

# Genetic diversity and reproductive biology in ecotypes of the facultative apomict *Hypericum perforatum* L.

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Apomixis is a mode of asexual reproduction through seed. Progeny produced by apomixis are clonal replicas of a mother plant. The essential feature of apomixis is that embryo sacs and embryos are produced in ovules without meiotic reduction or egg cell fertilisation. Thus, apomixis fixes successful gene combinations and propagates high fitness genotypes across generations. A more profound knowledge of the mechanisms that regulate reproductive events in plants would contribute fundamentally to understanding the evolution and genetic control of apomixis. Molecular markers were used to determine levels of genetic variation within and relationship among ecotypes of the facultative apomict *Hypericum perforatum* L. ( $2n = 4x = 32$ ). All ecotypes were polyclonal, being not dominated by a single genotype, and characterised by different levels of differentiation among multilocus genotypes. Flow cytometric analysis of seeds indicated that all ecotypes were facultatively apomictic, with

varying degrees of apomixis and sexuality. Seeds set by haploid parthenogenesis and/or by fertilisation of aposporic egg cells were detected in most populations. The occurrence of both dihaploids and hexaploids indicates that apospory and parthenogenesis may be developmentally uncoupled and supports two distinct genetic factors controlling apospory and parthenogenesis in this species. Cyto-embryological analysis showed that meiotic and aposporic processes do initiate within the same ovule: the aposporic initial often appeared evident at the time of megaspore mother cell differentiation. Our observations suggest that the egg cell exists in an active metabolic state before pollination, and that its parthenogenetic activation leading to embryo formation may occur before fertilisation and endosperm initiation.

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## Introduction

St John's wort (*Hypericum perforatum* L.) is a medicinal plant, which produces pharmaceutically important metabolites with antidepressive, anticancer and antiviral activities (Zanoli, 2004; Gartner *et al.*, 2005; Kubin *et al.*, 2005), and it is also regarded as a serious weed in many countries (Buckley *et al.*, 2003). It is capable of asexual reproduction by apomixis, whereby the embryo is formed without meiotic reduction and fertilisation, thus retaining the maternal genotype. Apomixis in this species is achieved through apomeiosis of the aposporic *Hieracium*-type, followed by parthenogenesis of the unreduced egg cell. Fertilisation of the central cell of aposporic embryo sacs by a pollen nucleus is normally required for endosperm formation (pseudogamy), although the endosperm rarely can also develop autonomously (Matzk *et al.*,

2001). *H. perforatum* is a facultative apomict since sexual and aposporic processes can occur in the same plant. The development of the sexual embryo sac is often terminated at the megaspore mother cell (MMC) or reduced megaspore stage, while one or more somatic aposporic initial cells differentiate from the nucellar tissue to initiate unreduced embryo sacs directly by mitosis (Mártonfi *et al.*, 1996). In tetraploids, sexually produced seeds retain the standard 2:1 (ie 4:2) maternal to paternal genome ratio in the hexaploid endosperm, whereas this ratio is unbalanced and variable in apomictic seeds (eg 8:2 and 8:0 with pseudogamy and autonomy, respectively), the result of differential fertilisation potential during embryo and endosperm formation.

Apomixis in *H. perforatum* was first described in the pioneering work of Noack (1939), but only recently characterised in great detail by Matzk *et al.* (2001) using the flow cytometric seed screen (FCSS). This latter study identified 11 different mechanisms of seed formation, in addition to low numbers of obligate sexually and apomictically reproducing plants: embryos of sexual origin were mainly diploid ( $2n = 2x = 16$ ), while apomictically formed embryos were tetraploid ( $2n = 4x = 32$ ) or hexaploid ( $2n = 6x = 48$ ). The different modes of reproduction in this species have been confirmed numerous times by examining molecular marker segregation in

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maternal plants and their progenies (Halušková and Čellárová, 1997; Arnholdt-Schmitt, 2000; Steck *et al*, 2001). DNA fingerprinting techniques in conjunction with cytological investigations have furthermore demonstrated low frequencies of recombinant offspring from otherwise apomictic mother plants (Halušková and Košuth, 2003; Mayo and Langridge, 2003).

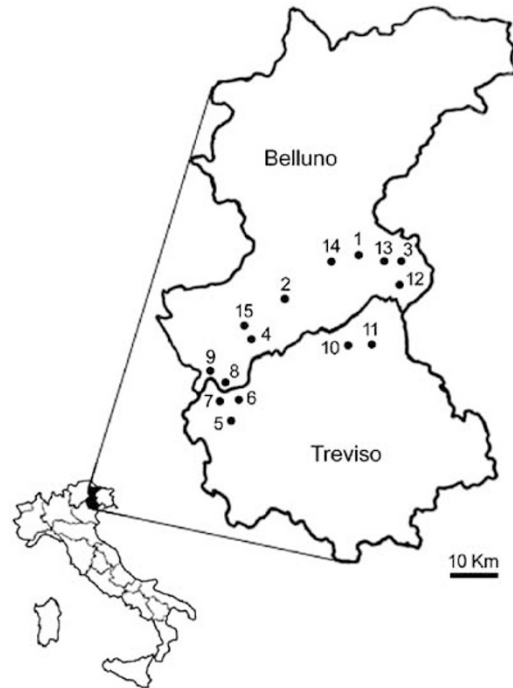
Apomictic taxa are expected to be characterised by higher inter- *versus* intrapopulation differentiation, the result of limited gene flow via pollen, genetic drift in disjunct populations, founder effects (ie genetic bottlenecks) and clonal reproduction (Carino and Daehler, 1999). Various experimental studies have reported the existence of distinct multilocus genotypes (ie clones) in apomictic plant populations (Ellstrand and Roose, 1987; Noyes and Soltis, 1996; Chapman *et al*, 2000; Van Der Hulst *et al*, 2000), although the source of this genetic diversity remains largely unexplained. Clonal diversity within a population reflects the sexual genetic pool from which the clones originated, the frequency of clonal origin, and the somatic mutations that subsequently accumulate in established clones (Meirmans and Van Tienderen, 2004). The diversity arising from the clonal origin is directly dependent on reproductive mode, and hence varying degrees of apomixis between *H. perforatum* landraces may have a significant impact on the diversity of local populations.

The aim of this work was to gain an insight into the reproductive dynamics and the developmental events of apomixis in *H. perforatum*, a species recently considered as a favourable model for apomixis research (Matzk *et al*, 2001, 2003). A survey of the genetic variation and differentiation occurring within and among natural populations was carried out using multilocus PCR-based molecular markers. The mode of reproduction was also investigated using the FCSS analysis to reconstruct the patterns of seed formation and to infer the sources of genetic variation at the population level. The reproductive biology of individual plants was analysed through cytohistological investigations in order to characterise the developmental pathway of the ovule from megasporogenesis to the early stages of embryogenesis.

## Materials and methods

### Plant material

Seed stocks of 15 local populations of *H. perforatum* L. collected in Northern Italy, provinces of Belluno and Treviso (Figure 1; Table 1), were used to perform plot trials according to a randomized complete block experimental design with three replicates. The Polish cultivar Topas (Seidler-Lozykowska and Dabrowska, 1996) was adopted as reference standard. Each population was characterised by variable degrees of uniformity for plant growth, leaf and flower characteristics, and flowering time (unpublished observations). We first investigated interpopulation genetic variation between pooled samples (12 randomly chosen plants per population) from each population. Based on this analysis, the nine most differentiated populations were selected along with the cultivar Topas, and 30 plants from each were grown in the greenhouse and used to assess within population genetic variation.



**Figure 1** Information on the *Hypericum perforatum* ecotypes. Map of the collection sites of the 15 ecotypes of *Hypericum perforatum* from the provinces of Treviso and Belluno (North-Eastern Italy). Numbers refer to the collection sites listed in Table 1.

**Table 1** Information on the collection sites of the 15 populations of *Hypericum perforatum* included in the analysis

No.	Collection sites	Province <sup>a</sup>	Altitude (m)
1	Cima i Prà-Ponte nelle Alpi	BL	336
2	Santa Giustina	BL	276
3	Irrighe-Chies d'Alpago	BL	1000
4	Cellarda-Feltre	BL	224
5	Casera Corpon-Crespano del Grappa	TV	720
6	Castel Cesil-Cavaso del Tomba	TV	1050
7	Bocca di Forca-Alano di Piave	BL	1250
8	Casera Domador-Alano di Piave	BL	1180
9	Malga Valderoa-Alano di Piave	BL	1300
10	Fais-Vittorio Veneto	TV	750
11	Costa-Vittorio Veneto	TV	200
12	Piano del Cansiglio-Tambre d'Alpago	BL	1020
13	Plois-Pieve d'Alpago	BL	1000
14	Tisoi-Belluno	BL	560
15	Vellai-Feltre	BL	360

<sup>a</sup>BL: Belluno; TV: Treviso (in the Veneto region, North-Est Italy).

### Genomic DNA isolation

Approximately 0.5 g of leaf tissue was collected from healthy plants and frozen in liquid nitrogen. Total genomic DNA from leaf samples was isolated according to the protocol described by Barcaccia and Rosellini (1996). The DNA pellet was washed twice with 70% ethanol, dried and redissolved in a standard Tris-EDTA buffer. The concentration of DNA samples was determined by optical density reading at 260 nm (1 OD = 50 µg/ml) and their purity calculated by the OD<sub>260</sub>/OD<sub>280</sub> ratio and the OD<sub>210</sub>-OD<sub>310</sub> pattern (Sambrook *et al*, 1989). An aliquot of genomic DNA was also assayed by electrophoresis on 1% agarose gels.

### Molecular markers

PCR parameters and gel electrophoresis conditions for the RAPD analysis were those described by Barcaccia *et al* (1997a). A list of the 10-mer primers used is reported in Table 2. Inter-microsatellite markers were assayed using seven different ISSR primers (synthesized by Life Technologies, Inc.) anchored at the 3' or 5' terminus of the simple CA repeat and extended into the flanking sequence by two or three nucleotide residues (Table 2). The protocol used for detecting ISSR polymorphisms was that reported by Barcaccia *et al* (2000a). Photographs (DC120 camera, Kodak) of the polymerized DNA fragments were taken after staining of the 2% agarose gels with ethidium bromide.

Restriction-ligation, preamplification and hot-PCR experiments were performed according to the AFLP protocol of Barcaccia *et al* (1998). The analysis of DNA fingerprints was based on the amplification of *EcoRI*/*MseI* and *PstI*/*MseI* genomic restriction fragments with four different primer combinations having three selective bases (Table 2). An aliquot of each reaction mixture was analysed by 4% denaturing polyacrylamide gel (Acrylamide/Bis 19:1 solution) electrophoresis according to Sambrook *et al* (1989). Genomic DNA fingerprints were visualized by autoradiogram (Biomax MR-1 film, Kodak) after 12 h exposure of gel blots at  $-80^{\circ}\text{C}$  using intensifying screens.

**Table 2** List of the ISSR and RAPD primers, and AFLP primer combinations used

Primer	Sequence (5'-3')
<b>ISSR</b>	
I-18	GTGC(TC) <sub>7</sub>
I-28	(GT) <sub>6</sub> CG
I-29	(GT) <sub>6</sub> CA
I-32	(AGC) <sub>4</sub> C
I-34	(AGC) <sub>4</sub> GG
I-39 <sup>a</sup>	(AGC) <sub>4</sub> AC
I-50 <sup>a</sup>	CCA(GCT) <sub>4</sub>
<b>RAPD</b>	
OP-A1	CAGGCCCTTC
OP-A4	AATCGGGCTG
OP-A9	GGGTAACGCC
OP-B12	CCTGACGCA
OP-B13	TTCCCCCGCT
OP-B19	ACCCCGAAG
OP-C6 <sup>a</sup>	GAACGGACTC
OP-C7 <sup>a</sup>	GTCCCGACGA
OP-C11	AAAGCTGCGG
OP-C19	GTTGCCAGCC
OP-D19 <sup>a</sup>	CTGGGGACTT
OP-D20	ACCCGGTCAC
OP-Q2	TCTGTCCGGTC
OP-Q11	TCTCCGCAAC
<b>AFLP</b>	
E+CAC/M+AAG	AGACTGCGTACCAATTCCAC GACGATGAGTCTGAGTAAAAG
E+CAC/M+ACT	AGACTGCGTACCAATTCCAC GACGATGAGTCTGAGTAAACT
P+AG/M+CCA <sup>a</sup>	GACTGCGTACATGCAGAG GACGATGAGTCTGAGTAACCA
P+AT/M+CAT <sup>a</sup>	GACTGCGTACATGCAGAT GACGATGAGTCTGAGTAACAT

<sup>a</sup>Primer used for the analysis of the genetic diversity within populations.

### Data analysis

A binary presence (1)–absence (0) matrix was created for all DNA markers and *H. perforatum* samples, with each genomic locus defined by a particular band size as identified by comparing sample lanes with known DNA ladders.

Dice's (1945) genetic similarity (*S*) estimates between populations or individuals was calculated in all possible pair-wise comparisons using the following formula:  $S_{ij} = 2M_{ij}/(2M_{ij} + M_i + M_j)$ , where  $M_{ij}$  represents the number of shared markers scored between the pair of samples (*i* and *j*) considered,  $M_i$  is the number of markers present in *i* but absent in *j*, and  $M_j$  is the number of products present in *j* but absent in *i*. Thus,  $S_{ij} = 1$  indicates complete identity between *i* and *j*, whereas  $S_{ij} = 0$  indicates no shared variants.

Phylogenetic analyses were performed using the unweighted pair-group arithmetic average method (UPGMA) clustering algorithm, and the dendrogram of single local varieties and centroids of all accessions were constructed from the symmetrical genetic similarity matrix. All calculations and analyses were conducted using the appropriate routines of the NTSYS version 2.1 software (Rohlf, 2000).

Different measures of genetic variability were used to estimate the levels of polymorphism among populations. If  $p_i$  denotes the frequency of the *i*th marker allele at the *j*th locus, then according to Kimura and Crow (1964) the effective number of alleles per locus ( $n_e$ ) was computed as  $n_e = 1/(\sum p_i^2)$ . The parameter  $n_e$  is a measure of diversity and is 1 for monomorphic marker loci and 2 for polymorphic loci that have marker alleles in equal frequencies. Total Nei's (1973) genetic diversity (*H*) was computed as  $H = 1 - \sum p_i^2$  over all populations. For a single locus and a dominant marker system, *H* ranges from 0 (monomorphic, noninformative) to 0.5 (highly discriminative, with the two possible marker alleles in equal frequencies). Calculations were conducted using the software POPGENE version 1.21 (Yeh *et al*, 1997).

### Flow cytometry

FCSSs were performed according to the procedure described by Matzk *et al* (2001). Bulks of 30 mature seeds each were used for the estimation of embryo and residual endosperm nuclear DNA contents of the 15 local populations and the cultivar Topas. Subsequently, 30 seeds were analysed individually to identify and quantify the different pathways of embryo and endosperm formation in the two populations that showed contrasting modes of reproduction according to the analysis of bulked seeds. Nuclei were isolated from crushed seeds using a lysis buffer (Matzk *et al*, 2001). After filtration of cellular debris, an equal volume of staining buffer was added to the suspensions of released nuclei. For the measurements, DAPI-stained nuclei suspensions were processed using a Facstar Plus flow cytometer and sorter (Becton-Dickinson, San José, CA, USA) equipped with an argon ion laser in UV mode (Barcaccia *et al*, 1997a).

Flow cytometry data from *H. perforatum* seed analysis were used to identify the relative proportions of different reproductive pathways. Although the embryo maintains the parental ploidy irrespective of its sexual or apomictic origin, amphimixis gives rise to a 6x endosperm, whereas apomictic reproduction leads to a 10x endosperm (in the

case of pseudogamy). The ratio between the numbers of seeds having 2C peaks (2x embryos) and those having 4C plus 2C peak sizes (sum of 4x and 2x embryos) was used to compute the proportion of seeds set by meiotic, haploid parthenogenesis. The ratio between the numbers of seeds having 10C peaks (10x endosperms) and those having 10C plus 6C peak sizes (sum of 10x and 6x endosperms) was assumed as an estimate of the proportion of seeds having originated through aposporic parthenogenesis, that is, apomixis. Of the seeds produced through sexual (amphimictic) reproduction, the analysis of DNA contents does not allow the discrimination between selfing and outcrossing origins because embryos and endosperms share the same ploidy, irrespectively of the gamete source (ie  $n+n$  and  $2n+n$ , respectively).

### Cytohological investigations

Megasporogenesis and megagametogenesis were analysed mainly using a cell stain-clearing technique in combination with a DAPI-based DNA staining standard procedure. Cytohological investigations were carried out using semithin sections of pistils.

Flowers from accessions 1 and 13, those that showed a contrasting mode of reproduction according to the flow cytometrical analyses, were harvested at different developmental stages (from small buds to open blooms), fixed in FAA solution and stored at 4°C. After dehydration in ethanol and xylene series and inclusion in paraplast (Citterio *et al*, 2005), flower sections (7µm) were cut with a microtome (model RM2135, Leica, Nussloch, Germany), dried on slides, deparaffinized in xylene and then treated with Safranin T and DAPI, according to standard biological staining procedures. The stain-clearing methodology of Stelly *et al* (1984) was adopted for thicker sections (14µm) following the protocol reported by Barcaccia *et al* (1997b), optimized for *H. perforatum* specimens by reducing the time of staining, destaining and clearing steps.

Slides were observed at light microscope (Ortholux 2, Leitz, Wetzlar, Germany) and, for DAPI-stained samples,

with fluorescence microscopy (Leica DMR). Photographs were taken with a DC300F digital camera (Leica).

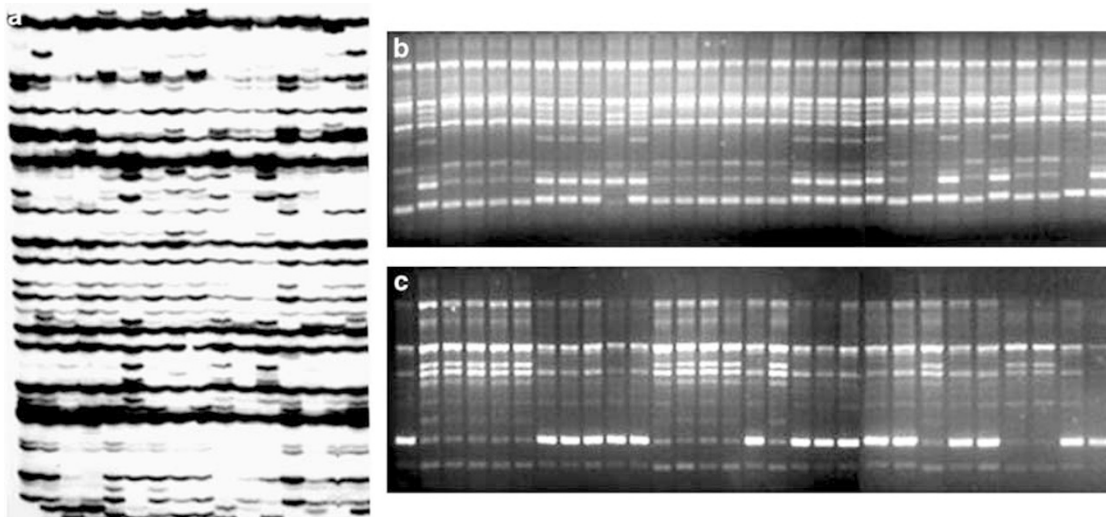
## Results

### Molecular markers

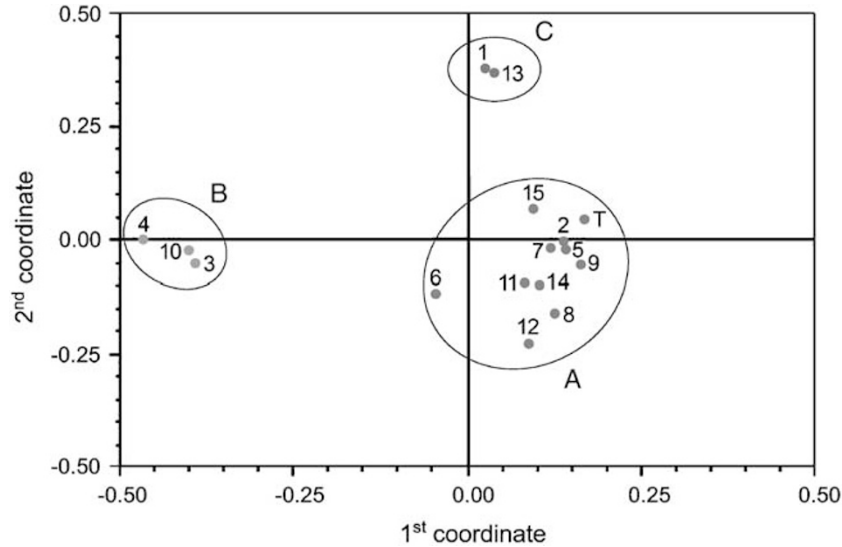
The genetic relationships among the 15 Italian populations of *H. perforatum* and the cultivar Topas were preliminarily assessed using one bulked DNA sample from each entry. RAPD, ISSR and AFLP fingerprints were generated using 14, 7 and 4 primer combinations, respectively, that proved to be useful in distinguishing the *H. perforatum* gene pools. A total of 102 RAPD, 35 ISSR and 274 AFLP marker loci were scored, 250 (60.8%) of which were polymorphic over all accessions. An example of AFLP banding patterns related to the local populations and the cultivar Topas is shown in Figure 2a.

A principal coordinate analysis (PCA) based on all scored polymorphisms enabled the differentiation of all accessions into three distinct subgroups (Figure 3). The first three coordinates explained about 54% of the total genetic variation. The first coordinate, which explained 26% of the variation, discriminated subgroup B (populations 3, 10 and 4) and population 6 from the rest of the accessions. The second coordinate, which explained 18% of the variation, clearly discriminated subgroup C (populations 1 and 13).

Subgroup A was composed of 11 accessions, and was characterised by inter-accession genetic similarity estimates (average = 0.854, ranging from 0.828 to 0.941) that were lower than those calculated between accessions within the subgroups B and C (average = 0.931 and 0.963, respectively). The mean genetic similarity between subgroups A and C was 0.814, whereas comparisons between A and B, and C and B were almost identical (0.769 and 0.768, respectively). Considering all markers together, the most similar gene pools were those of populations 1 and 13 (0.963) while 4 and 9 represented the most dissimilar populations (0.731). The mean genetic similarity estimate over all local populations was 0.821.



**Figure 2** Molecular polymorphisms detected among and within ecotypes. (a) AFLP banding patterns obtained in the 15 ecotypes (from left to right) of *Hypericum perforatum* and the cultivar Topas (last lane) with the primer combination Pst + AG/Mse + CCA; RAPD profiles generated by the individuals of the ecotype 10 (b) and ecotype 4 (c) with the primer OP-C7.



**Figure 3** Genetic relationships among ecotype gene pools. Centroids based on principal coordinate analysis showing the genetic differentiation and relatedness among the 15 ecotypes and the cultivar Topas (T).

Genetic relationships among *H. perforatum* accessions are summarized in the Dice's genetic similarity matrix (Supplementary Table 1S). The Mantel's test showed no significant correlation between genetic and geographic distances over all accessions ( $R^2 = 0.0195$ ,  $P = 0.1073$ ).

On the basis of the data collected in the preliminary analysis, nine local populations (30 plants per population), representing the total genetic variability among accessions were selected for further investigation, along with the cultivar Topas. In order to maximize the resolving potential of the genetic analysis, the marker loci that showed the highest information content (2 AFLP primer combinations, 3 RAPD and 2 Inter-SSR primers) within and among local populations were selected (Figure 2b and c).

On average, 18.5% of analysed markers were polymorphic per accession, and ranged from 4.1% (population 3) to 43.4% (population 4). The analysis of single plant DNAs showed high levels of uniformity within all *H. perforatum* local populations, as genetic similarity estimates ranged from 0.857 (population 4) to 0.994 (population 14), with a total average of 0.965 (0.996 for the Topas cultivar). All local populations sampled were shown to be multiclonal (Figure 4), with a mean number of detectable genotypes equal to 9, and ranging from 2 (population 4) to 15 (populations 12 and 13) (Table 3).

Nei's genetic diversity values were lowest in populations 3 and 14 (0.014 and 0.009, respectively) and comparable with that found in the cultivar Topas (0.013). By contrast, population 4 showed the highest genetic diversity (0.177), the result of comparable frequencies of two genetically differentiated genotypes (Table 3). Mean genetic diversity over all local populations was equal to  $H = 0.062$  ( $SD = 0.124$ ).

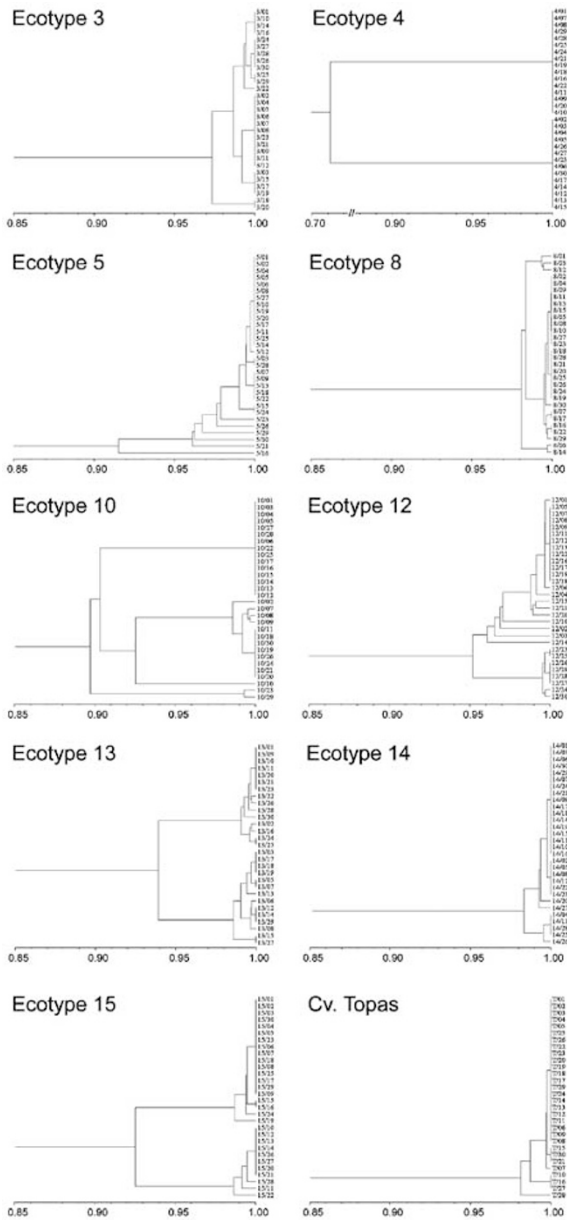
#### FCSS analysis

The reproductive behaviour of the accessions was investigated with the FCSS method, using bulked and single seeds collected from open-pollinated plants. This method allowed the indirect assessment of the events of

embryo and endosperm formation on the basis of their nuclear DNA contents. Most embryos were  $2n = 4x$  (tetraploid) as expected irrespective of their reproductive origin (apomictic  $2n + 0$  or sexual  $n + n$ ), whereas endosperms proved to be either decaploid ( $10x$ , ie 8x fusion nucleus fertilized by a reduced  $2x$  sperm nucleus) or hexaploid ( $6x$ , ie 4x fusion nucleus fertilized by a reduced  $2x$  sperm nucleus), depending on whether the seeds were derived apomictically or sexually, respectively. Octoploid ( $8x$ , ie 8x fusion nucleus not fertilized by any sperm nucleus) peaks were not found in any accession. Thus, pseudogamy was the predominant mode of endosperm formation.

The analysis of bulked seeds showed that plants from most of the accessions reproduce through facultative apomixis. The ratio between the 10C peak size and the sum of the 10C and the 6C peak sizes was assumed as an estimate of the degree of apomixis (Figure 5a and b). Among the *H. perforatum* ecotypes, the relative frequency of the apomictically derived seeds ranged from 0% (population 13) to 40% (population 1), being equal to 23% on average. The occurrence of haploid parthenogenesis was shown to be 7% on average. Seeds from sexual reproduction were detected with a relative frequency ranging from 49 to 88% (Table 4). The degree of sexuality may, in some cases, have been overestimated because 6C peaks may represent endosperms from sexual reproduction (balanced  $B_{II}$  hybrids and selfed progenies) and/or embryos from the fertilisation of unreduced egg cells (unbalanced  $B_{III}$  hybrids), while 10C peaks refer only to endosperm. *H. perforatum* embryos contain relatively more nuclei than endosperm residuals, and thus the occurrence of  $B_{III}$  hybrids in a population can bias the ratio of 10C–6C peaks in the sample of pooled seeds. On the basis of the bulked seed analysis, population 13 was classified as purely sexual, whereas population 1 was predominantly apomictic.

In order to investigate the true level of unreduced egg cell fertilisation and to precisely estimate the degree of apomixis/sexuality, single seed analyses were performed in populations 1 and 13, those which showed the most



**Figure 4** Genetic variation and genotype composition of the ecotypes. Dendrograms showing the genetic similarity estimates within ecotypes of *Hypericum perforatum* and the cultivar Topas (T). Ecotype 4 exhibited the lowest mean genetic similarity and therefore it was set a more broad x-axis scale of its plot.

contrasting modes of reproduction (Figure 5c–e). Seeds collected from plants of population 13 were derived from both sexual and apomictic reproduction. This population exhibited a reduced level of parthenogenesis in aposporic embryo sacs, as indicated by the high frequency of  $B_{III}$  hybrids. Overall, the frequency of events associated with sexual reproduction (meiosis and/or fertilisation) was equal to 45.5%. In contrast, a high incidence of asexual reproduction was observed in population 1, as was evidenced by 82.4% of the analysed seeds being derived from pseudogamous apomixis.

Endosperm ploidy predominantly reflected fertilisation of the unreduced polar nuclei, and hence most seeds were characterised by pseudogamy. However,

autonomous endosperm development was occasionally observed. Both populations revealed comparable frequencies of balanced  $B_{II}$  hybrids (9.1% in population 13 and 8.8% in population 1). Dihaploids resulting from parthenogenesis of meiotic-derived, normally reduced egg cells were identified, in addition to twins having embryos with distinct ploidy levels.

### Cytohistological investigations

Flower development before anthesis was subdivided into 11 different stages on the basis of bud length (Supplementary Figure 1S), each of which was analysed separately. The final, freshly opened bloom stage was also included in the investigations. In total, five different ovule developmental stages were observed. The anatropous ovules of *H. perforatum* showed a standard morphology (Figure 6a), but were usually characterised by the formation of an embryo sac from a nucellar cell (aposporic embryo sac) rather than a reduced embryo sac developing from the functional meiotic megaspore.

In buds up to 2.5 mm in length (stage 0, not shown), the ovule begins to develop from the placenta of the ovary wall, and the archesporial cell differentiates directly below the apex of the nucellus, which shows a prominent nucleus (Figure 6b and c). The archesporial cell (or primary sporogenic cell) developed directly into a single MMC, as the ovule of *H. perforatum* is tenuinucellar (Figure 6d). In buds of 3 mm in length (stage 1), the megaspore was observed in all ovules along with one or more aposporic initials usually positioned deep in the chalaza. Like the developing MMC, the voluminous initial cells were characterised by dense and vacuolized cytoplasm and large nuclei (Figure 6e and f). In buds of 4 mm in length (stage 2), megasporogenesis progressed completely up to the formation of a tetrad of reduced megaspores (Figure 6f and g) of which only the chalazal one survived. During this stage, the inner and outer integuments progressively enveloped the nucellus and the ovule rotated toward the placenta of the ovary to become anatropous (see Figure 6a).

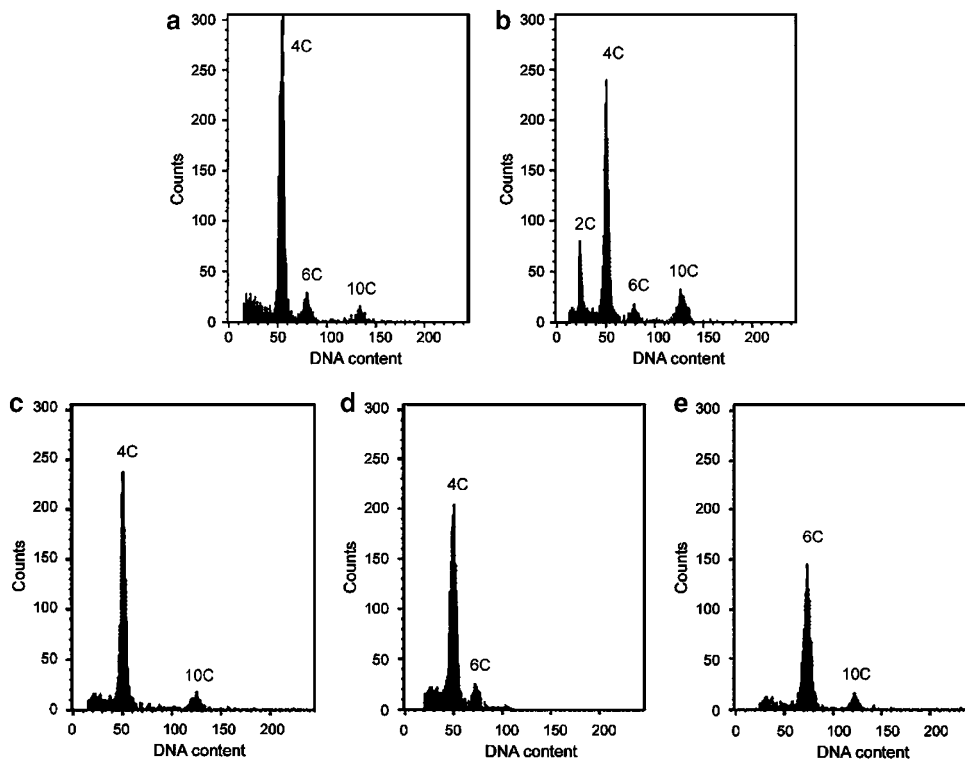
At the bud length of 5–6 mm (stages 3 and 4) all the ovules exhibited a cellularising, round-shaped embryo sac. The vast majority of them were found in a central position of the ovule, which suggested an aposporic origin (Figure 7a and b). It has been observed that the first mitotic division is parallel to the long axis of the cell and usually close to the micropylar region of the one-nucleated embryo sac (Figure 7c–e). After the first division, one nucleus migrated to the chalazal pole of the developing embryo sac. Cellularisation of the embryo sac was completed at a bud length of 6–8 mm (stages 5–7), and all the embryo sacs showed an extended shape (Figure 7f). The resultant mature ovule was longitudinally flanked by the funiculus, the structure connecting the lowest part of the chalaza to the placenta (Figure 8a).

Very few ovules showed more than one developing embryo sac. When two aposporic embryo sacs were observed in a given ovule (see Figure 7c), they exhibited a different orientation with respect to the micropylar–chalazal axis, and usually differentiated heterochronically with respect to each other. In only one case was an ovule with both sexual and aposporic embryo sacs observed, whereby two embryo sacs were clearly distinguishable on the basis of their micropylar and

**Table 3** Summary of genotypic variability, genetic similarity and genetic diversity statistics within populations of *Hypericum perforatum*

Accession	N	G	S $\pm$ SD	H $\pm$ SD	P
3	29	7	0.989 $\pm$ 0.010	0.014 $\pm$ 0.072	0.041
4	30	2	0.857 $\pm$ 0.139	0.177 $\pm$ 0.203	0.434
5	30	11	0.983 $\pm$ 0.022	0.058 $\pm$ 0.134	0.236
8	30	12	0.993 $\pm$ 0.009	0.035 $\pm$ 0.104	0.112
10	30	9	0.941 $\pm$ 0.047	0.099 $\pm$ 0.167	0.301
12	30	15	0.973 $\pm$ 0.022	0.080 $\pm$ 0.156	0.231
13	29	15	0.965 $\pm$ 0.035	0.070 $\pm$ 0.153	0.189
14	30	6	0.994 $\pm$ 0.007	0.009 $\pm$ 0.043	0.056
15	30	8	0.962 $\pm$ 0.037	0.063 $\pm$ 0.148	0.184
Topas	30	5	0.996 $\pm$ 0.006	0.013 $\pm$ 0.061	0.062
All populations	298	9	0.965 $\pm$ 0.033	0.062 $\pm$ 0.124	0.185

N, number of individuals sampled; G, number of multilocus genotypes detected; S, Dice's genetic similarity; H, Nei's genetic diversity; P, proportion of polymorphic loci.



**Figure 5** Flow cytometry histograms from cell nuclei of bulked and single seeds. Facultative apomictic ecotypes (no. 7 and no. 1) composed of tetraploids which set seeds by (a) amphimixis (4C:6C) and pseudogamous apomixis (4C:10C) or by (b) haploid parthenogenesis (2C:6C), amphimixis (4C:6C) and pseudogamous apomixis (4C:10C). (c) Apomictically derived seed (4C embryo plus 10C endosperm) belonging to ecotype 1; (d) Sexually-derived seed (4C embryo plus 6C endosperm), likely a B<sub>II</sub>-type hybrid seed belonging to ecotype 1; (e) Hexaploid B<sub>III</sub>-type hybrid seed belonging to ecotype 13 derived from the double fertilization of an unreduced egg cell (6C embryo plus 10C endosperm).

more chalazal position, respectively (see Figure 7a). At this stage, the egg cell and unfused polar nuclei (with large nucleoli) could be clearly differentiated as the former cell was typically pear shaped, larger and more strongly staining (Figure 8b and c). While synergids were well-developed, at this stage viable antipodals were found in few ovules. It is therefore likely that the antipodals degenerate very soon during embryo sac maturation. The synergids and the egg cell were usually positioned at the micropylar apex, but the usual triangular organization of the egg apparatus was not always conserved as the unreduced egg cell often localized in a more lateral position with respect to one of the

synergids. The distribution of the cytoplasm within the egg cell was highly polarized, a result of the presence of a large vacuole at the micropylar end that restricted the nucleus and most of the cytoplasm to the periphery of the cell (Figure 8d and e).

At the floret length of 8–10 mm (stages 8–10), embryo sacs retained the previously described morphology. The synergids typically degenerated prior to fertilisation, collapsing and leaving thin remnants tightly surrounding the egg cell (Figure 8f). Fusion of the polar nuclei was not observed at this time, and hence it is unclear whether this is important for the initiation of endosperm development in *H. perforatum*.

Embryo and endosperm development were additionally investigated in pistils harvested shortly after anthesis. Three stages of embryo development were observed, ranging from the early globular stage to the early heart stage. The endosperm was never observed, most likely since its development depends on fertilisation (Figure 8g–i). It appears that embryos initiated their development prior to fertilisation, and hence they arose from parthenogenetic activation of the egg cell.

**Table 4** Estimates of the rates (%) of seeds originated through haploid parthenogenesis, apomixis and amphimixis in the ecotypes of *Hypericum perforatum*

Accession	Haploid parthenogenesis	Apomixis	Amphimixis
1	11.6	39.6	48.8
2	ND	ND	ND
3	5.4	12.4	82.2
4	7.1	24.2	68.7
5	7.4	22.4	70.3
6	5.5	22.0	72.5
7	3.4	25.2	71.4
8	6.3	20.0	73.6
9	8.4	17.6	74.0
10	4.3	30.2	65.5
11	8.3	27.3	64.4
12	5.9	15.9	78.2
13	11.6	0.0	88.4
14	7.6	24.7	67.7
15	8.2	35.8	56.0
T	2.0	25.9	72.1
Mean	6.9	22.9	70.3

ND: not determined.

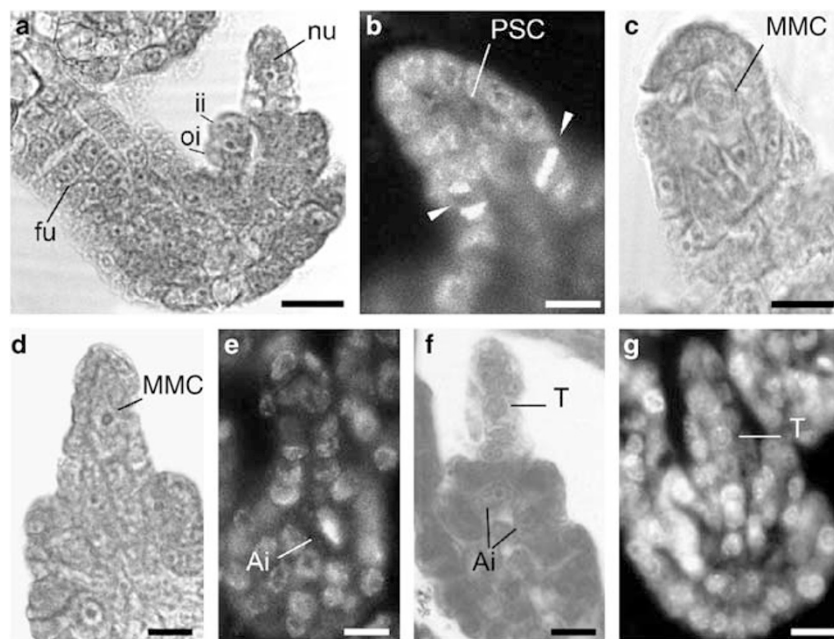
## Discussion

St John's wort deserves the attention of plant scientists not only for its pharmaceutically important metabolites but also for its remarkable evolutionary and adaptive capacities. This species has evolved into an extremely successful widespread weed, and a major characteristic of this success appears to be reproductive versatility coupled with genomic plasticity. *H. perforatum* reproduces mostly through apomixis, but offspring can also be generated sexually. From an adaptive point of view, facultative apomixis can be considered a means of exploiting genotypes adapted to current environmental conditions, while also ensuring a source of genetic variation upon which natural selection can act.

The asexual potential of a given genotype can be estimated from the mother plant, through the quantification of unreduced egg cell production (apospory) and/or failure of egg cell fertilisation (parthenogenesis), or through progeny tests. A more difficult task is the definition of the mode of reproduction at the population level: both cyto-histological investigations and molecular analysis such as seed DNA contents and plant DNA polymorphisms can be useful for assessing the reproductive pattern of single individuals and the extent of genetic variation among individuals.

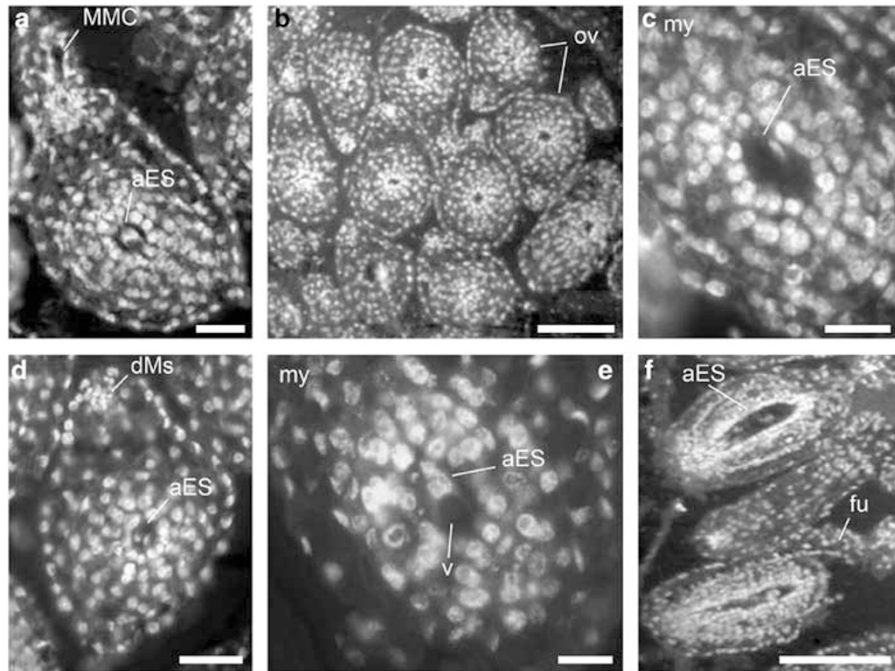
### Plant DNA polymorphisms and seed DNA content

Molecular markers have been used to differentiate between the sexually and apomictically generated progeny of *H. perforatum* (Halušková and Čellárová, 1997; Arnholdt-Schmitt, 2000; Mayo and Langridge, 2003). The results presented here represent the first report on the



**Figure 6** Development of ovules and aposporic initials, and megasporogenesis pathway in *Hypericum perforatum*. (a) Ovule showing an asymmetric growth of the integuments giving rise to its characteristic anatropous shape; Bar = 30  $\mu$ m. (b) Ovule primordium (prior to stage 1), bearing a primary sporogenic cell and showing two basal cells undergoing mitosis (indicated by arrows); Bar = 20  $\mu$ m. (c, d) Ovules at stage 1 bearing a megaspore mother cell; Bar = 20  $\mu$ m; (e–g) Ovules at stage 2 showing a megaspore mother cell or a linear tetrad of megaspores together with one or more aposporic initials in the deeper part of the chalaza; Bar = 30  $\mu$ m (nu, nucellus; fu, funiculus; PSC, primary sporogenic cell; MMC, megaspore mother cell; T, tetrad; Ai, aposporic initial; ii, inner integuments; oi, outer integuments).





**Figure 7** Megagametogenesis in *Hypericum perforatum*. (a) Ovule at stage 3–4 with a sexual and an aposporic embryo sac; Bar = 40  $\mu$ m. (b) Ovary showing several ovules at stage 3–4 with developing round-shaped embryo sacs; Bar = 200  $\mu$ m. (c) Ovule at stage 3–4 bearing two aposporic embryo sacs; Bar = 50  $\mu$ m. (d) A binucleated embryo sac centrally positioned into the chalaza; Bar = 80  $\mu$ m. (e) Ovule at stage 4–5 showing an embryo sac shortly after the first mitotic division (the two nuclei are indicated by arrows); Bar = 40  $\mu$ m. (f) Ovules at stage 6–7 showing cellularising embryo sacs with an extended shape; Bar = 200  $\mu$ m (my, micropyle; ch, chalaza; fu, funiculus; dMs; degenerating megaspores; sES, sexual embryo sac; aES, aposporic embryo sac; v, vacuole).

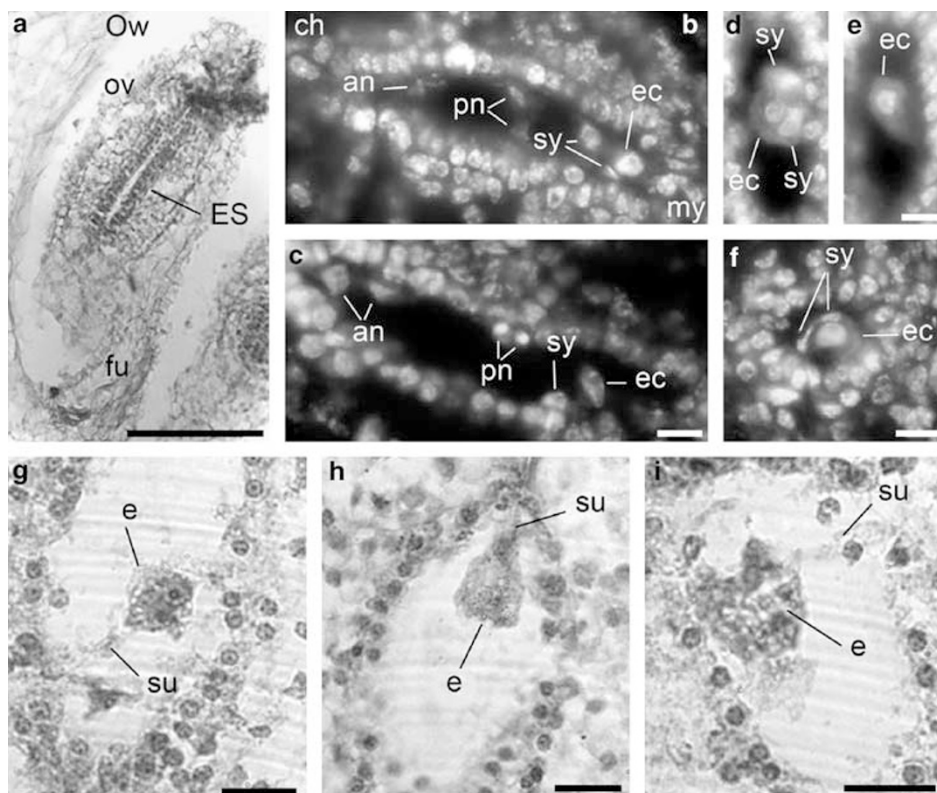
genetic diversity exhibited by natural populations of *H. perforatum* using molecular markers.

In the analysis of diversity among populations, high levels of genetic differentiation along with low estimates of genetic similarity were observed. The clearly distinguishable subgroups identified in the centroid analysis were characterised by high within subgroup genetic similarity, although genetic relatedness was not correlated with geographic distance between collection sites (ie no isolation by distance). This is exemplified by the Polish cultivar Topas, which was not differentiated from the Italian local populations. Clear genetic differentiation among clusters and the high similarity within single subgroups could suggest that the three sets of populations belong to different subspecies or varieties of *H. perforatum*. Based on the overall morphological variation of *H. perforatum*, Robson (2002) identified four subspecies of which the subsp. *perforatum* and subsp. *veronense* and several varieties are found in Italy. Furthermore, as *H. perforatum* appears to be an allopolyploid, which has originated from a cross between *H. maculatum* subsp. *immaculatum* and *H. attenuatum* (Robson, 2003), it is possible that the distinct *H. perforatum* groups identified in this analysis have originated through multiple hybridization events. Based on the data presented here, we cannot exclude the influence of other factors (eg postglacial dynamics, genetic drift, etc.) on our observed levels of genetic differentiation among populations.

The analyses of genetic diversity within populations demonstrated that they were all multiclonal. The presence of plant groups sharing the same multilocus genotype in a given population is often the most robust

and significant evidence of agamic reproduction (Van Der Hulst *et al*, 2000). Considering the total number of different genotypes observed in the analysed populations, 43.5% (37/85) were represented by more than one individual, a result inconsistent with what would be expected from a purely sexually reproducing population. Although the numbers of over-represented genotypes indicate that the predominant mode of reproduction is apomixis, sexual recombination has substantially contributed to genetic variation within these populations. Sexual reproduction was particularly evident in populations 12 and 13, where about half of the plants showed different genotypes.

With the exception of population 4, none of the analysed populations were dominated by a single genotype or composed of several genotypes with the same frequency. Population 4 was characterised by equal frequencies of two distinct genotypes, suggesting that sexually derived genetic variation has become fixed through apomixis. In contrast, population 14 was characterised by the highest genetic similarity, and included six low frequency genotypes that differ from one another by very few marker alleles. These genotypes could represent a single relatively old apomictic clonal lineage that has undergone divergence through time (Douhovnikoff and Dodd, 2003). Although it exhibited the highest genetic similarity values, even the cultivar Topas appeared to be multiclonal since two of the five detected genotypes were represented by one individual. On the whole, the amount of genetic diversity detected within *H. perforatum* populations is not unusual in comparison to values reported for other apomicts. The mean number of genotypes



**Figure 8** Embryo sac organization and early embryogenesis in *Hypericum perforatum* (stages 5–7 and following, where not specified). (a) A mature ovule with the axis of the nucellus parallel to the funiculus (anatropous ovule); Bar = 200  $\mu$ m. (b) A mature embryo sac with antipodal residuals, unfused polar nuclei and oangic apparatus with a strongly staining egg cell; Bar = 20  $\mu$ m. (c) A mature embryo sac with two visible antipodals, unfused polar nuclei attached to the embryo sac wall and part of the egg cell apparatus; Bar = 20  $\mu$ m. (d, e) Organization of the oangic apparatus of an aposporic embryo sac: the egg cell is flanked by the synergids in a triangular arrangement; the same oangic apparatus at a different level (adjacent section) exhibiting a pear-shaped egg cell; Bar = 20  $\mu$ m. (f) Oangic apparatus of an aposporic embryo sac: the egg cell shows a big nucleus and a large vacuole, whereas the synergids appear collapsed leaving thin remnants surrounding the egg cell; Bar = 30  $\mu$ m. (g–i) Embryo sacs showing embryonic development in ovules at stage 11: three different stages of embryogenesis are visible: an early globular stage (g), a transition stage (h) and an heart stage (i); Bars = 30  $\mu$ m (Ov, ovule; fu, funiculus; ES, embryo sac; my, mycophile; ch, chalaza; aES, aposporic embryo sac; sES, sexual embryo sac; ec, egg cell; sy, synergids; a, antipodals; pn, polar nuclei; e, embryo; su, suspensor).

detected per accession (=9.0) is lower than the corresponding value (11) found for the clonal species *Pilosella officinarum* (Chapman *et al*, 2000) but higher than those observed in *Erigeron compositus* (3.4) and *Bryonia alba* (6.4) (Noyes and Soltis, 1996; Novak and Mack, 2000).

The occurrence of sexuality in our populations of *H. perforatum*, as suggested by the level of genomic DNA polymorphism among individual plants, was confirmed by flow cytometric estimation of the nuclear DNA content of seed bulks. FCSS analysis confirmed that facultative apomixis is the prevalent mode of reproduction in *H. perforatum* populations, although high variability in the different reproductive pathways was observed. Populations 1 and 13, the most genetically similar populations according to the bulk sample analyses, were characterised by differing modes of reproduction when analysed both in bulk and as single seed. The correlation between genetic diversity and reproductive potential is exemplified by population 13, which was characterised by the highest number of genotypes and, as expected, very low levels of apomixis. Many procedures have been adopted to identify and quantify apomixis (reviewed in Leblanc and Mazzucato,

2001). For example, molecular markers in conjunction with flow cytometry have been used in the facultative apomict *Poa pratensis* to identify maternal plants, assess the genetic origin of aberrant plants and quantify the contribution of parental genomes (Barcaccia *et al*, 1997a, 2000b). In this study, the occurrence of sex and variable mechanisms of apomixis have been documented by means of FCSS in all analysed populations, and corroborates the data of Matzk *et al* (2001).

Our molecular and cytometric investigations have demonstrated large numbers of apomictic clonal lineages, as has been found in other apomictic species (Noyes and Soltis, 1996; Chapman *et al*, 2000; Van Der Hulst *et al*, 2000). Clonal diversity is likely to be correlated with the facultative nature of apomixis, as occasional sex leads to genetic diversity that is later fixed by apomixis. In addition, genetic variation could have also arisen from single clonal lineage origin followed by mutation accumulation (Ellstrand and Roose, 1987), although certain apomictic populations harbour genotypes resulting from segregation and/or recombination rather than differentiation via somatic mutation (Van Der Hulst *et al*, 2000).

### Cytohystological investigations

Cytohystological analysis was carried out to describe female sporogenesis and gametogenesis, and documented the different steps of MMC differentiation, meiosis, embryo sac formation from a nucellar cell, and the primary stages of embryogenesis. We could identify no differences between the two selected populations of this study, although the flowers were not sampled with the aim of performing a comparative analysis.

Some original observations were nonetheless made. For example, the aposporic initial often appeared evident and active even at the time of MMC differentiation and, subsequently, the vast majority of embryo sacs most likely had an aposporic origin because they were centrally located within the ovule. Thus, the sexual process is usually terminated soon after the aposporic initial cell differentiates in *H. perforatum*, as has been observed in most apomictic *Hieracium* (Koltunow *et al.*, 1998, 2000; Bicknell *et al.*, 2003). Our observations are consistent with the hypothesis that the aposporic initial might directly influence the sexual product degradation at this early stage (Tucker *et al.*, 2003).

Pollination timing has been proposed as a possible influence on the fate of aposporic embryo sacs in *H. perforatum* (Brutovská *et al.*, 1998). Our observations demonstrate that the egg cell was generally characterised by a strong DAPI stain when compared to the other cells of the embryo sac and the surrounding cells of the nucellus. This is consistent with the hypothesis that the mature aposporic egg cell, in contrast to the sexually derived egg cell, is in a highly active metabolic state even before pollination (Naumova and Vielle-Calzada, 2001). Moreover, it has also been observed that parthenogenetic activation of the egg cell can likely occur before polar nuclei fertilisation and/or endosperm initiation. Changes in the timing of developmental events could thus represent a mechanism that prevents fertilisation of the unreduced egg cell in *H. perforatum*. Precocious embryonic development has also been observed in aposporic species, including *Panicum maximum* (Naumova and Willemse, 1995), *Brachiaria brizantha* (Alves *et al.*, 2001), some highly parthenogenetic *Triticum* strains (Naumova and Matzk, 1998) and the diplosporic apomict *Tripsacum dactyloides* (Grimanelli *et al.*, 2003). Alternatively, a cell wall might impede fusion of the second sperm cell with the unreduced egg cell (Savidan, 1989) in order to avoid egg cell fertilisation, as has been demonstrated in *Pennisetum ciliare* by Vielle-Calzada *et al.* (1995), who identified the presence of a complete egg cell wall before pollen tube arrival. Thus, the apomictic egg cell, unlike the sexual one, can initiate embryogenesis before pollination and fertilisation of the embryo sac central cell. As a consequence, autonomous embryo initiation and development is not necessarily dependent on endosperm cues (Chaudhury *et al.*, 2001; Grossniklaus, 2001).

This study represents the first detailed report on the structural characteristics of female sporogenesis and gametophyte development in apomictic *H. perforatum*. The dissection of the apomictic pathway into distinct developmental steps is a required prerequisite for the understanding of its molecular basis, and the discrimination between particular ovule stages (eg premeiosis, meiosis, megagametogenesis and embryogenesis) could thus facilitate the identification of genes that are

differentially expressed between apomictic and sexual plants.

### Patterns of evolution and adaptedness

This study has demonstrated that certain populations of *H. perforatum* are polyclonal (ie not dominated by a single genotype), composed of overrepresented genotypes, and characterised by both apomictic and sexual reproduction. It appears that facultative apomixis has led to this diversity rather than the populations being a mixture of purely sexual and apomictic individuals. In the short-term apomixis can be advantageous since it preserves locally adapted gene combinations, although this may be disadvantageous in the long-term since apomictic clones may not adapt well to changing environments and will accumulate deleterious mutation. Therefore, while apomixis ensures the multiplication of high fitness genotypes, the retained sexual behaviour of facultative apomicts enables occasional hybridization, which may lead to recombinant genotypes upon which natural selection can act. In this way, facultative apomicts not only follow the strategy adopted by strict selfers but also ensure maintenance of high levels of heterozygosity and polyploidy. Such an hypothesis has been put forward for the two apomictic genera *Taraxacum* and *Chondrilla*, as it is thought that gene flow between apomicts and their sexual relatives has enabled apomixis to be maintained for longer evolutionary periods than originally predicted (van Dijk, 2003).

Reproductive mode may furthermore be sensitive to environmental conditions (Barcaccia *et al.*, 1997b; Mazzucato *et al.*, 1997), and thus the induction of recombinant genotypes should be considered during cultivar selection and multiplication if the working environment is likely to differ from that of germplasm collection or cultivar constitution. For example, the Polish cultivar Topas, although characterised by a within-population genetic similarity higher than Italian ecotypes, was composed of multiclinal genotypes rather than being genetically uniform as expected. Such a result could be the consequence of years of seed multiplication in a southern Mediterranean environment, which is quite different from that of its origin in central Europe.

The high occurrence of non-maternally derived seeds in a seed stock does not necessarily reflect a genetically variable field population, nor can the high occurrence of sexually derived plants in an experimental trial be directly used to estimate levels of apomixis. Since apomixis can lead to irregular embryo and seedling formation, unbalanced chromosome numbers, mutation accumulation and sterility, discrepancies observed between molecular polymorphisms scored within single populations and the relative proportions of seeds derived by apomictic or sexual processes may result from differential seed vitality and plant fertility. The relative fitness of sexually and apomictically derived embryos is furthermore correlated with the environment of evaluation (Mazzucato *et al.*, 1997).

In conclusion, our data highlight reproductive mechanisms and adaptive strategies of *H. perforatum*, and suggest that the frequency of facultative apomixis is influenced by both genetic and environmental factors. The cytological pathway of aposporic embryo sac formation appears to be stable and under a strict genetic

control, but seeds can be set not only through parthenogenesis of the unreduced egg cell coupled with pseudogamous endosperm development, but also through sexual processes including the autonomous development of meiotic, normally reduced egg cells and fertilisation of both reduced and unreduced egg cells. Thus, the mode of reproduction in this species seems to be much more dynamic and versatile than previously expected, although the environment most likely plays a role in buffering recombination potential. The picture that emerges from our survey suggests that experimental trials performed with a few genotypes in a chosen environment can lead to results that deviate significantly from the dynamics of the same genotypes in their source populations.

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