratory

19   experiments on inbreeding and beetle flight.

22 **INTRODUCTION**

23 Burying beetles (genus *Nicrophorus*) are relatively unusual among insects in providing elaborate

24 parental care for their developing larvae. Reproduction centres on the fresh carcass of a small

25 vertebrate (like a songbird or mouse), which adults locate by flight. If more than one adult of the

26 same sex finds the carcass, they will commonly fight to determine ownership (e.g. Otronen,

27 1988; Scott, 1994). Defeated subordinates may stay nearby: males may sneak matings with the

28 dominant female, while females might become intraspecific brood parasites (e.g. Müller, Eggert

29 & Dressel, 1990; Hopwood et al., 2015). The dominant individuals of each sex then pair up and

30 together prepare the carcass for reproduction (although mated females can singlehandedly raise

31 young: Müller et al., 2007). They remove the fur or feathers, roll the flesh into a ball and bury it

32 below ground in a shallow grave. During this time, parents defend their carcass breeding

33 resource from attack by conspecifics, congenerics and other carrion-feeding insects (Robertson,

34 1993; Trumbo, 1990). Eggs are laid in the soil near the carcass. Newly hatched larvae crawl into

35 a crater on the brood ball and there they solicit attention from their parents, who also stay to

36 protect them from attack (Trumbo, 2007). About a week after the larvae hatch, the carcass is

37 entirely consumed. The beetle parents then fly off to search for new mating opportunities or fresh

38 carrion and the larvae disperse into the soil to pupate (Scott, 1998).

39 Burying beetles thus play a key ecological role as nutrient recyclers (e.g. Royle,

40 Hopwood & Head, 2013). Furthermore, their relatively unusual natural history, and the ease with

41 which they can be bred in the lab, means that several have become popular study species in

42 experimental analyses of social evolution, the evolution of parental care and mating systems (e.g.

43   Royle, Hopwood & Head, 2013; Scott, 1998). Nevertheless, despite their widespread use in the

44   lab, relatively little work has focused on the burying beetles' ecology in nature. This is because it

45   is difficult to track marked burying beetles through their life course to understand patterns of

46   dispersal (e.g. Attisano & Kilner, 2015), the likely extent of competition for limited carrion

47   resources (e.g. Kilner et al., 2015), the degree of connectivity between populations and therefore

48   the potential for inbreeding (e.g. Pilakouta et al., 2015). Yet this knowledge is key for

49   interpreting the results of experiments carried out in the laboratory (e.g. Kilner et al., 2015,

50   Attisano & Kilner, 2015, Pilakouta et al., 2015).

51   Furthermore, habitat fragmentation has recently been suggested to influence population

52   structure in beetles (Keller & Largiader, 2003; Suzuki & Yao, 2014). Habitat fragmentation by

53   deforestation has specifically been hypothesised to influence dispersal of *N. americanus* in the

54   USA (Creighton et al., 2009) and *N. quadripunctatus* populations in Japan (Suzuki & Yao,

55   2014). Yet behavioural work on flight performance shows that burying beetles are capable of

56   sustained flight over tens of kilometres (Attisano & Kilner 2015). Therefore a key aim of this

57   study was to determine the extent to which burying beetles disperse in nature over relatively

58   short distances, using genetic techniques (cf Houston et al., 2015).

59   Recently, molecular resources have been developed for *N. vespilloides*, including a

60   genome, epigenome (Cunningham et al., 2015) and transcriptomes (e. g. Parker et al., 2015;

61   Palmer et al., 2016). We took advantage of these newly available molecular tools to develop

62   microsatellite markers. We used the markers to determine the extent of genetic differentiation

63   between natural populations of *N. vespilloides*, deliberately choosing sites on a local scale, no

64   more than 21km apart (Fig.1) to test the power of the markers we developed. These four

65   populations were all in south Cambridgeshire and at: Waresley Woods (Latitude: 52.17487°;

66   Longitude: -0.17354º), Gamlingay Woods (Latitude: 52.15555º; Longitude: -0.19286º), Byron's

67   Pool (Latitude: 52.17305º; Longitude: 0.10196º) and Madingley Woods (Latitude: 52.22658º;

68   Longitude: 0.04303º). These four populations inhabit patches of woodland that are islands in a

69   landscape dominated by arable farmland and urban development. Their close proximity enabled

70   us to determine whether habitat fragmentation or beetle flight performance could better explain

71   population structure on a local scale.

72

73   **MATERIAL AND METHODS**

74   *Beetles*

75   Adult beetles from the four south Cambridgeshire populations (Fig.1): Gamlingay (n=40),

76   Waresley (n=40), Byron's Pool (n=33) and Madingley (n=26) were collected during May-

77   October 2016 using Japanese beetle traps. Male and female beetles, sampled in equal numbers,

78   were brought back to the laboratory alive and preserved in absolute ethanol for genetic analysis.

79   In order to avoid collecting related individuals, periodical sampling was performed and only

80   adult beetles (not larvae) were analysed. These were males and females attracted at random to

81   the carcasses provided in the beetle traps.

82   In an earlier pilot study, we tested microsatellite markers from *N. quadripunctatus*,

83   (Suzuki & Yao, 2014) in *N. vespilloides*, but these failed to amplify reliably (Matthew Schrader,

84   pers. comm.). We therefore developed new markers specifically for *N. vespilloides*.

85

86   *DNA extraction, microsatellite amplification and analysis*

87   Total genomic DNA (n=139) was extracted individually from beetle heads using the DNeasy

88   Tissue Kit (Qiagen). Molecular markers were developed with the program msatcommander

89 (Faircloth, 2008), using the publicly available *N. vespilloides* genome (NCBI Bioproject number

90 PRJNA284849; Cunningham et al., 2015) and transcriptome (NCBI Bioproject PRJNA285436;

91 Parker et al., 2015). Twenty potential microsatellites were tested and optimised. From these, 14

92 polymorphic microsatellite markers were amplified using three primer mixes with the Qiagen

93 Multiplex PCR kit, following the manufacturer's instructions, to a final volume of 10 µL. The

94 fluorescent M13 tail single-reaction nested PCR method (Schuelke, 2000) using four tails

95 (Tysklind, 2009) was used to amplify the loci. An initial denaturation step of 15 minutes at 95°C

96 was followed by 13 cycles at 94°C for 30 s, 62°C for 90 s, and 72°C for 60 s. In order to attach

97 the dye tails to the PCR product, an extra 31 cycles at 94°C of 30 s, 50°C for 90 s and 72°C for

98 60 s were performed and followed by a final extension at 60°C for 30 minutes. Extension

99 products were resolved on an ABI 3730 instrument at the Edinburgh Genomics Institute Sanger

100 Sequencing Centre with GeneScan 500 LIZ (Applied Biosystems) as internal size standard.

101 Alleles were scored and checked using Peak Scanner v.1.0 (Applied Biosystems).

102  GenAlEx version 6.5 (Peakall & Smouse 2012) and FSTAT (Goudet, 1995) were used to

103 generate descriptive statistics (e.g. number of alleles, allelic frequencies, mean number of alleles

104 per locus and observed (H_0) and expected heterozygosity (H_E)). Tests for deviations from Hardy-

105 Weinberg proportions and genotypic linkage equilibrium were estimated using GENEPOP

106 (Raymond & Rousset, 1995; Rousset, 2008). CERVUS v3.0.7 (Kalinowski et al., 2007) was used

107 to test for null alleles. Estimates of F_{ST}, F_{IS}, population pairwise F_{ST} and their significance per

108 population over all loci were calculated using FSTAT (Goudet, 1995).

109

110 **RESULTS**

111 *Microsatellite development*

112   We screened the *N. vespilloides* genome (4660 contigs; NCBI Bioproject number

113   PRJNA284849) for microsatellite markers with at least 8 repeats for di- and trinucleotides and at

114   least 6 repeats for tetra-, penta- and hexanucleotides, and identified 1818 sequences containing

115   repeats. A total of 5547 microsatellites were present and 4515 primer pairs were obtained using

116   Primer3 (Rozen & Skaletsky, 2000) incorporated into msatcommander (Faircloth, 2008). Similar

117   searches to the transcriptome identified 263 microsatellites and 69 primer pairs were designed.

118   To maximize the potential for amplification, we rejected primers of low quality, and that were

119   likely to self-anneal. To facilitate multiplexing, we chose markers from 100-500bp that varied in

120   size and the number of repeat motifs. To avoid linkage, we chose sequences with just one

121   marker. Twenty microsatellites were chosen for molecular marker optimization (16 from the

122   genome and 4 from the transcriptome). From these, a robust suite of 14 reliable microsatellites

123   was derived for the population genetic analysis (see Supplementary information). By 'robust' we

124   mean that they i) amplified reliably in all populations and in the majority of the samples; ii) did

125   not show secondary amplification; iii) were polymorphic in all populations; and iv) were

126   relatively easy to score (e.g. did not show stuttering).

127

128   *Microsatellite analysis*

129   All of the 139 *N. vespilloides* collected from the four Cambridgeshire populations were

130   genotyped for the 14 microsatellites. All 14 loci were polymorphic for the tested populations

131   and the number of alleles per locus ranged between 5 and 13, with a total number of 134 alleles

132   in the global sample (Table 1). The level of genetic variability was similar across loci. The

133   expected heterozygosity (H_E) per locus ranged from 0.272 to 0.841, and the observed

134   heterozygosity (H_O) ranged from 0.247 to 0.825 (Table 1). All individual loci but one showed

135   lower observed heterozygosity than expected heterozygosity (over all loci H_E=0.714 and

136   H_O=0.652). The expected heterozygosity across all loci per population ranged from 0.696 to

137   0.706, while the observed heterozygosity ranged from 0.638 to 0.679 (Table 2).

138   Tests for concordance with Hardy-Weinberg equilibrium (HWE) revealed deviations

139   from HWE in locus Nvesp_D, Nvesp_F, Nvesp_I, Nvesp_G and Nvesp_H (Table 1). Testing

140   HWE for individual populations and loci revealed that this disequilibrium remained significant

141   within populations (Nvesp_D: significant in all populations but Waresley; Nvesp_F: significant

142   for Byron's Pool only; Nvesp_I: significant in Waresley only; Nvesp_G: significant in Waresley

143   only; Nvesp_H: significant in Gamlingay and Byron's Pool) (Table 2). The frequency of null

144   alleles was low across loci (Table 1) and, overall, close to zero (indicating absence of null

145   alleles). However, three of the loci exhibiting deviations from HWE (Nvesp_D, Nvesp_I and

146   Nvesp_H) showed some of the highest null allele frequencies (>0.05).

147   Evidence of linkage disequilibrium was observed in pairwise loci Nvesp_Q/Nvesp_E and

148   Nvesp_E/ Nvesp_G in the global population test. Global F_{IS} value was 0.085, suggesting some

149   heterozygous deficiency. A pattern of genetic differentiation (global F_{ST}= 0.023) was observed in

150   the sampling area, with all but one significant population-pairwise F_{ST} after Bonferroni

151   correction (α = 0.008333). In order to assess whether there were potential biases in the markers

152   that exhibit deviations from HWE or the presence of potential null alleles, the analyses were run

153   with and without these markers. We found that the two runs rendered similar results (Fig. 1).

154

155   **DISCUSSION**

156   We developed microsatellite markers to infer details of the burying beetle's ecology that cannot

157   be deduced through simple observation, but which are becoming increasingly important for the

158   interpretation of experiments on this species in the laboratory. The process of microsatellite

159   development was greatly facilitated by the existing *N. vespilloides* genome (Cunningham et al.,

160   2015) and transcriptome (Parker et al., 2015). The available genomic tools, however, are still

161   poorly annotated and so further detailed characterisation of the sequences containing the markers

162   is still limited (see Supplementary information). Nevertheless, our analyses suggest that the

163   markers are predominantly unlinked. We rapidly developed a set of 14 (out of 20) reliable

164   polymorphic markers for the species: i.e. 70% were successful. This proportion of successful

165   markers is similar to that obtained for *N. quadripunctatus* (Suzuki & Yao, 2014), although these

166   authors used enriched genomic libraries for marker development.

167   Our genetic analyses revealed significant deviations from Hardy-Weinberg equilibrium at

168   five loci. This is most likely due to an excess of homozygotes at these loci but could also be due

169   to the presence of null alleles in three of these markers. The only other marker with a putatively

170   high null allele frequency (>0.05) was Nvesp_E. However, the high number of homozygotes for

171   this marker is unlikely to be due to null alleles because it is in HWE (Kalinowski et al., 2007).

172   We are confident that our results are not biased by including markers that deviated from HWE,

173   or potential null alleles, because we obtained similar results when we excluded these markers

174   from our analyses. In general, the microsatellite markers showed lower observed heterozygosity

175   than expected and the global F_{IS} value (0.085) also suggested some heterozygosity deficiency.

176   We infer from these findings that there is limited gene flow between our study populations, and

177   potentially some inbreeding as well. The consequences of inbreeding in *N. vespilloides* have

178   recently been analysed experimentally in the laboratory (e.g. Mattey, Strutt & Smiseth, 2013;

179   Pilakouta et al., 2015; Pilakouta & Smiseth, 2016) but to our knowledge, our study provides the

180   first indication that *N. vespilloides* might breed with relatives in nature and it matches previous

181   results obtained for *N. quadripunctatus* (Suzuki & Yao, 2014).

182   Consistent with our interpretation of limited gene flow between populations, we found

183   significant pairwise population differentiation between all but one pair of populations (Waresley-

184   Gamlingay; approximately 2.5 km apart) despite the low geographical separation between the

185   sampling sites (maximum 20.3 km). This genetic differentiation may be the result of neutral

186   population differentiation, or the effects of selection acting on functional genes correlated with

187   the neutral markers (e.g. Rousset & Raymond, 1995). We think the former possibility is more

188   likely because we obtained similar results when we ran the analyses with and without markers in

189   HWE and because a BLASTx search showed that the markers did not generally correspond to

190   (albeit limited) known coding genes in *N. vespilloides*.

191   Previous work on flight performance of *N. vespilloides* showed that these beetles have a

192   wide distribution of flight distances in the laboratory ranging from 68 m to 26 km. Furthermore,

193   flight durations ranged from 61 s to 6.5 h under laboratory conditions (Attisano & Kilner, 2015).

194   Beetles tethered in flight mills may be able to fly for greater distances than naturally flying

195   beetles because they bear less of their weight in flight. But even if we assume that beetles in

196   natural flight cover only a third of the distance that they achieved in a flight mill, our data

197   suggest that population differentiation within the sampling area is not solely attributable to the

198   flight range of the burying beetle.

199   We suggest instead that habitat fragmentation has driven the fine-scale population

200   structure we report here by imposing barriers that limit dispersal (cf Pascoal et al., 2009; Kanno

201   et al., 2011; Valtonen et al., 2014). Our analyses suggest that each 'island' population of burying

202   beetles is increasingly reproductively isolated with increasing geographic distance between

203   woodlands. Perhaps *N. vespilloides* is unwilling, or unable, to undertake flights across open

204   fields and through housing. Studies of congeneric species (*Nicrophorus marginatus*, *N.*

205   *tomentosus*, *N. orbicollis* and *N. defodiens*) found that the size of these woodland fragments is

206   known to affect the abundance and reproductive success of the resident burying beetle

207   populations (Trumbo & Bloch, 2000; Gibbs & Stanton, 2001). Our analyses suggest that the

208   extent of their connectivity might also be an important factor for promoting gene flow and

209   preventing populations from becoming smaller and more inbred. If our interpretation is correct,

210   then it has important conservation implications because it suggests that there is a threshold size

211   of woodland required to sustain an outbreeding burying beetle population. It also might help

212   explain why populations of American burying beetles (*N. americanus*) have collapsed so

213   spectacularly in recent years following substantial deforestation, causing it to become

214   endangered (Anderson, 1982; Creighton et al., 2009).

215   Although Single Nucleotide Polymorphism (SNPs) are starting to be the tool of choice

216   for population genetics/genomics studies, microsatellites still provide a cheaper alternative.

217   Nevertheless, we anticipate that the microsatellites we have developed will prove most useful in

218   future work for assigning parentage (Pascoal et al., in prep) because the large brood size

219   typically seen in *N. vespilloides* still makes other techniques prohibitively expensive. *N.*

220   *vespilloides* mates rampantly and promiscuously in the laboratory (e.g. House et al., 2009) and in

221   natural populations (e.g. Müller et al., 2007). Although several previous studies have analysed

222   strategies used by males for securing paternity (e.g. Eggert, 1992; House et al., 2009), parentage

223   has never before been assigned using microsatellites. The microsatellites we have developed here

224   thus pave the way for more detailed analyses of the evolutionary causes and consequences of

225   promiscuity in this species.

226

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341 beetles for the genetic analysis.

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351 **FIGURES**
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	W	G	BP	MD
W	0	0.0015 (0.0013)	0.0319 (0.0353)	0.0219 (0.0301)
G	ns	0	0.0292 (0.0298)	0.0237 (0.0324)
BP	**	**	0	0.0415 (0.0578)
MD	**	**	**	0

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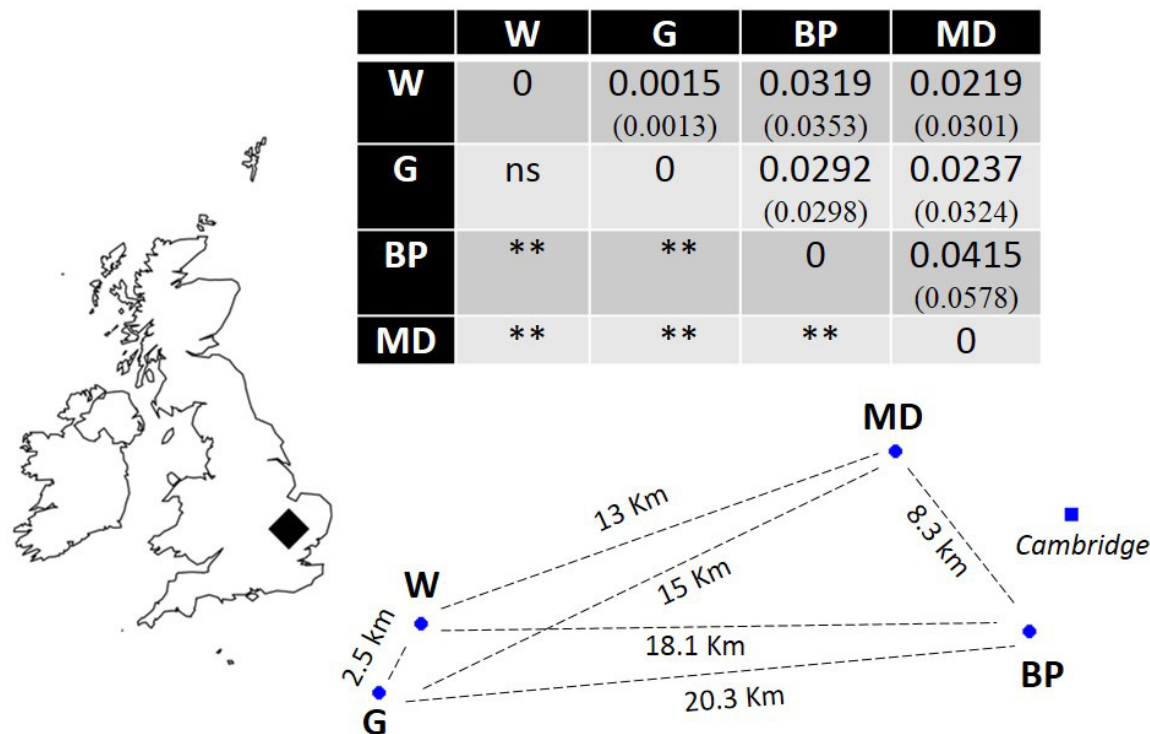
356 **Figure 1.** Sampling location in south Cambridgeshire and genetic differentiation in pairwise

357 comparisons between populations. *P* values for pairwise F_{ST} estimates across all loci are

358 indicated below the diagonal, after Bonferroni correction ($\alpha = 0.008333$). Values in brackets

359 represent the pairwise F_{ST} values using only the 9 markers in HWE; the significance levels were

360 identical in both runs. W: Waresley; G: Gamlingay; BP: Byron's Pool; MD: Madingley.

361 Distances are "as the crow flies" distances calculated using online tools. The map and

362 population's spatial representation (using the sites geographical coordinates) were produced in R.

363 **TABLES:**

364 **Table 1.** Main genetic variability measures by locus of *N. vespilloides* from Cambridgeshire. T(ºC): annealing temperature; bp: base

365 pairs; G: genome; T: transcriptome; Na: number of alleles found per locus; H_E: expected heterozygosity; H_O: observed heterozygosity;

366 F_{IS}: standardized genetic variance within populations at each locus; F_{ST}: standardized genetic variance among populations at each

367 locus; Null: frequency of null alleles per locus; HW: Hardy-Weinberg P values.

Locus	Primer sequence 5'-3'	Product size (bp)	Repeat motif	T (ºC)	PCR	Source	Na	H_E	H_O	F_{ST}	F_{IS}	Null
Nvesp_A	F: Fam-CTACGGCGTGCAGAATTACC	138	(AAC)9	62	Mix1	G	13	0.757	0.770	0.002	-0.026	-0.0114
	R: AACTCTCTGGTGTCGACGTC											
Nvesp_D	F: Pet-TACGTGCGGTAATGAGGCG	201	(AAC)11	62	Mix1	G	8	0.829	0.585	0.016	0.287	0.1701
	R: ACGCCCTGCTCCCTATTTAG											
Nvesp_J	F: Vic-TGTGTGTAGAGTGGACGGG	303	(AAAG)7	62	Mix1	G	9	0.657	0.631	0.035	0.010	0.0131
	R: TGGACGAGTTGAAGACGAGG											
Nvesp_M	F: Ned-CCAGCAACCCACAAAGAAGC	373	(AG)10	62	Mix1	G	11	0.841	0.825	0.019	0.039	0.0244
	R: ATACCACAAGTCCCGACCTG											
Nvesp_Q	F: Fam-ATGCGGCTTTGATATCCAGG	428	(AAT)8	62	Mix1	G	8	0.516	0.494	0.005	0.075	0.0560
	R: TCAGATTCCGCTCTCCTTCC											
Nvesp_B	F: Fam-GTTGTTTCCGGTTGTTTGCG	158	(AC)8	62	Mix2	G	10	0.723	0.701	0.021	0.035	0.0266
	R: TTCGAAGTTAAACGGCCGTG											
Nvesp_F	F: Pet-TAAAGGGTTGGGAGGTTGGC	216	(AC)10	62	Mix2	G	10	0.802	0.723	0.023	0.075	0.0458
	R: CACGATCCATACACGTGCAC											
Nvesp_I	F: Vic-CTGATCACCGGAACCCTCTC	286	(AG)8	62	Mix2	T	6	0.569	0.498	0.006	0.135	0.0793
	R: GAATTCCCGGGTTTATGCCG											
Nvesp_P	F: Fam-TGGTGATGCAATTGTGAGGC	410	(ATC)8	62	Mix2	G	9	0.809	0.741	0.028	0.082	0.0498
	R: CGGTTGGCAGACGATGTAAC											

Nvesp_E	F: Pet-ATGGATGGATGGAGAGTGGC R: TTGATGGTTTCGAAAGGGCG	201	(AC)8	60	Mix3	T	11	0.787	0.680	0.092	0.139	0.1109
Nvesp_G	F: Fam-CGTGTGCGTGTTTCTACCTC R: ATGGGCACGTATCCATACCC	224	(AT)8	60	Mix3	T	12	0.834	0.776	0.013	0.064	0.0351
Nvesp_H	F: Vic-TCGTAGATGTCTCGTGCCTG R: CAGTTTGAAGGTGGTGGCTG	283	(AG)9	60	Mix3	G	12	0.840	0.737	0.013	0.120	0.0669
Nvesp_K	F: Ned-GCTCTCATTCTCCCAAACGC GTGGACGCGCATAAGTTGTC	334	(AGG)8	60	Mix3	G	5	0.272	0.247	-0.002	0.085	0.0328
Nvesp_O	F: Fam-ATGCCAATTAACGCGTCGAG CATCGTTACCTGTGCGACTG	395	(AAG)8	60	Mix3	G	10	0.760	0.718	0.013	0.054	0.0387
All							134	0.714	0.652	0.023	0.085	

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375 **Table 2.** Main genetic variability measures for Cambridgeshire populations. W: Waresley; G: Gamlingay; BP: Byron's Pool; MD:

376 Madingley; N: mean number of samples per locus; N-all: mean number of alleles per locus; H_E: expected heterozygosity; H_O:

377 observed heterozygosity; A-O refers to Nvesp_A-Nvesp_O.

					Hardy-Weinberg P values													
Pop	N(±SD)	N-all(±SD)	H_E(±SD)	H_O(±SD)	A	D	J	M	Q	B	F	I	P	E	G	H	K	O
W	38.143 (0.619)	7.571 (0.453)	0.699 (0.047)	0.679 (0.046)	0.255	0.141	0.632	0.228	0.529	0.180	1.000	**0.009**	0.834	0.328	**0.004**	0.297	1.000	0.544
G	37.214 (0.639)	7.714 (0.474)	0.706 (0.043)	0.638 (0.045)	0.665	**0.000**	0.969	0.115	**0.036**	0.267	0.535	0.282	0.164	0.221	0.351	**0.000**	0.064	**0.028**
BP	29.714 (0.485)	7.429 (0.581)	0.702 (0.043)	0.664 (0.049)	0.428	**0.016**	0.908	**0.022**	0.191	0.855	**0.000**	0.206	0.256	0.083	0.136	**0.017**	0.436	0.904
MD	19.286 (0.606)	6.429 (0.343)	0.696 (0.044)	0.649 (0.045)	0.288	**0.037**	0.059	0.936	0.598	0.607	0.102	0.518	0.103	0.571	0.183	0.100	0.232	0.071

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