

**Preliminary study to detect genetic
variation within *Dactylorhiza viridis* using
plastid microsatellites**

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Introduction

In order to make a preliminary assessment of the potential genetic diversity in *Dactylorhiza viridis* populations in England, the genetic variation between *D. viridis* samples from other geographical locations was investigated in this study. The genetic variation between *D. viridis* and other *Dactylorhiza* species was also investigated. The plastid genome was analysed for the presence of polymorphic microsatellite loci in four different regions of the genome.

Materials and Methods

Sampling

The samples of *D. viridis* analysed in this study were all from European countries with the exception of four individual samples from China. A large proportion of the samples analysed was from Italy and Estonia, with other countries including Austria, France, Ireland, Russia and Sweden being represented by two samples each. Two British samples were included in this analysis, one sample was from England (near Crich, Derbyshire) and the other was from Scotland. Other individuals from within the genus *Dactylorhiza* were also included in this study in order to compare the genetic diversity between the different species. These individuals came from across Europe, including samples from England. (See **Table 1** for list of samples.)

Plastid genome regions

Ten regions of the *D. viridis* plastid genome were amplified and sequenced as these regions commonly contain microsatellites in many taxa. From these regions, four were selected as they contained potentially variable microsatellites in *D. viridis*. Forward (F) and reverse (R) primers were designed for each of the four regions in order to detect variable microsatellites during sequencing. The forward primers were fluorescently labelled so that the size of the microsatellite could be detected during genotyping. (See **Table 2** for a list of the four plastid regions and the primers used to amplify each region.)

Microsatellite detection and analysis

The four microsatellite loci were analysed for 42 samples of *D. viridis* and 20 samples of other *Dactylorhiza* species. The microsatellite loci were amplified by PCR and the products were run on an ABI 3100 DNA analyser. The size of each microsatellite was determined in base pairs by using Genotyper 2.0 software (Applied Biosystems, Inc.).

A matrix was prepared, using different letters for alleles of differing lengths of microsatellite regions. Haplotypes were defined from the different combination of alleles for the four loci. (See **Table 1** for full details.)

Haplotype Analysis

The program NETWORK 4.5.1.0. (www.fluxus-engineering.com) was used to construct a median-joining (MJ) network based on the plastid DNA haplotypes of the *D. viridis* samples. All characters were equally weighted in this analysis.

Results

These markers allowed us to identify 32 different haplotypes, of which 25 were only found once. Haplotypes 1-14 were found exclusively in samples of *D. viridis* and all but one of the haplotypes identified in the other *Dactylorhiza* species were only found in one species. (See **Table 1** for details of the allelic compositions of the haplotypes.)

Of the 14 different haplotypes (1-14) found in *D. viridis*, eight were only represented by a single individual. The most common haplotype (H1) was found in 15 of the samples, including eight from Estonia, four from Italy, both of the Irish samples and the only Scottish individual. The second most common haplotype (H9) was found in six samples, all of which were from Estonia. Haplotype H2 occurred in five Italian samples and was unique to that country. Haplotype H3 was found in the single English sample, one Italian sample, two Austrian samples and one Russian sample. Two of the samples from France had haplotype H11 and the other French sample had the unique H12 haplotype. The four samples from China each had different haplotypes (H5-8), none of which was found in any other sample. The remainder of the *D. viridis* samples all had unique haplotypes.

Of the other *Dactylorhiza* species analysed in this study, all had unique haplotypes except the two samples of *D. lapponica* which shared the H32 haplotype and samples of *D. maculata* from Norway and *D. fuchsii* from Sweden which shared H19.

Haplotype network

Two versions of the MJ network were produced in this analysis. The first MJ network includes all of the 14 *D. viridis* haplotypes identified by plastid microsatellite analysis (Fig. 1). The second MJ network (Fig. 2) excluded the four unique Chinese haplotypes as there is evidence of morphological and phylogenetic differences between Chinese and European populations of *D. viridis*.

The MJ network shown in Fig. 1 has a torso made up of predominantly central European haplotypes and it also includes two of the Chinese haplotypes (H6 and H8). The four British samples also lie within the torso or close to it (H1 and H3). The two French haplotypes (H11 and H12) are found at opposite poles of the network.

When the four Chinese haplotypes are excluded from the analysis (Fig. 2), the overall structure of the network does not change significantly. Haplotypes from central Europe make up the torso of the network and the two French haplotypes occur on opposite branches. Haplotype H3, which includes the English sample, becomes part of the torso in this network.

Discussion

Dactylorhiza viridis

From this limited sampling, it seems that the *D. viridis* populations in Italy and China are genetically diverse, with four different haplotypes occurring in each country. However, only two haplotypes occur among the 14 Estonian samples (H1 and H9). The most common haplotype found in this study (H1) is found over a wide geographic area, in Italy, Estonia, Scotland and Ireland. The presence of some geographical structure in the results may allow, with increased sampling, the migration routes taken by this species after the last glacial maximum to be established. As only four polymorphic loci were used in this study, it was surprising to obtain this much structure in the median-joining networks (14 polymorphic loci gave a similar level of structure for *Cypripedium calceolus*; Fay *et al.*, 2009).

Other *Dactylorhiza* species

The number of unique haplotypes found is an indication of genetic diversity between the different species of the genus *Dactylorhiza*. Another indicator of this is the failure of some of the primers to amplify the microsatellite regions in some of the different species. This may have been due to the DNA sequences at the primer sites of these regions being too different in these species and therefore amplification could not take place as the primers failed to anneal.

FUTURE WORK

We have already initiated a further round of investigation to identify additional length-variable sections of plastid DNA. In addition to this, we intend to investigate the possibility of identifying nuclear microsatellite loci. If successful, this will provide biparental information to supplement the plastid data.

With the existing plastid markers we now have a data matrix to which additional samples can be added when these become available. Samples provided by Natural England will be treated as a priority in the new financial year.

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REFERENCE

Fay, M. F., Bone, R., Cook, P., Kahandawala, I., Greensmith, J., Harris, S., Pedersen, H. Æ., Ingrouille, M. J. & Lexer, C. (2009). Genetic diversity in *Cypripedium calceolus* (Orchidaceae) with a focus on northwestern Europe, as revealed by plastid DNA length polymorphisms. *Annals of Botany* 104: 517-525.

Table 1: List of samples used in this study, including accession numbers, country of origin and haplotype details.

| | | Accession | Country | Haplotype ¹ | Haplotype No. |
|----|------------------------|-----------|---------------|------------------------|---------------|
| 1 | <i>D. viridis</i> | 19420 | Austria | EDBC | 3 |
| 2 | <i>D. viridis</i> | 19421 | Austria | EDBC | 3 |
| 3 | <i>D. viridis</i> | 13070 | China | EFBB | 5 |
| 4 | <i>D. viridis</i> | 13074 | China | GFAE | 6 |
| 5 | <i>D. viridis</i> | 13075 | China | EGCA | 7 |
| 6 | <i>D. viridis</i> | 13079 | China | GEAE | 8 |
| 7 | <i>D. viridis</i> | 35736 | Estonia | FEBC | 1 |
| 8 | <i>D. viridis</i> | 35734 | Estonia | FEBC | 1 |
| 9 | <i>D. viridis</i> | 35732 | Estonia | HEAC | 9 |
| 10 | <i>D. viridis</i> | 35729 | Estonia | HE?C | 9 |
| 11 | <i>D. viridis</i> | 35739 | Estonia | FE?C | 1 |
| 12 | <i>D. viridis</i> | 35738 | Estonia | FEBC | 1 |
| 13 | <i>D. viridis</i> | 35737 | Estonia | FEBC | 1 |
| 14 | <i>D. viridis</i> | 35733 | Estonia | FEBC | 1 |
| 15 | <i>D. viridis</i> | 35731 | Estonia | HEAC | 9 |
| 16 | <i>D. viridis</i> | 35730 | Estonia | FEBC | 1 |
| 17 | <i>D. viridis</i> | 35728 | Estonia | HEAC | 9 |
| 18 | <i>D. viridis</i> | 35727 | Estonia | HEAC | 9 |
| 19 | <i>D. viridis</i> | 35726 | Estonia | HEAC | 9 |
| 20 | <i>D. viridis</i> | 35735 | Estonia | FEBC | 1 |
| 21 | <i>D. viridis</i> | 17601 | Eu. Russia | EDBB | 10 |
| 22 | <i>D. viridis</i> | 17606 | Eu. Russia | EDBC | 3 |
| 23 | <i>D. viridis</i> | 15985 | France | EFAC | 11 |
| 24 | <i>D. viridis</i> | 31340 | France | JIAD | 12 |
| 25 | <i>D. viridis</i> | 36651 | France | EFAC | 11 |
| 26 | <i>D. viridis</i> | 36649 | Ireland | FEBC | 1 |
| 27 | <i>D. viridis</i> | 36650 | Ireland | FEBC | 1 |
| 28 | <i>D. viridis</i> | 35420 | Italy | FEBC | 1 |
| 29 | <i>D. viridis</i> | 35421 | Italy | GE?C | 2 |
| 30 | <i>D. viridis</i> | 35422 | Italy | GEBC | 2 |
| 31 | <i>D. viridis</i> | 35423 | Italy | GEBC | 2 |
| 32 | <i>D. viridis</i> | 35424 | Italy | GEBC | 2 |
| 33 | <i>D. viridis</i> | 35425 | Italy | GEBC | 2 |
| 34 | <i>D. viridis</i> | 35426 | Italy | EDBC | 3 |
| 35 | <i>D. viridis</i> | 35427 | Italy | FEBC | 1 |
| 36 | <i>D. viridis</i> | 35428 | Italy | HE?B | 4 |
| 37 | <i>D. viridis</i> | 35429 | Italy | FEBC | 1 |
| 38 | <i>D. viridis</i> | 35430 | Italy | FEBC | 1 |
| 39 | <i>D. viridis</i> | 15982 | Scotland | FEBC | 1 |
| 40 | <i>D. viridis</i> | 15979 | Sweden | GDBC | 13 |
| 41 | <i>D. viridis</i> | 15980 | Sweden | HEBC | 14 |
| 42 | <i>D. viridis</i> | 0-1396 | England | EDBC | 3 |
| | Other species | | | | |
| 43 | <i>D. incarnata</i> | 0-937 | England | ?EFB | 15 |
| 44 | <i>D. incarnata</i> | 3987 | Sweden | KCDA | 16 |
| 45 | <i>D. incarnata</i> | 13751 | Wales | KACA | 17 |
| 46 | <i>D. maculata</i> | 11798 | Iceland | BMGB | 18 |
| 47 | <i>D. maculata</i> | 5553 | Norway | DJFB | 19 |
| 48 | <i>D. maculata</i> | 11795 | Portugal | BNGB | 20 |
| 49 | <i>D. saccifera</i> | 15986 | Greece | IMFB | 21 |
| 50 | <i>D. saccifera</i> | 18113 | Greece | IOEB | 22 |
| 51 | <i>D. saccifera</i> | 11800 | Turkey | IHFB | 23 |
| 52 | <i>D. fuchsia</i> | 10241 | Wales | DKFB | 24 |
| 53 | <i>D. fuchsia</i> | 3996 | Sweden | DJFB | 19 |
| 54 | <i>D. foliosa</i> | 14695 | Madeira | APGA | 25 |
| 55 | <i>D. foliosa</i> | 0-537 | Madeira (Kew) | APFA | 26 |
| 56 | <i>D. purpurella</i> | 13758 | Wales | AI?? | 27 |
| 57 | <i>D. alpestris</i> | 14698 | Switzerland | FMFB | 28 |
| 58 | <i>D. praetermissa</i> | 17050 | England | ?B?E | 29 |
| 59 | <i>D. occidentalis</i> | 30964 | Ireland | ?B?B | 30 |
| 60 | <i>D. occidentalis</i> | 30965 | Ireland | AL?? | 31 |
| 61 | <i>D. lapponica</i> | 4000 | Sweden | CJFB | 32 |
| 62 | <i>D. lapponica</i> | 5552 | Sweden | CJFB | 32 |

? – indicates unknown allele

¹ ? – unknown allele due to amplification failing for one or more loci.

Table 2: List of the four plastid regions and the primers used to amplify each region.

| Region | Primer Direction | Primer Sequence |
|---------------|-------------------------|----------------------------|
| Dvir accD psa | F | GCA TCC CTC TCT TTA CAA GT |
| | R | CGC GAC TTT AAC CAA TCT TA |
| Dvir trnL | F | CGA AAT CGG TAG ACG CTA CG |
| | R | CCA TCG AGT CTC TGC ACC TA |
| Dvir trnY-T A | F | TAT GAC TAG ATC CGA GGA GT |
| | R | GGG ATA GCT CAT TCA TGA AC |
| Dvir trnY-T T | F | AGA TGA GTT AGA CCT ATC GC |
| | R | GAT GAG GGA TTT GTA ACC TG |

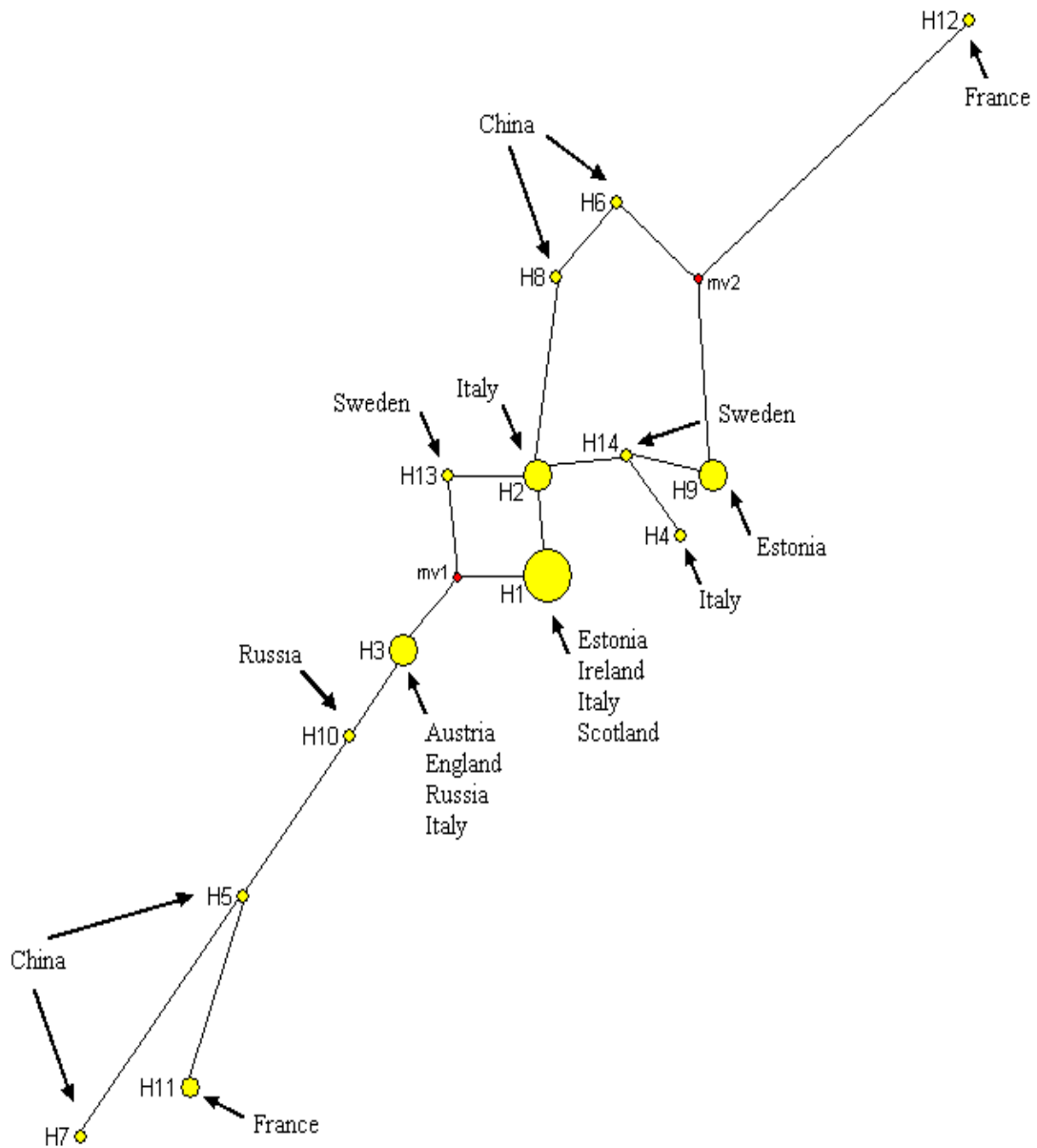


FIG. 1. Median-joining network for plastid DNA haplotypes in *Dactylorhiza viridis*. The haplotypes are represented by the circles, the size of each circle being proportional to the observed frequency of each haplotype. Median vectors are labelled mv1 and mv2; these represent possible unsampled or extinct haplotypes. Information on the geographic locations where each haplotype is found is provided.

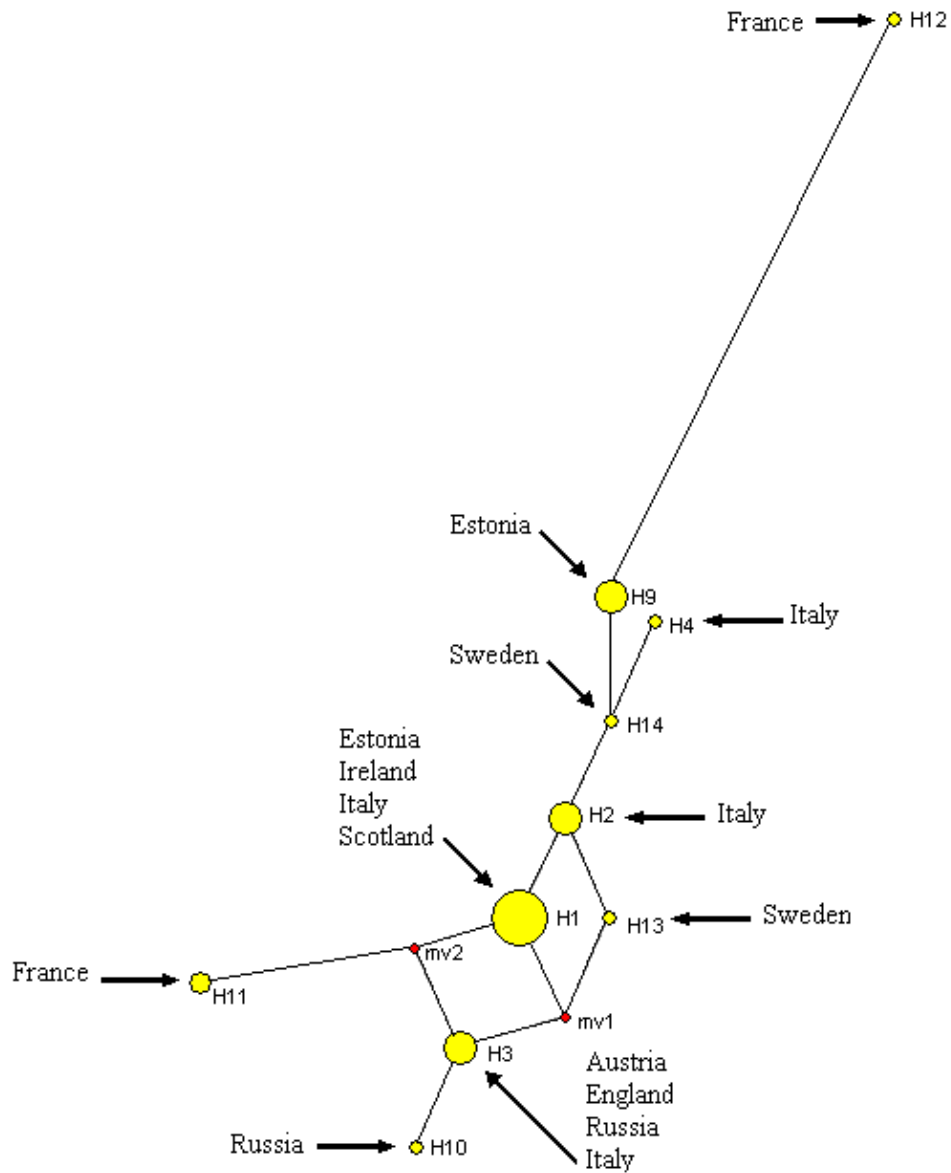


FIG. 2. Median-joining network for plastid DNA haplotypes in European samples of *Dactylorhiza viridis*. This analysis excludes haplotypes found in China. The haplotypes are represented by the circles, the size of each circle being proportional to the observed frequency of each haplotype. Median vectors are labelled mv1 and mv2; these represent possible unsampled or extinct haplotypes. Information on the geographic locations where each haplotype is found is provided.