

## ORIGINAL ARTICLE

Adaptive divergence and speciation among sexual and pseudoviviparous populations of *Festuca*T Chiurugwi<sup>1</sup>, MA Beaumont<sup>1,3</sup>, MJ Wilkinson<sup>2</sup> and NH Battey<sup>1</sup><sup>1</sup>School of Biological Sciences, University of Reading, Reading, UK; <sup>2</sup>Institute of Biological Environmental and Rural Sciences, Aberystwyth University, Aberystwyth, UK and <sup>3</sup>Schools of Mathematics and Biological Sciences, University of Bristol, Bristol, UK

Pseudovivipary is an environmentally induced flowering abnormality in which vegetative shoots replace seminiferous (sexual) inflorescences. Pseudovivipary is usually retained in transplantation experiments, indicating that the trait is not solely induced by the growing environment. Pseudovivipary is the defining characteristic of *Festuca vivipara*, and arguably the only feature separating this species from its closest seminiferous relative, *Festuca ovina*. We performed phylogenetic and population genetic analysis on sympatric *F. ovina* and *F. vivipara* samples to establish whether pseudovivipary is an adaptive trait that accurately defines the separation of genetically distinct *Festuca* species. Chloroplast and nuclear marker-based analyses revealed that variation at a geographical level can exceed that between *F. vivipara* and *F. ovina*. We deduced that *F. vivipara* is a recent species that frequently arises

independently within *F. ovina* populations and has not accumulated significant genetic differentiation from its progenitor. We inferred local gene flow between the species. We identified one amplified fragment length polymorphism marker that may be linked to a pseudovivipary-related region of the genome, and several other markers provide evidence of regional local adaptation in *Festuca* populations. We conclude that *F. vivipara* can only be appropriately recognized as a morphologically and ecologically distinct species; it lacks genetic differentiation from its relatives. This is the first report of a 'failure in normal flowering development' that repeatedly appears to be adaptive, such that the trait responsible for species recognition constantly reappears on a local basis.

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## Introduction

Environmental conditions, especially temperature, rainfall and photoperiod, vary with time and location. Plants can change their phenotype in tune with variations in environmental conditions. In some cases, these adaptive changes are genetically controlled such that the progeny of individuals from different environments retain their different characteristics when grown together. If this differential adaptation to the environment occurs within a species, races with distinct ecological preferences emerge (Jump *et al.*, 2006). These races can in turn evolve into new species.

In many temperate grasses, marginal floral inductive conditions (mainly photoperiod and temperature) induce proliferation of inflorescences in which flowering is aborted and leafy shoots are produced instead of sexual reproductive structures (Arber, 1934; Beetle, 1980) (Figure 1). This phenotype, known as pseudovivipary, generally prevails in wet areas, mainly in alpine and arctic regions, where the leafy propagules can survive and establish successfully (Wycherley, 1953a, 1954; Lee and Harmer, 1980; Heide, 1994; Elmqvist and Cox, 1996). Pseudovi-

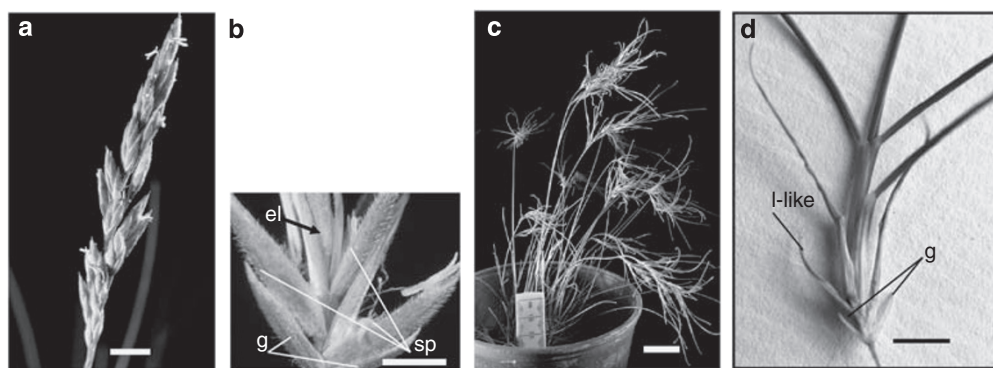
vipary is thought to be a genetically assimilated form of asexual reproduction that arises from habitually seed-producing (seminiferous) species (Heide, 1988).

The distribution of constantly pseudoviviparous forms often overlaps with that of closely related seminiferous taxa, with the asexual forms typically occupying environments that do not favour the production of viable seed. Examples of such sympatric seminiferous–pseudoviviparous pairings include *Festuca vivipara* (L.) Sm. with *Festuca ovina* L. *sensu stricto* (hereafter termed *F. ovina*), and *Poa bulbosa* L. *sensu stricto* with *Poa bulbosa* var. *vivipara* Koel. (Wycherley, 1953a; Beetle, 1980; Heide, 1989). The stability of the pseudoviviparous trait after transplantation to a common growing environment (Youngner, 1960; Moore and Doggett, 1976; Beetle, 1980; Heide, 1988, 1989, 1994) suggests a genetic rather than a plastic (environmentally induced) maintenance of the phenotype. Grass forms exhibiting phenotypically fixed pseudovivipary are generally taxonomically recognized at infraspecific ranks, although there is little consensus between taxa and researchers (Wycherley, 1953b; Youngner, 1960; Beetle, 1980). *F. vivipara* is an exception to this rule, as it is a species whose defining characteristic is pseudovivipary (Beetle, 1980), although the taxon has been recognized at several infraspecific ranks (Wycherley, 1953b; Beetle, 1980; Frederiksen, 1981; Wilkinson and Stace, 1991). Researchers have postulated that *F. vivipara* arose from *F. ovina* through mutation, hybridization and/or polyploidy (Arber, 1934; Flovik, 1938; Wycherley, 1953b; Beetle, 1980).

Correspondence: Dr T Chiurugwi, School of Biological Sciences, University of Reading, Philip Lyle Building, Reading, RG6 6BX, UK.

E-mail: t.chiurugwi@reading.ac.uk

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**Figure 1** Comparison of *F. ovina* and *F. vivipara* spikes and spikelets. (a, b) *F. ovina* spike and spikelet (sp); the spike is made up of spikelets of three florets each. The spikelet meristem terminates in an empty lemma (el) (arrow). (c, d) In *F. vivipara* spikelets, florets are replaced by shoots. Scale bar = 5 mm. g, glume; l-like, leaf-like lemma.

Pseudovivipary can be experimentally induced in a wide range of seminiferous grasses by growing them under suboptimal floral inductive conditions; moreover, in facultative pseudoviviparous forms, sexual reproduction can be partially restored under optimal flowering conditions (Moore and Doggett, 1976; Heide, 1989). In addition, pseudoviviparous plants produce fertile flowers in areas in which pseudoviviparous and seminiferous populations overlap occasionally (Wycherley, 1953a, 1954; Lee and Harmer, 1980). This phenomenon, present in a number of species of *Poa* and *Festuca*, indicates that sexuality is not totally suppressed in all pseudoviviparous individuals (Wycherley, 1953a, 1954; Beetle, 1980; Lee and Harmer, 1980; Heide, 1988). These observations led Beetle (1980) to suggest that pseudoviviparous forms arose in response to prolonged exposure to conditions favourable for their development. Thereafter, these forms spread to occupy their current range. According to this hypothesis, pseudoviviparous members of the same species share a monophyletic origin. The alternative, polyphyletic theory proposes that pseudovivipary is constantly evolving among seminiferous populations that are found in conditions that are not conducive for normal flowering (Wilkinson and Stace, 1991). Determining the actual origin of pseudovivipary is central to establishing a common practice for the taxonomic treatment of these taxa, and for understanding the ecological and evolutionary role of the phenomenon (Wilkinson and Stace, 1991).

Thus far, studies of pseudovivipary have focused on describing the phenotype and the conditions that induce it, and on exploring its ecological significance (Wycherley, 1954; Beetle, 1980; Lee and Harmer, 1980; Heide, 1994). Although information about the cause(s) of pseudovivipary has been widely reported, little is known about how it is controlled and maintained at a molecular or genetic level (Wycherley, 1953b; Youngner, 1960; Beetle, 1980; Lee and Harmer, 1980; Heide, 1989). Determining the phylogeographic origin of pseudoviviparous grasses is a first natural step towards answering this question. To do this, we performed a phylogeographic study of *F. vivipara* and sympatric *F. ovina* from the United Kingdom using amplified fragment length polymorphism (AFLP) and sequence variation of chloroplast DNA loci (cpDNA).

We integrated population genetic and phylogenetic analysis to explain the geographic distribution of

genealogical lineages among *F. ovina* and *F. vivipara* samples, and to investigate the genetic polymorphism linked to pseudovivipary. Similar approaches, relying on the correlation between gene clines and selective environmental transition, have been used in a range of situations (Beaumont and Nichols, 1996; Beaumont and Balding, 2004; Jump *et al.*, 2006; Bonin *et al.*, 2007; Caballero *et al.*, 2008; Herrera and Bazaga, 2008). Our data are more consistent with the hypothesis that UK *F. vivipara* repeatedly arises *de novo* from local seminiferous populations of *F. ovina*.

## Materials and methods

### Plant material

Paired samples of *F. vivipara* (L.) Sm. with *F. ovina* L. *sensu stricto* plants (growing within 5 m of each other) were collected from over 50 localities around three mountains in the United Kingdom: Ben Lawers (Mid Perthshire, Scotland), Snowdon (Caernarvonshire, North Wales) and Craig Cerrig Gleisiad (Breconshire, South Wales) (Supplementary Figure 1). At locations at which either *F. ovina* or *F. vivipara* was absent, samples of the species present were collected. In total, more than 120 samples were collected.

Samples used to assemble cpDNA sequence data comprised of 48 individuals from Snowdon, 23 from Ben Lawers and 9 from Craig Cerrig Gleisiad. The AFLP analysis included 26 samples from Snowdon, 16 from Ben Lawers and 14 from Craig Cerrig Gleisiad. All plant samples were confirmed to be either *F. vivipara* or *F. ovina* by morphological and ploidy level analysis (Wilkinson and Stace, 1991).

### DNA extraction

DNA was extracted from 0.1 g of fresh leaves using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The samples used for both cpDNA and AFLP analyses were chosen to represent all three collection sites, and wherever possible to include paired neighbouring samples of *F. vivipara* and *F. ovina*. DNA quality and quantity were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies LLC, Wilmington, DE, USA), and the concentration adjusted to 5–10 ng  $\mu\text{l}^{-1}$  for cpDNA sequencing and to 10–15 ng  $\mu\text{l}^{-1}$  for AFLP analysis.

### Chloroplast DNA sequence analysis

Initially, three non-coding cpDNA regions, *trnCF-rpoB*, *trnS(GCU)-psbD* and *trnT2-rps4* (Saltonstall, 2001), were amplified and assessed for sequence variation among the following eight samples: four *F. vivipara* and four *F. ovina* from Snowdon and Ben Lawers using primers published by Saltonstall (2001). These cpDNA regions have been previously shown to be polymorphic in *Festuca pratensis* (Fjellheim et al., 2006). For PCR, 25 µl reaction mixes comprising 12.5 µl Biomix (Bioline, London, UK), 25 pmol of each primer and 2.5 µl of DNA template were assembled. The reaction mix was then subjected to 1 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 40 s at 56 °C and 1.5 min at 72 °C, culminating with 10 min at 72 °C. PCR amplicons were sequenced directly, twice in both directions, using the amplification primers after cleaning with a NucleoFast 96 PCR Cleanup kit (Macherey-Nalgene, Düren, Germany). Cleaned PCR products were sent to MacroGen Inc. (Korea) for cycle sequencing and product fractionation using dye terminator chemistry with an ABI3730XL unit (Sanger et al., 1977; MacroGen Inc., Seoul, Korea). Sequences were aligned using ClustalW2 (Larkin et al., 2007) and the alignments edited using MacClade 4.07 software package (Maddison and Maddison, 2002; Sinauer Associates, Sunderland, MA, USA).

Following the initial screen, the two variable loci (*trnCF-rpoB* and *trnT2-rps4*) were used to obtain sequence in both directions for the remaining samples. For the larger screen, cpDNA sequence data were also obtained from GenBank for *Agrostis stolonifera*, *F. pratensis* haplotype A, B and C (from Georgia, Bulgaria and the United Kingdom, respectively), Norwegian *Festuca gigantea*, *Festuca arundinacea* and *Lolium perenne*; these were used as reference samples for the phylogenetic analysis (Fjellheim et al., 2006; Sasaki et al., 2007). *A. stolonifera* was used as the out-group sample.

Parsimony analysis to construct a phylogeny was performed using Phylogenetic Analysis Using Parsimony\*4.0b10 (Swofford, 1998). Heuristic parsimony search was conducted using 100 random addition sequence replicates and tree bisection reconnection branch swapping, retaining no more than 10 trees per replicate. Bootstrap analysis was carried out using Phylogenetic Analysis Using Parsimony implementing the same heuristic search strategy and character type settings as in the phylogeny construction and 100 bootstrap replicates. The structure of the tree results from Phylogenetic Analysis Using Parsimony was further explored in MacClade. The level and distribution of genetic variation in *F. ovina* and *F. vivipara* populations observed from cpDNA sequence data was further explored in AFLP analysis.

### AFLP analysis

Fluorescent-AFLP analysis was performed using the AFLP Core Reagents and AFLP Primer Starter kits (Invitrogen, Paisley, UK). Selective amplicons generated using 6-carboxyfluorescein (FAM)- and 6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein (HEX)-labelled primers were fractionated by capillary electrophoresis on an ABI PRISM 3100 capillary electrophoresis unit. Initially, a total of six primer pairs were screened: (1) M-CAA:E-ACG, (2) M-CTC:E-ACG, (3) M-CTC:E-GAC\*, (4) M-CAT:E-GAC\*, (5) M-CAT:E-GCA, (6) M-CAA: E-GCAFAM\*. Three of these

(asterisked) that produced consistent and scorable profiles were selected for further analysis. Electropherogram traces were analysed using GeneMarker Version 1.71 (Softgenetics LCC, State College, PA, USA).

AFLP profiles were scored for the presence or absence of amplicon peaks between 80 and 500 bp in size using GeneMarker software (Softgenetics LCC). Peaks of similar size and intensity were assumed to be homologous.

### AFLP marker diversity among *F. ovina*/*F. vivipara* populations

To estimate genetic diversity and population differentiation, analysis of molecular variance (AMOVA) was carried out on the binary data set derived from GeneMarker using GenAlex software, version 6.2 (Peakall and Smouse, 2006; The Australian National University, Canberra, Australia). We used the binary genetic distance option, which results in the  $\phi_{PT}$  estimator, an analogue of  $F_{ST}$ . Six populations defined by mountain and species, pooled into three regions defined by geographic origin were compared in this analysis. Principal coordinate analysis was used to graphically represent the genetic distance matrices generated from AMOVA.

### Identification of candidate loci for selection

Outlier loci (those deemed potentially under selection) were identified using DFDIST software ([www.rubic.rdg.ac.uk/~mab/stuff/](http://www.rubic.rdg.ac.uk/~mab/stuff/)) (Beaumont and Nichols, 1996). The sub-populations compared with DFDIST were as follows: (1) Three populations defined by mountain (*F. ovina* and *F. vivipara* pooled together on each mountain); (2) For *F. ovina*, three populations defined by mountain; (3) For *F. vivipara*, three populations defined by mountain; and (4) For each mountain, two populations of *F. ovina* and *F. vivipara*. A total of 50 000 realizations were performed using the default settings of the DFDIST program with a smoothing proportion of 0.04, target average  $F_{ST}$  equal to the trimmed mean  $F_{ST}$  (from Ddatacal software, distributed with DFDIST; <http://www.rubic.rdg.ac.uk/~mab/stuff/>), a  $\theta$ -value of 0.06 and maximum allowable frequency of 0.99 pooled across samples. The Benjamini and Hochberg false discovery rate (FDR) correction method was used to correct for the occurrence of false positives in loci identified as under selection (Benjamini and Hochberg, 1995). The  $P$ -values used in the FDR analysis were derived from the three  $P$ -values ( $P_1$ ,  $P_2$  and  $P_3$ ) produced by the pv program (distributed with DFDIST; <http://www.rubic.rdg.ac.uk/~mab/stuff/>). The reason DFDIST produces three  $P$ -values is because of the presence of ties in the data. The empirical probability of observing simulated  $F_{ST}$  values as small as or smaller than the data point is given by  $P_1$ , and the probability of observing values greater than or equal to the data point is given by  $P_2$ . A third  $P$ -value,  $P_3$ , is also given, which is defined as follows: if  $P_1 \geq 0.5$ , then the greater of  $P_1$  and  $P_2$ ; if  $P_1 < 0.5$ , the lesser of  $P_1$  and  $P_2$ . However, in this study, we used  $P_x$ , computed as  $P_x = P_1 - 0.5(P_1 + P_2 - 1)$ , which was then converted into a two-tailed  $P$ -value by computing  $1 - 2 \times \text{abs}(P_x - 0.5)$  (Beaumont and Nichols, 1996; Beaumont and Balding, 2004). We used  $P_x$  because it is based on the midpoint of the ties, and has a relatively uniform simulated distribution, in line with the assumption of methods that compute the FDR. Loci with

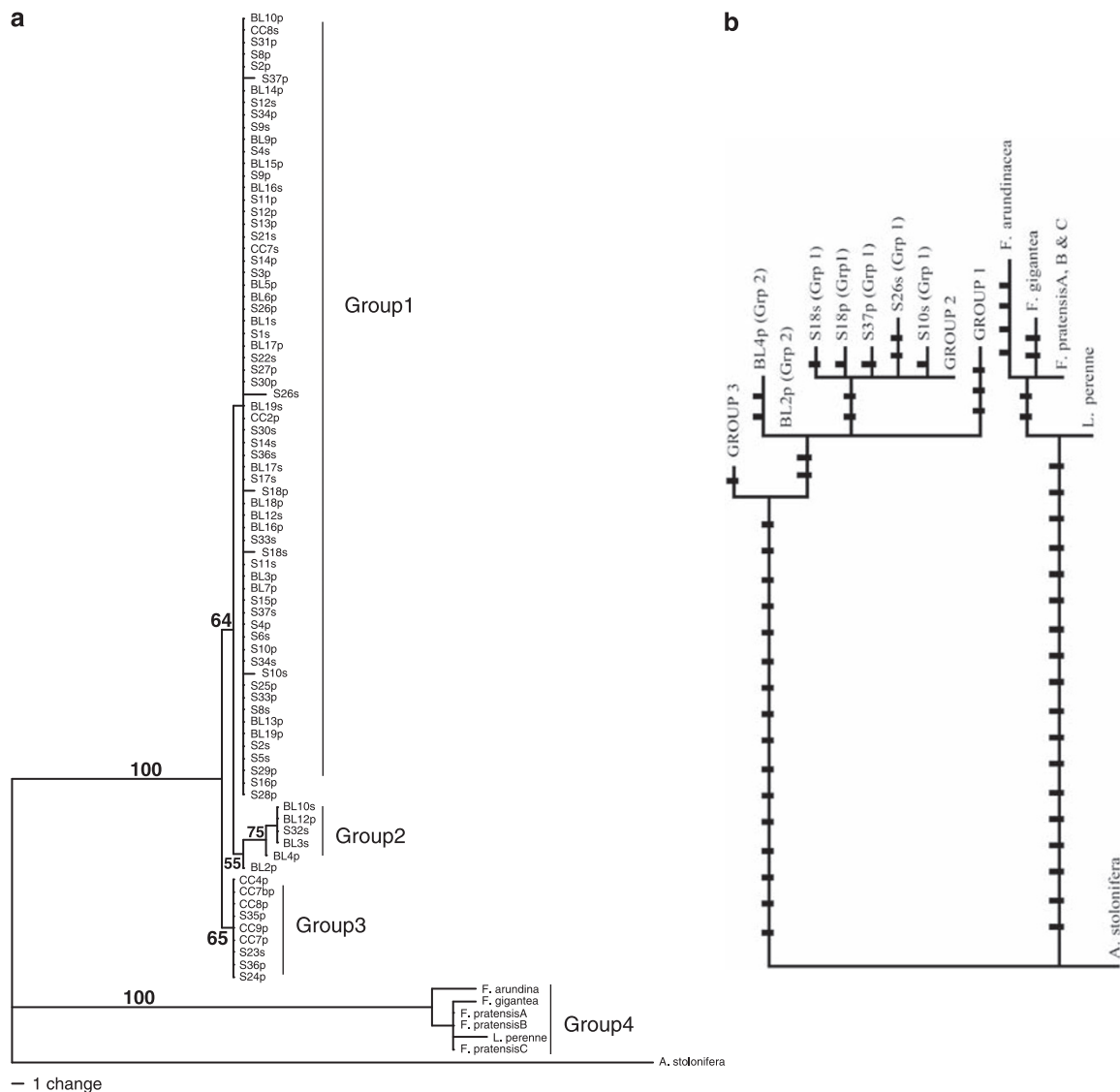
significant *P*-values at a FDR threshold of 50% were identified using the Benjamini and Hochberg method.

## Results

### Genetic diversity and differentiation among *F. ovina*/*F. vivipara* populations

**Chloroplast sequence analysis:** The cpDNA sequence alignment produced a data matrix of 1055 characters. In the strict consensus tree from parsimony analysis, the samples were separated into four major clades: group 1, 2 and 3 containing all *F. ovina* and *F. vivipara* samples, and group 4 with all other *Festuca* spp. and *L. perenne* samples from GenBank (Figure 2). The *Festuca ovina-vivipara* samples under study separated into group 3, comprising *F. vivipara* samples from Craig Cerrig Gleisiad and Snowdon; group 2, comprising *F. vivipara* samples from Ben Lawers and one

*F. ovina* sample from Snowdon; and group 1 comprising the remaining samples. The *F. ovina*/*F. vivipara* samples were found to comprise 10 chloroplast haplotypes, separated among them by between 1 and 10 unambiguous changes, and collectively separated by more than 10 changes from samples in group 4 (Figure 2b). Representative sequences for each haplotype were submitted to the GenBank genetic sequence database (*trnCF-rpoB* and *trnT2-rps4*, respectively): S18p—accession numbers HM807585 and HM807595; S10s—accession numbers HM807586 and HM807596; CC7s—accession numbers HM807587 and HM807597; S37p—accession numbers HM807588 and HM807598; S26s—accession numbers HM807589 and HM807599; S18s—accession numbers HM807590 and HM807600; S37p—accession numbers HM807591 and HM807601; BL4p—accession numbers HM807592 and HM807602; S10s—accession numbers HM807593 and HM807603; BL2p—accession numbers HM807594 and HM807604.



**Figure 2** Results of parsimony analysis of *Festuca* cpDNA sequence data. (a) Strict consensus tree from parsimony analysis of cpDNA sequence data. The samples are separated into four groups: groups 1–4. Groups 1, 2 and 3 had modest bootstrap support. Scale bar represents one change. (b) Consensus tree from (Figure 1a) condensed in MacClade. Groups 1 and 2 are separated from group 3 by 10 unambiguous changes, whereas *F. ovina* and *F. vivipara* samples in groups 1 and 2 are separated by one or two base changes. Horizontal bars represent the number of unambiguous changes on each branch. BL, Ben Lawers; CC, Craig Cerrig Gleisiad; p, *F. vivipara*; s, *F. ovina*; S, Snowdon.

It was clear from the cpDNA data that the *F. ovina*/*F. vivipara* complex is separate from the other *Festuca* spp. included in the study. The chloroplast haplotypes observed here could not be used to demarcate clades defined by species (*F. vivipara* or *F. ovina*). The strongly supported clusters recovered from the analysis were moderately concordant with geographic origin, but not with species identity. Therefore, we further explored the genetic diversity and differentiation among the *F. ovina*/*F. vivipara* populations using AFLP analysis.

**AFLP analysis:** We generated 105 repeatable AFLP bands (loci) of consistent intensity for the 56 samples used in the study. Of the 105 loci, 65 were found to be polymorphic between samples. The AFLP data were used to explore the level of genetic diversity and its distribution among the *Festuca* populations.

**Genetic differentiation among *Festuca* populations:** AMOVA with six populations, defined by sample origin and species, and three regions, defined by the mountains, showed that 5% of the AFLP variation among samples was due to differences between the mountains, 3% due to variation among the populations and the remaining 92% was due to variation within populations (Table 1).

In almost all the pairwise comparisons between the six populations, variation was highly significant ( $P < 0.01$ ) between populations from different locations. Within locations, significant variation was only observed between *F. ovina* and *F. vivipara* among samples from Craig Cerrig Gleisiad (Table 2).

The results from AMOVA were supported by a plot of the first two principal coordinates extracted from the principal coordinate analysis (Figure 3a). Here, *F. ovina* and *F. vivipara* samples from the same mountain did not form distinct clusters and, whereas Ben Lawers and Snowdon samples were separate from each other, the Craig Cerrig Gleisiad cluster overlapped with the former two. The plot is reminiscent of the phylogenetic tree based on cpDNA variation, as the samples separated into location-based groups separated by statistically significant genetic differentiation.

**Identification of outlier loci:** Further examination of the AFLP data using DFDIST sought to determine whether, given the relatively low differentiation between populations of *F. ovina* and *F. vivipara* within the same mountain, there was evidence of any highly differentiated loci. In the within-mountain comparisons carried out here, an outlier locus was only detected among Craig Cerrig Gleisiad samples (Figure 3b). In addition, this locus (50) was only an outlier in this

comparison, not detected in other comparisons (Figures 3b–d), and was well within the 50% FDR threshold ( $P = 0.006$ ).

With samples subdivided into three populations per species (defined by mountain of origin), outlier loci were only observed from the *F. vivipara* comparisons: loci 63, 2, 24 and 3 fell within the 50% threshold in FDR analysis with  $P$ -values of 0.004, 0.005, 0.006 and 0.016 (Figure 3c). One of the loci detected here (locus 2) was the same as that detected in an analysis in which the species were pooled into one population for each mountain (Figures 3c and d). A similar analysis with all *F. ovina* and *F. vivipara* samples pooled together revealed no outliers at the 50% FDR threshold, although locus 2 was borderline ( $P = 0.008$ ). Thus, we find evidence of adaptive divergence among mountains at some loci in *F. vivipara*, but not in *F. ovina*. Furthermore, evidence of adaptive divergence at these loci disappears when the two species are pooled within each mountain.

## Discussion

Several authors have suggested that, in most cases, pseudovivipary is merely a floral or inflorescence reversion that arises spontaneously in several grass species in montane-artic conditions and that has unclear ecological or taxonomic significance (Beetle, 1980; Lee and Harmer, 1980). Only in *F. vivipara* has the trait led to the definition of a new species. We investigated the genealogical and population genetic relationships between sympatric stands of *F. vivipara* and seminiferous *F. ovina* to gain insights into the genetic control of pseudovivipary and the role it has in speciation.

**Table 2** Results of AMOVA conducted on AFLP variation among and within populations of *F. vivipara* and *F. ovina* from Snowdon, Ben Lawers and Craig Cerrig Gleisiad

BLp		0.039				
BLs	0.022					
CCp	0.102*	0.186**		0.061		
CCs	0.053	0.062	0.095*			
Sp	0.086**	0.055	0.153*	0.015		0.010
Ss	0.085**	0.053	0.134	-0.006**	0.009	
	BLp	BLs	CCp	CCs	Sp	Ss

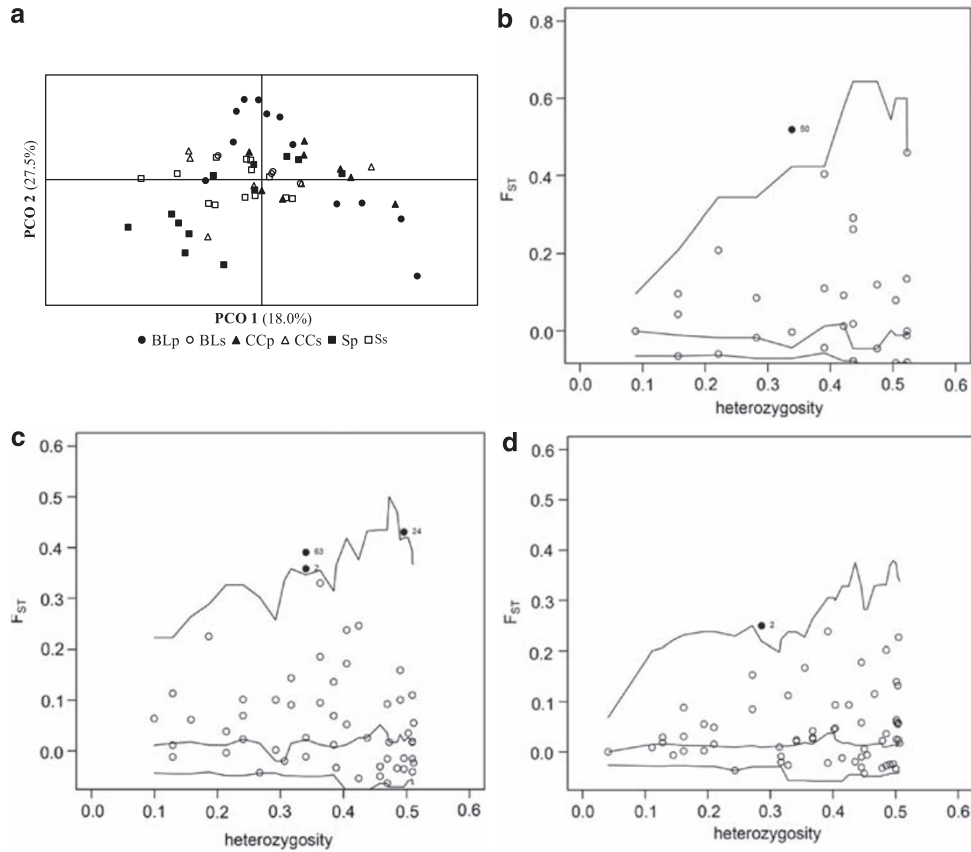
Abbreviations: AMOVA, analysis of molecular variance; BL, Ben Lawers; CC, Craig Cerrig Gleisiad; p, *F. vivipara* (pseudoviviparous); s, *F. ovina* (seminiferous); S, Snowdon.

The lower diagonal half of the matrix shows the pairwise  $\phi_{IT}$  estimates across the six populations. \* $P < 0.5$  and \*\* $P < 0.01$ . The upper diagonal half of the matrix shows the pairwise DFDIST  $F_{ST}$  estimates from Ddatacal.

**Table 1** Results of AMOVA conducted on AFLP variation among and within populations of *F. vivipara* and *F. ovina* from Snowdon, Ben Lawers and Craig Cerrig Gleisiad

Source	d.f.	Sum of squares	Variance components	% of variation	P
Among mountains	2	52.104	0.631	5%	$\phi_{RT} = 0.052$ 0.000
Among populations	3	43.265	0.384	3%	$\phi_{PR} = 0.033$ 0.038
Within populations	49	547.577	11.175	92%	$\phi_{PT} = 0.083$ 0.000
Total	54	642.945	12.191	100%	

Abbreviations: AFLP, amplified fragment length polymorphism; AMOVA, analysis of molecular variance; d.f., degree of freedom;  $\phi_{PR}$ , the level of differentiation among populations;  $\phi_{RT}$ , level of differentiation among mountains.



**Figure 3** Analysis of AFLP variation among *Festuca* populations. (a) A plot of *Festuca* samples from Snowdon (S), Ben Lawers (BL), and Craig Cerrig Gleisiad (CC) produced from principal coordinate analysis. p, pseudoviviparous; s, seminiferous. The percentages of total variation accounted for by the first two principal coordinates (PCO 1 and PCO 2) are shown in brackets. (b) Comparison of the distribution of per-locus  $F_{ST}$  of *F. ovina* and *F. vivipara* samples from Craig Cerrig Gleisiad ( $n=14$ ). Estimated values of  $F_{ST}$  from 56 AFLP loci are plotted as a function of heterozygosity. In (b–d), solid lines denote 0.995th, 0.5th and 0.005th quantiles of the conditional distribution obtained from DFDIST simulation and outlying loci are highlighted in black. (c) Comparison of the distribution of per-locus  $F_{ST}$  of *F. vivipara* samples from Snowdon, Ben Lawers and Craig Cerrig Gleisiad ( $n=32$ ). Estimated values of  $F_{ST}$  from 64 AFLP loci are plotted as a function of heterozygosity. (d) Comparison of the distribution of per-locus  $F_{ST}$  of *F. ovina* and *F. vivipara* samples from Snowdon, Ben Lawers and Craig Cerrig Gleisiad ( $n=55$ ). Estimated values of  $F_{ST}$  from 65 AFLP loci are plotted as a function of heterozygosity.

### *F. vivipara* and *F. ovina* are not genetically distinct

The phylogeny of pseudoviviparous and seminiferous *Festuca* plants from three locations across the United Kingdom, compiled on the basis of two intergenic chloroplast loci (*trnT2-rps4* and *trnCF-rpoB*), revealed only a low level of variation between samples, with just 10 distinct haplotypes observed in total. Nevertheless, there was sufficient structuring between locations to allow a loose classification based on geographic origin, but not to consistently distinguish *F. ovina* from *F. vivipara*. Although more regions of the chloroplast could be usefully deployed to further resolve the sequence-based phylogeny, it is likely that most additional loci that could be used for this purpose would be less informative than those already selected. For this reason, we elected to use AFLP data to provide finer resolution of genetic relationships between samples.

In common with the cpDNA sequence data, the AFLP data revealed separation of samples into groups on the basis of geographical origin, supported by AMOVA, principal coordinate analysis and DFDIST analysis. Similar levels of differentiation could not be detected between the two *Festuca* species. The AFLP data failed to

clearly separate samples according to species identity for all but one mountain studied here. This is in line with the cpDNA sequence data. The results point to significant levels of genetic exchange between the two species, making it impossible to distinguish between them, except at Craig Cerrig Gleisiad, where one locus was highly differentiated between the two species. Although this locus could be a false positive, it provides evidence for a genetic basis for the differentiation between *F. vivipara* and *F. ovina* at one site. The fact that this species-specific polymorphism does not hold in the two other sites clearly indicates that the genetic variant is not itself directly causal of pseudovivipary. Nevertheless, it does not rule out the possibility that this marker may be linked to such a locus. Under this scenario, it can be speculated that directional selection around this locus leads to variation in the mode of reproduction among *F. ovina* plants, giving rise to *de novo* formation of *F. vivipara*. It would be interesting to observe whether this inference holds, or more loci can be identified, with a larger sample size. If the inference holds, characterization of this and similar loci may ultimately lead to the identification of the genetic basis of pseudovivipary.

### Adaptive differentiation and speciation

The species-by-species analysis demonstrated that it is possible to distinguish *F. vivipara* populations from different locations on the basis of both cpDNA and AFLP data. Analysis of AFLP data shows that genetic exchange is greater among *F. ovina* than among *F. vivipara* plants, an observation clearly in agreement with the two species' modes of reproduction. Interestingly, the locus identified as distinguishing between *F. vivipara* and *F. ovina* (locus 50) is not recovered as an outlier in intermountain comparisons. This suggests that there were genetic differences between populations of *F. vivipara* progenitors (*F. ovina*) from the three different locations, as shown by loci 2, 3, 24 and 63, but some of this has been lost over time. Because of limited gene flow between populations, only allowed by the occasional flowering of *F. vivipara* (Wycherley, 1954; Wilkinson and Stace, 1991), most of the genetic differences have been retained by local *F. vivipara* populations. This deduction provides further support for the local evolution of *F. vivipara* from *F. ovina*. Therefore, it would appear that, on encountering conditions unfavourable for normal flowering, *F. ovina* is replaced by *F. vivipara*, not from other areas but from within its population. There exist in *F. ovina* stands small amounts of genetic differentiation linked to propensity to pseudoviviparous development, for example, around locus 50 in this analysis. Montane-arctic conditions would clearly confer a selective advantage to individuals possessing this propensity, leading to their proportionate increase in these environments. For these newly established populations to continue to adapt to montane-arctic conditions, which are unfavourable to flowering, there is a presumed requirement for gene flow from other similarly adapted populations (Jump *et al.*, 2006). This does not happen, however, as our DFDIST analysis of *F. vivipara* populations revealed; there is limited genetic exchange between *F. vivipara* populations from geographically separate locations. This limit on genetic exchange makes long-term genetic fixation of the pseudoviviparous trait impossible and could explain why habitually pseudoviviparous grasses partially revert to normal flowering under conducive flowering conditions (Heide, 1989). The mountain-by-mountain AFLP data analysis thus suggests that *F. vivipara* is constantly arising from *F. ovina* locally.

### *F. vivipara* is a morphologically and ecologically distinct species

The low levels of differentiation between seminiferous and pseudoviviparous *Festuca* plants are consistent with a polyphyletic origin for *F. vivipara*. A polyphyletic origin does not necessarily preclude species recognition, however, as many hybrids accorded recognition at specific rank are nevertheless known to have polyphyletic origins, for example, *Helianthus deserticola* (Gross *et al.*, 2003). *F. vivipara* is common in areas of high rainfall and low sunlight, which include rocky crevices, and rocky scree slopes (Wycherley, 1953a; Wilkinson and Stace, 1991). These conditions are thought to be conducive for the survival and establishment of *F. vivipara*'s non-dormant, leafy propagules while making impossible regular sexual reproduction in seminiferous grass species (Wycherley, 1953a; Lee and Harmer, 1980). In this

study, we did not uncover evidence of any consistent genetic differentiation that separates *F. ovina* from *F. vivipara*. This does not necessarily mean that *F. vivipara* is not a species, as there are no set levels of genetic differentiation required to support species-level taxonomic delineation (Lowe *et al.*, 2004). Even though these two *Festuca* taxa are not genetically different and lack complete reproductive isolation from each other, they can be considered to be different species separated by ecological and morphological differentiation (Lowe *et al.*, 2004). Although the cpDNA and AFLP data analyses suggest that *F. vivipara* is not defined by major evolutionary genetic processes, ecological processes that make impossible sexual reproduction have clearly led to its development from *F. ovina*. In addition, *F. vivipara* can be defined as a morphological species; when *F. vivipara* and *F. ovina* are grown under common environmental conditions, they remain morphologically distinct.

In summary, we demonstrate a lack of clear genetic differentiation between *F. vivipara* and *F. ovina*. This is coupled with a shared genetic structuring indicating repeated local emergence of pseudovivipary from seminiferous *F. ovina*. Furthermore, the limited geographic scale over which genetic differentiation becomes apparent between *F. ovina* and *F. vivipara* suggests a high propensity for transition from seminiferous to pseudoviviparous states. For this reason, we argue that the link between this high propensity to proliferate and spikelet meristem reversion warrants further investigation.

### Conflict of interest

The authors declare no conflict of interest.

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