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Taxonomy, phylogeny and reproductive ecology of *Gentiana lutea* L.

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Abstract

This research focuses on taxonomy, phylogeny and reproductive ecology of *Gentiana lutea*. Taxonomic analysis is a critical step in botanical studies, as it is necessary to recognise taxonomical unit. Herbarium specimens were observed to assess the reliability of several subspecies-diagnostic characters. The analysis of *G. lutea* genetic variability and comparison with that of the other species of sect. *Gentiana* were performed to elucidate phylogenetic relationships among *G. lutea* subspecies and to propose a phylogenetic hypothesis for the evolution and the colonization dynamics of the section. Appropriate scientific information is critical for the assessment of species conservation status and for effective management plans. I carried out field work on five natural populations and performed laboratory analyses on specific critical aspects, with special regard to *G. lutea* breeding system and type and efficiency of plant-pollinator system.

Bracts length is a reliable character to identify subsp. *vardjanii*, but not exclusive, hence to clearly identify it, other traits have to be considered. The phylogenetic hypotheses obtained from nuclear and chloroplast data are not congruent. Nuclear markers show a monophyly of sect. *Gentiana*, a strongly species identity of *G. lutea* and clear genetic identity of subsp. *vardjanii*. The little information emerging from plastid markers indicate a weak signal of hybridization and incomplete sorting of ancestral lineages. *G. lutea* shows a striking variation in intra-floral dichogamy probably evolved to reduce pollen-stigma interference. Although the species is partially self-compatible, pollen vectors are necessary for a successful reproduction, moreover it shows a strong inbreeding depression. *G. lutea* is a generalist species: within its spectrum of visitors is possible to recognize "nectar thieves" and pollinators with sedentary or dynamic behaviour. Pollen limitation is frequent and it could be mainly explained by poor pollen quality.

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Introduction

1.1 Background

1.1.1 Genus *Gentiana* L.

Gentiana L. is the largest genus in the family Gentianaceae, comprising about 361 species. These species mostly grow in temperate and alpine regions: widely in Asia (312 ssp.), less frequently in Europe and North - Central America (29 and 35 ssp., respectively), and sporadically in South America (3 ssp.), Africa (2 ssp. in Morocco only) and Eastern Australia (1 sp.). According to Yuan et al. (1996), two centres of diversity can be recognized: the main one in the Southwest mountains of China and adjacent North-East Burma, where, among 190 species, about half are endemics; the second in the Alps and in the Pyrenees, with 27 species, including 17 endemics.

The taxonomy of the genus has changed dramatically since its first description. *Gentiana sensu lato* is a very heterogeneous assemblage of morphologically different groups, including *Tripterospermum*, *Crawfurdia*, *Megacodon* and *Gentianella sensu lato*, further consisting of *Gentianella sensu stricto*, *Comastoma*, *Gentianopsis* and *Pterygocalyx*, which are nowadays considered as genera by many taxonomists (Ho et al., 1996).

At present, most authors (e.g. Ho et al., 1996; Ho and Liu, 2001; Gielly and Taberlet, 1996; Hungerer and Kadereit, 1998; Struwe and Albert, 2002; Mishiba et al., 2009) accept the circumscription of the genus, based on sub-

genus *Eugentiana* as described by Kusnezow (1896), excluding all the other genera mentioned above. This concept is narrower than *Gentiana sensu lato*, but much broader than *Gentiana sensu stricto* as defined by Löve and Löve (1972) and other authors (see Yuan et al., 1996 and references therein), who restricted *Gentiana* to the five species treated by Tutin et al. (1972) as sect. *Gentiana*.

Here I follow the classification proposed by Ho and Liu (1990), who recognized 15 sections within genus *Gentiana* (Table 1.1). Based on geographical pattern, Yuan et al. (1996) suggested a parallel diversification in the two centres of diversity: sections *Gentiana*, *Calathianae* and *Ciminalis* may have evolved from the European diversity centre while all other sections from the Asian one, except sect. *Pneumonanthe*, whose origin remains unclear.

Section	**Sp.**	**Distribution**
Gentiana L.	5	Europe, Turkey
Ciminalis (Adans.) Dum.	7	Europe
Calathianae Froel.	8	Europe, NE America, N and W Asia, NW Africa
Chondrophyllae Bunge	158	Europe, Asia, N and C America, NW Africa, Australia
Cruciata Gaudin	21	Europe, Asia
Kudoa Masam. = *Monopodiae* (Harry Sm.) TN Ho	37	Kashmir, China, E Asia, Malaysia, Indonesia
Otophora Kusn.	12	Himalayas, India, China, Myanmar
Isomeria Kusn.	18	E and NE Asia, Himalayas, NW and N America
Microsperma TN Ho	10	Nepal, Bhutan, SW China
Frigidae Kusn.	18	Europe, Asia, N America
Phyllocalyx TN Ho	1	SW China
Dolichocarpa TN Ho	14	Europe, Asia, N C and S America
Fimbricorona TN Ho	4	Himalayas, SW China
Stenogyne Franch.	14	Myanmar, China, Thailand
Pneumonanthe (Gled.) Gaudin	42	Europe, W, N and C Asia, N and C America

Table 1.1: Sections of genus *Gentiana*: number of species and geographical distribution. Table modified from Ho and Liu (1990).

1.1.2 Section *Gentiana* L.

Section *Gentiana* includes five species (*G.lutea* L., *G. burseri* Lapeyrouse, *G. punctata* L., *G. purpurea* L. and *G. pannonica* Scopoli) and five subspecies

(in addition to the nominal subspecies). The basic chromosome number is 2n=40 for all taxa (Yuan et al., 1996). The section is distributed throughout Europe. All species and subspecies (except *G. burseri* subsp. *burseri* and *G. lutea* subsp. *montserratii*, both endemic to the Central Pyrenees) occur in the Alpine Region, so the Alps are undoubtedly the main diversity centre of the section, although we cannot deduce whether it is a centre of diversification or a survival (glacial refuge) area.

Within the genus natural hybrids are infrequent: just one hybrid is described for sect. *Ciminalis*, and one/two for sect. *Calathianae*. From this perspective, sect. *Gentiana* represents an exception since seven spontaneous hybrids are described (Figure 1.1). Reflecting this pattern, the species belonging to this section clearly have a high genetic affinity (Anchisi et al., 2010).

Some phylogenetic studies have been conducted to reconstruct the relationships within genus *Gentiana*. According to Müller (1982), sect. *Gentiana* is considered ancestral within the genus and sect. *Pneumonanthe* is considered sister to sections *Calathianae*, *Cruciata*, *Frigidae* and *Ciminalis*. Similar conclusions have been drawn by Carbonnier et al. (1977) based on phytochemical analyses, even though many Asian sections were not taken into account. Phylogenetic analysis by Ho et al. (1996), based on 61 informative characters from morphology, palynology and cytology, showed that the genus is first split to perennial and annual clades. In the perennial clade, sect. *Pneumonanthe* is considered the most primitive, followed by the Asian and the European clades, arisen after sect. *Pneumonanthe*. In the European subclade, sect. *Gentiana* emerges and is considered a highly specialized and rather isolated group, while sect. *Ciminalis* and *Calathianae* are closely connected. The phylogeny inferred from Internal Transcribed Spacer (ITS) sequence data by Yuan et al. (1996) is congruent with morphological classifications, except for *G. asclepiadea*, which appears to be closely related to sect. *Gentiana*. *G. asclepiadea* is included in sect. *Pneumonanthe*, but its chromosome number (2n=44) differs from all the other members of the section (2n=26). Based on this distinctive feature Löve and Löve (1976) elevated the species to the genus rank (*Dasystephana*). Phytochemical evidences contradicted this conclusion: the three European sections contain xantone-O-glycoside, while it

is absent in *G. asclepiadea* which presents xantone-C-glycoside instead: this trait (xantone-C-glycoside) is apparently plesiomorphic in the entire genus (Yuan et al., 1996 and references therein). In the same study authors did not consider sect. *Gentiana* as ancestral. Gielly and Taberlet (1996) inferred a phylogeny of the European gentians from chloroplast *trn*L (UAA) intron sequences. A peculiar position of *G. lutea* subsp. *montserratii*, in polytomy with the clade including all other species of sect. *Gentiana* is found congruent with the morphological, palynological and ecological data of Vivant (1975), and the phytochemical data of Massias et al. (1987). They also confirmed the close relationship between *G. asclepiadea* and the species of sect. *Gentiana*. Two recent studies (Mishiba et al., 2009 and Davitashvili and Karrer, 2010), support the taxonomic position of *G. asclepiadea* within sect. *Gentiana*, basing their conclusions respectively on chloroplast markers and seed morphology.
Although several species of sect. *Gentiana* were considered in phylogenetic studies, no detail on the relationships among them is currently available.

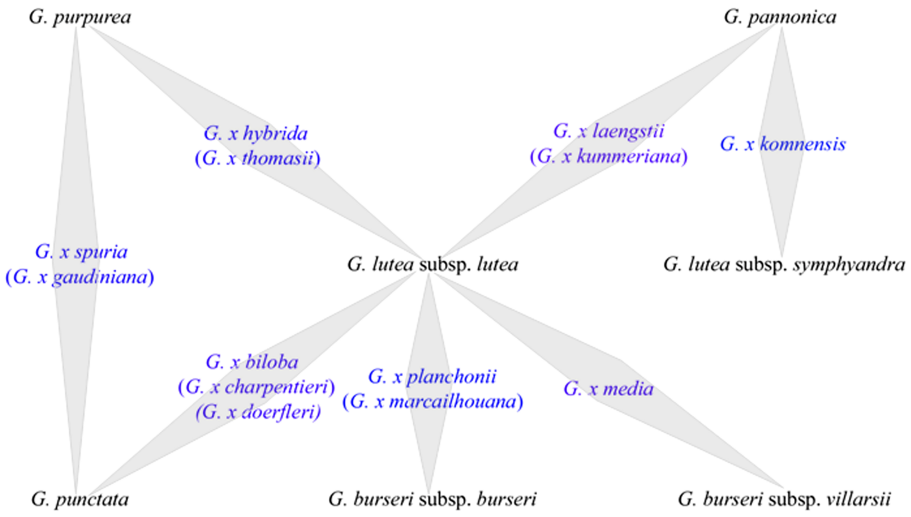

Figure 1.1: Spontaneous hybrids of sect. *Gentiana*. Species in black, hybrids in blue. Picture modified from Anchisi et al., 2010.

1.2 Study species

1.2.1 *Gentiana lutea* L.

Gentiana lutea L. is a long-lived scapose hemicryptophyte. It presents an unbranched stout stem, growing up to 2 metres tall. Basal leaves are glaucous, decussate, lanceolate-elliptic to broadly ovate with 5-7 strong veins; stem leaves are narrower and stalkless (Tutin et al., 1972). In June the plant produces a new sterile or flowering stem (Appendix - Figure 1). Flowering stems carry up to 10 pseudo-whorls containing numerous pedicellate flowers (about 20). The inflorescence develops essentially in basipetal direction and in centrifugal way within pseudo-whorl (Kozuharova, 1994). Each flower shows a split calyx, with 2-7 minute teeth and a yellow gamopetalous corolla with 3-9 deeply engraved lobes (Appendix - Figure 2). Stigma is bilamellate and anthers are usually free, except for *G. lutea* subsp. *symphyandra*. Five nectaries occur between stamen filaments and corolla attachment point. Flower bracts are green and almost equal in length to pseudo-whorls, except for *G. lutea* subsp. *vardjanii*. Flowering begins after 10 years (Yankova et al., 2010) and occurs between June and July. Fruit is a many seeded capsule (Struwe and Albert, 2002) composed of two carpels and ripening in August, (Appendix - Figure 3). Seeds are circular to elliptic, flattened and winged (Appendix - Figure 4); the wing is often absent at the hilum/micropile. According to Struwe and Albert (2002) wind is the main dispersal vector of seeds: the species grows in open vegetation, where no tree restricts anemochory. Müller-Schneider (1986) reports dysochorous dispersal by snow finches as an alternative type of dispersal (Struwe and Albert, 2002).

G. lutea multiplies through vegetative propagation: the spreading of rhizome assures population persistence and growth (Hesse et al., 2007), (Appendix - Figure 5), so even large populations are often represented by few individuals (Georgieva, 2007). Vegetative stems show internodes, except for *G. lutea* subsp. *vardjanii* (Appendix - Figure 6).

The species grows in grassy alpine and sub-alpine pastures, usually on calcareous soils, at altitudes ranging from 800 to 2500 metres a.s.l.. The species

is distributed in the South European high mountains, from Spain to Greece up to the North-West part of Turkey (Figure 1.2).
G. lutea is an important medicinal plant whose rhizome contains numerous principles used as a remedy for digestive disorders (it increases gastric secretions and whets appetite). This is mainly due to the presence of bitter tasting secoiridoid-glycosides (e.g. swertiamarin, gentiopicroside, amarogentin and sweroside), which show cholagogue, hepatoprotective and wound-healing properties. Other constituents are relevant as well, as the iridoid loganic acid (anti-inflammatory activity), xanthone glycoside (gentioside) and xanthones like gentisin and isogentisin (Aberham et al., 2007). For these reasons the species has a long history of use in pharmaceutical industry, liquor-production and decoction preparation.
The collection of the rhizome without regulation is the main threat to *G. lutea*, and that is the reason why some protection measures were assumed. The species is listed in the *"Council Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora (Habitat Directive) – Annex V"*, where are included animal and plant species of Community interest whose taking in the wild and exploitation may be subject to conservation measures. In addition, the species is included in the *"Council Regulation (CE) No. 338/97 on the protection of species of wild fauna and flora by regulating trade therein - Annex D"*, that lays down the provisions for import and export, indicates procedures and documents required for such trade and regulates the movement of live specimens (in respect of *G. lutea* the provisions are applied even to dried material). In Italy *G. lutea* is locally protected by regional regulations.
G. lutea includes four subspecies: *G. lutea* subsp. *lutea*; *G. lutea* subsp. *symphyandra* (Murb.) Hayek; *G. lutea* subsp. *vardjanii* Wraber and *G. lutea* subsp. *montserratii* (Vivant ex Greuter) Romo. A brief description follows here.

G. lutea* subsp. *lutea matches the description above. It is distributed all over the range of the species except for the Balkan Peninsula (Tutin et al., 1972) and the Eastern Alps (Wraber, 1986).

G. lutea* subsp. *symphyandra shows the anthers connate in a tube (Appendix - Figure 7); it grows in the Eastern part of the Alps and in the Balkan Peninsula (Tutin et al., 1972).

G. lutea* subsp. *vardjanii differs from subsp. *lutea* in the presence of yellowish green floral bracts longer than pseudo-whorls and in the presence of vegetative stemless shoots - rosette type (Appendix - Figure 8 and Figure 9). Flowering stem is shorter (about 80 cm) and flowers are smaller than in subsp. *symphyandra* (Vender et al., 2010). Pseudo-whorls are more compact and flower peduncles are shorter than in other subspecies (personal observation). It is surely present in the South-Eastern Alps (in Italy, Carinthia and Slovenia) but its exact distribution is still to be confirmed. It grows sympatrically with subsp. *symphyandra* but its flowering occurs 2-3 weeks in advance (Wraber, 1986; Vender et al., 2010).

G. lutea* subsp. *montserratii is about one metre tall. It presents 6-7 ovate-elliptic corolla lobes; anther filaments are longer than anthers; floral peduncles are longer and pollen grains are bigger than subsp. *lutea* (Vivant, 1975), (Appendix - Figure 10 and Figure 11). According to Anchisi et al. (2010) it is endemic to small areas of Pre-Pyrenees (Sierre de Leyre, San Juan de la Peña d'Oroel and Cadì), and of Central Pyrenees (Ordesa, Vall de Boì and Val Ferrera).

1.2.2 Other species of sect. *Gentiana*

A brief description of the other species belonging to sect. *Gentiana* follows here:

***Gentiana burseri* Lapeyrouse**

Plant up to 80 cm tall with upright and unbranched stem; lower leaves stalked from elliptic-lanceolate to ovate-elliptic with 5-7 distinct veins; stem leaves shorter and broader progressively becoming stalkless upwards; flowers in dense clusters in the upper leaf-axils, sessile; papery calyx with the

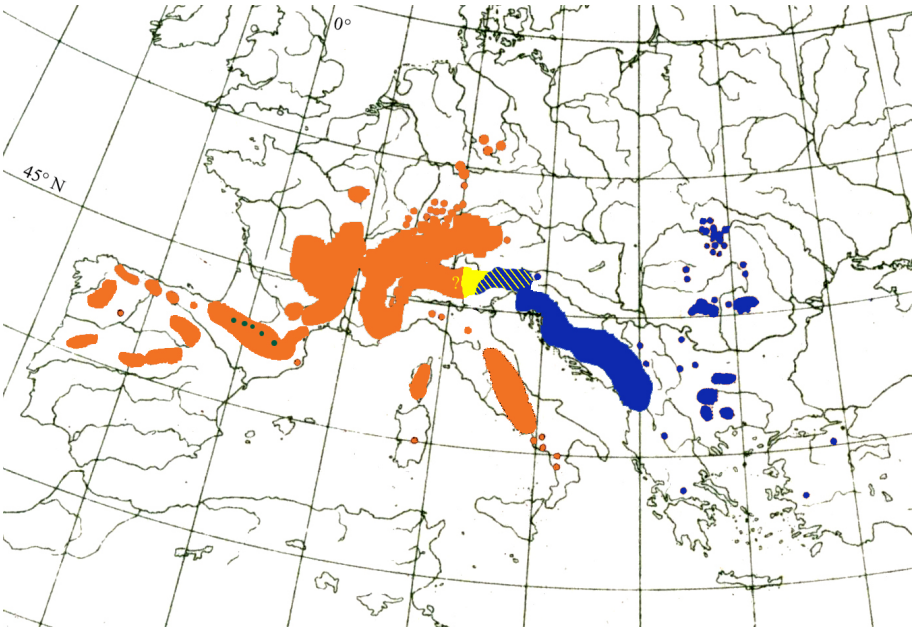

Figure 1.2: Geographical distribution of *G. lutea* subspecies. Orange, blue, yellow and green refer, to the distribution of subsp. *lutea*, subsp. *symphyandra*, subsp. *vardjanii* and subsp. *montserratii*, respectively. Picture modified from Meusel et al. (1978).

tube split down one side, with minute lobes; corolla campanulate, with 5-7 lobes as long as the tube, pale yellow or greenish-yellow towards the base, often with brown spots, lobes triangular with the sinuses between them with an acute appendage); anthers connate; capsule shortly stalked (Tutin et al., 1972). Blooming time between July and August. It grows in grassy alpine and sub-alpine pastures, usually on acid or neutral soils at altitude ranging from 1000 to 2400 (2700) metres a.s.l. (Ågren and Schemske, 1993). *G. burseri* includes three subspecies: *G. burseri* subsp. *burseri*; *G. burseri* subsp. *villarsii* (Grisebach) Rouy and *G. burseri* subsp. *actinocalyx* Polidori.

G. burseri* subsp. *burseri follows the description above, (Appendix - Figure 12 and Figure 13). Its distribution range covers French and Spanish Pyrenees.

G. burseri* subsp. *villarsii shows obtuse or sub-acute corolla lobes and the sinuses between them have a truncate appendage (Tutin et al.,

1972), (Appendix - Figure 14 and Figure 15). It is present in the South-West Alps, both in Italy and France (Anchisi et al., 2010).

G. burseri* subsp. *actinocalyx according to Polidori (2008), differs from subsp. *villarsii* in the presence of one-piece or incompletely divided calyx with (3)5-8 teeth (in subsp. *villarsii* the calyx is split down to the base). Calyx margin shows numerous cone or club shaped papillae (about 0.1 mm); club shaped papillae are also present in flower margins, (Appendix - Figure 16, Figure 17 and Figure 18). The subspecies is endemic of a small part of Ligurian and Maritime Alps.

***Gentiana punctata* L.**

Plant up to 60 cm tall, stem erect with a metallic tinge; basal leaves stalked, elliptic abruptly acute, with 5-7 veins; stem leaves narrower, becoming progressively more shortly stalked upwards; flower sessile, crowded in terminal and axillary clusters; calyx tube with 5-8 teeth, erect, acute, green; corolla broadly tubular with 5-8 pale greenish-yellow lobes, usually with dark purple spots; lobes shorter than the tube, spreading; plicae small, obtuse; anthers connate at first, later free, (Appendix - Figure 19 and Figure 20). Flowering occurs from July to August (Tutin et al., 1972). It vegetates on sub-alpine meadows, grasslands and shrub-lands, preferably on acid soils, at 1400-2700 metres of altitude. It is mainly distributed in south-east European mountains (Alps, Carpathian), towards South up to Albania, Macedonia and Bulgaria (Balkans), towards North-West up to Ukraine (Anchisi et al., 2010).

***Gentiana purpurea* L.**

Plant up to 60 cm tall; stem simple, erect, sometimes reddish; leaves lanceolate to broadly ovate, with 5-7 strong longitudinal veins; flowers sessile, in small terminal clusters, sometimes also in few-flowered axillary whorls; calyx membranous, split down to the base; corolla reddish purple with dark purple spots, lobes ovate; anthers connate, (Appendix - Figure 21 and Figure 22). It blooms from July to September (Tutin et al., 1972). *G. purpurea* grows between 1200 and 2600 metres above sea level and in the same habitat and soils of *G. punctata*. The species is distributed in the Alps, in the Northern

Apennines and in the South of Norway (Anchisi et al., 2010).

***Gentiana pannonica* Scopoli**

Similar to *G. punctata*; stem without metallic tinge; calyx with recurved teeth; corolla purple with reddish-black spots; anthers connate; capsule shortly stalked, (Appendix - Figure 23 and Figure 24). The species flowers between June and September (Tutin et al., 1972). Alpine grasslands and secondary mountain meadows are typical habitats of this species. The centre of its distribution is situated in the Eastern Alps, where it occurs on calcareous and sometimes also on neutral bedrock at altitude ranging from 1300 to 2300 metres a.s.l. Apart from the Alps, the species occurs also in the Bohemian Forest (Hofhanzlovà and Fèr, 2009).

1.3 Taxonomic analysis

Nowadays plant identification is mainly based on morphological characters. These characters have been used for a long time as data source for taxonomical analyses, in order to identify and name species (or more generically taxa), and to arrange them into a classificatory system (Judd et al., 2007). Taxonomic analysis is a critical step in phylogenetic studies: before carrying out molecular analyses, prior investigations are necessary, to clearly recognise taxonomical units, and thereby to ensure an adequate sampling (Hungerer and Kadereit, 1998).

Within *G. lutea*, the following subspecies-diagnostic characters have been recognised: free/connate anthers, anthers length, stamen filament length, stigma shape after anthesis (Pignatti, 1982); corolla lobes shape, floral peduncles, stamen filament longer/shorter than anther (Vivant, 1975); bracts length, vegetative stems with/without internodes (Wraber, 1986). Nevertheless, the reliability of some of these diagnostic characters has never been proven. Details on species description are given in paragraph 1.2.1.

1.4 Phylogenetic analysis: molecular tools

The use of both nuclear and chloroplast markers may be suitable to elucidate phylogenetic relationships among species and to highlight speciation and colonization dynamics.

1.4.1 Nuclear markers

The Internal and External Transcribed Spacers (ITS and ETS) are part of the 18S-5.8S-26S region of the nuclear ribosomal DNA. In the last decade, the use of Internal Transcribed Spacer region (ITS) has revolutionized plant phylogeny. Since concerted evolution has generally homogenized sequence variation among ITS copies within individuals, direct sequencing of this region is possible for most systems (Kay et al., 2006). This feature, coupled with the availability of universal primers (White et al., 1990; Muir and Schlötterer, 1999; Blattner, 1999) and high substitution rate (especially compared to most chloroplast regions), make them accessible and appropriate for resolving inter-specific phylogenetic relationships (Baldwin et al., 1995). At present some authors have suggested using the ITS region for barcoding, to provide greater taxonomic resolution than can be obtained using chloroplast markers alone (Lia et al., 2011). However, their reliability as the sole source of phylogenetic evidence has come under criticism because of their evolution, given that a number of molecular genetic processes impact ITS sequences in ways that may mislead phylogenetic inference (Alvarez and Wendel, 2003). Despite this, ITS sequences remain one the most efficient loci to infer species-level phylogenetic relationships (Kay et al., 2006). Several molecular studies, based on ITS, were carried out on genus *Gentiana* (Yuan and Küpfer, 1995; Yuan et al., 1996; Diadema et al., 1997; Hungerer and Kadereit, 1998; Yuan and Küpfer, 2005) and in almost all cases they are congruent with both morphological phylogeny and phylogenies inferred with other markers.
According to Baldwin et al. (1995) ITS sequences do not always show sufficient variation for robust resolution of some generic and subgeneric relationships and External Transcribed Spacer (ETS) represents an additional fragment for augmenting ITS data. ETS region appears to evolve at least as

rapidly as ITS regions and moreover many studies reveal their faster evolution compared to ITS (Baldwin and Markos, 1998; Kadereit et al., 2007; Timme et al., 2007). The primary barrier to using ETS as a molecular marker is the lack of a highly conserved region flanking the 5' end of the spacer, due to the presence of the highly variable Non-Transcribed Spacer (NTS), bordering its 5' ends and rapidly evolving in sequence and length (Baldwin and Markos, 1998). For this reason internal primer construction is often necessary. ETS evolution may however evolve under similar constraints of ITS (Baldwin and Markos, 1998 and references therein).

1.4.2 Chloroplast markers

Noncoding sequences of the chloroplast genome are a primary source of data for molecular systematic, phylogeographic and population genetic studies of plants. Even if several guidelines on the variability of plastid regions have been drawn, suitable markers across all taxonomic lineages do not exist, hence a preliminary screening is recommended.
Shaw et al. (2007) suggested *rpl*32-*trn*L intergenic spacer as the best chloroplast region of the 34 surveyed, suitable for low-level molecular studies. Shaw et al. (2007) and Štorchovà and Olson (2007) suggested *psb*A-*trn*H intergenic spacer as a useful marker for DNA barcoding, basing on three reasons: it is highly variable, it is a relative short region across angiosperms and published primers seem to be universal; Ma et al. (2010) highlighted its discriminate power for species identification within pteridophytes.
However, despite the extremely high utility of noncoding regions our knowledge about their evolution is far from complete, in particular chloroplast capture (hybridization), deep coalescence and incomplete sorting of ancestral lineages could confound phylogenetic inference (Gurushidze et al., 2010). Shaw and Small (2005) have highlighted how recent histories of hybridization in closely related species can homogenize or even uncouple plastid genome phylogenies from species phylogenies. If study species do not represent genetically and reproductively isolated lineages and moreover introgressive hybridization phenomena occur, due to the lack of chloroplast genome recombination, plas-

tid genome can represent an error source in molecular systematics (Gabrielsen et al., 1997). In addition, both deep coalescence (the merging of genetic lineages backwards to a most recent common ancestor) and incomplete sorting of ancient lineages (a common ancestor undergoes several speciation events in a short time period and ancestral polymorphism is not fully resolved into two monophyletic lineages when the second speciation occurs) can be misleading in phylogenetic reconstructions (Gurushidze et al., 2010 and references therein).

1.5 Reproductive ecology

1.5.1 Dichogamy and herkogamy

Most flowering plant species produce hermaphrodite flowers, however many floral traits evolved to overcome the peculiarities related with housing male and female function within the same flower. Dichogamy and herkogamy are two common examples of such traits (Sargent et al., 2006).
According to Lloyd and Webb (1986), intra-floral dichogamy is a temporal separation of sexual functions: anthers can expose viable pollen before stigma receptivity (protandry) or, otherwise, stigma can become receptive before anthers dehiscence (protogyny); the simultaneous presentation of pollen and stigma is described as adichogamy (or homogamy). When there is no overlap in the presentation of pollen and stigma, the dichogamy is complete, otherwise, if there is overlap it is incomplete. Generally, the lifespan of male and female functions are timed in absence of pollinator visits, but in nature the periods of effective presentation are likely to be shorter and more variable than their potential, primary due to plant-pollinator interaction (Lloyd and Webb, 1986).
Herkogamy is the spatial separation of sexual functions. Webb and Lloyd (1986) distinguished several classes of herkogamy; referring to "unordered herkogamy" when the distance between male and female structures is small than pollinator size, and pollinator behaviour is not constrained by the blossom; to "ordered herkogamy" when both pollen and stigma are positioned

into position along the pathway the visitor takes on legitimately approaching the blossom; to "reciprocal herkogamy" when there are two or three different forms of blossom are present, either on the same plant or on separate plants. Dichogamy and herkogamy are usually interpreted as mechanisms to reduce self fertilization. However, a considerable number of angiosperms shows more than one "outcrossing mechanisms" such as dichogamy, herkogamy, self-incompatibility and unisexuality. There are two possible explanations for the combinations of outcrossing features: from one side each of them alone might be insufficient to totally prevent self-fertilization, on the other side, selection could promote different floral features, including dichogamy and herkogamy, that reduce pollen-stigma interference (Lloyd and Webb, 1986; Webb and Lloyd, 1986). Sargent et al. (2006) developed a population genetic model to examine the influence of anther-stigma interference and inbreeding depression on the evolution of dichogamy. Their model predicts that both forces can drive the evolution of dichogamy within a single species, however anther-stigma interference represents a key force in the evolution of dichogamy since it has impact both in self-compatible and self-incompatible species.

1.5.2 Plant breeding system

Plant breeding system is a key trait that affects both ecological aspects (individual fitness, dependence on pollinators for sexual reproduction, plasticity in response to environmental shifts, population genetics) and evolutionary dynamics. Plants show a wide pattern of breeding systems ranging from enforced outbreeding of single-sex (dioecious) plants or of plants with a genetic self-incompatibility system, to agamospermy, through different degrees/modes of self-pollination (Charlesworth, 2006).
Lloyd and Schoen (1992) proposed a classification of self-pollination into six modes, based on number and kinds of flowers involved, role of pollinators and timing of self-pollination relative to cross-pollination. In particular they distinguished cleistogamy, geitonogamy, facilitate self-pollination (both pollinator mediated) and autonomous selfing (prior, competing and delayed compared to cross-pollination). Focusing on the mechanisms me-

diated by pollinators, facilitate self-pollination occurs in flowers presenting simultaneously pollen and receptive stigma, while geitonogamy involves transfer of pollen between flowers of the same plant, presenting the ecological properties of cross-fertilization and the genetic properties of self-fertilization. The amount of these two modes of selfing varies enormously depending on pollinator behaviour (the way they move, the time they spend on each flower/inflorescence) and on intrinsic flower/inflorescence features (herkogamy and dichogamy). Furthermore, inter-flower interference not only may carry the costs of self-fertilization, but also reduces the amount of pollen available for export (so-called "pollen discounting"; Harder and Wilson, 1998). As pollen discounting diminishes outcross siring success, the avoidance of interference may be an important evolutionary force in floral biology (Barrett, 2002).

The success of cross-pollen vs. self-pollen, once deposited on the stigma, is determined by two post-pollination mechanisms: self-incompatibility and inbreeding depression.

Self-incompatibility is a barrier based on a genetic system of self-recognition that reduces the frequency of self-fertilization (De Nettancourt, 1997). Within angiosperms three types of self-incompatibility (SI) have been described: sporophytic SI (SSI), gametophytic SI (GSI) and ovarian SI (OSI). In SSI the incompatibility is determined by recognition of the diploid genotype of pollen parent at the stigmatic level of the receipt plant (De Nettancourt, 1997), while in GSI is determined by recognition of the haploid genotype at style level (Hiscock and McInnis, 2003). Recently, the concept of SI has been expanded to include apparent pre-zygotic ovarian SI where proper ovules development depends on whether self or outcross pollen is present. However, the absence of differential cross and self pollen tube growth makes it difficult to ascertain when self-recognition occurs (pre or post zygotic level). In post-zygotic OSI a late-acting self-incompatibility ceases embryos development before the occurrence of early-acting inbreeding depression (Seavey and Bawa, 1986).

The number and the quality of offspring can be regulated even after fertilization. An important form of post-zygotic selection is inbreeding depression (re-

duced fitness of inbred offspring compared to that derived from out-breeding). According to Charlesworth and Charlesworth (1987) inbreeding depression is due to the presence of recessive (or partially recessive) deleterious mutations, in homozygous state. Mortality may be obligate and reflect the expression of recessive lethal alleles. In contrast to late-acting SI, inbreeding depression is mainly characterized by reduced fitness or death at various developmental stages (Sage et al., 2005). Due to genetic drift, small or isolated populations may be subject to loss of genetic variability and consequent higher inbreeding depression, however in long-lived species these negative consequences may not become obvious for a long time (Conte and Cristofolini, 1992), nevertheless reproduction may be affected much earlier than population survival (Oostermeijer et al., 1992).
For these reasons, deposition of self-pollen within a plant may have costs on both male and female fitness.

1.5.3 Resource allocation to reproduction

To complete their life cycle, plants must function as a balanced system in term of resource uptake and use. In accordance with this sight, resources extracted from the environment and manufactured within the plant are allocated to different plant structures and functions as growth, reproduction and defence. Resource allocation to growth and defence of vegetative parts ensures the presence of specialized reproductive structures (Bazzaz and Grace, 1997). Resources build-up is particularly important in mountain environments characterized by challenging environmental conditions (short growing season, long and cold winters) since it provides plant support to vegetative regrowth, ability to bridge temporal gaps without resources, support of sexual and/or vegetative reproduction and resistance to natural calamities (Lütz, 2012). Severe climatic conditions can hamper sexual reproduction limiting flowering and seed production. In these environments vegetative propagation may be more advantageous than sexual reproduction as it assures population maintenance in place and time, sharing resources through clonal integration and reducing the mortality of genets (Körner, 2003; Lütz, 2012). However,

seeds are essential for population dynamics, allowing the establishment of new individuals, via dispersal, both in space and time (Hesse et al., 2007).
Concerning reproduction Darwin (1859) already noted that reduced investment in one reproductive function could be compensated by left additional resources to other sexual function. Although sexual allocation remains difficult to analyse, some attempts have been undertaken. In order to highlight this topic, Darwin's idea of trade-off was translated in evolutionary models by several authors (Charlesworth and Charlesworth, 1981; Charnov, 1982; Campbell, 2000 and references therein). In the classic form, sex allocation was simply evaluated as the proportion of resources invested by the plant in androecium rather than in gynoecium, while recent theories separate allocation basing on the timing of investment (Campbell, 2000 and references therein). In particular, maternal investment can be regulated in three sequential stages such as: flowers determination, ovaries development (in hermaphrodite flowers concurrent to pollen grains production) and fruit maturation.
Several evolutionary hypotheses have been proposed to explain the evolutionary significance in investing resources for flower determination such as: "pollinator attraction hypothesis", "bet hedging hypothesis"', "mate selection hypothesis" and "pollen donation hypothesis" (Berjano et al., 2011 and references therein). In particular, the "bet hedging hypothesis" considers extra flowers as ovules reserve, reducing possible risks arising from environmental conditions (Stephenson, 1981) and allowing unpredictable fertilisation opportunities (Burd et al., 2009).
In hermaphrodite flowers, investment in ovules is concurrent with pollen grains production, so resource allocation can be oriented in female and male functions, respectively. Cruden (1977) described the pollen-ovule ratio (P/O) as an indirect indicator of breeding systems. Based on the observation that flowers of self-incompatible species produced more pollen grains than closely related self compatible taxa with similar ovule number, he hypothesized that the higher is the P/O ratio, the higher is the number of pollen grains required to achieve successful pollination, hence the lower is the efficiency. Cruden himself considered that P/O ratio should be correlated with habitat or successional stages and recently Baker et al. (2005) affirmed that sexual

allocation may vary in time, and that different amounts of resources might be invested to pollen grains or ovules in early or late flowering season. In accordance with this assumption Burd (2011) identified a complex web of selective factors that potentially affect pollen and ovule numbers and the resulting P/O ratio, for instance pollen presentation and dispensing, patterns of pollen receipt, pollen tube competition and female mate choice through embryo abortion.
Last maternal investment is in fruit production. Ghazoul and Satake (2009) proposed the "sacrificial sibling hypothesis" to explain why some taxa retain low quality fruit. Selection is expected to favour early abortion of inbred zygotes, in order to minimize loss of energetic resources; however, in many species, this does not occur. According to these authors, the large proportion of developing fruits can be selected to dilute the impact of pre-dispersal seed predators, acting as seed predator sinks, and thereby increasing survival probabilities of viable seeds. In their dissertation they examined both selfed and seedless fruits production, considering the last as more efficient decoys since they only require investment in dry weight and do not limit the potential for outcrossed fruit.

1.6 Plant-pollinator interactions

1.6.1 Pollinator behaviour

Reproduction in entomophilous plants is determined not only by breeding system but also by interactions with pollen vectors. The study of behavioural patterns of pollinators is a key argument for pollination biology studies as it gives crucial information about pollen deposition, dispersal and carry-over, pollination efficiency, resource utilization by foragers, advertisement and visitation frequency and pollinator community composition. Observation of pollinator behaviour into sequential stages such as activity prior to floral contact, behaviour within flower and inflorescence as well as movement among conspecific and heterospecific plants, is useful to know plant-pollinator interface, since each component has important implications for floral attractiveness,

resources utilization, pollination efficiency, pollen flow, breeding system and population structure (Potts, 2005).
This interaction may be particularly complex, since pollinator behaviour can be influenced by floral and plant structure (inflorescence architecture and daily flower number) and other biotic and abiotic factors (Brunet, 2005 and references therein). Foraging pollinators behaviour, meant as number and position of consecutively visited flowers, can influence both geitonogamy spread (De Jong et al., 1993) and pollen discounting (Harder and Wilson, 1998), modulating the balance between selfing and cross-fertilization (Brunet, 2005), with important reproductive and evolutionary consequences (Ohashi and Yahara, 1998). In particular, pollen discounting occurs more frequently when selfing results from geitonogamy, since this process relies in pollinators in the same way as outcrossing does, and shows high levels in plant that simultaneously display many flowers (Brunet, 2005 and references therein). Nevertheless, pollinator directionality, inflorescence development, quantity and quality of nectar may contribute to limit both geitonogamy (Fisogni et al., 2011) and pollen discounting.

1.6.2 Floral rewards

The concept of co-evolution between food-rewarding flowers and their pollinators was first proposed by Darwin (1859) and was explicitly developed in his following Darwin (1862). He famously predicted that *Angraecum sesquipedale*, a long-spurred Malagasy orchid, must be pollinated by a hawkmoth with an exceptionally long tongue. His prediction had gone unverified until 21 years after his death when the moth (*Xanthopan morgani predicta*) was discovered (Johnson and Anderson, 2010). From that moment plant-pollinator system was considered as a co-evolving mutualism: from one side plants offer rewards, supplying essential needs of consumers and promoting their repeated visits; from the other side, pollinators directly or indirectly depose pollen on a compatible stigma, giving an unwilling fundamental service to plants (Dafni et al., 2005).
Pollen and nectar are the main floral rewards, nevertheless there are other

both nutritive and non nutritive minor rewards. Among nutritive rewards are glower tissue, food tissue (food scales, food bodies, non fertile pollen and pseudo pollen), stigmatic fluid, fatty oils, while non nutritive ones are, for example, nest material (trichomes, resins, waxes and corolla parts), shelter, sexual attractants and mating sites (Dafni et al., 2005).
Pollen is consumed by numerous kinds of insects including coleopterans, flies, butterflies, bees and wasps. It represents the main source of protein and nitrogen. Pollen can be directly eaten by visitors, or collected as larval food, as bees do: in this case the actively collected pollen is stored in specific body structures (corbiculae or legs-scopae) and is not available for pollination (Westerkamp, 1996). From the plant point of view, pollen is the vehicle for male gametes. Almost all angiosperms pollinated by animals present an adhesive material around pollen grains (pollenkitt). According to Pacini and Hesse (2005), pollenkitt has many functions, some of which are strictly related with pollinators interaction: it facilitates pollen dispersal promoting adhesion to insects body, it keeps together pollen grains during transport, it renders pollen attractive to animals and more or less visible to animal eyes, it avoids predation through smell, it enables pollen packaging by bees and form corbiculae and it provides a digestible reward for pollinators. From the plant perspective pollenkitt holds pollen in the anther until dispersal, enables secondary pollen presentation, protects pollen from water loss, ultra-violet radiation, fungi and bacteria attacks, maintains sporophytic protein responsible for pollen stigma recognition, protects pollen from hydrolysis, enables pollen clumps to reach stigma and facilitate adhesion, allows self-pollination and facilitates pollen rehydration.
Nectar is the most important reward offered by flowering plants to their visitors. It is secreted by specialized organs (nectaries). According to Galetto and Bernardello (2005) (see also references therein) two main types of nectaries can be recognised: entra-floral and floral nectaries. Extra-floral nectaries protect vegetative and reproductive structures from predators, they are located in vegetative organs or outer floral parts and are never involved in pollen transfer. Floral nectaries are located within flowers and they are involved in pollination process; if their nectar production goes on after anthesis up to

fruit development, they are thought to protect developing seeds (post-floral nectaries). Nectar may be considered as phloem fluid (Fahn, 1979), modified during secretion and converted into a mixture of sucrose, fructose and glucose in varying proportions; minor sugars, such as sorbitol, melibiose, maltose, and mannitol are usually also present. Although sugars represent the major energetic source, many other substances are found, such as amino acids, lipids, phenols and antioxidants (imparting a specific taste or odour for pollinators attraction), as well as alkaloids, saponins or non-protein amino acids, that can turn it toxic or repellent (Galetto and Bernardello, 2005 and references therein). A few of the non-toxic non-protein amino acids, including β-alanine, ornithine, homoserine, and γ-aminobutyric acid (GABA) are known to accumulate in nectar and it is apparent that they are consistent and sizable components of certain floral nectars, but whether they have any role in attraction of pollinators must await further studies (Nicolson and Thornburg, 2007). According to Petanidou (2007), hexose-rich nectar is easy to digest and adapted to consumption by an extensive array of mainly non-specialized pollinators (short-tongued bees, wasps, beetles, butterflies and flies), while high-sucrose nectars are better adapted to more specialized pollinators, such as long-tongued bees, able to perform sucrose digestion (hydrolysis).

1.6.3 Pollen limitation

Scarcity of pollinators or inefficient pollination may create conditions where pollen is a limiting factor for plant reproductive success: the consequence is a reduced fruit and/or seed set. Pollen limited systems are well known for outcrossing perennials, but they also occur in self-compatible annuals and are characterized by increased reproductive output following pollen addition (Brunet, 2005). The deposition of insufficient compatible pollen on the stigma may result from a variety of factors limiting pollen quantity or quality. Inadequate pollinators visits or low pollen transfer effectiveness are more often implicated, and this pollinator limited reproduction can select for reproductive assurance through autogamy or mixed mating (Martinell et al., 2011). Pollen limitation can also result from poor pollen quality: in this case,

geitonogamy or heterospecific transfer by biotic or abiotic vectors may be responsible for low pollen viability and reduced compatibility (Kephart, 2005). Both pollen and pollinator limitation may lead to reduction in seed production with consequent effects on the demographic structure of populations, especially in species highly dependent on seed for propagation and survival which can incur high risks for population persistence (Bond, 1994). Plant isolation and decrease in population size may result in a greater likelihood of pollen limitation (Wagenius and Lyon, 2010). Furthermore, different ecological perturbations such as habitat fragmentation, loss of pollinators, resource availability and presence of invasive plants, can act towards a disruption of plant-pollinator interface leading to pollinator limitation (Knight et al., 2005).

Chapter 2

Aims

This research focuses on taxonomy, phylogeny and reproductive ecology of *Gentiana lutea*.

Taxonomic analysis is a critical step in botanical studies, as it is necessary to recognise taxonomical units. It was carried out on one hundred herbarium specimens in order to i) check historic data on the geographical distributions of *G. lutea* subspecies and ii) test the reliability of several subspecies-diagnostic characters.

Several evolutionary processes contribute to determine the extent and the distribution of the genetic variability within a species (e.g. habitat fragmentation, population isolation, mutation, genetic drift, mating system, gene flow, selection etc.). The knowledge of *G. lutea* genetic variability and the comparison with that of the other species belonging to sect. *Gentiana*, could contribute to understand the evolutionary dynamics of this section. At a later time, this knowledge may lead to infer the evolutionary process of species with similar reproductive and ecological features. Phylogenetic study was carried out both with nuclear (ITS and 3' ETS) and chloroplast (*rpl*32-*trn*L and *psb*A-*trn*H) markers in order to i) evaluate congruences between nuclear and chloroplast sequences in inferring phylogeny, ii) assess their utility as phylogenetic data source, iii) elucidate phylogenetic relationships among *G. lutea* subspecies, with special regard to the little known subsp. *vardjanii* and to the controversial position of subsp. *montserratii*, iv) propose a phylogenetic

hypothesis for the evolution of sect. *Gentiana*, and v) infer colonization dynamics of the section during the Quaternary.

Appropriate scientific information is critical for the assessment of species conservation status (Gauthier et al., 2010) and for the effective management and conservation plans. Nevertheless, at present, data on the biology and ecology of plant species are often missing. I carried out field work on five natural populations, belonging to different subspecies, and performed laboratory analyses on specific critical aspects, with special regard to *G. lutea* breeding system and interactions with pollinators. I specifically wanted to: i) compare flower phenology of *G. lutea* subspecies, ii) assess plant breeding system and reproductive success, iii) study sexual resource allocation, iv) compare fitness traits of seed derived from different pollination treatments, v) evaluate the effects of inbreeding depression, vi) describe spectrum of pollinators, vii) quantify their role in *G. lutea* pollination, viii) quantify nectar standing crop and identify nectar constituents, ix) evaluate the presence of pollen limitation.

Chapter 3

Materials and methods

Unless indicated otherwise, all statistical analyses were performed with R Development Core Team software, version 2.14.0 (released on 2011.10.31).

3.1 Taxonomy of *G. lutea*

To test the reliability of subspecies-diagnostic characters and to check historic data on the geographical subspecies distributions, one hundred herbarium specimens of *G. lutea* from the Italian Central Herbarium of Florence and from the University Herbarium of Trieste (Italy) were observed (87 and 13 samples, respectively; Figure 3.1). Totally, 70 herbarium specimens of subsp. *lutea*, 18 of subsp. *symphyandra* and 12 of subsp. *vardjanii* were examined. The following characters were observed: anthers length (3 measures for each specimens); bracts length (classified as longer than pseudo-whorls and shorter/as long as pseudo-whorls); stigma shape after anthesis (classified as spirally coiled and erecto-patent). Due to the small number of specimens, comprehensive of both vegetative and flowering stems, features of vegetative stems were not considered.

3.1.1 Statistical analyses

Normality of the data sets was tested using a Shapiro-Wilk test. Differences among subspecies were checked using one-way ANOVA followed by Tukey's

Figure 3.1: *G. lutea* subsp. *vardjanii* - *Isotypus* - herbarium specimen from University of Ljubljana, collector: T. Wraber, 28.07.1985.

pairwise comparisons (quantitative data), or using Chi-squared test (qualitative data).

3.2 Phylogeny of sect. *Gentiana*

The phylogenetic study was carried out at the Institut für Spezielle Botanik - Johannes Gutenberg Universität - Mainz (Germany), under the supervision of Prof. Joachim Kadereit and co-workers.

3.2.1 Plant material

In order to elucidate the phylogenetic structure of sect. *Gentiana*, leaf material from every taxon described in literature was collected in the field during summers 2009 and 2010, and dried with silica gel. To clarify its problematic phylogenetic position, a population of *G. asclepiadea* was sampled and inserted in the study. For each sampled population, a specimen was collected and conserved at Bologna (BOLO) Herbarium as voucher, Table 3.1. In order to have a good geographical representation of the more widespread species, I

sampled more than one population per species. Six herbarium samples were added coming from Herbarium of Bologna (1), Mainz (2), Munich (2) and Oslo (1). The analysis involved one individual per population for a total of 28 samples including 4 outgroups (*G. acaulis* - sect. *Ciminalis*; *G. verna* - sect. *Calathianae*; *G. pneumonanthe* - sect. *Pneumonanthe*; *G. cruciata* - sect. *Cruciata*). Sampling details are given in Table 3.1.

3.2.2 DNA extraction

Extraction was carried out from 1-2 segments of leaf material (ca. 5mg), previously pulverised by ball mill (MM301, Retsch GmbH, Germany) and afterwards extracted using the DNeasy Plant Mini Kit™ (Qiagen GmbH, Germany) following the manufacturer's protocol. Quality and quantity of DNA was checked comparing 5µl of DNA extract (mixed with 3µl of loading buffer) with 6µl of Generuler™ DNA Ladder (MBI Fermentas, Germany), on 0.8% agarose gel stained with ethidium bromide.

3.2.3 DNA amplification

To reconstruct phylogenetic relationships among taxa, I employed both nuclear and chloroplast markers.

3.2.3.1 Nuclear markers

The Internal Transcribed Spacer region (including ITS1, 5.8S and ITS2) and the External Transcribed Spacer (3' ETS) were chosen as nuclear markers and analysed for nucleotide sequence variation. Universal primers 18S (Muir and Schlötterer, 1999) and ITS B (Blattner, 1999) were employed to amplify the region:

- 18S: 5'-CCTTMTCATYTAGAGGAAGGAG-3'
- ITS B: 5'-CTTTTCCTCCGCTTATTGATATG-3'

The PCR reaction was prepared with 0.5µl of template (ca. 50ng), 1.25µl 50mM $MgCl_2$, 0.25µl 20mM dNTPs (Peqlab TM, Germany), 0.5µl 50µM of

Species	Sampl.	Location/Collectors	Latitide	Longitude	Alt., Exp.	Voucher	Cod.
G. lutea subsp. *lutea*	21.07.10	Puerto del Portalet, Huesca–Spain/MR-AF	42°47'52"N	0°24'36"W	1770m-S	504367	PDP
G. lutea subsp. *lutea*	14.07.10	Mt. Vettore, AP–Italy/MR-AF	42°48'18"N	13°15'46"E	1880m-SE	504368	MV
G. lutea subsp. *lutea*	24.07.10	Mt. Terminillo, RI–Italy/MR-LM	42°28'12"N	13°07'20"E	1860m-NE	504369	MT
G. lutea subsp. *symphyandra*	04.08.09	Mt. Grande, BO–Italy/MR-VL	44°08'57"N	10°52'10"E	1400m-E	502430, 502428	MGR
G. lutea subsp. *symphyandra*	21.07.10	Mt. Nanos, Notranjska–Slovenia/MG-GC-SC	45°47'02"N	14°01'44"E	890m-NW	504362	MN
G. lutea subsp. *symphyandra*	07.12.10	Mts. Cozia, Călimăneşti–Romania/	45°21'09"N	24°23'44"E	700m-E	-	MCO
G. lutea subsp. *vardjanii*	30.07.09	Passo Lusia, TN–Italy/MR-GC-VL	46°20'15"N	11°41'55"E	1880m-SE	504364	PL
G. lutea subsp. *vardjanii*	09.07.10	Mt. Guglielmo, BS–Italy/AF	45°45'13"N	10°10'16"E	1800m-NW	504363	MGU
G. lutea subsp. *vardjanii*	27.07.10	Mt. Pegherolo, BG–Italy/MR-AF	46°02'21"N	9°41'58"E	1800m-E	504365	MP
G. lutea subsp. *montserratii*	20.07.10	Peña d'Oroel, Huesca–Spain/MR-AF	42°31'43"N	0°31'51"W	1200m–E	504366	PO
G. burseri subsp. *actinocalyx*	24.07.10	Pèpin Peak, Nice–France/MG-GC-SC	44°08'56"N	7°36'00"E	2250m-N	504352	PP
G. burseri subsp. *villarsii*	24.07.10	Colle di Tenda, CN–Italy/MG-GC-SC	44°08'46"N	7°33'11"E	1940m-N	504353	CT
G. burseri subsp. *burseri*	21.07.10	Puerto del Portalet, Huesca-Spain/MR-AF	42°47'52"N	0°24'36"W	1770m-N	504351	PDP
G. punctata	21.07.09	Passo Rolle, TN–Italy/MR-GC-UM	46°17'60"N	11°48'00"E	2160m-S	502431	PR
G. punctata	17.07.10	Pudria Valley, TN–Italy/GC	46°19'59"N	10°37'42"E	2140m-N	504356	PV
*G. punctata**	30.07.09	Fellhorn, Oberstdorf–Germany	-	-	-	Ma:	FE
G. purpurea	12.08.09	Corno alle Scale, BO–Italy/MR-GC-VL	44°07'24"N	10°49'52"E	1945m-peak	502429	CS
*G. purpurea**	30.07.09	Fellhorn, Oberstdorf–Germany	-	-	-	Ma:	FE
G. purpurea	10.07.10	Mt. Cusna, RE–Italy/MR-LM	44°16'43"N	10°23'16"E	1750m-E	504370	MCU
*G. purpurea**	05.08.02	Hordaland-Voss	-	-	-	Os:	HO
G. pannonica	22.07.10	Soriška Planina, Soriška–Slovenia/MG-GC-SC	46°14'13"N	14°00'22"E	1325m-N	504355	SP
*G. pannonica**	19.08.90	Kitzbühel Alps, Tirol-Austria	-	-	1850m-N	M	KA
*G. pannonica**	05.08.93	Schachten, Bayern-Germany	-	-	1125m-/	M	SC
G. asclepiadea	03.09.09	Dardagna River, BO–Italy/MR-AF-VL	44°08'00"N	10°49'01"E	1525m-SE	502437	DR
G. acaulis	07.07.10	Passo Lusia, TN–Italy/MR-MG-SC	46°20'22"N	11°42'00"E	1980m-E	504357	PL
G. verna	10.07.10	Mt. Cusna, RE–Italy/MR-LM	44°16'05"N	10°24'47"E	1840m-SW	504354	MCU
G. pneumonanthe	06.09.10	Colle il Castellaccio, PI–Italy/MG-AF	44°40'19"N	9°41'38"E	1250m-/	505072	CC
*G. cruciata**	10.08.10	Mt. Nanos, Notranjska–Slovenia	-	-	-	-	MN

Table 3.1: Molecular analysis, sampling details: species (asterisks represent herbarium samples), sampling date (sampl.), location and collectors (MR: M Rossi; AF: A Fisogni; MG: M Galloni; GC: G Cristofolini; SC: S Crema; UM: U Mossetti; LM: L Moretti; VL: V Lucchetta), latitude, longitude, altitude and exposure (Alt.-Exp.), sample code (Cod.) used in following phylogenetic trees. Vauchers are deposited in BOLO unless indicated otherwise and represent sampled population (abbreviations: M=Munich, Os=Oslo, Ma=Mainz).

each primer, 0.2µl 5U/µTaq-Polymerase (NEB GmbH, Germany), 2.5µl Polymerase Buffer 10x (supplied with Taq) and sterile water, for a final volume of 25µl. The cycler profile was: 1' at 94°C; 35 cycles of 20" at 94°C, 30" at 57°C, 1' at 72°C; followed by a final step of 20" 94°C, 1' 20" at 57°C and 8' at 72°C (Biometra T gradient thermocycler, also used for the following analyses). Because of the difficulty in reading sequences, ITS fragments of *G. punctata* were cloned using the pGEM-T Easy Vector (Promega UK), following the specified protocol. After cloning, DNA was purified with Plasmid Miniprep Kit II (PeqGOLD Biotechnologie GmbH - PEQLAB), and three clones were randomly chosen for further analyses.
The entire IGS sequence (InterGenic Spacer) was obtained just for 4 samples (*G. burseri* subsp. *villarsii*, *G. lutea* subsp. *lutea* – Puerto del Portalet, *G. burseri* subsp. *burseri* and *G. punctata* – Passo Rolle), using 18S-IGS and 26S-IGS primers by Baldwin and Markos (1998):

- 18S-IGS: 5'-GAGACAAGCATATGACTACTGGCAGGATCAACCAG-3'
- 26S-IGS: 5'-GGATTGTTCACCCACCAATAGGGAACGTGAGCTG-3'

PCR reaction was prepared with 2µl of template (ca. 200ng), 1.25µl 50mM $MgCl_2$, 0.25µl DMSO 1%, 0.25µl 20mM dNTPs (Peqlab TM, Germany), 0.5µl 50µM of each primer, 0.25µl U Taq-Polymerase (NEB GmbH, Germany), 2.5µl Polymerase Buffer 10x (supplied with Taq) and sterile water, for a final volume of 25µl. The cycler was programmed as described by Kadereit et al. (2007), with a 68°C annealing temperature. PCR products were sequenced and aligned (see next paragraphs), together with ETS sequence of *Gentianella chathamica* (Genbank accession GQ281766.1). In order to amplify ETS sequence of all samples, three new internal primers were designed:

- ETS-*Gentiana*1: 5'-TTTYGTGGCTTTCGTGCCCAGC-3'
- ETS-*Gentiana*2: 5'-CGGATGCATTGCGAACGTGATGG-3'
- ETS-*Gentiana*3: 5'-TTGGCCGGTGTCGGTCGGACGA-3'

The amplified region is gradually shorter using ETS-*Gentiana*1, 2 and 3; ETS-*Gentiana*3 is the only primer complementary with *G. chathamica* sequence. Since all new primers give good PCR products, in order to study the longest fragment, successive amplifications were performed using the primer combination ETS-*Gentiana*1 and 18S-IGS, under the same PCR conditions and the same cycler profile of ITS (annealing temperature of 66°C).

3.2.3.2 Chloroplast markers

Eight samples from different taxa were tested for 7 chloroplast markers: *trn*L-F, Taberlet et al. (1991); *atp*B-*rbc*L spacer, Xu et al. (2000); *psb*D-*trn*T, *rpl*32-*trn*L, *ndh*F-*rpl*32, *psb*J-*pet*A, Shaw et al. (2007); *psb*A-*trn*H, Hamilton (1999) and Demesure et al. (1995). According to their molecular variability, two markers were selected:

*rpl*32-*trn*L:

- *trn*L (UAG): 5'-CTGCTTCCTAAGAGCAGCGT-3'
- *rpl*32-F: 5'-CAGTTCCAAAAAAACGTACTTC-3'

*psb*A-*trn*H:

- *trn*H (GUG): 5'-ACGGGAATTGAACCCGCGCA-3' (Demesure et al., 1995)
- *psb*A: 5'-CGAAGCTCCATCTACAAATGG-3' (Hamilton, 1999)

PCR reaction was prepared with 1µl of template (ca. 100ng), 1.5µl 50mM $MgCl_2$, 0.3µl 20mM dNTPs (Peqlab TM, Germany), 0.5µl 50µM of each primer, 0.25µl Phusion High-Fidelity DNA polymerase (New England Bio-Labs, Ipswich, MA), 5µl Polymerase Buffer (supplied with Taq) and sterile water for a final volume of 25µl. Cycler program was 30" at 98°C; 30 cycles of 10" at 98°C, 30" at 62°C (*rpl*32-*trn*L) or 61°C (*psb*A-*trn*H), 30" at 72°C; followed by a final step of 10' 72°C.

3.2.4 Sequencing reaction

Amplified products were length checked on a 0.8% agarose gel stained with ethidium bromide, under ultraviolet light. PCR products were cleaned using spin filter columns (UltraClean PCR Clean-Up Kit, MO BIO Laboratories Inc., CA, USA). Forward and reverse strands were sequenced with the same PCR primers, except for clones, where P7 and SP6 primers by pGEM-T Easy Vector (Promega UK) were employed. Protocol of BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, Hayward, California, USA) was followed to prepare the sequencing reaction (cycler profile: 30 cycles at 96°C for 10" and 55°C for 4'). The products were purified using Sephadex G-50 (GE Healthcare Europe GmbH, Germany) on multi-screen-HV (96-well filtration plate; Millipore Corporation, USA) following the standard protocols. Samples were finally run on an ABI 3730 capillary sequencer at GENterprise GmbH (Germany).

3.2.5 Statistical analyses

A double strand *consensus sequence* was automatically edited with Sequencher 4.1 (GeneCodes Corp., Ann Arbor, Michigan, U.S.A.) and manually adjusted where needed. Nuclear electropherograms were closely examined for double peaks and coded following IUPAC nucleotide code. All sequences were aligned by hand using MacClade 4.1 (Maddison and Maddison, 2000), excluding that portions of matrix where a certain alignment was not achieved. In particular, according to Štorchovà and Olson (2007) *psb*A-*trn*H alignment was carefully examined. Indels and inversions were scored separately and added to the data-matrix, except for indels involved in *psb*A-*trn*H secondary structure and for microsatellite indels, considered under selective constraint (Štorchovà and Olson, 2007), and supposedly affected by size homoplasy (Hale et al., 2004), respectively. Nuclear data were analysed with and without indels.
Molecular data were analysed using parsimony heuristic searches (MP) and maximum Likelihood (ML). Maximum parsimony was implemented in PAUP* 4.10b (Swofford, 2002), heuristic search was set with 1,000 random taxon addition and tree bisection-reconnection (TBR) branch swapping and gaps

treated as missing data; MulTrees was turned on and multistate taxa were considered as polymorphisms. Parsimony bootstrap searches were conducted with 1,000 bootstrap replicates, random taxon addition replicates, MulTrees on, and rearrangements limited to 1,000,000 per replicate for both nrDNA and cpDNA analyses. Maximum likelihood heuristic searches were performed using RAxML version 7.2.8: HPC2 on teragrid (Stamatakis, 2006; Stamatakis et al., 2008) in the Cipres Portal (Miller et al., 2009). The searches were run with 1,000 rapid bootstrap replicates and gaps treated as missing data. The GTRCAT model with 25 rate categories was used for the bootstrap search and the GTRGAMMA model was used for the final tree as recommended by Stamatakis et al. (2008). Bootstrap values were obtained by constructing majority-rule consensus trees in PAUP*.
Phylogenetic trees were rooted with *G. acaulis*, *G. verna* and *G. cruciata*, while all species belonging to sect. *Gentiana*, together with *G. asclepiadea* and *G. pneumonanthe* were considered as ingroup.
Chloroplast data were analysed using network analysis, using TCS version 1.21 with gaps treated as missing data (Clement et al., 2000) using the statistical parsimony algorithm (Templeton et al., 1992). Indels (except polyT stretches) were coded as single additional binary characters (Simmons and Ochoterena, 2000).

3.3 Reproductive ecology

3.3.1 Study sites

Studies were carried out from 2009 to 2011 in five natural populations of *G. lutea*, belonging to different subspecies. Populations' extent of occurrence has been measured by the sum of minimum convex polygons (IUCN, 2001).

G. lutea* subsp. *lutea

The target population of *G. lutea* subsp. *lutea* is located in the Central Apennines, on the South-East side of Mount Vettore (Ascoli Piceno - Italy) between 1850 and 2300 metres above sea level (Figure 3.2), within the Na-

tional park "Parco Nazionale dei Monti Sibillini" (inside the IT5340014 SIC - Monte Vettore e Valle del Lago di Pilato). Here *G. lutea* grows in a grassy alpine/sub-alpine pasture. Individuals are patchily distributed in three main sub-populations on a total surface of about 3 hectares. Investigations were carried out on the sub-population at the lower altitude (latitude 42°48'18" N; longitude 13°15'46" E), approximately 430 and 750 metres far from the others. Estimate population size is about 5-8 hundred flowering individuals.

G. lutea* subsp. *symphyandra

G. lutea subsp. *symphyandra* population occurs in the East side of Mount Grande (Bologna - Italy) within the IT4050002 - SIC-ZPS – Corno alle Scale (latitude 44°8'57" N, longitude 10°52'10" E), between 1380 and 1460 metres above sea level (Figure 3.3). The population is sited in a clearing within a *Fagus sylvatica* forest, where it covers an area of about 4,500 m^2, probably preserved by the steepness of the mountain. The number of reproductive individuals varies between years: 2009 n=80; 2010 n=330; 2011 n=113 and the proportion of flowering stems on vegetative ones, evaluated in one patch (5x5 m), shows that on average 10 percent of individuals is reproductive (14% - 2009; 11% - 2010; 6% - 2011). The peculiarity of this population is to be outside the known distribution range of subsp. *symphyandra*.

As control, another population, placed in Mount Nanos (Notranjska – Slovenia; latitude 45°47'2" N, longitude 14°1'44" E; North-West exposure) at 750-1050 metres of altitude, was chosen for minor surveys (Figure 3.4). Few tens of thousands individuals make up the whole population, covering an area of approximately 300 hectares. The habitat consists of mountain grasslands.

G. lutea* subsp. *vardjanii

The studied population of *G. lutea* subsp. *vardjanii* is located at Passo Lusia (Trento - Italy) (latitude 46°20'15" N, longitude 11°41'55" E; South-East exposure; Figure 3.5). It vegetates between 1920 and 2040 metres of altitude, in an alpine pasture and is more or less densely distributed over an area of approximately 10 hectares. The number of flowering stems was 782 in 2009, 430 in 2010 and 1377 in 2011, with a percentage of reproductive individuals

(assessed in 5x5 m patch) of 16, 2 and 23%, respectively (13.5% mean).

G. lutea* subsp. *montserratii

Minor surveys were carried out in the population of *G. lutea* subsp. *montserratii*, which occurs at the *locus classicus* (Vivant, 1975) at San Juan de la Peña d'Oroel (Huesca – Spain; latitude 42°31'43" N, longitude 0°31'51" W; 1200 m a.s.l.; North-East exposure; Figure 3.6). Few hundreds individuals grow on the hill inside *Pinus sylvestris* sparse woodland.

Figure 3.2: Study population of *G. lutea* subsp. *lutea*, Mt. Vettore (Ascoli Piceno - Italy).

Figure 3.3: Study population of *G. lutea* subsp. *symphyandra*, Mt. Grande (Bologna - Italy).

Figure 3.4: Study population of *G. lutea* subsp. *symphyandra*, Mt. Nanos (Notranjska – Slovenia).

Figure 3.5: Study population of *G. lutea* subsp. *vardjanii*, Passo Lusia (Trento - Italy).

Figure 3.6: Study population of *G. lutea* subsp. *montserratii*, San Juan de la Peña d'Oroel (Huesca – Spain).

3.3.2 Flower phenology

Anthesis was studied in subsp. *lutea*, subsp. *symphyandra* and subsp. *vardjanii* during July 2011. For each subspecies 4/5 developmental phases were recognised and their development was monitored three times a day, for two days (10AM, 14PM and 18PM), during flower lifespan (Figure 3.7, Table 3.2). Morphological aspect and anther and stigma maturity were recorded as well as temperature and relative humidity.

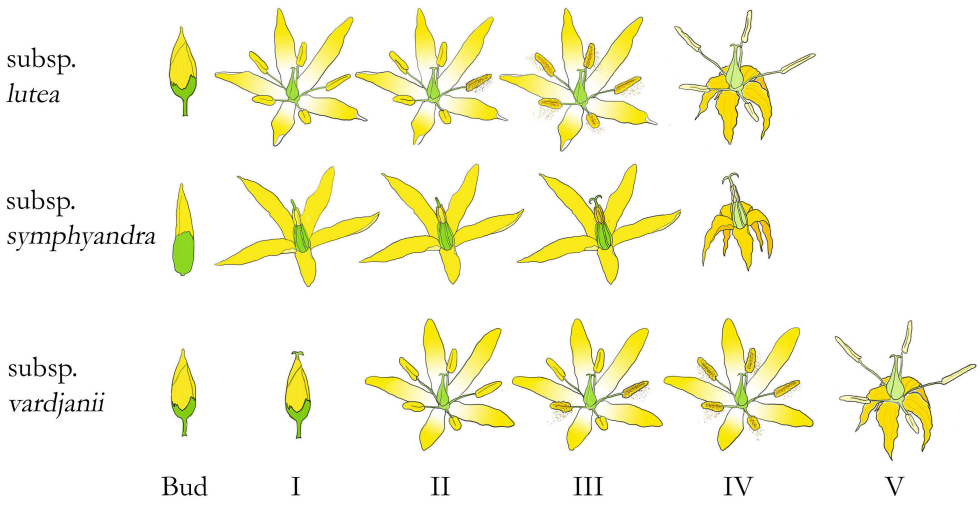

Figure 3.7: Drawings of flower developmental phases (by M. Albertini). Phase description is given Table 3.2.

Ages	Description	fl (st)
	subsp. *lutea*	
	Bud	-
I	Open flower, stigma hardly bilamellate	17 (8)
II	Open flower, stigma bilamellate, 1-4 dehisced anthers	26 (8)
III	Open flower, stigma bilamellate, complete anthers dehiscence	26 (8)
IV	Perianth withered	12 (4)
	subsp. *symphyandra*	
	Bud	
I	Open flower	10 (4)
II	Open flower, stigma undivided or hardly bilamellate, 1-4 dehisced anthers	20 (5)
III	Open flower, stigma bilamellate, complete anthers dehiscence	23 (5)
IV	Perianth withered	17 (4)
	subsp. *vardjanii*	
	Bud	-
I	Bud, stigma bilamellate poked out through the top of the corolla	21 (9)
II	Open flower, stigma bilamellate	28 (9)
III	Open flower, stigma bilamellate, 1-4 dehisced anther	33 (10)
IV	Open flower, stigma bilamellate, complete anther dehiscence	24 (7)
V	Perianth withered	11 (3)

Table 3.2: Description of flower developmental phases. For each phase sample sizes used to estimate flower lifespan is given (fl=number of flowers, st=n. of stems).

Stigma receptivity was assessed, for 9-13 flowers of each developmental phase, by peroxidases test (Macherey-Nagel Peroxtesmo KO peroxidases test paper), following the method described by Dafni and Motte-Mauès, 1998 (Table 3.2, Figure 3.8). Flowers from different stems were used. Two classes of stigma receptivity were identified: stigma hardly bilamellate with receptivity limited to a small apical area of stigmatic surface (class II), and stigma bilamellate, with widespread receptivity to the entire stigmatic surface (class I).With respect to male function, a qualitative pollen viability test (Sigma Fast™ 3.3′-diaminobenzidine, DAB tablets set, Dafni et al., 2005), was performed on dehisced anthers (0-3h, 3-6h, 8-11h, 24-30h and more than 35h old; Figure 3.9). For each age, 2-6 flowers belonging to different stems were examined (for detail see Table 4.4). Viability was assessed on one hundred pollen grains per flower (microscope Nikon Eclipse E600) on two replicates. Three classes of pollen viability were identified: highly viable pollen, from 100%

to 80% (I class); viable pollen, from 79% to 50% (II class, lower threshold corresponds to pollen viability duration by Kumar et al., 1995); scarcely viable pollen, under 49% (III class). Description of flower ages and details on sample size are given in Table 3.2 and Figure 3.7. For each subspecies the maturity of reproductive structures was assessed and the mean flower lifespan (± standard error) was calculated.

Figure 3.8: Stigma receptivity: Peroxtesmo test; blue colour indicates the presence of peroxidases.

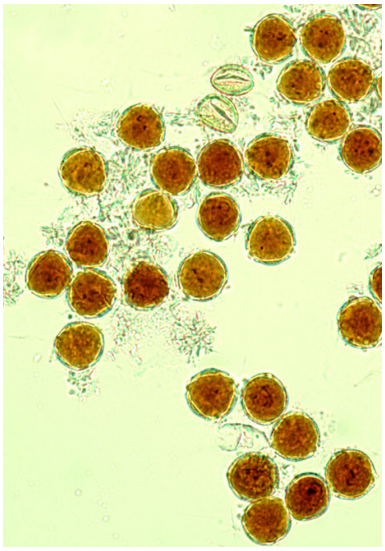

Figure 3.9: Pollen viability: DAB test. A dark brown-purple-red indicates the presence of peroxidases.

3.3.3 Breeding system

To study *G. lutea* breeding system I carried out different pollination treatments (studied flowers were randomly chosen, marked with flower plastic markers and followed during their development). Tests were performed during summer 2010-2011 (subsp. *lutea*) and 2009-2010 (subsp. *symphyandra* and subsp. *vardjanii*). Agamospermy (A) was tested in subsp. *vardjanii* by cutting off stigma surface before anthesis (10 stems, 30 flowers). Non-manipulated flowers, where open-pollination was allowed, were chosen as controls (C). To assess the occurrence of self-pollination and the degree of

self-compatibility, two treatments were performed on the same stems: spontaneous selfing (SS), in which pollinator visits were excluded, and hand-self pollination (HS), in which receptive stigmas were hand-pollinated (twice) with dehiscent anthers excised from different flowers of the same stem. In both treatments stems were previously bagged with nonwoven fabric before flower opening and bags were left until complete fruit development. Details on sample size, for each treatment, are given in Table 3.3. For subsp. *montserratii* and subsp. *symphyandra* (Mt. Nanos population), only reproductive success in open pollinated flowers was monitored; in Mt. Nanos population 30 additional flowers were randomly taken to estimate ovules numbers.
In subsp. *vardjanii* obligate hand-cross pollination was performed on 10 flowers (2 stems): flowers were bagged at the bud stage and emasculated before anthers dehiscence. Pollination was performed on receptive stigmas with anthers excised from 3-5 different plants collected at least 50 metres away, in order to limit genetic affinities.
Fruits were harvested prior to opening (approximately one month after anthesis) and brought to the laboratory. Predation, unfertilized ovules and seeds number were assessed for each fruit, using a dissecting microscope, and viable seeds per capsule were weighted using a high precision electronic balance. According to Petanidou et al. (1995), filled seeds were considered viable while empty and shriveled ones were considered aborted. The sum of viable seeds, aborted seeds and unfertilized ovules was considered to represent the total initial number of ovules in the ovary. Since fruit recovery was 100%, the fruit set, meant as fruit:flower ratio, did not give any information on breeding system. In order to obtain comparable data, two different categories of fruits were considered: aborted fruits (*fa*: fruits without viable seeds) and seeded fruits (*fs*: fruits containing viable seeds).
According to Zapata and Arroyo (1978), two indexes were employed to describe the breeding system.

- Index of Automatic Self-pollination (IAS=percent SS fruit set : percent HS fruit set): fully autogamous species score 1; partially autogamous plants take values less than 1 and greater than 0; self-compatible species, mechanical prevented from intra-floral selfing, score 0.

- Index of Self-Incompatibility (ISI=HS seed set : hand cross-pollination seed set): completely self-compatible species score 1; incompletely compatible species take values less than 1 and greater than 0.2; species showing self-incompatibility score values less than or equal to 0.2. To calculate this index I used seed set of the pollen-augmented flowers instead of the seed set of hand cross-pollination, since no significant differences were found between these two treatments (see paragraph 4.4.1).

	A		**C**		**SS**		**HS**		**S**	
subsp.	fl	st	fl	st	fl	st	fl	st	fl	st
lutea	-	-	19	85	6	28	6	26	20	86
2010/2011	-/-	-/-	10/9	49/39	-/6	-/28	-/6	-/26	10/10	50/36
symph.	-	-	20	60	10	30	9	30	20	58
2009/2010	-/-	-/-	10/10	30/30	-/10	-/30	-/9	-/30	10/10	28/30
vardjanii	10	30	20	80	15	79	12	38	20	68
2009/2010	10/-	30/-	10/10	30/50	5/10	49/30	-/12	-/38	10/10	30/38

Table 3.3: Sample sizes (fl=number of flowers, st=n. of stems) of open pollinated flowers (C) and pollination treatments (A=agamospermy, SS=spontaneus selfing, HS= hand-self pollination, S=pollen augmented flowers - see paragraph 3.6.6), over the two years of study, in subsp. *lutea*, *symphyandra* (*symph.*) and *vardjanii*.

3.3.3.1 Statistical analyses

Fruit set (*fs*:*fs*+*fa*) and seed set (seeds:ovules ratio for capsule, hereafter indicated as s:o) were considered in order to assess whether there were differences among non-manipulated flowers, self pollination and hand-self pollination treatments, and between pollen augmented flowers (see paragraph 3.6.6) and hand-cross pollinated flowers.

Fitness parameters as predation, fruit set, ovules number and seed set were compared to detect differences among populations. Differences in fruit sets were tested using Chi-squared test. Normality of the data sets was evaluated using a Shapiro-Wilk test. Differences between mean values were tested either by Student's t test (data normally distributed or data normality achieved with $\sqrt{}$arcsinx transformation), or by non-parametric Kruskal-Wallis test and

post-hoc pairwise comparisons performed by Mann-Whitney U-tests (data not normally distributed data sets).
Similarly, correlation between seed number and mean seed weight was calculated using Pearson's or Spearman's correlation, for data normally and not-normally distributed, respectively.

3.3.4 Resource allocation to sexual function

Pollen:ovule ratio (i.e. the estimated number of pollen grains per flower divided by the number of ovules per flower) was calculated on a total of 15 flower buds (5 per subspecies). Buds were brought to the laboratory; ovules number per ovary were counted using a stereo microscope and a manual counter; anthers were stored in microcentrifuge tube with 400 µl of preserving solution ($\frac{1}{2}$ glycerin and $\frac{1}{2}$ ethanol 70%). I followed the protocol suggested by Dafni et al. (2005) and modified by Galloni et al. (2007) to estimate the number of pollen grains. Pollen was collected from anthers using an ultrasonic water bath for 30 minutes and pollen-free anthers were removed. Due to the high number of pollen grains, 10 µl of solution were diluted in 200 µl of preserving solution to obtain an intermediate dilution. Two aliquots of 2 µl per flower were placed on microscope slides, melted with 10 µl of Calberla solution, mounted with a cover glass and sealed with nail varnish. Pollen grains were counted with an optical microscope (Nikon Eclipse E600) and a manual counter. The total number of pollen grains is given by the result obtained multiplied by the dilution factors. Since two aliquots per flower were counted, mean value was considered.

3.3.4.1 Statistical analyses

To check differences in P/O values among subspecies, non-parametric Kruskal-Wallis test and Mann-Whitney U-tests post-hoc pairwise comparisons were performed (data sets not normally distributed - Shapiro-Wilk test).

3.4 Seed germination

Seed germination were performed in 2010 and 2011 in order to compare fitness traits of seeds resulting from autogamy to those of seeds from controls and pollen-augmented flowers (see paragraph 3.6.6). In subsp. *montserratii* and subsp. *symphyandra* (Mt. Nanos population) seed germination was checked on open pollinated flowers, Table 3.4. Twenty-five percent of seeds, from each capsule, were randomly taken, with an upper and lower threshold of 10 and 3 seeds, respectively. Since *G. lutea* forms a short-term persistent seed bank (Hesse et al., 2007), germination tests were carried out within one year from the seeds collection date. Totally, the performance of 503 seeds of subsp. *lutea* (174 C, 247 S, 82 SS and HS), 603 of subsp. *symphyandra* (217 C, 264 S, 122 SS and HS), 551 of subsp. *vardjanii* (174 C, 226 S, 151 SS and HS), 277 of subsp. *montserratii* and 249 of subsp. *symphyandra* (Mt. Nanos population), was tested. Details on collection date, tests date and sample sizes, are given in Table 3.4.

Prior to germination experiments, seeds were weighted, disinfected by immersion in a 1% sodium hypochlorite solution for 3 minutes, followed by 10 minutes of thorough wash with flowing distillate water and finally placed in Petri dishes (11 cm diameter) containing a disk of filter paper (Whatman Filter Paper n. 1). Following the protocol suggested by Kèry et al. (2000), in order to break dormancy, 5 ml of gibberellic acid solution (1mg GA_3 ml^{-1} of sterile water; Gibberellic Acid tech. - Lancaster Synthesis), were added. Dishes, containing up to 60 seeds, were randomly placed inside a climatic chamber (Multitemp CA 7000 - Andreaus, Frigomeccanica s.r.l.) and kept in the dark, at 17-20°C. Germination status was monitored every 2-3 days, adding 2-3 ml of sterile water when required. Seeds were considered germinated when the emergent radical reached 2 mm length. Seeds showing fungal attack were removed, independently of their germination status. Dishes were randomly repositioned in the chamber, in order to avoid possible position effects. Germination was assessed after 31 days (subsp. *lutea* and subsp. *symphyandra*), 16 days (subsp. *vardjanii*) and 36 days (subsp. *montserratii*). For each test mean germination rate and mean germination time (i.e. the time

to germinate 50% of the seed) was calculated.

Subsp.	Collect.	Test	C		S		SS + HS	
			st (fr)	seeds	st (fr)	seeds	st (fr)	seeds
lutea	Aug, 11	Oct, 11	9 (22)	174	9 (26)	247	6 (38)	82
symphyandra	Jul, 10	May, 11	10 (27)	217	10 (30)	264	9 (39)	122
vardjanii	Jul, 09	Apr, 10	8 (16)	174	10 (20)	226	6 (42)	151
montserratii	Jul, 10	May, 11	10 (29)	277	- (-)	-	- (-)	-
sympyandra, Nanos	Aug, 11	Oct, 11	10 (29)	249	- (-)	-	- (-)	-

Table 3.4: Seed germination: sample sizes (st=number of stems, fr=n. of fruits, seeds) for each treatment (C=open pollinated flowers, S=pollen augmented flowers, SS=spontaneus selfing, HS= hand-self pollination); periods of seed collection (collect.) and test performance (test).

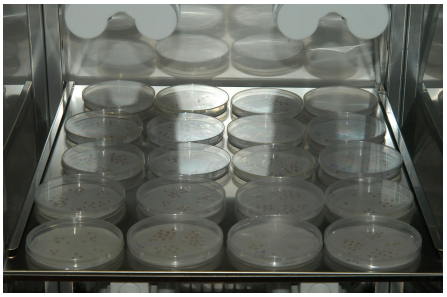

Figure 3.10: Germination test: climatic chamber.

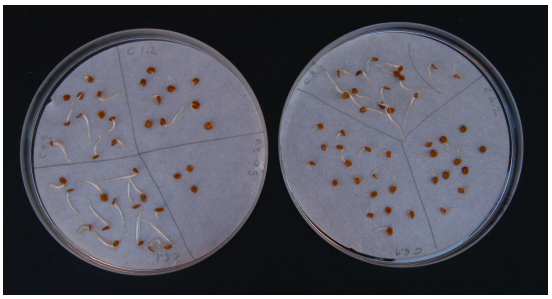

Figure 3.11: Germination test: germinated seeds.

3.4.1 Statistical analyses

To evaluate differences in germination rate among seeds resulting from autogamy, open pollinated and pollen-augmented flowers, non-parametric Kruskal-Wallis test and post-hoc pairwise comparisons with Mann-Whitney U-tests were performed (data sets not normally distributed, Shapiro-Wilk test). The same analysis was carried out to estimate differences among seeds both from non-manipulated flowers and from autogamy of all the studied populations as well as differences in germination time among subspecies.

3.5 Inbreeding depression

Inbreeding depression was calculated for each subspecies, according to Ågren and Schemske (1993), as $\delta = 1 - (Ws/Wo)$ when $Ws < Wo$ and $\delta = (Wo/Ws) - 1$ when $Wo < Ws$, where Ws and Wo are the mean fitness of selfed and outcrossed offspring, respectively (for the reason discussed in paragraph 4.4.1, data set of pollen augmented flowers was used instead of that of hand-cross pollination data). Because higher values of germination time represent reduced fitness performance, for this trait I followed the formula suggested by Ramsey and Vaughton (1996): $\delta = 1 - (Wo/Ws)$ when $Ws < Wo$ and $\delta = (Ws/Wo) - 1$ when $Wo < Ws$. Cumulative inbreeding depression was calculated by multiplying all fitness values for each cross-type progeny and then applying the formula above (Goodwillie and Knight, 2006). Positive δ values indicate inbreeding depression, whereas negative values mean outbreeding depression. The δ values for maternal reproductive success (fruit set and seed set) and progeny fitness traits (seed weight, germination rate and germination time) were calculated.

3.5.1 Statistical analyses

Normality of the data sets was tested using a Shapiro-Wilk test: appropriate transformations (arcsin transformation for proportions and log transformation for proportional variables) were not useful to achieve normality. Differences between selfed and outcrossed offspring for each fitness trait were verified by Chi-squared test (qualitative data) and by Student's t test or non-parametric Mann-Whitney U-tests (quantitative data normally and not normally distributed, respectively).

3.6 Plant – pollinator interactions

3.6.1 Flower pollinators

To assess the spectrum of pollinators I followed the protocol used for the European ALARM Project by Westphal et al. (2008), modified by Fisogni

et al. (2011).
Surveys were performed during two flowering seasons (2010 and 2011) under suitable weather conditions for pollinators (minimum of 15°C, low wind, no rain). Four intervals of observations (15 minutes each one) were performed twice a day (AM and PM), each followed by 15 minutes breaks. I considered four observation intervals as a single observation unit (2h observation overall). For each insect visit, the collected floral reward (nectar or pollen), the insect behaviour towards both stigma (touch, not touch), and stem (dynamic or sedentary activity) were reported. Concerning sedentary insects the number of individuals at the beginning and at the end of observation were considered and mean value was used for further analyses. After every observation unit, 30 minutes were spent in collecting insects visiting the flowers of *G. lutea* throughout the population. Specimens were then determined to the family, genus or species level, and conserved at the BES Department, University of Bologna.
The relative abundance of pollinators was then calculated. The total time of insects' observations was 13 hours and 15 minutes (5h for subsp. *lutea*, 3h 30m for subsp. *symphyandra*, 4h 45m for subsp. *vardjanii*). Details are reported in Table 3.5.
Due to the inflorescence morphology of *G. lutea*, all visiting insects were considered as pollinators and classified as active pollinators (touching the stigmas) or as occasional pollinators, (they did not touch the stigmas, but it is impossible to state it could not happen).

Population	**Days**	**Observations**	**Patches**	**Stems**	**Flowers**
Mt. Vettore (2010/2011)	1/2	2h/3h	2/1	3/5	100/300
Mt. Grande (2010/2011)	1/2	30m/3h	1/1	2/3	150/120
Passo Lusia (2010/2011)	2/2	2h 30m/2h 15m	2/1	3/8	170/700

Table 3.5: Days, time of insects' observations (hours, minutes), number of patches observed, together with number of stems and open flowers, over the two years of study .

3.6.2 Pollinator fidelity

To assess the fidelity of potential pollinators, the pollen loads from sampled insects were analysed. Pollen grains were removed from insect body under a stereo microscope, placed on a microscope-slide, melted with 10 µl of Calberla solution, mounted with a cover glass and sealed with nail varnish. One hundred grains per slide (or all grains, if less than 100) were observed under optical microscope (Nikon Eclipse E600). *G. lutea* pollen, sampled from each study population, was used as reference. The Pollen Dispersal Units (PDU), as dyads, up to pollinaria, were considered as a single unit, since They result from one pollinator visit. The fidelity of each pollinator taxon was evaluated as the mean percentage of *G. lutea* pollen, on total pollen load. Pollen baskets were excluded from analysis, as this pollen is unlikely to be available for pollination.

3.6.3 Index of Pollinator Importance

In order to evaluate quality and quantity components of pollinator's performance and the role pollinators played in *G. lutea* reproduction, the Index of Pollinator Importance (PI) by Galloni et al. (2008) was computed:

$$PI = fv{*}F$$

where, for a given taxon:

- fv is the frequency of visits based on observations;
- F is the mean pollinator fidelity as described by Gibson et al. (2006). According with the authors, only insects carrying at least five grains of a given pollen species were considered to be carriers of that species. Fidelity value equal to zero was assigned to sampled insects that did not result carriers of *G. lutea* pollen. Taxa with less than two specimens were not considered.

Positive PI values were assigned to dynamic pollinators whereas negative values to sedentary ones (that would so increase out-crossing and geitonogamy,

respectively). The index was calculated per groups of related species, showing similar behaviour (i.e. genus, family).

3.6.4 Pollenkitt

According to Pacini and Hesse (2005), by virtue of its lipid composition, pollenkitt stains with all Sudan dyes. Fresh pollen grains were melted with a saturated solution of Sudan IV (Scarlet R) in 70% ethanol, filtered with Whatman Filter Paper n. 1, and observed immediately under an optical microscope (Nikon Eclipse E600).

3.6.5 Nectar analyses

3.6.5.1 Nectar standing crop

Nectar standing crop (i.e. the amount of nectar in a flower exposed to pollinators at a given moment; Galetto and Bernardello, 2005) was performed in 2011 in population of subsp. *vardjanii*, following the protocol by Fisogni et al. (2011). Nectar volumes were estimated using Drummond Microcaps (0.5, 1 µl, Drummond Scientific Co., U.S.A.); nectar concentration, expressed as % on a w/w basis of an equivalent sucrose solution, was measured by hand held refractometers EBS45-03 and EBS45-05 (Bellingham & Stanley Eclipse, Bellingham + Stanley LTD., U.K.) and the International Temperature Correction for °Brix scale was applied. Flowers were sampled only once, without being removed from the plants. Nectar standing crop was evaluated three times a day (12:00AM, 15:00PM, 18:00PM), on 20-22 flowers from 5-9 different stems each interval, over 2 days.

3.6.5.1.1 Statistical analysis Differences in nectar volume and nectar concentration among intervals were tested with Kruskal-Wallis test Mann-Whitney post-hoc pairwise comparisons or with one-way ANOVA followed by Tukey's pairwise comparisons (data sets not normally/normally distributed, respectively - Shapiro-Wilk test).

3.6.5.2 Nectar chromatography

In order to evaluate the sugar composition and to detect the presence of alcohols and amino-acids, three nectar samples were collected, each from a study population. Sampling was carried out on flowers from 6-8 different stems, previously bagged with nonwoven fabric. Nectar uptake was performed using micropipette ranging from 10-100µl. Totally, more than 0.5ml of nectar per population was collected. Samples were stored at -20°C and HPLC analysis was performed by Massimo Nepi's research group (Department of Environmental Sciences "G. Sarfatti", University of Siena, Via P. A. Mattioli 4, 53100 Siena, Italy). In particular isocratic HPLC was performed to detect sucrose, glucose, fructose as well as ethanol and methanol amount, while gradient HPLC was carried out to research presence of protein amino acids and some non protein ones (β-alanine, citrulline, L-homoserine, α-aminobutyric acid, γ-aminobutyric acid, hydroxyproline, ornithine and taurine).

3.6.6 Pollen limitation

In order to assess the presence and degree of pollen limitation, hand-cross pollination treatment was performed on different stems, over two years (2010 and 2011 in Mt. Vettore population; 2009 and 2010 in Mt. Grande and Passo Lusia populations). Receptive stigmas were cross pollinated with dehiscent anthers excised from 3-5 different stems. To limit genetic affinities among parents, anthers were collected from stems at least 50 metres away from treated ones. Open pollinated plants were taken as controls. In total 86 flowers (36 stems) of Mt. Vettore population, 58 flowers (20 stems) of Mt. Grande population and 68 flowers (20 stems) of Passo Lusia population were pollen-augmented. Details on sample size over the years are showed in Table 3.3. Fruits were collected before opening and brought to the laboratory. Predation, unfertilized ovules and seeds number were assessed for each fruit; fruit set and mean seed set were calculated (details in paragraph 3.3.3).

3.6.6.1 Statistical analyses

Chi-squared test (qualitative data) and Student's t test or Mann-Whitney tests (quantitative data normally or not normally distributed, respectively), were performed to test for differences in fruit set and seed set, between controls and pollen augmented flowers. Normality of the data sets was tested using a Shapiro-Wilk test, in some cases data normality was achieved with $\sqrt{\text{arcsinx}}$ transformation.

Chapter 4

Results

4.1 Taxonomic analysis

Depending on condition and on the anthesis stage of the specimens, all characters considered, or a part of them, were examined.
Stigma shape after anthesis was erecto-patent in subsp. *symphyandra* (n=5) and spirally coiled in subsp. *vardjanii* (n=11). Subsp. *lutea* showed both spirally coiled and erecto-patent stigmas (n=14 and 18, respectively). Significant differences in stigma shape were revealed among subspecies (X^2=16.44, df=2, $p < 0.001$) and breaking down the degrees of freedom, differences were found between subsp. *lutea* and subsp. *vardjanii*, and between subsp. *symphyandra* and subsp. *vardjanii* (X^2=13.13, df=1, $p < 0.001$), while subsp. *lutea* and *symphyandra* did not show significant differences.
Mean anthers length was 8.23±0.96mm in subsp. *lutea*, 9.31±0.84mm in subsp. *symphyandra* and 7.02±0.89mm in subsp. *vardjanii*, measured on 61, 16 and 12 samples, respectively. One-way ANOVA (F=20.72, df=2 and 86, $p < 0.001$) and Tukey's pairwise comparison revealed significant differences in this trait (subsp. *lutea* vs. *symphyandra* Q=5.00, $p < 0.01$; subsp. *lutea* vs. *vardjanii* and subsp. *symphyandra* vs. *vardjanii* Q=5.54 and 10.54, $p < 0.001$).
The bracts length was observed in 55 specimens of subsp. *lutea*, 13 of subsp. *symphyandra* and 11 of subsp. *vardjanii*. The number of specimens showing

respectively bracts longer than pseudo-whorls – shorter/as long as pseudo-whorls, was 18 – 37 in subsp. *lutea*, 3 – 10 in subsp. *symphyandra* and 11 – 0 in subsp. *vardjanii*. Statistical differences were found among subspecies (X^2=18.97, df=2, $p < 0.001$): breaking down the degrees of freedom, no significant differences were highlighted between subsp. *lutea* and subsp. *symphyandra*, while both subsp. *lutea* and subsp. *symphyandra* differed significantly from subsp. *vardjanii* (X^2=18.77, df=1, $p < 0.001$).
The known geographical distributions of each subspecies was confirmed by historic data of specimens.

4.2 Phylogeny of sect. *Gentiana*

4.2.1 Nuclear markers

The alignment of ITS1, 5.8S, ITS2 and 3' ETS of all taxa has 1199 positions (661 and 538 bases, respectively), of which 11.0% are parsimony informative characters (2.8% concerning just sect. *Gentiana*). Details on constant characters, parsimony-uninformative variable characters, parsimony-informative characters, indels and percentage of parsimony informative characters, for each marker, are given in Table 4.1.
The maximum parsimony strict consensus tree was obtained from 652 most parsimonious trees. Since no significant difference in tree topology was found between ML and MP (analyses performed without indels), maximum likelihood tree, is shown (Figure 4.1).
Nuclear markers clearly distinguish sect. *Gentiana* (bootstrap support values MP 100/ML 93, hereafter indicates as 100/93). The section is composed of a polytomy of three clades, the first of which contains all *G. lutea* populations, the second one groups *G. pannonica* populations, and the third one includes *G. purpurea*, *G. burseri* and *G. punctata*. *G. lutea* species identity is well supported (bootstrap values: 83/94), and within it, relationships among subspecies are not resolved except for subsp. *vardjanii*, which populations group together (bootstrap values: 89/92). *G. pannonica* is also well supported (bootstrap values: 100/100) while within *G. burseri*, *G. purpurea*

and *G. punctata* clade, only *G. punctata* is monophyletic (bootstrap values: 51/80). In particular within *G. burseri*, subsp. *burseri* (endemic of the Pyrenees) does not group with subsp. *villarsii* and *actinocalyx*, and similarly within *G. purpurea*, population coming from Scandinavian peninsula does not cluster with the others from Central-Southern Europe. Relationships among species are poorly resolved, the only information concerns *G. purpurea* and *G. punctata* (sister species; bootstrap value 51/81), which form a poorly supported clade.

The analysis of nuclear markers confirms the problematic phylogenetic position of *G. asclepiadea*: it clusters in polytomy with sect. *Gentiana* far from *G. pneumonanthe*.

The topologies of the maximum likelihood tree performed with and without indels are consistent. The tree obtained including indels data (data not shown) resolves *G. asclepiadea* position, which clusters with sect. *Gentiana* species (bootstrap value: 100) and *G. pannonica* relationships (it seems to be basal of *G. burseri*, *G. purpurea* and *G. punctata* group - bootstrap value 67); in addition *G. punctata* species identity is better supported (bootstrap value 90).

Group	***c***	***pu***	***pi***	***in***	***%***	***c***	***pu***	***pi***	***in***	***%***	***c***	***pu***	***pi***	***in***	***%***
	ITS1+5.8S+ITS2					**3' ETS**					**combination**				
all taxa	578	36	47	14	7.1	390	63	85	13	15.8	968	99	132	27	11.0
Gentiana	635	9	17	3	2.6	516	6	16	3	3.0	1151	15	33	6	2.8
	***rpl*32-*trn*L**					***psb*A-*trn*H**					**combination**				
all taxa	907	72	38	24	3.7	326	37	25	12	6.4	1233	109	63	36	4.5
Gentiana	992	21	4	3	0.4	370	13	5	1	1.3	1362	34	9	4	0.6

Table 4.1: Characteristics of DNA sequences in nuclear (ITS1, 5.8S, ITS2 and 3' ETS) and chloroplast markers (*rpl*32-*trn*L, *psb*A-*trn*H) in all analysed taxa (outgroups included) and in sect. *Gentiana*. Abbreviations: *c*: constant characters, *pu*: parsimony-uninformative variable characters, *pi*: parsimony-informative characters, *in*: indels, *%*: percentage of parsimony informative characters, based on bases mutation (indels excluded).

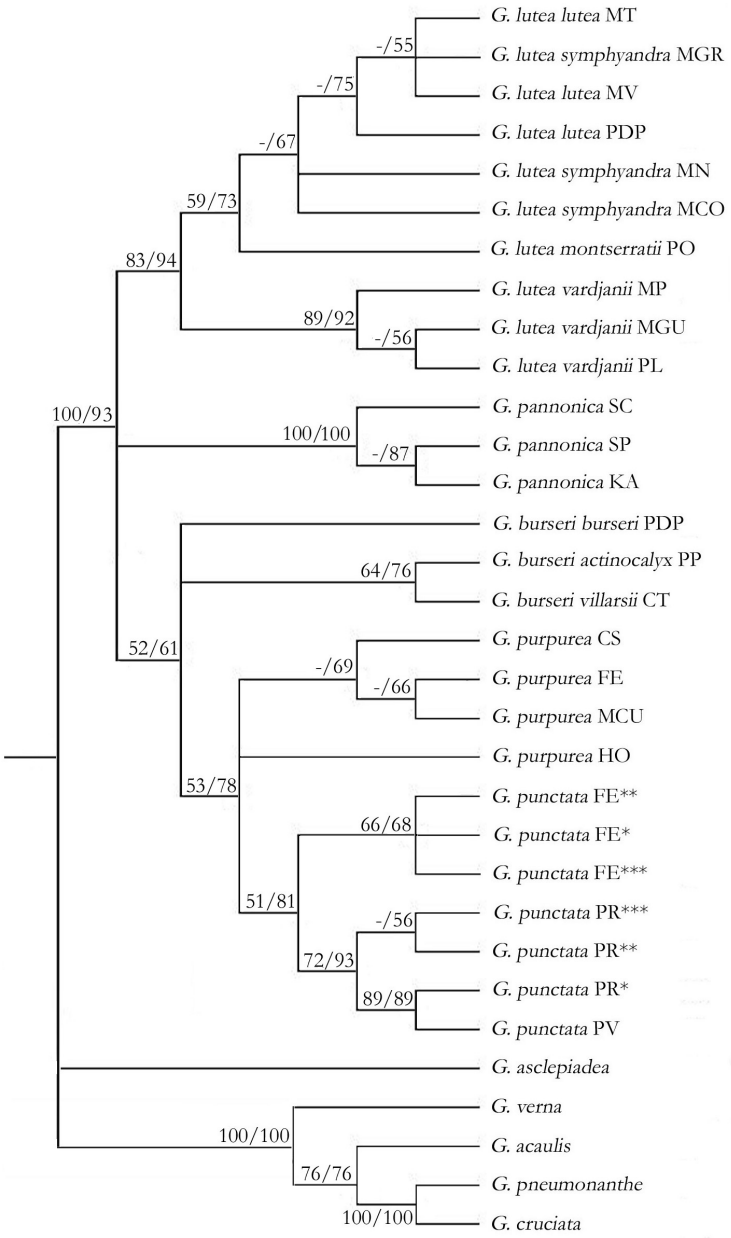

Figure 4.1: Nuclear maximum likelihood tree performed without indels. Bootstrap values are indicated above branches (MP value/ML value). Sample codes are given in Table 3.1; *, **, *** correspond to first, second and third clone, respectively.

4.2.2 Chloroplast markers

The screening of seven chloroplast markers highlights a low genetic variability within the sect. *Gentiana* plastid genome.
The alignment of both *rpl*32-*trn*L and *psb*A-*trn*H has 1405 positions (1017 and 388 bases, respectively) of which 4.5% and 0.6% are parsimony informative in all taxa and in sect. *Gentiana* alone, respectively. Details on constant characters, parsimony-uninformative variable characters, parsimony-informative characters, indels and percentage of parsimony informative characters, for each marker, are given in Table 4.1.
A maximum parsimony strict consensus tree was obtained from 1423 most parsimonious trees and no significant difference in tree topology was found between MP and ML analyses, hence ML trees is shown (Figure 4.2). The phylogenetic hypothesis obtained from chloroplast markers is not congruent with both phylogeny obtained from nuclear data set and with morphological classification.
Results indicate that sect. *Gentiana* is not supported as being monophyletic: a basal polytomy includes a cluster with all populations of the section, *G. lutea* subsp. *montserratii*, *G. asclepiadea* and *G. verna* (bootstrap values: 95/86). Excluding *G. lutea* subsp. *montserratii*, all other populations of the sect. *Gentiana* group together in a cluster characterised by a basal polytomy (bootstrap support values: 89/93). Within this cluster, the haplotype network shows two main haplotypes (Figure 4.3, Table 4.2): haplotype A which groups two populations of *G. lutea* subsp. *vardjanii* sited in Central-Eastern Alps; and haplotype B (grouping *G. lutea* subsp. *symphyandra*, *G. lutea* subsp. *lutea* and *G. pannonica* samples) mainly distributed in Eastern Europe. Five haplotypes derive from haplotype A, one of which (a5) is peculiar, since it includes samples from Central Alps and from Pyrenees, belonging to several different species. Six haplotypes derive from haplotype B, mainly distributed in Western Alps and Apennines, except for b1, representative of *G. purpurea* population coming from Norway. In particular haplotype b4 (represented by *G. lutea* subsp. *symphyandra*, Northern Apennines), derives from b3, which includes two *G. purpurea* populations located just north.

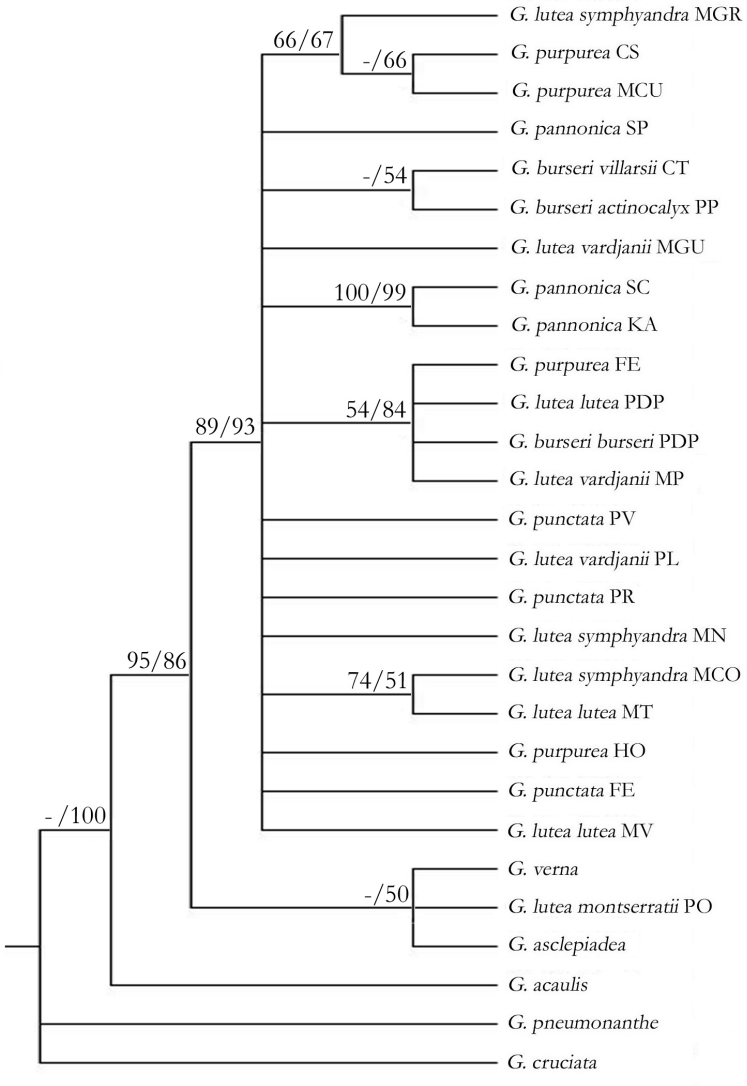

Figure 4.2: Chloroplast maximum likelihood tree. Bootstrap values are indicated above branches (MP value/ML value). Sample codes are given in Table 3.1.

Haplotype	**Species**	**Locality**
A	*G. lutea vardjanii* MGU	Central Alps
	G. lutea vardjanii PL	Eastern Alps
a1	*G. punctata* PV	Eastern Alps
a2	*G. punctata* PR	Eastern Alps
a3	*G. pannonica* KA	Eastern Alps
a4	*G. pannonica* SC	Bavaria
a5	*G. lutea lutea* PDP	Central Pyrenees
	G. burseri burseri PDP	Central Pyrenees
	G. lutea vardjanii MP	Central Alps
	G. purpurea FE	Central Alps
B	*G. lutea lutea* MV	Central Apennines
	G. lutea symphyandra MN	Dinaric Alps
	G. lutea symphyandra MCO	Eastern Carpathians
	G. pannonica SP	Eastern Alps
b1	*G. purpurea* HO	Norway
b2	*G. burseri villarsii* CT	Western Alps
	G. burseri actinocalyx PP	Western Alps
b3	*G. purpurea* MC	Northern Apennines
	G. purpurea CS	Northern Apennines
b4	*G. lutea symphyandra* MG	Northern Apennines
b5	*G. lutea lutea* MT	Central Apennines
b6	*G. punctata* FE	Central Alps

Table 4.2: List of haplotypes: for each haplotype corresponding samples (samples codes are given in Table 3.1) and geographical area are given.

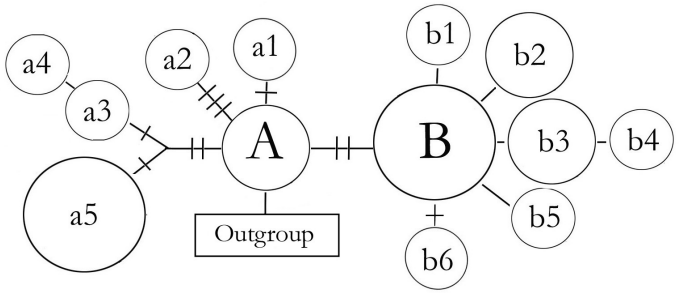

Figure 4.3: Section *Gentiana* haplotype network of plastid markers: haplotype codes are given in Table 4.2, perpendicular segments represent hypothetical haplotypes, nucleotide substitutions are represented by each segment between haplotypes, circle sizes represent the number of individual(s), outgroup includes *G. lutea* subsp. *montserratii*.

4.3 Flower phenology

G. lutea showed asynchronous dichogamy within a stem (personal observation).

The following results concern intrafloral dichogamy. Mean temperature and mean relative humidity recorded during the two days of observations were T=19.5 °C and UR=51.5% for subsp. *lutea*, T=21.8°C and UR=56.3% for susp. *symphyandra* and T=21.0°C and UR=34% for subsp. *vardjanii*. Mean flower lifespan was about 3 days and it slightly varied among the populations depending on environmental variables. Developmental phases are listed in Table 3.2 and their duration is given in Table 4.3.

Stigmatic receptivity begins gradually together with the separation of the two stigmatic lobes, increasing quite quickly and lasting until flower withering (Table 4.3). Pollen was highly viable up to 11 hours after anthers opening in Mt. Vettore and up to 6 hours in Passo Lusia populations, and in both cases remained viable up to 30 hours after anthers dehiscence. Pollen from Mt. Grande population showed lower viability compared to that of others populations: from anthers opening up to 10 hours after dehiscence its viability resulted lower than 80%. Results on pollen viability are given in table Table 4.4.

On average, the presence of fresh pollen was recorded for ca. 1 day (n=25),

Phase	subsp. *lutea*		subsp. *symphyandra*		subsp. *vardjanii*	
	lifespan (n)	recep. class (n)	lifespan (n)	recep. class (n)	lifespan (n)	recep. class (n)
I	9h 24m (17)	II (10)	1h 36m (10)	- -	9h 6m (21)	I (9)
II	8h 18m (26)	I (10)	8h (20)	II (13)	14h (28)	I (10)
III	28h 36m (26)	I (10)	21h 36m (23)	I (10)	5h 12m (34)	I (10)
IV	41h 18m (12)	I (10)	29h 12m (17)	I (11)	17h 18m (24)	- -
V					24h 42m (11)	I (10)
Total	**87h 36m**		**60h 24m**		**70h 18m**	

Table 4.3: Duration and stigma receptivity for each developmental phase (sample sizes in brackets). Classes of stigma receptivity: class II - stigma hardly bilamellate with receptivity limited to a small apical area of stigmatic surface; class I - stigma bilamellate, with widespread receptivity to the entire stigmatic surface. Different developmental ages show error rate ranging from 2h 18m to 42m (data not shown).

3 hours (n=10) and 11 hours (n=23) in Mt. Vettore, Mt. Grande and Passo Lusia populations, respectively; elapsed this time its presence was not appreciable due both to pollinators activity and to anthers desiccation.

All information above is resumed in Figure 4.4. As the figure shows, *G. lutea* revealed a striking variation in dichogamy. Subsp. *lutea* and subsp. *vardjanii* presented an incomplete protogyny, as there was overlap between pollen presentation and stigma receptivity (Lloyd and Webb, 1986). However this condition differed between the two subspecies: subsp. *lutea* appears to be functionally adichogamous, being the stigma only slightly receptive before anthers dehiscence, while full receptivity occurs at the same time as male phase (Figure 4.4). By contrast, in subsp. *vardjanii*, anthesis begins with female phase that lasts approximately 24 hours before anthers dehiscence, showing incomplete protogyny.

In subsp. *symphyandra*, young flowers (age I, Figure 4.4), present unreceptive/limited receptive stigma and dehiscing anthers with fresh pollen exposed. The period of effective pollen presentation in nature conditions tends to be shorter than the potential one, due to environmental variables and pollinators

	Anther age				
	Pollen viability (%) – Class				
Subsp.	*0-3h*	*3-6h*	*8-10h*	*24-30h*	*> 35h*
lutea	**95.7 – I** (n=5)	**93.0 – I** (n=5)	**87.4 – I** (n=5)	**67.1 – II** (n=5)	**32.1 – III** (n=5)
symphyandra	**66.5 – II** (n=5)	**75.5 – II** (n=2)	**53.3 – II** (n=4)	**38.9 – III** (n=5)	**21.0 – III** (n=5)
vardjanii	**89.3 – I** (n=5)	**85.4 – I** (n=5)	**65.1 – II** (n=5)	**64.5 – II** (n=5)	**10.6 – III** (n=6)

Table 4.4: Pollen viability (%) and viability class at different anther ages. Class I: highly viable pollen (100%-80%); class II: viable pollen (79%-50%); class III: scarcely viable pollen (<49%). Sample sizes in brackets. Anther age $> 35h$ corresponds to 52-57h for subsp. *lutea*, 44-55h for subsp. *symphyandra* and 35-45h for subsp. *vardjanii*.

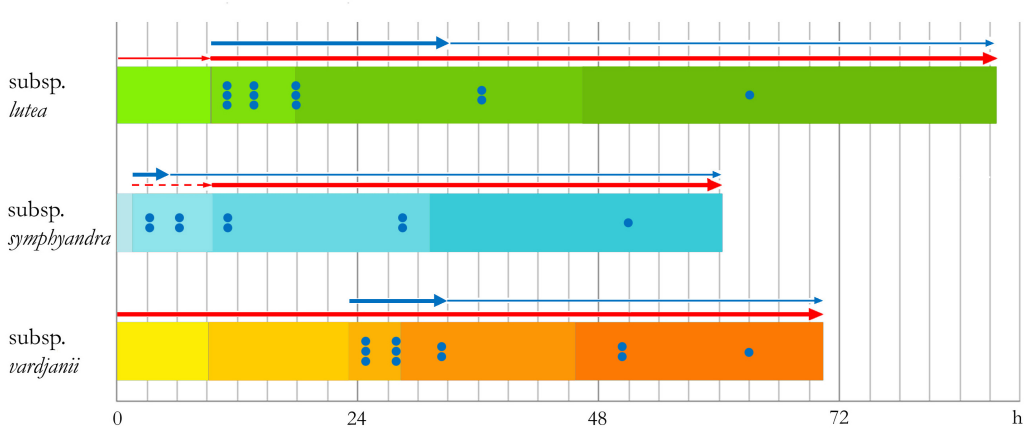

Figure 4.4: Temporal patterns of flower development (h= hours). Colour gradations indicate the duration of each floral phase, from I (lighter) to V (darker), in subsp. *lutea* (green), *symphyandra* (blue) and *vardjanii* (orange). Red arrows indicate female phases (stigmatic receptivity): thin: class II, stigma hardly bilamellate, receptivity limited to a small apical area (dotted line: co-presence of stigma undivided and class II of receptivity); thick: class I, stigma bilamellate, with widespread receptivity. Blue arrows indicate the abundance of exposed pollen: thick: abundant presence of fresh pollen; thin: presence not appreciable. Blue circles indicate pollen viability: class I - 3 circles, highly viable pollen (100%-80%); class II, 2 circles, viable pollen (79%-50%); class III - 1 circle, scarcely viable pollen (<49%).

collecting activity. In subsp. *symphyandra*, although pollen presentation and stigma receptivity overlap, complete stigma receptivity occurs when anthers are mostly empty. For this reason subsp. *symphyandra* could be considered completely protandrous (see also Kozuharova and Anchev, 2006).

4.4 Reproductive ecology

4.4.1 Breeding system

Data sets from different years do not show significant differences in reproductive output of controls and pollination treatments, hence results concerning the two-years period are showed (except for seed set of subsp. *lutea*, where data set of 2011 was used).

The test for agamospermy indicated that seed production without fertilization is not possible, as none of the treated flowers developed into seeded fruits (n=28).

Fruit set from open pollinated flowers was 0.96, n=47 in subsp. *lutea*; 0.95, n=59 in subsp. *symphyandra*; 0.97, n=34 in subsp. *vardjanii*, (Figure 4.5). Both spontaneus and hand self pollination led to fruit production (Figure 4.5, Table 4.5), but in the first case fruit set was significantly lower than in hand-self pollination and controls, while no differences were found between controls and hand-self pollination. Details on fruit set values, sample sizes, X^2 values and p-values are given in Table 4.5.

Though fruit set indicated self-compatibility (Table 4.5), seed set in controls was significantly higher than that resulting from both spontaneus selfing and hand-self pollination (Figure 4.5). These differences were confirmed by Kruskal-Wallis test and pairwise Mann-Whitney comparisons, that revealed significant differences in seed set between controls and spontaneus selfing and between controls and hand-self pollination, while no differences were highlighted between the two selfing treatments. Details on seed set values ($\pm$ standard error), sample sizes, Kruskal-Wallis and Mann-Whitney results (together with p-values) are given in Table 4.6.

In Passo Lusia population seed set of pollen augmented flowers did not differ

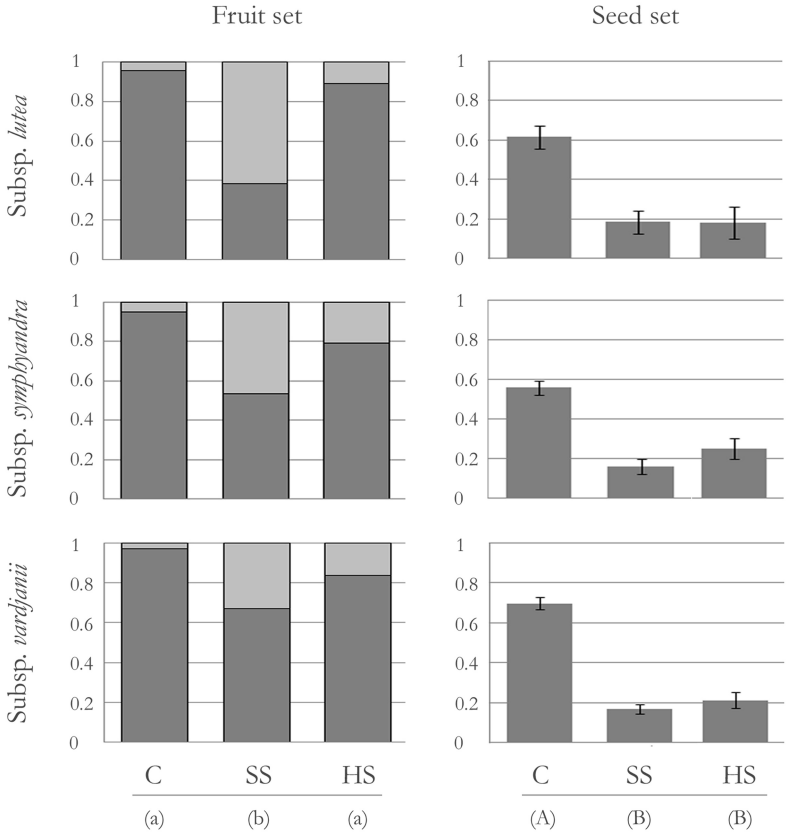

Figure 4.5: Fruit set and seed set, in open pollinated flowers (C) and pollination treatments (SS = spontaneous selfing, HS = hand-self pollination). Light gray bars: aborted fruit; dark gray bars: seeded fruit. Different letters in brackets indicate significant differences among treatments, error bars represent standard errors.

from hand-cross pollinated flowers (M-W, p: ns).

Seed set of spontaneus selfing did not differ among subspecies (K-W, p: ns). The Index of Automatic Self-pollination (IAS) is 0.43 in subsp. *lutea*, 0.67 in subsp. *symphyandra* and 0.80 in subsp. *vardjanii* (mean=0.63), showing that *G. lutea* is partially autogamous.

The Index of Self-Incompatibility has been calculated for subsp. *lutea*, *symphyandra* and *vardjanii* (ISI=0.25, 0.34 and 0.27, respectively; mean=0.27): in all cases it indicates values close to the lower threshold of incompletely compatible species .

Fruit set						
	Treatments			X^2 Test		
Subsp.	C	SS	HS	C-SS-HS (2)	C-HS (1)	C,HS - SS (1)
lutea	0.96 n=47	0.38 n=47	0.89 n=18	X^2=40.79 $p < 0.001$	X^2=0.02 ns	X^2=40.50 $p < 0.001$
symph.	0.95 n=59	0.53 n=30	0.79 n=29	X^2=21.95 $p < 0.001$	X^2=0.03 ns	X^2=18.93 $p < 0.001$
vard.	0.97 n=34	0.67 n=76	0.84 n=31	X^2=13.08 $p < 0.01$	X^2=0.01 ns	X^2=11.44 $p < 0.001$

Table 4.5: Fruit set, sample sizes (C=open pollinated flowers, SS=spontaneus selfing, HS=hand-self pollination) and statistical analysis of variance. X^2 values and p-values are given for test among all treatments (C-SS-HS), and for pairwise comparisons (C-HS and C,HS-SS); degrees of freedom in brackets. Abbreviations: *symph.*: *symphyandra*, *vard.*: *vardjanii*.

Seed set							
	Treatments			K-W test and post-hoc M-W			
Subsp.	C	SS	HS	C-SS-HS	C-SS	C-HS	SS-HS
lutea	0.62 (±0.06) n=45	0.18 (±0.06) n=18	0.18 (±0.08) n=16	K-W=21,74 $p < 0.001$	M-W $p < 0.001$	M-W $p < 0.001$	M-W ns
symph.	0.56 (±0.04) n=56	0.16 (±0.04) n=16	0.25 (±0.05) n=23	K-W=31.58 $p < 0.001$	M-W $p < 0.001$	M-W $p < 0.001$	M-W ns
vard.	0.70 (±0.03) n=32	0.17 (±0.02) n=51	0.21 (±0.04) n=26	K-W=57.46 $p < 0.001$	M-W $p < 0.001$	M-W $p < 0.001$	M-W ns

Table 4.6: Seed set (± standard error), sample sizes for each subspecies, (C=open pollinated flowers, SS=spontaneus selfing, HS=hand-self pollination) and statistical analysis of variance. Kruskal-Wallis values (C-SS-HS), Mann-Whitney post-hoc pairwise comparisons (C-SS, C-HS, SS-HS), and p-values, are given. Abbreviations: *symph.*: *symphyandra*, *vard.*: *vardjanii*.

Several fitness parameters as: predation, fruit set, ovules number and seed set, were compared to study inter-populations differences on reproductive success. In these analyses data of the two-years period were considered.

Predation had a great impact on fruit production: in Mt. Vettore and Passo Lusia populations, fruit set was reduced by 45.0% (n=85) and 55.0% (n=80), respectively, while in the other populations its impact was negligible (1.6% in Mt. Grande, n=60; 0.0% in San Juan de la Peña d'Oroel, n=30 and 3.3%

in Mt. Nanos, n=30).

Fruit set was not significantly different among populations (X^2, df=4, p:ns). Statistical analyses revealed that number of ovules is not a conserved character in *G. lutea*. Both Mt. Vettore and Passo Lusia populations showed significant differences between study years (Mt. Vettore: ovules=98.2±2.2 in 2010 and 92.1±2.1 in 2011, t-test, t=2.0, $p < 0.05$; Passo Lusia ovules=59.1±1.3 in 2009 and 68.0±1.9 in 2010, t-test, t=-4.0, $p < 0.001$), while years did not differ in Mt. Grande population (t-test, p: ns). Kruskal-Wallis revealed differences among populations (K-W H=271.8, $p < 0.001$) and all Mann-Whitney test highlighted significant differences in all pairwise comparisons ($p < 0.001$). Mean ovules number, over the study years, was 95.5±1.5 (n=152) in Mt. Vettore, 80.8±1.2 (n=186) in Mt. Grande, 57.4±2.3 (n=56) in Mt. Nanos, 65.7±1.2 (n=250) in Passo Lusia and 112.5±4.1 (n=30) in San Juan de la Peña d'Oroel populations (Figure 4.6).

Seed set does not differ significantly among populations (K-W H=7.69,

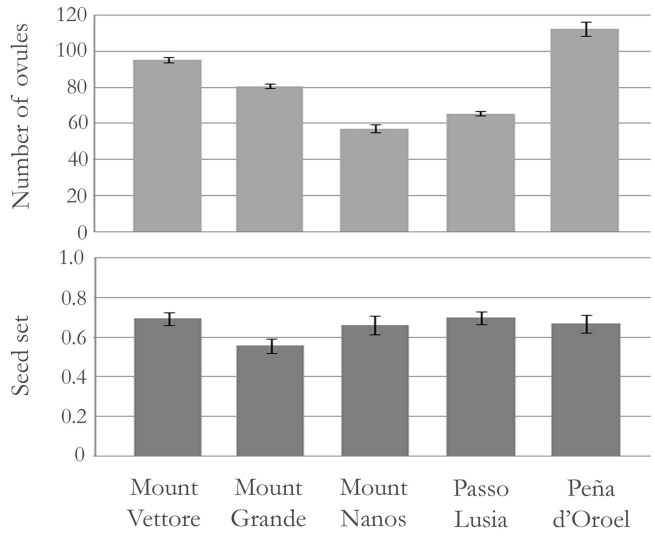

Figure 4.6: Number of ovules and seed set for each study population, error bars represent standard error.

p= ns), ranging from 0.70±0.03 of Passo Lusia population to 0.56±0.04 of Mt. Grande population (Mt. Vettore s:o=0.70±0.03, n=45; Mt. Nanos

s:o=0.66±0.05, n=29; Peña d'Oroel s:o=0.67±0.04, n=29, Figure 4.6). Post-hoc Mann-Whitney pairwise comparisons revealed significant differences between Mt. Grande population and both Passo Lusia and Mt. Vettore populations (M-W U test, $p < 0.05$).
Seed number was negatively related with mean seed weight in Mt. Grande population (rs=-0.21, $p < 0.01$, n=153), while no correlation was detected in Mt. Vettore and Passo Lusia populations.

4.4.2 Resource allocation to sexual function

The investment in pollen and ovules was analysed in order to study the energetic allocation in male and female function. Mean P/O values for each subspecies are here reported: subsp. *lutea*: 9371.1±1495.1, range: 5460.0-12048.1; subsp. *symphyandra*: 12900.6±1352.6, range: 8929.3-16426.1; subsp. *vardjanii*: 5432.9±979.7, range: 3092.3-8984.9. Kruskal-Wallis test revealed differences among subspecies (K-W H=7.7, $p < 0.05$) and post-hoc pairwise comparisons highlighted that subsp. *vardjanii* differed significantly from subsp. *symphyandra* (M-W, $p < 0.05$); and that p-value between subsp. *vardjanii* and *lutea* was close to the limit of significance (M-W, $p = 0.06$), while subsp. *lutea* and *symphyandra* did not show significant differences.

4.5 Seed germination

Germination rate for seeds resulting from controls, pollen augmented flowers and selfing treatments was: 95.7±3.5%, 96.5±1.4% and 48.4±9.4% in Mt. Vettore population.; 25.1±4.5%, 28.4±5.3% and 15.8±4.5% in Mt. Grande population; 91.9±2.5%, 92.8±3.0% and 34.8±5.9% in Passo Lusia population (Figure 4.7, sample sizes Table 3.4).
Germination rate differed among treatments (K-W H=20.07, $p < 0.001$ Mt. Vettore; H=6.33, $p < 0.05$, Mt. Grande; H=36.02, $p < 0.001$, Passo Lusia) and post hoc Mann-Whitney U-test revealed significant differences between controls vs. autogamy and between cross-pollination vs. autogamy, while no significant difference was found between controls and cross-pollination.

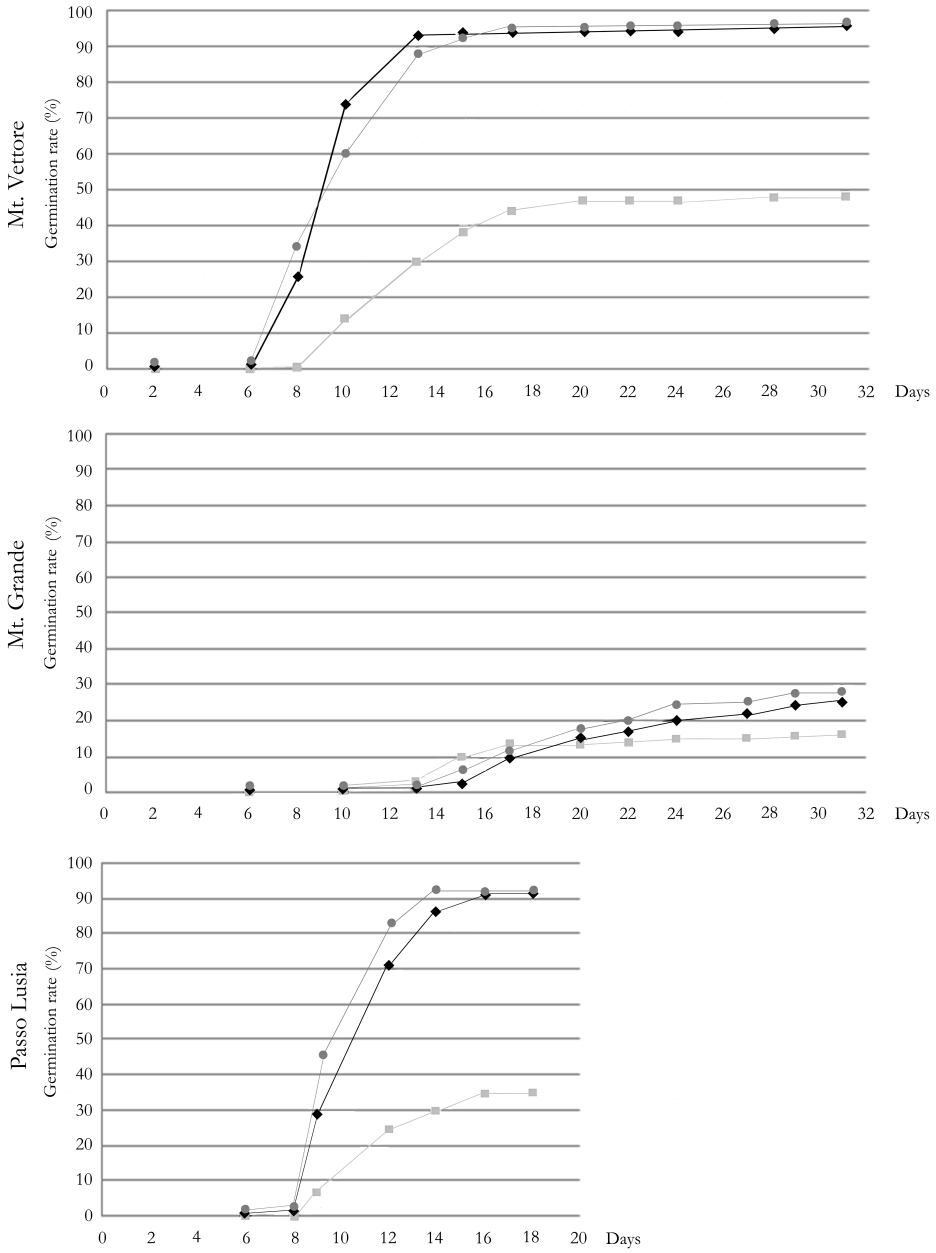

Figure 4.7: Germination rate (%) of seeds obtained from different treatments in the studied populations: Mt. Vettore, Mt. Grande and Passo Lusia. Black rhombs: open pollination (controls); dark grey circles: pollen augmentation treatment; light grey squares: autogamy treatments (spontaneus selfing and hand-self pollination).

Although this trend was found in all populations the level of significance of p-value was higher in Mt. Vettore and Passo Lusia populations (M-W, $p < 0.001$) compared to Mt. Grande population (M-W, $p < 0.05$).
Seed germination rate (controls) of Mt. Nanos and San Juan de la Peña d'Oroel populations, was 73.1±5.8% and 85.7±2.4%, respectively.
Considering all studied populations, Kruskal-Wallis test revealed significant differences in germination rate of open pollinated flowers (K-W H=63.28, $p < 0.001$) and Mann-Whitney pairwise comparisons pointed out the lower performance of Mt. Grande seeds compared to other populations and the higher germination rate of seeds from Mt. Vettore population compared to Mt. Nanos and San Juan de la Peña d'Oroel ($p < 0.001$).
Seeds resulting from autogamy in different populations showed different germination rates (K-W H=7.43, $p < 0.05$) and Mann-Whitney post-hoc comparisons highlighted that Mt. Grande population presented lower values compared to both those of Mt. Vettore and Passo Lusia ($p < 0.01$ and $p < 0.05$, respectively).
Considering the seeds derived from natural pollination, the time to germinate was different depending on subspecies: germination time was 9.1±0.2 days for subsp. *lutea*; 19.0±0.7 and 19.0±0.9 days for subsp. *symphyandra* (Mt. Grande and Mt. Nanos populations, respectively); 10.7±0.4 days for subsp. *vardjanii* and 15.7±0.7 days for subsp. *montserratii*. Kruskal-Wallis test revealed differences in germination time among subspecies (K-W H=73.8, $p < 0.001$) and Mann-Whitney confirmed it showing differences in all pairwise comparisons ($p < 0.01/p < 0.001$), except between the two populations of subsp. *symphyandra*.

4.6 Inbreeding depression

Inbreeding depression index (Ågren and Schemske, 1993) highlighted a marked advantage of outcrossed offspring compared to selfed in the three study populations (δ=0.87 in Mt. Vettore, 0.94 in Mt. Grande, 0.96 in Passo Lusia, mean=0.92). Fruit set, seed set and germination rate are the fitness traits that more contribute to cut fitness of selfed offspring. Details on δ value for

each fitness trait, statistical test used to check differences between selfed and outcrossed progeny (together with p-value) and cumulative δ values are given in Table 4.7.

	Mt. Vettore - 2011				Mt. Grande - 2010				Passo Lusia - 2009			
	Self	**Cross**	δ	**test**	**Self**	**Cross**	δ	**test**	**Self**	**Cross**	δ	**test**
fruit set	0.33	1	0.67	X^2 ***	0.53	1	0.47	X^2 ***	0.77	1	0.23	X^2 *
seed set	0.30 (0.18)	0.71 (0.04)	0.57	MW ns	0.16 (0.04)	0.74 (0.05)	0.79	MW ***	0.14 (0.03)	0.83 (0.03)	0.83	MW ***
seed weig.	1.12 (0.14)	0.78 (0.02)	-0.30	MW ns	0.61 (0.06)	0.60 (0.03)	-0.02	MW ns	0.91 (0.04)	1.10 (0.05)	0.18	MW **
germ. rate	48.4 (9.4)	96.5 (1.4)	0.50	MW ***	15.8 (4.5)	28.4 (5.3)	0.44	MW *	34.8 (3.0)	92.8 (5.9)	0.63	MW ***
germ. time	11.8 (0.8)	9.3 (0.5)	0.21	MW **	16.3 (1.0)	18.0 (1.0)	-0.09	t ns	10.4 (0.3)	9.8 (0.3)	0.06	MW ns
Cum.	*64.8*	*496.2*	**0.87**		*13.3*	*225.6*	**0.94**		*35.3*	*829.3*	**0.96**	

Table 4.7: Inbreeding depression (δ) in Mt. Vettore, Mt. Grande and Passo Lusia populations. Observed fitness traits: fruit set, seed set, mean seed weight (mg), seed germination rate (%) and seed germination time (days). For each trait mean (standard error in brackets), δ value, test used to detect differences between selfed and outcrossed offspring (X^2: Chi-squared, MW: Mann-Whitney, t: Student's t test) and p-values (*: $p < 0.05$, **$p < 0.01$, ***: $p < 0.001$) are given. In the last row cumulative inbreeding depression.

4.7 Plant - pollinator interactions

4.7.1 Flower pollinators

The total time of insects' observations was 13 hours and 15 minutes (details are given in Table 3.5).
In general pollinators belong to four orders: Hymenoptera, Diptera, Coleoptera and Lepidoptera. Hymenoptera, Diptera and Coleoptera were found in all studied populations (although with different families), while Lepidoptera were seldom observed in Mt. Grande and Passo Lusia populations. Sampled specimens belonging to genus *Bombus* were predominantly males.

In Mt. Vettore population the majority of pollinators were hymenopterans, suborder Apocrita (83.3%), with Formicidae the most represented family (68.0%); dipterans and coleopterans were less common with 15.2% and

Mt. Vettore						
Pollinator	**Visits 2010/2011**	**Total %**	**Stigma touch**	**N/P**	**Behaviour**	**N**
Hymenoptera						
Apidae						
Bombus ssp. - *B. ruderarius* Müller * - *B. rupestris* Fabricius **	12/11	2.3	✓	N-P	dynamic	4
Bombus ssp. - *B. terrestris* L. - *B. vestalis* Geoffroy	-/32	3.3	✓	N	dynamic	3
Vespidae						
Polystes ssp. - *P. biglumis bimaculatus* - *P. sulcifer*	16/45	6.2	✓	N	dynamic	4
Ichneumonidae						
Ichneumoninae – 3 spp.	2/33	3.5	✓	N	dynamic	5
Formicidae – 1 sp.	537/131	68.0	✓	N	sedentary	7
Diptera						
Muscomorpha – 1 sp.	25/124	15.2	✓	N-P	sedentary	2
Coleoptera						
Coleoptera – 1 sp.	5/-	0.5		N	sedentary	1
Coleoptera – 1 sp.	6/-	0.6		N	sedentary	3
Other – 2 spp.	1/3	0.4				

Table 4.8: Mount Vettore - list of pollinators: total number of visits in 2010 and 2011, respectively; relative abundance (%) in the two-years period; pollinator behaviour towards stigma (✓: stigma touch observed); collected floral rewards (P: pollen, N: nectar); pollinator behaviour towards inflorescence (dynamic/sedentary activity); N: number of sampled insects; * subgenus *Thoracobombus* Dalla Torre, ** subgenus *Psithyrus* Lepeletier (nowadays still considered as genus by Williams, 1994).

1.1% of visits, respectively. Not all pollinators touched flower reproductive structures (anthers and stigma), thereby only some of them were considered as active pollinators, while the others were regarded as occasional pollinators. Nectar is the main floral reward, however *Bombus rupestris*, *B. ruderarius* and Muscomorpha collected pollen as well. In particular flies were observed while removing pollen directly from receptive stigmas. Bees, wasps and ichneumon wasps are dynamic pollinators, while ants, flies and beetles are sedentary. All information is resumed in Table 4.8. Pictures of pollinators in Appendix (Figure 25 and Figure 26).

Mt. Grande						
Pollinator	**Visits 2010/2011**	**Total %**	**Stigma touch**	**N/P**	**Behaviour**	**N**
Hymenoptera						
Apidae						
Bombus ssp.	4/60	32.2	✓	N	dynamic	9
- *B. lapidarius* L. *						
- *B. rupestris* Fabricius **						
Bombus ssp.	-/41	20.5	✓	N-P	dynamic	6
- *B. terrestris* L.						
- *B. lucorum* L.						
- *B. vestalis* Geoffroy **						
- *B. campestris* Panzer **						
Halictidae						
Lasioglossum						
L. albipes Fabricius	1/3	2.0	✓	(N)-P	dynamic	1
L. morio Fabricius	-/17	8.6	✓	N-P	dynamic	1
Diptera						
Muscomorpha – 1 sp.	3/24	13.6	✓	(N)-P	sedentary	2
Syrphidae – 2 spp.	1/1	1.0	✓	P	dynamic	2
- *Episyrphus balteatus* De Geer						
Coleoptera – 1 sp.	2/38	20.1	✓	N	sedentary	2
Lepidoptera						
Sphingidae						
Macroglossum stellatarum	2/-	1.0		N		-
Other – 2 spp.	-/3	1.0				

Table 4.9: Mount Grande population - list of pollinators: total number of visits in 2010 and 2011, respectively; relative abundance (%) in the two-years period; pollinator behaviour towards stigma (✓: stigma touch observed); collected floral rewards (P: pollen, N: nectar, in brackets rewards rarely collected); pollinator behaviour towards inflorescence (dynamic/sedentary activity); N: number of sampled insects; * subgenus *Melanobombus* Dalla Torre; ** subgenus *Psithyrus* Lepeletier (nowadays still considered as genus by Williams, 1994).

In Mt. Grande population the majority of pollinators belonged to the order of Hymenoptera (63.3%) and Apidae was the most represented family (52.7%). Recorded visits for dipterans, coleopterans and lepidopterans were 14.6%, 20.1% and 1.0%, respectively. All pollinators actively touched mature stigmas, except *Macroglossum stellatarum*. In particular, due to its sustained flying ability and to the floral structure of subsp. *symphyandra*, it likely would play a marginal role in pollination. Pollinators were observed seeking nectar

Passo Lusia						
Pollinator	**Visits 2010/2011**	**Total %**	**Stigma touch**	**N/P**	**Behaviour**	**N**
Hymenoptera						
Apidae						
Apis mellifera	13/54	8.7	✓	N-P	dynamic	7
Bombus spp.	-/16	2.1	✓	N-P	dynamic	7
- *B. terrestris* L.						
- *B. lucorum* L.						
- *B. hortorum* L. *						
- *B. pratorum* L. **						
Halictidae	2/-	0.3	✓	P	dynamic	8
Lasioglossum spp.						
- *L. albipes* Fabricium						
- *L.* sp. gr. *albipes*						
Tenthredinidae						
Aglaostigma sp.	1/6	0.9		-	dynamic	18
Diptera						
Muscomorpha – 3 spp.	173/391	73.0	✓	N-P	sedentary	13
***Syrphidae* 2spp.**	10/38	6.2	✓	P	dynamic	8
- *Scaeva pyrastri* L.						
- *Scaeva selenitica* Meigen						
Coleoptera						
Rutelidae						
Phylloperta sp.	17/26	5.6	✓	N-P	sedentary	6
Elateridae						
Ctenicera sp.	6/2	1.0		P	sedentary	3
Cetonidae – 1 sp.	1/-	0.1		-	sedentary	1
fam. *Coleoptera* - 1 sp	-/8	1.1	✓	N-(P)	sedentary	2
Lepidoptera						
Noctuidae						
Sideridis reticulata	-/1	0.1		N	dynamic	1
Other - 2 spp.	2/5	0.9				

Table 4.10: Passo Lusia population - list of pollinators: total number of visits in 2010 and 2011, respectively; relative abundance (%) in the two-years period; pollinator behaviour towards stigma (✓: stigma touch observed); collected floral rewards (P: pollen, N: nectar, in brackets rewards rarely collected); pollinator behaviour towards inflorescence (dynamic/sedentary activity); N: number of sampled insects; * subgenus *Megabombus* Dalla Torre; ** subgenus *Pyrobombus* Dalla Torre.

and pollen (details in Table 4.9) and both flies (Muscomorpha) and hoverflies (Syrphidae) fed on pollen directly from mature stigmas. The majority of visitors are dynamic, except flies (Muscomorpha) and beetles (Coleoptera).

Pictures of pollinators in Appendix (Figure 27 and Figure 28).
In Passo Lusia population 79.2% of pollinators was represented by dipterans among which Muscomorpha was the most abundant family (73.0%); hymenopterans, coleopterans and lepidopterans were less common with 12.0%, 7.8% and 0.1% of visits, respectively. Bees, halictid species, flies and beetles were observed actively touching mature stigmas and hence considered active pollinators, while the others were regarded as occasional pollinators. Both nectar and pollen were collected as floral rewards (details in Table 4.10) and flies (Muscomorpha), syrphids (Syrphidae) and beetles (Coleoptera) were observed feeding on pollen directly from mature stigmas. Visitors species belonging to Muscomorpha and Coleoptera are sedentary pollinators while those belonging to families Apidae, Halictidae (*Lasioglossum*), Tenthredinidae, Syrphidae and Noctuidae are dynamic. Pictures of pollinators in Appendix (Figure 29 and Figure 30).

4.7.2 Pollinator fidelity

Pollinator fidelity, as described by Gibson et al. (2006), was calculated for groups of related species (Table 4.11).
In Mt. Vettore population, *Bombus* species showed the highest value of fidelity (0.787), followed by Ichneumoninae, Coleoptera, Muscomorpha and Vespidae species. None of the ants sampled specimens presented more than 5 pollen grains of *G. lutea*, so they were not considered pollinators.
The fidelity of genus *Bombus* was the highest, compared with other insects, also in Mt. Grande population (0.514), followed by that of hoverflies, *Lasioglossum* ssp., beetles spp. and fly spp..
In Passo Lusia population two taxa (*Apis mellifera* and Coleoptera species) showed the highest value of fidelity (0.730). Lower values were found in genus *Bombus*, Muscomorpha ssp., genus *Lasioglossum*, Tenthrenidae ssp. and hoverflies. Only four individuals among 13 sampled specimens of Muscomorpha were carriers of *G. lutea* pollen.

Group	*N*	*np*	*fv*	*F*	*PI*
Mt. Vettore					
Bombus	7	7	0.056	0.787	+0.044
Vespidae	5	5	0.062	0.559	+0.035
Ichneumoninae	5	5	0.035	0.786	+0.028
Formicidae	7	0	0.680	0.000	0.000
Muscomorpha	2	1	0.152	0.618	-0.047
Coleoptera	4	4	0.011	0.777	-0.009
Mt. Grande					
Bombus	15	15	0.527	0.514	+0.271
Lasioglossum	2	2	0.106	0.460	+0.049
Muscomorpha	2	2	0.136	0.069	-0.009
Syrphidae	2	2	0.010	0.495	+0.005
Coleoptera	2	2	0.201	0.295	-0.059
Passo Lusia					
Apis mellifera	7	7	0.087	0.730	+0.064
Bombus	7	7	0.021	0.533	+0.011
Lasioglossum	8	8	0.003	0.370	+0.001
Tenthredinidae	18	18	0.009	0.362	+0.003
Muscomorpha	13	4	0.730	0.119	-0.087
Syrphidae	8	8	0.062	0.356	+0.022
Coleoptera	12	12	0.078	0.730	-0.057

Table 4.11: Index of Pollinator Importance (PI) calculated for groups of related species. *N*: individuals available; *np*: sampled specimens considered carriers of *G. lutea* pollen; *fv*: frequency of visits based on observations; *F*: mean pollinator fidelity as described by Gibson et al. (2006); *PI*: Index of Pollinator Importance (positive values: dynamic pollinators; negative values: sedentary pollinators).

4.7.3 Index of Pollinator Importance

Since each studied population shows its own frequency of pollinator visits, and given that simultaneous flowering of co-occurring species biases pollinators fidelity, values of Pollinator Importance index differ among populations (Table 4.11, Figure 4.11). However, in general, Muscomorpha and Coleoptera show negative PI values, in contrast with *Apis mellifera*, *Bombus* spp., *Lasioglossum* spp., Vespidae spp., Ichneumoninae spp., Tenthredinidae spp. and Syrphidae spp. which present positive values.

In Mt. Vettore population bumblebees species show the highest value of pollinator importance (PI=+0.044), followed by ichneumon wasps and wasps.

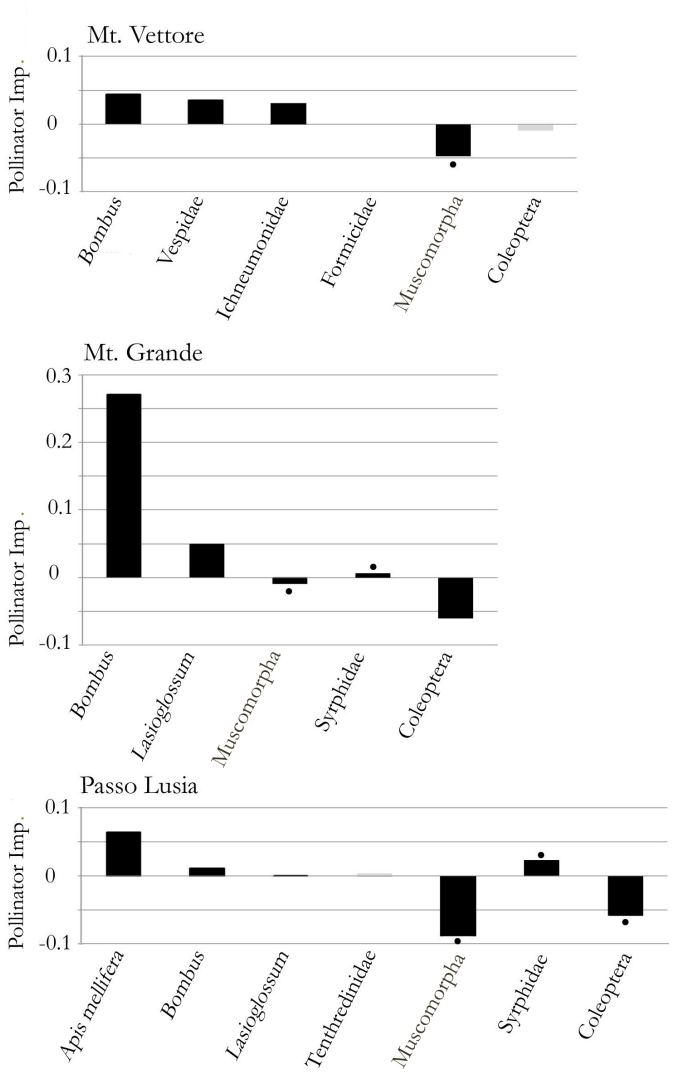

Figure 4.8: Index of Pollinator Importance (PI). Dark bars: active pollinators; light grey bars: occasional pollinators; dark spot: pollinators removing pollen from receptive stigmas.

Two of the three observed ichneumon wasps species did not touch mature stigmas, however the most frequent did, hence they are regarded as active pollinators. Formicidae sp. does not play any role on *G. lutea* pollination, mainly due to the small sizes and to the scarce presence of body hairs.
In Mt. Grande population genus *Bombus* shows the highest value of pollinator importance (PI=+0.271), followed by genus *Lasioglossum* and Syrphidae

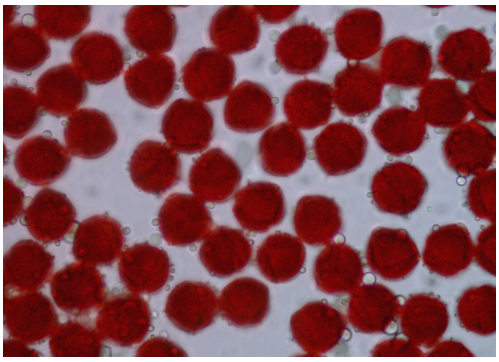

Figure 4.9: *G. lutea* pollen grains: presence of pollenkitt confirmed by lipid droplets adhered to pollen surface.

species.
In Passo Lusia population *Apis mellifera* has the highest positive value of pollinator Importance (PI=+0.064), followed by syrphids, bumblebees, Tenthredinidae spp. (occasional pollinators) and *Lasioglossum* species.

4.7.4 Pollenkitt

The presence of lipid droplets adhered to surface of *G. lutea* pollen grains indicate the presence of pollenkitt (Figure 4.9).

4.7.5 Nectar analyses

4.7.5.1 Nectar standing crop

Nectar standing crop was measured in Passo Lusia population: mean nectar volume per flower was 0.52±0.21µl (n=41) at 12AM, 0.12±0.02µl (n=41) at 15PM and 0.04±0.01µl (n=41) at 18PM. Kruskal-Wallis test revealed significant differences among intervals (K-W H=28.63, $p < 0.001$) and post-hoc pairwise comparisons highlighted differences between 12AM interval and both 15PM and 18PM intervals (M-W, $p < 0.001$).
Nectar concentration, expressed as % on a w/w basis of an equivalent sucrose solution, was generally low, ranging from 11.1% to 49.9%. Mean concentration was 35.7±1.8% (n=31) at 12AM, 31.7±1.6 (n=27) at 15PM and 28.1±3.0% (n=12) at 18PM. One-way ANOVA did not reveal significant differences among intervals (F=2.88, df=2 and 66, p: ns).

4.7.5.2 Nectar chromatography

Results of chromatographic analysis are shown in Table 4.12.
Sugar content of nectar did not differ among populations and consisted primarily of hexoses (glucose and fructose, mean=177.8±16.1 and 164.9±17.5 mg/ml, respectively), while sucrose showed the lower concentration values (mean=1.9±0.5 mg/ml). A total of 18 free amino acids were identified and results highlighted that *G. lutea* nectar is extremely rich in β-alanine and proline, (non-protein/protein amino acid; mean=2.2±0.2 and 1.1±0.1, respectively, Table 4.12).
Analyses did not reveal presence of alcohols (ethanol and methanol).

Constituents	**Mt. Vettore**	**Mt. Grande**	**Passo Lusia**	**Mean± st. er.**
Sucrose	1.3	1.6	2.8	1.9±0.5
Glucose	194.4	193.4	145.7	177.8±16.1
Fructose	180.2	184.5	129.9	164.9±17.5
β-alanine	2.2	1.8	2.7	2.2±0.2
Proline	1.0	0.9	1.2	1.1±0.1
Other aa	5.8	3.0	2.2	3.7±1.1
Alcohols	-	-	-	

Table 4.12: Nectar constituents as resulted by chromatographic analyses. Sugars (sucrose, glucose and fructose; mg/ml), amino-acids (β-alanine, proline and other amino acids=aa; mM) and alcohols concentration values are reported.

4.7.6 Pollen limitation

In all studied populations, over the two-years of surveys, natural pollinated and pollen augmented flowers did not show significant differences in fruit set (X^2, p:ns), while statistical analyses revealed significant differences in seed set. In particular, in both Mt. Vettore and Mt. Grande populations, pollen limitation was observed in one of the two years of study (2010: M-W U=193.5, $p < 0.001$ and M-W U=215, $p < 0.01$, respectively), while no difference was highlighted neither in 2011 (Mt. Vettore) nor in 2009 (Mt. Grande). Differently, in Passo Lusia population, seed set from open pollinated flowers was significantly lower compared to that from hand pollinated flowers

in both years of study (2009: t-test t=-2.38, $p < 0.05$; 2010: M-W U=84.5, $p < 0.05$). Results are resumed in Figure 4.10.

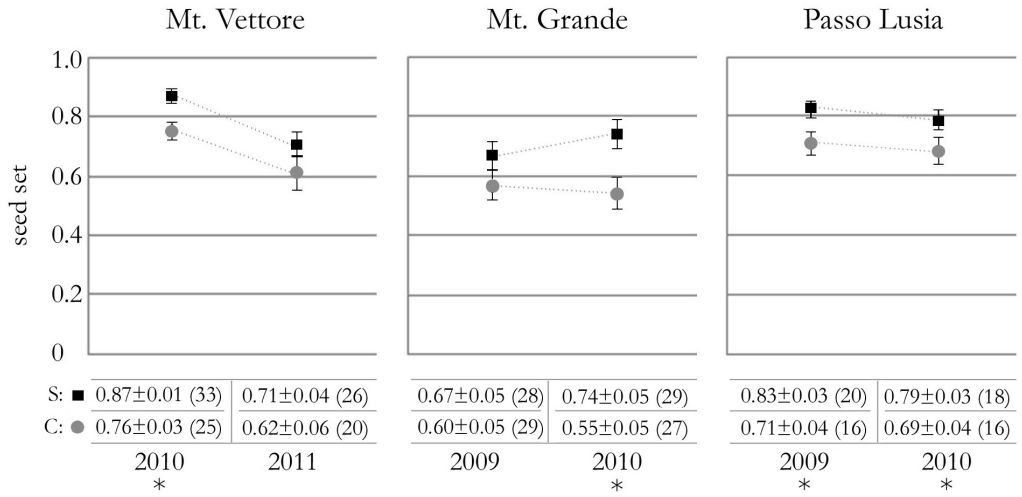

Figure 4.10: Pollen limitation in the study populations. Black squares: seed set of pollen augmented flowers; grey circles: seed set of open pollinated flowers; error bars represent standard errors. In table seed set values (± standard errors) and sample size (in brackets) are shown; asterisks indicate significant differences among treatments.

Chapter 5

Discussion

5.1 Taxonomy of *G. lutea*

Historical data confirm the known geographical distribution for each subspecies. Concerning subsp. *symphyandra*, Mt. Grande population is the only documented that occurs outside the known distribution range. Two different hypotheses could explain the presence of this population in the Northern Apennines. The first one is an historic hypothesis: since the Middle Ages mountain-dwellers used to plant this medicinal species close to their settlement (Rosenbauer, 1996), in this sight the population could have a synanthropic origin. Another hypothesis could imply a mutation in the genes involved in anthers development, which may have changed anthers arrangement, from free to connate. Nevertheless, this second explanation is less parsimonious than the first, since it foresees two separate mutational events: one in the known distribution area and one in Mt. Grande.

In contrast with Pignatti (1982), my observations show that stigma shape after anthesis is not a reliable character to distinguish subsp. *lutea* from subsp. *symphyandra*, but it may be used as an additional diagnostic character to identify both subsp. *symphyandra* (erecto-patent stigma) and subsp. *vardjanii* (spirally coiled stigma).

Although anthers length is significantly different among all subspecies, these differences are of the order of a few millimetres and thereby this trait is un-

suitable.
According to Wraber (1986) bracts length is a reliable character to identify subsp. *vardjanii*, however this is not exclusive: sporadically, bracts longer than pseudo-whorls occur also in subsp. *lutea*. For this reason, in order to undoubtedly identify subsp. *vardjanii*, other traits as bract colour, presence of vegetative stemless shoots and flowering time have to be considered.
For all observed traits, subsp. *lutea* shows an intermediate position or shares both character forms, compared to other subspecies, suggesting a basal phylogenetic position.

5.2 Phylogeny of sect *Gentiana*

The phylogenetic hypotheses obtained from the separate analysis of nuclear and chloroplast data sets are not congruent.
Both ITS and ETS are useful data sources in inferring phylogeny of sect. *Gentiana*, and between them 3' ETS region results more variable, especially at genus level. This finding confirms results by Baldwin and Markos (1998) and Kadereit et al. (2007), who stated that ETS region appears to evolve faster than ITS. Concerning Internal Transcribed Spacer, my results support the suggestion of Lia et al. (2011), who recommended ITS as additional marker to implement resolution power of DNA barcode.
The complete relations of sect. *Gentiana* have never been analysed. Previous phylogenetic considerations included just a few species of this section in a wider infrageneric panorama.
In the phylogeny inferred by Yuan et al. (1996) from ITS region, *G. lutea* and *G. punctata* were strongly related, within the group of European endemic sections. This strong relationship among species of sect. *Gentiana* is clearly confirmed by my findings: the phylogeny obtained from combination of nuclear markers, shows that sect. *Gentiana* is clearly monophyletic. Within the section, *G. lutea* identity is strongly supported and within it, subsp. *vardjanii* populations cluster together with a clear subspecies identity. This subspecies identity is conserved despite the fact that, at present, subsp. *vardjanii* occurs sympatrically with subsp. *symphyandra*, suggesting an actual presence

of reproductive barrier between these taxa. Flowering of subsp. *vardjanii* currently occurs 2-3 weeks before that of other subspecies (Wraber, 1986; Vender et al., 2010): this early anthesis can explain its present isolation, although it cannot be deduced whether it played a role in its divergence, or if other factors were involved (geographical, ecological, reproductive). Concerning its genetic identity and its actual reproductive isolation, *G. lutea* subsp. *vardjanii* could be considered as an example of speciation in progress.
Both *G. pannonica* and *G. punctata* show clear species identity, while *G. purpurea* and *G. burseri* are not monophyletic. Within *G. burseri*, subsp. *burseri*, does not cluster with subsp. *villarsii* and *actinocalyx*. Hungerer and Kadereit (1998) calibrated the molecular clock for sect. *Ciminalis* basing on ITS phylogeny and according to the authors, its speciation seems to have taken place largely during the climatic oscillations of the Quaternary. Most likely the same occurred for European sections *Gentiana* and *Calathianae*. From this sight, small relict populations of *G. burseri* may testify a continuous range of this species from Central to South-East Europe during the last glacial. Thereafter, during cold periods, populations could have migrated in mountain ranges with consequent lack of gene flow among the populations sited in the Eastern Alps and in the Pyrenees, that may have resulted in their genetic differentiation. This hypothesis, based on vicariance model of speciation, could explain differentiation among *G. burseri* subspecies. A similar argument could explain *G. purpurea* status. Two colonization hypotheses concerning the presence of arctic-alpine flora in Scandinavian peninsula could explain its status. On one side "nunatak hypothesis" or "glacial survival hypothesis" invoke the existence of glacial refugia within the North European ice sheet (Löve and Löve, 1963; Dahl, 1990; Gabrielsen et al., 1997), on the other "tabula rasa hypotesis" states that all plants migrated after last glaciation (Nordal, 1987). The climatic changes of the late Pleistocene probably happened rapidly, with conditions switching from glacial to near interglacial. Recently, paleobotanical data suggest that many arctic-alpine plants grew beyond the ice sheet during the Weichselian glaciation, colonizing open habitats as rapidly as they became available and showing a dynamic pattern of immigration, expansion and extinction (Gabrielsen et al.,

1997 and references therein). Both fossil records (Birks, 1994) and genetic studies (Gabrielsen et al., 1997) do not support the existence of glacial refugia hypothesis to explain the geographical distribution of artic-alpine plants. Although my findings reveal the presence of a genetic divergence between population from Scandinavian peninsula and those from Central-Southern Europe, they are not able to support one or the other hypothesis. Further studies on geographical structure of genetic variation within *G. purpurea* may highlight this topic.

According to Yuan et al. (1996) the European sections *Gentiana*, *Ciminalis* and *Calathianae* evolved in Europe from one common Asian ancestor with European distribution range. Hungerer and Kadereit (1998) hypothesised that the ancestor of sect. *Ciminalis* was calcicole and this assumption was corroborated by the facts that many species of sect. *Calathianae* are calcicole. My findings support this hypothesis: within sect. *Gentiana*, *G. lutea* and *G. pannonica* are mainly calcicole while *G. burseri*, *G. punctata* and *G. purpurea* are mainly calcifuge. Even if relationships among the species are poorly resolved, it is more parsimonious to assume that the ancestor was calcicole, rather than calcifuge (in the first case calcifuge habit would have evolved once; in the second, two state changes would have to be postulated). The low resolution of interspecies relationships, may reflect both consistent hybridization events among species and rapid speciation processes during the Quaternary.

Concerning *G. asclepiadea*, my finding confirms its problematic position: it results related to sect. *Gentiana*, rather than to *G. pneumonanthe*. This result, congruent with those of Yuan et al. (1996), Gielly and Taberlet (1996), Mishiba et al. (2009), Davitashvili and Karrer (2010), could let to elevate the species to higher taxonomic rank, up to consider *G. asclepiadea* as a monotypic section.

As mentioned above, there is no congruence between the phylogeny inferred from nuclear and chloroplast data sets.

Both chloroplast markers show a low genetic variability within sect. *Gentiana*, indicating a widespread homogeneity of plastid genome.

My findings confirm the peculiar position of *G. lutea* subsp. *montserratii*

found by Gielly and Taberlet (1996). This subspecies shares a nuclear genome of *G. lutea* and a plastid genome of another section (my results show both *G. verna* and *G. asclepiadea* in a polytomy with it). Because cpDNA is maternally inherited, subsp. *montserratii* may be considered as an intersectional hybrid. Similar position was found for *G. asclepiadea*.

Even if sample size is limited, the little information that emerges could indicate a weak signal of both geographical hybridization and incomplete sorting of ancestral lineages. Shaw and Small (2005) highlighted that recent histories of hybridization among closely related species can homogenize or even uncouple plastid genome phylogenies from species phylogenies. This is mainly due to the uniparental inheritance of chloroplast genome: if hybridization is frequent, closely related species can share the same chloroplast genome, thereby chloroplasts may be distributed geographically instead of taxonomically. This pattern of chloroplast distribution among related species was found by several authors: Shaw et al. (2007) on *Prunus*; Dumolin-Lapegue et al. (1997) on *Quercus*; Jackson et al. (1999) on *Eucalyptus*. Within sect. *Gentiana* haplotype b4 (represented by *G. lutea* subsp. *symphyandra*, Northern Apennines), derives from b3, which includes two *G. purpurea* populations located just North, highlighting a possible (past) regional hybridization. Nowadays sect. *Gentiana* shows seven natural hybrids (Anchisi et al., 2010): this current high level of hybridization still indicates how significant the role of hybridization may have been during Quaternary speciation dynamics. On the other side, both a5 and B haplotypes are shared by different taxa from distant localities (Pyrenees and Alps, Apennines and East Europe Mountains, respectively). This pattern of plastid genome variability in sect. *Gentiana* may represent an example of incomplete sorting of ancient lineages, which ancestral polymorphisms may have persisted through speciation events. This phenomenon occurs preferentially in young species groups (Jakob and Blattner, 2006) and in rapid speciation processes (Gurushidze et al., 2010). Sect. *Gentiana* shows both these features: as mentioned above speciation of European sections of genus *Gentiana* occurred in climatic oscillation of the Quaternary (Hungerer and Kadereit, 1998), so sect. *Gentiana* shows a young age; in addition, low phylogenetic resolution of interspecies relationships, may reflect rapid speci-

ation processes. Notwithstanding, according with Jakob and Blattner (2006) it is often impossible to distinguish hybridization and incomplete lineage sorting in phylogenetic analyses.

5.3 Reproductive ecology

5.3.1 Flower phenology

In *G. lutea*, mean flower lifespan is about 3 days; it slightly varies among the populations depending on environmental variables. Pollen is generally highly viable; the lower viability found in Mt. Grande population is to ascribe to the population status, with possible consequences related to reduced fitness (see paragraph 5.4) .

G. lutea shows asynchronous inter-floral dichogamy (personal observation). Within a stem pollen and stigma presentation of different blossom are not in phase with each other, leading to both geitonogamous self-pollination and pollen discounting (Lloyd and Webb, 1986). In *G. lutea* these negative effects increase depending on inflorescence compactness: more specifically, they should be greater in subsp. *varjanii*, where pseudo-whorls of flowers are more compact, compared to subsp. *lutea* and subsp. *symphyandra* (where pseudo-whorls are less dense).

All subspecies of *G. lutea* show unordered herkogamy (Webb and Lloyd, 1986). It can be considered less structured in subsp. *lutea* and subsp. *vardjanii* (which present free anthers), and more structured in subsp. *symphyandra* (which shows connate anthers), however the distance between male and female structures is small relative to pollinator size and behaviour. According to Webb and Lloyd (1986), unordered herkogamy does not require precise pollinator behaviour, thereby unspecialised visitors may be effective pollinators. This description is consistent with *G. lutea* spectrum of pollinators, set up by numerous unspecialised taxa belonging to four different orders of insects (see paragraph 5.6.1).

The species shows a striking variation in intra-floral dichogamy. In particular my finding indicates that subsp. *lutea* and subsp. *vardjanii* are incom-

pletely protogynous; this condition differs between the two subspecies: subsp. *lutea* appears to be functionally adichogamous, by contrast, subsp. *vardjanii* presents an incomplete protogyny. On the other side subsp. *symphyandra* shows a complete protandry. My observations on subsp. *lutea* and *symphyandra* are consistent with Kozuharova and Anchev (2006). Among angiosperms, dichogamy appears to be a continuously distributed trait within families (e.g. protandry in Compositae or Lobeliaceae) or even in higher categories (notably the widespread protogyny in Magnoliidae), however in most cases it is confined to a modest number of closely related species (Lloyd and Webb, 1986 and references therein). Few studies are currently known concerning variable dichogamy within species, for example Luijten et al. (1999) indicated a variable dichogamy both within individual and within population in *Gentianella germanica* (tribe Gentianeae), suggesting that this floral trait can be extremely variable. My finding indicates that within *G. lutea* dichogamy is not a conserved trait, however it results continuously distributed within subspecies. The model of Sargent et al. (2006) predicts that both anther-stigma interference and inbreeding depression play an important role in dichogamy evolution within a species, and stressed that anther-stigma interference alone is a strong selective force, which could drive the evolution of dichogamy. My finding are consistent with their hypothesis: the low level of *G. lutea* auto-compatibility contributes to reduce the success of self-pollination and strongly limits the deleterious effects of inbreeding depression (see paragraphs 5.3.2, 5.4 and 5.5). It would suggest that the avoidance of self-fertilization is not the most important force in the evolution of *G. lutea* dichogamy, which seems more likely advantageous in avoiding interference between male and female sexual functions. Moreover, plants with large inflorescences may suffer more from anther stigma interference, primarily due to geitonogamy and consequently by pollen discounting, therefore, dichogamy is more likely to evolve under strong selection (Harder et al., 2000). This hypothesis is also supported by the relation observed between temporal distance between anthers and stigma maturation and the degree of "anthium" compactness: this time-lag is in fact longer in subsp. *vardjanii* (which shows the more compact pseudo-whorls) compared to the other subspecies. Gen-

erally, more pronounced dichogamy may promote more efficient pollination, in alleviating physical interference between anther and stigma function (Sargent et al., 2006 and references therein). In this sight, pollination efficiency of subsp. *vardjanii* is confirmed by the lower pollen-ovule ratio compared to the other subspecies (see paragraph 5.3.3).
There are no universally applicable reasons why either protandry or protogyny should be more effective in avoiding pollen-stigma interference (Lloyd and Webb, 1986). Sargent et al. (2006) suggested that protandry may evolve more easily as a by-product of floral development, conversely protogyny requires a reversal in the order of flower whorl development, and therefore the mutations required for protogyny evolution may be less likely to occur. Reproductive assurance has been invoked to explain the evolution of protogyny instead of protandry: stigma presentation before anther dehiscence favours the occurrence of cross-pollination when selfing is not allowed. By contrast, protandry favours early pollen release, increasing male fitness (Bertin and Newman, 1993). In *G. lutea* both protandry and protogyny strategies occur, highlighting the possible achievement of equilibrium status in reducing pollen-stigma interference, following both "evolutionary directions".

5.3.2 *G. lutea* breeding system

The breeding system of *Gentiana lutea* has been studied with field experiments followed by laboratory analyses, carried out in five natural populations belonging to different subspecies, over a three-years period.
Seed production is not possible without fertilization, as none of the manipulated flowers produced apomictic seed.
My findings show that fruit recovery in *G. lutea* was always 100%, regardless of pollination treatment (including agamospermy study). The same result was found by Petanidou et al. (1995) in *Gentiana pneumonanthe* and by Luijten et al. (1998) in *Gentianella germanica*. Seedless fruits remain on the plant throughout the dispersal season and apparently do not contribute to parental fitness. According to Traveset (1993) and Fuentes and Schupp (1998), these deceptive fruits may play a role in reducing seed predation, as

decoys for insects. Moreover Zangerl et al. (1991) described the inability of insect predators to discriminate between aborted and seeded fruits. Ghazoul and Satake (2009) formalized the model of the "sacrificial sibling hypothesis", considering energetic costs of fruit production on fitness. These authors considered seedless fruits as very efficient decoys since they only require investment in dry weight and do not limit the potential for outcrossed fruit. This second "function" matches with the "bet hedging hypothesis" (Stephenson, 1981), considering extra-flowers as ovules reserve to unpredictable (stochastic) fertilisation opportunities (Burd et al., 2009). My studies reveal a high predation impact on fruit production (45-55%) in two study populations and confirm what formerly observed by Kozuharova (1994), who described predation in four *Gentiana* species (including *G. lutea*) by *Thricops* ssp. larvae. It is reasonable to think that the co-occurrence of high predation impact together with seedless fruits in *G. lutea* could be regarded as a further confirmation of the 'sacrificial sibling hypothesis'.

Contrary to what claimed by Hegi (1927) and lately cited by Kèry et al. (2000), my findings reveal that *G. lutea* is a self-compatible species. The lack of differences in fruit set between controls and hand-selfed flowers, together with the Index of Automatic Self-pollination (IAS), indicate *G. lutea* as partially autogamous (Zapata and Arroyo, 1978). Due to inflorescence morphology, *G. lutea* pollination unit (anthium) is represented by the single pseudo-whorl of flowers, rather than by the single flower, therefore it is not possible to discriminate between the contributes of intra-autogamy and geitonogamy to selfing rate. In this sight the higher IAS value shown by subsp. *vadjanii*, can be explained by the greater compactness of its pseudo-whorls. The lower seed production following both autogamy treatments compared to controls, along with the values of Index of Self-Incompatibility (ISI), reveal that *G. lutea* is quite close to the lower threshold of incompletely compatible species. All these results suggest the existence of a self-incompatibility system, however with the adopted method is not possible to assess at what stage it occurs.

These findings point out the importance of pollen vectors for a successful reproduction of *G. lutea*.

Ovule production is not a conserved character in *G. lutea* and it varies both over the years and among populations. Similar results were obtained by Petanidou et al. (1995) and Oostermeijer et al. (1998) for *Gentiana pneumonanthe* and by Hofhanzlovà and Křenovà (2007) for *Gentiana pannonica*. In particular, according to Oostermeijer et al. (1998), there is no correlation between ovules number and population size, while both Petanidou et al. (1995) and Hofhanzlovà and Křenovà (2007) observed a correlation, the former with flowering season (ovules reduction in the late flowering, reflecting unfavourable environmental condition) and the latter with strong drought (related with ovules reduction). Given that, shifts in flowering season and in environmental variables can be responsible of the observed variability in ovule number of *G. lutea*.

All studied populations, under natural conditions, show a high seed production. However, the lowest seed set was recorded for Mt. Grande population. The small population is outside the known distribution range of subsp. *symphyandra*: the low reproductive output could be due to genetic drift (founder effect or bottleneck) with consequent reduction of population genetic variability. Furthermore, due to vegetative propagation, even large populations of *G. lutea* are often represented by few individuals (Georgieva, 2007). In this sight, a higher level of autogamy due to cross-pollination among stems belonging to the same genet, can explain this result.

Seed number was negatively related with mean seed weight only in Mt. Grande population, while no correlation was detected in Mt. Vettore and Passo Lusia populations. A possible explanation is given by resources limitation. In contrast with the other populations, that occur in alpine pastures, Mt. Grande population grows in a clearing within a forest, characterized by high steepness, where rain falling may flow quickly over soil, taking away parts of nutrients. In addition, the soil is not deep, due to rocky outcrops, and the area is not used for cattle grazing.

5.3.3 Resource allocation to sexual function

Pollen:ovule ratio indicates obligate xenogamy for all subspecies of *G.lutea* (Cruden, 1977). The value found for subsp. *symphyandra* matches with that obtained by Kozuharova and Anchev (2006). According to Cruden (1977), obligate xenogamous species are primarily outcrossers, protandrous or self-incompatible and require pollinators for reproduction. Insect pollination is in fact indispensable for the reproductive success of *G. lutea*, but in contrast to P/O predictive theory, the species is partially compatible.
Statistical analyses reveal differences within species, in particular subsp. *vard-janii* shows a lower P/O value compared with the other subspecies, but always within obligate xenogamy species.

5.4 Seed germination

Self-fertilized seeds show low germination rates compared to outbred offspring, probably because of post-zygotic inbreeding depression, due both to the expression of the lethal alleles, and to the presence of recessive deleterious mutation in homozygous state (Charlesworth and Charlesworth, 1987). The advantages of seed production when pollinators are absent, are thus nullified. The lack of differences in germination rate of outbred progeny (controls and pollen augmented flowers) suggests that the genetic quality of the two offspring is comparable. Since outbred seeds show very high germination rate (close to 100%), far from the values recorded for self-fertilized seeds (35-48%), is reasonable to think that cross-pollen is more competitive than self-pollen; thereby, in natural conditions and in presence of pollen vectors, the rate of autogamy seems to be negligible.
These arguments are valid for Mt. Vettore and Passo Lusia populations, while Mt. Grande population presents very low seed germination rates, despite the pattern similar to other populations. Since inbreeding depression results both from selfing and mating between relatives, its effects are greater when populations are small and isolated, and mating is casual (Ferriol et al., 2011). Mt. Grande population perfectly matches this description: the population con-

sists of few hundred flowering individuals and is strongly isolated (it occurs outside the known distribution area of subsp. *symphyandra*).
As it is known, within population local adaptation may be result from micro-geographical differentiation in selection pressure (Turner et al., 1982). Outcrossing over long distances might lead to reduced fitness due to the disruption of co-adapted gene-complexes with the consequent decreased habitat adaptation (Templeton, 1986; Oostermeijer et al., 1982). Hence a test of outbreeding depression might be considered before planning conservation management practices of genetic rescue.
The time required for seed to germinate was significantly different among subspecies of *G. lutea*: in particular, subsp. *lutea* and subsp. *vardjanii* showed the shortest germination times, subsp. *montserratii* an intermediate time and subsp. *symphyandra* the longest.

5.5 Inbreeding depression

The cumulative index of inbreeding depression (Ågren and Schemske, 1993 and Goodwillie and Knight, 2006), highlights the advantage of outbred offspring compared to self-fertilized. Fruit set, seed set and germination rate are the traits that more contribute to reduce the fitness of selfed progeny, mainly limited by self-incompatibility and inbreeding depression.

5.6 Plant-pollinator interaction

5.6.1 Flower pollinators

The pollination system of *Gentiana lutea* has been studied with field observations followed by laboratory analyses, carried out in three natural populations, over two study-years each. All populations were actively visited by insects.
My findings show that *G. lutea* is a generalist species: numerous taxa, belonging to four different orders of insects, were observed visiting its flowers. Pollination syndrome is consistent with observations: flowers show short

corolla tube, both nectar and pollen are easily accessible by insects without need of pronounced coadaptive specialization.
Nevertheless, among the wide spectrum of visitors, it is possible to recognize two main categories: "nectar thieves" and pollinators, and within pollinators insects with sedentary or dynamic behaviour.
Where present, ants showed a very high frequency of visits. However, since they did not carry *G. lutea* pollen, they did not offer any pollination service. For this reason they can be considered as nectar thieves, acquiring nectar by foraging between corolla lobes in a non-destructive manner (Inouye, 1980). Nectar thieves may modify nectar quantity and quality: any modification could bias changes in pollinators foraging behaviour, which may alter the reproductive success of plants (Gonzàlez-Gòmez and Valdivia, 2005).
Bees, bumblebees, wasps, ichneumon wasps, sawflies and hoverflies showed a dynamic behaviour during their foraging activity. All of them actively touched reproductive structures, except for two species of ichneumon wasps (showing lower frequencies).
Bees (*Apis mellifera* and *Lasioglossum* species) revealed comparable behaviours, foraging for nectar and pollen. Bumblebees fed mainly on nectar, and rarely on pollen: nectar is used for self-feeding or for dampen pollen in nest cells, while pollen is collected by workers as larval food provisioning. In Mt. Vettore and Passo Lusia populations, among *Bombus*, both "true" bumblebees and "cuckoo" bumblebees occurred. Cuckoo bumblebees are a specialized lineage of social parasites which have lost both social behaviour and ability to collect pollen, and are instead cleptoparasites of the colonies of "true" bumblebees. The high percentage of male individuals, mainly belonging to cuckoo bumblebees, may explain the preference of nectar as floral reward. In addition, from field observations emerge their tendency to became sluggish. This peculiar behaviour was shown exclusively from individuals foraging for nectar (personal observation) and not from those seeking pollen. Due to this changeable behaviour, their pollinators role shifts from insects with dynamic activity, to insects with substantially sedentary behaviour. Similar observations were reported by Adler (2000), Jakuska et al. (2005) and Herrera et al. (2008) who indicated alcohols (derived from nectar micro-

bial fermentation), as the main cause of insect behaviour. Staphenson (1981) observed disorientation and narcosis related with iridoid glycosides in *Catalpa speciosa*.

Ichneumon wasps fed on nectar. These insects are important parasitoids, whose common hosts are larvae and pupae of Coleoptera, Hymenoptera, and Lepidoptera. According to Kozuharova et al. (1994) they limit the reproduction of pests in both *G. lutea* and *G. punctata*, nevertheless their presence is not connected with pollination but rather with biological defence against pests. This result is partially coherent with my findings: although not all species are active pollinators, both the frequency of visits and fidelity suggest a role in *G. lutea* pollination. Wasps foraged for nectar and touched reproductive structures. Similarly to what I found for bumblebees, wasps also include an obligate social parasite (*P. sulcifer* - Cervo, 2006). Sawflies had a marginal role in pollination and hoverflies fed on pollen both from dehiscent anthers and mature stigmas. Due to this foraging behaviour, hoverflies may be viewed with a critical sight: on one side they contribute to cross-pollination, on the other they can feed on viable pollen directly from mature stigmas contributing to pollen discounting and decreasing the likelihood of pollen germination.

Two taxa show sedentary activity: flies (Muscomorpha) and coleopterans. All observed species usually creep inside the flower, or from one to another, however mostly within the same pseudo-whorl. Flies visit *G. lutea* flowers both for nectar and pollen, actively touching reproductive structures during their foraging activity. Their frequency of visits can reach very high values (as noted in Passo Lusia population), however due to their small body size and the scarcity of body hairs, their pollination role is inconstant. Similarly, coleopteran species feed on nectar and/or pollen, actively or occasionally touching floral reproductive structures. The behaviour of both taxa reveal a negative pollination performance, since they play a potential role in increasing both pollen discounting and geitonogamy. In particular, given the weak level of self-compatibility of *G. lutea*, sedentary pollinators might mainly damage male fitness components by increasing pollen discounting. My finding on the pollination role of flies is in contrast with Kozuharova,

who indicated flies (*Thricops* spp.) as active visitors and main pollinators of *G. lutea*, and described their positive role as pollinators in cool and wet weather, when bumblebees are absent (Kozuharova et al., 1994). Moreover, according to Kozuharova et al. (2005), since they are pollen eaters, they may contribute to the separation of male and female stages favouring prevention of intra-flower self pollination.

My observations reveal that a great part of *G. lutea* fruits were destroyed by larvae, probably belonging to Muscomorpha (personal observation). Similar results were found by Kozuharova (1994), who reported predation by *Thricops* spp. in *G. lutea*, *G. punctata*, *G. asclepiadea* and *G. cruciata*. All these findings highlight the detrimental effect of Muscomorpha both during pollination process and fruit production (pre-dispersal seeds predation).

G. lutea is seldom visited also by specialized pollinators such as the lepidopterans *Sideridis reticulata* and *Macroglossum stellatarum*, which would play a marginal role in pollination.

Difference in PI values among populations depend on the frequency of pollinator visits, which in turn are influenced by the abundance of rewarding co-flowering species.

Mt. Vettore population was characterized by a high percentage of ants visits, which may negatively affect population reproductive success by disrupting the visits of effective pollinators and thus reducing pollination likelihood (ants may act as antagonists). However this population did not show a lower reproductive success compared to the others, highlighting the neutral effect of ants on pollination dynamics. Bumblebees showed the highest PI value, followed by ichneumon wasps and common wasps: these taxa represent the effective pollinators, which mainly contribute to increase cross-pollination. Among pollinators with sedentary behaviour, flies (Muscomorpha) revealed a high PI value, similar to that of bumblebees.

In Mt. Grande population, the spectrum of pollinators includes bumblebees, halictid bees and hoverflies as dynamic pollinators while flies (Muscomorpha) and beetles as sedentary ones. The Pollinator Importance index indicated a very high importance of bumblebees, due both to frequency of visits and fidelity, and a lower importance of halictid bees. This population shows a

spectrum of pollinators mainly set up by dynamic insects and efficient pollen vectors.
By contrast, Passo Lusia population shows an important presence of sedentary pollinators. Flies (Muscomorpha) show the highest PI value, followed by beetles, with PI value comparable to that of the main dynamic pollinator (*Apis mellifera*). Syrphids, bumblebees and halictid bees exhibited the lowest values of pollinator importance.
In conclusion, among the study populations, the one of Mt. Grande shows the best pollinators performance, followed by Mt. Vettore population (where sedentary pollinators show a limited impact) and Passo Lusia population, where the importance of sedentary pollinators exceeds that of dynamics. All these findings confirmed what was assessed by Herrera (1990) and Navarro (1999): not always the most effective pollinator is the most abundant floral visitor and, moreover, in some plant-pollinator systems the most abundant floral visitor is not actually a pollinator.

5.6.2 Floral rewards: nectar

Nectar standing crop analysis and pollinator surveys indicate nectar as main reward. The lower nectar volumes found in the afternoon suggest a connection with pollinators activity, which in turn depends on environmental variables, such as temperature, relative humidity, wind speed and solar radiation (Potts, 2005).
Chromatographic analysis reveals that *G. lutea* nectar is rich in hexose. According to Petanidou (2007) hexose-rich nectar is easy to digest and adapted to consumption by an extensive array of mainly non-specialized pollinators. This observation is perfectly consistent with spectra of pollinators of all study populations, however in Mt. Grande population a majority of more specialized pollinators (bumblebees) occurs.
Nectar composition showed a significant presence of β-alanine and proline, (non-protein/protein amino acid, respectively). Bertazzini et al. (2010) performed dual choice feeding tests, highlighting a clear preference of forager honeybees for nectar containing proline compared to that containing only

sugars; similar behaviours were found out by Rathman et al. (1990) and by Erhardt and Rusterholz (1998). Proline has been proposed as energy substrate to fuel the earliest or most expensive stages of insect flight activity: in this sight proline content in nectar could be regarded as coevolution strategy to increase plant visitation and thereby plant fitness (Bertazzini et al., 2010 an references therein). Concerning non-toxic amino acids, a few of them, including β-alanine, are known to accumulate in nectar, but whether they have any role in attraction of pollinators must await further studies (Nicolson and Thornburg, 2007). Additional studies of nectar preference (nectar proline and β-alanine rich vs. nectar containing only sugars) carried out with bumblebees, could clarify their role in pollinators attraction. However, the effect of proline and β-alanine on cuckoo bumblebees would be hard to investigate due to their ecology and to the difficulty to use test-colonies in controlled conditions.

5.6.3 Pollen limitation

In addition to surveys on pollinators behaviour, I studied the occurrence of pollen limitation. My findings reveal the evidence of pollen limitation along time and space: in 2010 for Mt. Vettore and Mt. Grande populations and in both study years for Passo Lusia population. Reduced seed set can be a consequence of low pollen quantity or quality, and hence it can occur also after sufficient pollinator visits (Kephart, 2005). The high pollinators activity observed in all populations over the study period, leads to think that pollen limitation could be mainly explained by plant-pollinator interference rather than by insufficient visits. In particular, the composition of pollinator spectrum and pollinator dynamism may change the contribution of geitonogamous pollination. In this sight, in Passo Lusia population -where the importance of sedentary pollinators exceeds that of dynamics- pollen limitation was observed in both years, while in Mt. Vettore and in Mt. Grande populations pollen limitation was detected only in one of the two study years. According to Cosacov et al. (2008) self-incompatible species with multiple flowers per plant may be particularly prone to pollen limitation by quality,

if geitonogamous incompatible pollen is deposited on their stigmas. This description of pollen limitation in multiple flowers species fits very well with *G. lutea*: in fact despite the species shows an incomplete compatibility, it is close to the lower threshold of compatibility.

Chapter 6

Conclusions

Historical data, obtained from taxonomic analysis, confirm the known geographical distribution of *G. lutea*, thereby actually, Mt. Grande is the only documented population occurring outside the known distribution range. This could be due to a synanthropic origin of the population, or to a mutation event in the genes involved in anthers development. Considering subspecies diagnostic-characters, stigma shape after anthesis may be used as an additional feature to identify subsp. *symphyandra* and subsp. *vardjanii*, while bracts length represents a reliable character (but not exclusive) to identify subsp. *vardjanii*, hence in order to undoubtedly identify the subspecies, other traits have to be considered.

The phylogenetic hypotheses obtained from nuclear and chloroplast data sets are not congruent. The phylogeny obtained from nuclear sequences shows that sect. *Gentiana* is monophyletic, and within it, *G. lutea* identity is strongly supported. Similarly, subsp. *vardjanii* shows a clear subspecies identity, despite the sympatry with subsp. *symphyandra*. As anthesic periods do not overlap, a reproductive barrier can explain this present isolation: this feature combined with its genetic identity, could lead to consider subsp. *vardjanii* as an example of speciation in progress. Both *G. pannonica* and *G. punctata* show species identity; while *G. purpurea* and *G. burseri* are not monophyletic: a vicariance model of speciation could explain the genetic differentiation within these two species. Even if relationships among species of

sect. *Gentiana* are poorly resolved, my findings support the hypothesis of Hungerer and Kadereit (1998), according to which the European sections evolved from a calcicole ancestor. The problematic position of *G. asclepiadea* was confirmed: this result is congruent with those of other authors and could lead to elevate *G. asclepiadea* to higher taxonomic rank, up to consider the species as a monotypic section.
Phylogeny inferred from plastid markers confirms the peculiar position of subsp. *montserratii*, which may represent an intersectional hybrid. The little information that emerges from chloroplast phylogeny could indicate a weak signal of both geographical hybridization and incomplete sorting of ancestral lineages. The current high level of hybridization still indicates how significant the role of hybridization may have been within sect. *Gentiana*.
G. lutea presents asynchronous inter-floral dichogamy. All subspecies show unordered herkogamy, which can be considered less structured in subsp. *lutea* and subsp. *vardjanii*, and more structured in subsp. *symphyandra*. The species shows a striking variation in intra-floral dichogamy: subsp. *lutea* appears to be functionally adichogamous, subsp. *vardjanii* presents an incomplete protogyny and subsp. *symphyandra* shows a complete protandry. The low level of *G. lutea* auto-compatibility reduces the success of self-pollination and strongly limits the deleterious effects of inbreeding depression. It would suggest that the avoidance of self-fertilization is not the most important force in the evolution of dichogamy, which seems more likely advantageous in limiting pollen-stigma interference. This hypothesis is supported by the fact that this time-lag is longer in subsp. *vardjanii*, which shows the more compact pseudo-whorls.
Seed production is not possible without fertilization, while fruit recovery was always 100%. The co-occurrence of high predation impact combined with seedless fruits could be regarded as a confirmation of the "sacrificial sibling hypothesis". *G. lutea* is a self-compatible species, however the lower production of seeds derived from autogamy indicates an incomplete-compatibility, suggesting the existence of a self-incompatibility system. Ovule production is not a conserved character in *G. lutea*: shifts in flowering season and in environmental variables may be responsible for this variability. Pollen:ovule

ratio indicates obligate xenogamy for all subspecies: insect pollination is in fact indispensable for the reproductive success, however in contrast to P/O predictive theory, the species is partially compatible.
Self-fertilized seeds show low germination rates compared to outbred offspring, probably due to post-zygotic inbreeding depression, thereby the advantages of seed production when pollinators are absent, are thus nullified.
Concerning reproductive success, Mt. Grande population shows the lowest seed production and the lowest seed germination rate. Given the population small size and isolation, it could be due to genetic drift with consequent effects of inbreeding depression. Hence a test of outbreeding depression might be considered as the first step before planning conservation management practices of genetic rescue.
My findings show that *G. lutea* is a generalist species: numerous taxa, belonging to four different orders of insects, were observed. Nevertheless, among the wide spectrum of visitors, it is possible to recognize "nectar thieves" and pollinators (with sedentary or dynamic behaviour). Among the study populations, the one of Mt. Grande shows the best pollinators performance, followed by Mt. Vettore population (where sedentary pollinators show a limited impact) and Passo Lusia population, where the importance of sedentary pollinators exceeds that of dynamics.
My findings reveal the evidence of pollen limitation along time and space. The high pollinators activity observed in all populations, and the greater impact of pollen limitation in Passo Lusia population, lead to think that it could be mainly explained by poor pollen quality rather than by insufficient visits.
Chromatographic analysis reveals that *G. lutea* nectar is rich in hexose and shows a significant presence of β-alanine and proline. Additional studies of nectar preference carried out with bumblebees, could clarify their role in pollinators attraction.

Appendix

Figure 1: *G. lutea* subsp. *lutea*: flowering stems.

Figure 2: *G. lutea* subsp. *lutea*: flower.

Figure 3: *G. lutea* subsp. *lutea*: capsule.

Figure 4: *G. lutea* subsp. *montserratii*: seeds.

Figure 5: *G. lutea* subsp. *lutea*: rhizome.

Figure 6: *G. lutea* subsp. *lutea*: vegetative stem with internodes.

Figure 7: *G. lutea* subsp. *symphyandra*: flower with connate anthers.

Figure 8: *G. lutea* subsp. *vardjanii*: flowering stem with yellowish floral bracts longer than pseudo-whorls.

Figure 9: *G. lutea* subsp. *vardjanii*: vegetative stem without internodes.

Figure 10: *G. lutea* subsp. *montserratii*: flowering stem.

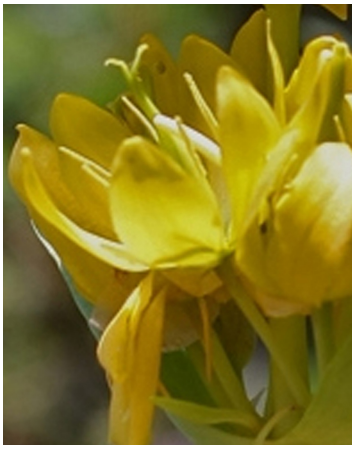

Figure 11: *G. lutea* subsp. *montserratii*: flower with ovate corolla lobe.

Figure 12: *G. burseri* subsp. *burseri*: flowering stem.

Figure 13: *G. burseri* subsp. *burseri*: flower.

Figure 14: *G. burseri* subsp. *villarsii*: flower.

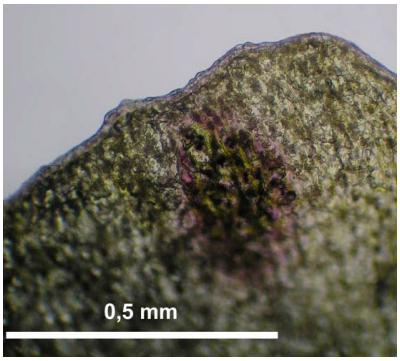

Figure 15: *G. burseri* subsp. *villarsii*: margin of calyx (Polidori, 2008).

Figure 16: *G. burseri* subsp. *actinocalyx*: flowers (Polidori, 2008).

Figure 17: *G. burseri* subsp. *actinocalyx*: calyx (Polidori, 2008).

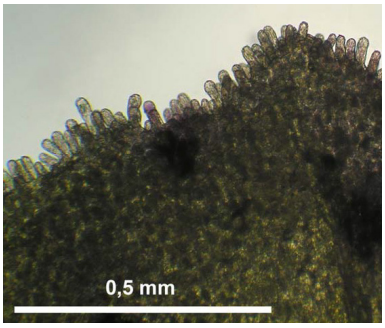

Figure 18: *G. burseri* subsp. *actinocalyx*: corolla papillae (Polidori, 2008).

Figure 19: *G. punctata*: flowering stems.

Figure 20: *G. punctata*: flowers.

Figure 21: *G. purpurea*: flowering stems.

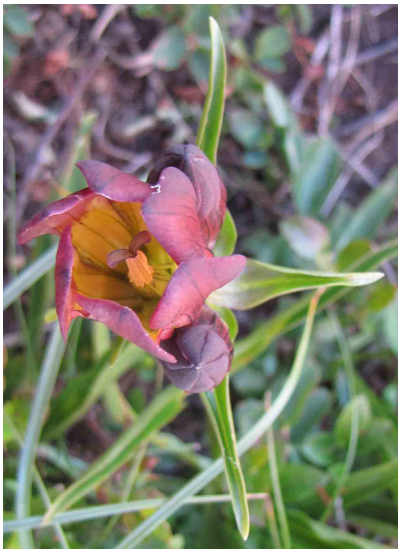

Figure 22: *G. purpurea*: flowers.

Figure 23: *G. pannonica*: flowering stems.

Figure 24: *G. pannonica*: flowers.

Figure 25: Ichneumoninae sp. in *G. lutea* subsp. *lutea*.

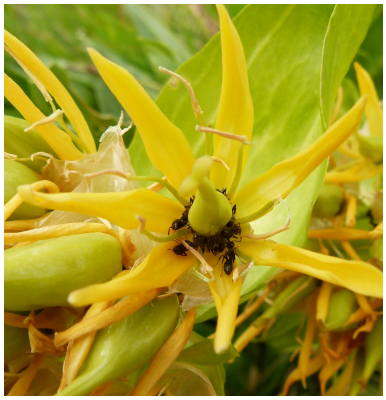

Figure 26: Formicidae sp. in *G. lutea* subsp. *lutea*.

Figure 27: *Bombus lapidarius* in *G. lutea* subsp. *symphyandra*.

Figure 28: *Episyrphus balteatus* in *G. lutea* subsp. *symphyandra*.

Figure 29: Syrphidae sp. in *G. lutea* subsp. *vardjanii*.

Figure 30: Muscidae sp. in *G. lutea* subsp. *vardjanii*.

Bibliography

338/97 Regulation (EC). 3.3.1997. *Council Regulation 338/97 of 9 December 1996 on the protection of species of wild fauna and flora by regulating trade therein.* OJL, 61.

92/43/EEC Directive. 22.07.1992. *Council Directive 92/43/EEC of 21 May 1992 on the Conservation of Natural Habitats and of Wild Fauna and Flora.* OJL, 206:7–50.

Aberham A, Schwaiger S, Stuppner H, and Ganzera M. 2007. *Quantitative analysis of iridoids, secoiridoids, xanthones and xanthone glycosides in Gentiana lutea L. roots by RP-HPLC and LC-MS.* Journal of Pharmaceutical and Biomedical Analysis, 45:437–442.

Adler LS. 2000. *The ecological significance of toxic nectar.* Oikos, 91:409–420.

Ågren J and Schemske DW. 1993. *Outcrossing rate and inbreeding depression in two annual monoecious herbs, Begonia hirsuta and B. semiovata.* Evolution, 47:125–135.

Alvarez I and Wendel JF. 2003. *Ribosomal ITS sequences and plant phylogenetic inference.* Molecular Phylogenetics and Evolution, 29(3):417–434.

Anchisi E, Bernini A, Cartasegna N, and Polani F. 2010. *Genziane d'Europa.* Verba&Scripta, Pavia.

Baker AM, Burd M, and Climie KM. 2005. *Flowering phenology and sexual allocation in single-mutation lineages of Arabidopsis thaliana.* Evolution, 59:970–978.

Baldwin BG and Markos S. 1998. *Phylogenetic utility of the external transcribed spacer (ETS) of 18S-26S rDNA: congruence of ETS and ITS trees of Calycadenia (Compositae).* Molecular Phylogenetics and Evolution, 10: 449–463.

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, and Donoghue MJ. 1995. *The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny.* Annals of the Missouri Botanical Garden, 82(2):247–277.

Barrett SCH. 2002. *Sexual interference of the floral kind.* Heredity, 88: 154–159.

Bazzaz FA and Grace J. 1997. *Plant Resource Allocation (Physiological Ecology).* Academic Press, San Diego - California - USA.

Berjano R, Arista M, Ortiz PL, and Talavera S. 2011. *Persistently low fruiting success in the Mediterranean pipevine Aristolochia baetica (Aristolochiaceae): a multi-year study.* Plant Biology, 13 (1):109–117.

Bertazzini M, Medrzycki P, Bortolotti L, Maistrello L, and Forlani G. 2010. *Amino acid content and nectar choice by forager honeybees (Apis mellifera L.).* Amino Acids, 39:315–318.

Bertin RI and Newman CM. 1993. *Dichogamy in angiosperms.* The Botanical Review, 59:112–152.

Birks HH. 1994. *Plant macrofossils and the nunatak theory of perglacial survival.* Dissertationes botanicae, 234:129–143.

Blattner FR. 1999. *Direct amplification of the entire ITS region from poorly preserved plant material using recombinant PCR.* Biotechniques, 27:1180–1186.

Bond WJ. 1994. *Do mutualisms matter? Assessing the impact of pollinator and disperser disruption on plant extinction.* Philosophical Transactions of the Royal Society, 344:83–90.

Brunet J. 2005. *Plant breeding systems and pollen dispersal - Plant-pollinator interactions an pollen dispersal. In: Dafni A, Kevan PG, Husband BC (Eds) Practical pollination biology.* Enviroquest, Ltd., Cambridge, Ontario, Canada, pp 56-82.

Burd M. 2011. *Are relationships between pollen-ovule ratio and pollen and seed size explained by sex-allocation?* Evolution, 65 (10):3002–3005.

Burd M, Ashman TL, Campbell DR, Dudash MR, Johnston MO, Knight TM, Mazer SJ, Mitchell RJ, Steets JA, and Vamosi JC. 2009. *Ovule number per flower in a world of unpredictable pollination.* American Journal of Botany, 96:1159–1167.

Campbell DR. 2000. *Experimental tests of sex-allocation theory in plants.* Trends in Ecology and Evolution, 15 (6) - review:227–231.

Carbonnier J, Massias M, and Molho D. 1977. *Importance taxonomique du schèma de substitution des xanthones chez Gentiana L.* Bullettin du Museum National d'Historie Naturelle (Paris) - sèrie 3 n. 504 - Sciences physique-chimique, 13:23–40.

Cervo R. 2006. *Polistes wasps and their social parasites: an overview.* Annales Zoologici Fennici, 43:531–549.

Charlesworth D. 2006. *Evolution of Plant Breeding Systems - Review.* Current Biology, 16:R726–R735.

Charlesworth D and Charlesworth B. 1981. *Allocation of resources to male and female function in hermaphrodites.* Biological Journal of the Linnean Society, 15:57–74.

Charlesworth D and Charlesworth B. 1987. *Inbreeding depression and its evolutionary consequences.* Annual Review of Ecology and Systematics, 18:237–268.

Charnov EL. 1982. *The theory of sex allocation.* Princeton University Press, Princeton, New Jersey, USA.

Clement M, Posada D, and Crandall KA. 2000. *STCS: a computer program to estimate gene genealogies.* Molecular Ecology, 9:1657–1659.

Conte L and Cristofolini G. 1992. *Assessment of RAPD variation in Abies nebrodensis (Lojac.) Mattei (Pinaceae) using haploid tissue analysis.* Israel Journal of Plant Sciences, 51(3):199–206.

Cosacov A, Nattero J, and Cocucci AA. 2008. *Variation of pollinator assemblages and pollen limitation in a locally specialized system: the oil-producing Nierembergia linariifolia (Solanaceae).* Annals of Botany, 102:723–734.

Cruden RW. 1977. *Pollen-Ovule ratios: a conservative indicator of breeding systems in flowering plants.* Evolution, 31(1):32–46.

Dafni A and Motte-Mauès MM. 1998. *A rapid and simple procedure to determine stigma receptivity.* Sexual Plant Reproduction, 11:171–180.

Dafni A, Pacini E, and Nepi M. 2005. *Pollen and stigma biology. In: Dafni A, Kevan PG, Husband BC (Eds) Practical pollination biology.* Enviroquest, Ltd., Cambridge, Cambridge, Ontario, Canada, pp 83-146.

Dahl E. 1990. *History of the Scandinavian alpine flora. In Gjærevoll O - Maps of distribution of Norvegian vascular plants. II - Alpine plants.* Tapir Publications, Trondheim.

Darwin CR. 1859. *On the Origin of Species.* John Murray, London.

Darwin CR. 1862. *On the various contrivances by which British and foreign orchids are fertilized by insects.* John Murray, London.

Davitashvili N and Karrer G. 2010. *Taxonomic importance of seed morphology in Gentiana (Gentianaceae).* Botanical Journal of the Linnean Society, 162:101–115.

De Jong TJ, Waser NM, and Klinkhamer PGL. 1993. *Geitonogamy – the neglected side of selfing.* Trends in Ecology and Evolution, 8:321–325.

De Nettancourt D. 1997. *Incompatibility in angiosperms.* Sexual Plant Reproduction, 10:185–199.

Demesure B, Sodzi N, and Petit RJ. 1995. *A set of universal primers for amplification of polymorphic noncoding regions of mitochondrial and chloroplast DNA in plants.* Molecular Ecology, 4:129–131.

Diadema K, Bretagnolle F, Affre L, Yuan YM, and Medail F. 1997. *The monophyly and rapid evolution of Gentiana sect. Chondrophyllae Bunge s.l. (Gentianaceae) evidence for nucleotide sequences of the internal transcribed spacers (ITS) of nuclear ribosomal DNA.* Botanical Journal of the Linnean Society, 123:25–43.

Dumolin-Lapegue S, Demesure B, Fineschi S, Le Corre V, and Petit RJ. 1997. *Phylogeographic structure of white oaks throughout the European continent.* Genetics, 146:1475–1487.

Erhardt A and Rusterholz HP. 1998. *Do peacock butterflies (Inachis io L.) detect and prefer nectar amino acids and other nitrogenous compounds?* Oecologia, 117:536–542.

Fahn A. 1979. *Secretory tissues in plants.* Academic Press, London, UK.

Ferriol M, Pichot C, and Lefèvre F. 2011. *Variation of selfing rate and inbreeding depression among individuals and across generations within an admixed Cedrus population.* Heredity, 106 (1):146–157.

Fisogni A, Cristofolini G, Rossi M, and Galloni M. 2011. *Pollinator directionality as a response to nectar gradient: promoting outcrossing while avoiding geitonogamy.* Plant Biology, 13(6):848–856.

Fuentes M and Schupp E. 1998. *Deceptive fruits reduce seed predation by birds in Juniperus osteosperma.* Evolutionary Ecology, 12:823–827.

Gabrielsen TM, Bachmann K, Jakobsen KS, and Brochmann C. 1997. *Glacial survival does not matter: RAPD phylogeography of Nordic Saxifraga oppositifolia.* Molecular Ecology, 6:831–842.

Galetto L and Bernardello G. 2005. *Rewards in flowers - nectar. In: Dafni A, Kevan PG, Husband BC (Eds) Practical pollination biology.* Enviroquest, Ltd., Cambridge, Cambridge, Ontario, Canada, pp 261-328.

Galloni M, Podda L, Vivarelli D, and Cristofolini G. 2007. *Pollen presentation, pollen-ovule ratios, and other reproductive traits in Mediterranean Legumes (Fam. Fabaceae - Subfam. Faboideae).* Plant Systematics and Evolution, 266:147–164.

Galloni M, Podda L, Vivarelli D, Quaranta M, and Cristofolini G. 2008. *Visitor diversity and pollinator specialization in Mediterranean legumes.* Flora, 203:94–102.

Gauthier P, Debussche M, and Thompson JD. 2010. *Regional priority setting for rare species based on a method combining three criteria.* Biological Conservation, 143:1501–1509.

Georgieva E. 2007. *Ecological, biological and phytochemical peculiarities of Gentiana lutea L. and Gentiana punctata L. in Bulgaria.* D. Phil. Thesis, Sofia.

Ghazoul J and Satake A. 2009. *Nonviable seed set enhances plant fitness: the sacrificial sibling hypothesis.* Ecology, 90 (2):369–377.

Gibson RH, Nelson IL, Hopkins GW, Hamlett BJ, and Memmott J. 2006. *Pollinator webs, plant communities and the conservation of rare plants: arable weeds as a case study.* Journal of Applied Ecology, 43:246–257.

Gielly L and Taberlet P. 1996. *A phylogeny of the European gentians inferred from chloroplast trnL (UAA) intron sequences.* Botanical Journal of the Linnean Society, 120:57–75.

Gonzàlez-Gòmez PL and Valdivia CE. 2005. *Direct and indirect effects of nectar robbing on the pollinating behavior of Patagona gigas (Trochilidae).* Biotropica, 37:693–696.

Goodwillie C and Knight MC. 2006. *Inbreeding depression and mixed mating in Leptosiphon jepsonii: a comparison of three populations.* Annals of Botany, 98:351–360.

Gurushidze M, Fritsch1 RM, and Blattner FR. 2010. *Species-level phylogeny of Allium subgenus Melanocrommyum: incomplete lineage sorting, hybridization and trnF gene duplication.* Taxon, 59(3):829–840.

Hale ML, Borland AM, Gustafsson MHG, and Wolff K. 2004. *Causes of size homoplasy among chloroplast microsatellites in closely related Clusia species.* Journal of Molecular Evolution, 58:182–190.

Hamilton MB. 1999. *Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation.* Molecular Ecology, 8:513–525.

Harder LD and Wilson WG. 1998. *A Clarification of pollen discounting and its joint effects with inbreeding depression on mating system evolution.* The American Naturalist, 152 (5):684–95.

Harder LD, Barrett SCH, and Cole WW. 2000. *The mating consequences of sexual segregation within inflorescences of flowering plants.* The Royal Society, 267:315–320.

Hegi G. 1927. *Illustrierte Flora Von Mitteleuropa.* JF Lehmenns-Verlag, München, Germany.

Herrera CM. 1990. *Daily patterns of pollinator activity, differential pollinating effectiveness, and floral resource availability, in a summer-flowering Mediterranean shrub.* Oikos, 58:277–288.

Herrera CM, Garcìa IM, and Pèrez R. 2008. *Invisible floral larcenies: microbial communities degrade floral nectar of bumble bee-pollinated plants.* Ecology, 89(9):2369–2376.

Hesse E, Rees M, and Müller-Schàrer H. 2007. *Seed bank persistence of clonal weeds in contrasting habitats: implications for control.* Plant Ecology, 190: 233–243.

Hiscock SJ and McInnis SM. 2003. *The diversity of Self-Incompatibility systems in flowering plants.* Plant Biology, 5:23–32.

Ho TN and Liu SW. 1990. *The infrageneric classification of Gentiana (Gentianaceae).* Bullettin of the British museum (Natural History) - Botany series, 20:169–192.

Ho TN and Liu SW. 2001. *A wordwide monograph of Gentiana.* Science Press, Beijing and New-York.

Ho TN, Liu SW, and Lu XF. 1996. *A phylogenetic analysis of Gentiana (Gentianaceae).* Acta Phytotaxonomica Sinica, 34(5):505–530.

Hofhanzlovà E and Fèr T. 2009. *Genetic variation and reproduction strategy of Gentiana pannonica in different habitats.* Flora, 204:99–110.

Hofhanzlovà E and Křenovà Z. 2007. *Pollination strategy and reproductive success of Gentiana pannonica in a natural population.* Silva Gabreta, 13 (2):83–94.

Hungerer KB and Kadereit JW. 1998. *The phylogeny and biogeography of Gentiana L. sect. Ciminalis (Adans.) Dumort.: a historical interpretation of distribution ranges in the European high mountains.* Perspectives in Plant Ecology, 1(1):121–135.

Inouye DW. 1980. *The terminology of floral larceny.* Ecology, 61:1251–1252.

IUCN. 2001. *IUCN Red List categories and criteria: version 3.1, IUCN Species Survival Commission.* Glan, Switzerland and Cambridge, UK.

Jackson HD, Steane DA, Potts BM, and Vaillancourt RE. 1999. *Chloroplast DNA evidence for reticulate evolution in. Eucalyptus (Myrtaceae).* Molecular Ecology, 8:739–751.

Jakob SS and Blattner FR. 2006. *A chloroplast genealogy of Hordeum (Poaceae): long-term persisting haplotypes, incomplete lineage sorting, regional extinction, and the consequences for phylogenetic inference.* Molecular Biology and Evolution, 23(83):1602–1612.

Jakuska A, Przado D, Steininger M, Aniol-Kwiatkowska J, and Kadej M. 2005. *Why do pollinators become 'sluggish'? Nectar chemical constituents from Epipactis helleborine (L.) Crantz (Orchidaceae).* Applied Ecology and Environmental Research, 3(2):29–38.

Johnson SD and Anderson B. 2010. *Coevolution between food-rewarding flowers and their pollinators.* Evolution: Education and Outreach, 3:32–39.

Judd WS, Campbell CS, Kellog EA, Stevens PF, and Donoghue MJ. 2007. *Taxonomy. In Plant Systematics - A Phylogenetic Approach, Third Edition.* Sinauer Associates, Sunderland.

Kadereit G, Ball P, Beer S, Mucina L, Sokoloff D, Teege P, Yaprak AE, and Freitag H. 2007. *A taxonomic nightmare comes true: phylogeny and biogeography of glassworts (Salicornia L., Chenopodiaceae).* Taxon, 56(4): 1143–1170.

Kay KM, Whittall JB, and Hodges SA. 2006. *A survey of nuclear ribosomal internal transcribed spacer substitution rates across angiosperms: an approximate molecular clock with life history effects.* Evolutionary Biology, 6(36):1–9.

Kephart S. 2005. *Pollen-pollinator limitation - Plant-pollinator interface. In: Dafni A, Kevan PG, Husband BC (Eds) Practical pollination biology.* Enviroquest, Ltd., Cambridge, Cambridge, Ontario, Canada, pp 364-370.

Kèry M, Matthies D, and Spillmann HH. 2000. *Reduced fecundity and offspring performance in small populations of the declining grassland plants Primula veris and Gentiana lutea.* Journal of Ecology, 88:17–30.

Knight TM, Steets JA, Vamosi JC, Mazer SJ, Burd M, Campbell DR, Dudash MR, Johnston MO, Mitchell RJ, and Ashman TL. 2005. *Pollen limitation of plant reproduction: pattern and process.* Annual Review of Ecology, Evolution, and Systematics, 36:467–497.

Körner C. 2003. *Alpine plant life, 2nd edn.* Springer, Berlin.

Kozuharova EK. 1994. *Modes of polination of some Gentiana L. species distributed in Bulgaria.* Annuals of the University of Sofia.

Kozuharova EK and Anchev ME. 2006. *Nastic corolla movements of nine Gentiana species (Gentianaceae), presented in the Bulgarian flora.* Phytologia Balcanica, 12(2):255–265.

Kozuharova EK, Shishinjova MD, and Tonkov SB. 1994. *Contribution to the investigation on pollination of populations of Gentiana punctata L. and Gentiana lutea L. in Vitosha Mountains.* Annuals of the University of Sofia.

Kozuharova EK, Lavchiev V, and Lennartsson T. 2005. *Flies of the genus Trichops (Muscidae, Diptera) as pollinators of plants with bell-shaped flowers in the mountains of Bulgaria.* Studia Dipterologica, 12:409–418.

Kumar A, Chowdhury RK, and Dahisa OS. 1995. *Pollen viability and stigma acceptivity in relation to meteorological parameters in pearl millet.* Seed Science Technology, 23:147–156.

Kusnezow NI. 1896. *Subgenus Eugentiana Kusnez. generis Gentiana Tournef.* Acta Horti Petropolitani, 15:1–507.

Lia DZ, Gaoa LM, Lia HT, Wanga H, Gea XJ, Liua JQ, Chena ZD, Zhoua SL, Chena SL, Yanga JB, Fua CX, Zenga CX, Yana HF, Zhua YJ, Suna YS, Chena SY, Zhaoa L, Wanga K, Yanga T, and Duana GW. 2011. *China Plant BOL Group - Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants.* Proceedings of the National Academy of Sciences, 108(49):19641–19646.

Lloyd D and Schoen D. 1992. *Self-and cross-fertilization in plants. I. Functional dimensions.* International Journal of Plant Sciences, 153:358–369.

Lloyd GD and Webb CJ. 1986. *The avoidance of interference between the presentation of pollen and stigmas in angiosperms I.Dicogamy.* New Zealand Journal of Botany, 24:135–162.

Löve Á and Löve D. 1963. *North Atlantic Biota and Their History.* Pergamon Press, Oxford.

Löve A and Löve D. 1972. *Favargera and Gentianodes, two new genera of alpine Gentianaceae.* Botaniska Notiser, 125:255–258.

Löve A and Löve D. 1976. *The natural genera of Gentianaceae.* Kachroo P, ed. Recent advances in botany, Delhi: Dehra Dun, 205-222.

Luijten S, Oostermeijer G, Ellis-Adam A, and den Nijs H. 1999. *Variable herkogamy and autofertility in marginal populations of Gentianella germanica in The Netherlands.* Folia Geobotanica, 34:483–496.

Luijten SH, Oostermeijer JCB, Ellis-Adam AC, and den Nijs HCM. 1998. *Reproductive biology of the rare biennial Gentianella gemanica compared with other gentians of different life history.* Acta Botanica Neerlandica, 47:325–336.

Lütz C. 2012. *Plants in alpine regions: cell physiology of adaption and survival strategies.* Springer, Dordrecht, Heidelberg, London, New York, Berlin.

Ma XY, Xie CX, Liu C, Song JY, Yao H, Luo K, Zhu YJ, Gao T, Pang XH, Qian J, and Chen SL. 2010. *Species identification of medicinal pteridophytes by a DNA barcode marker, the chloroplast psbA-trnH intergenic region.* Biological and Pharmaceutical Bulletin, 33(11):1919–1924.

Maddison WP and Maddison DK. 2000. *MacClade: analysis of phylogeny and character evolution, version 4.0.* Sunderland Sinauer Associates.

Martinell MC, Rovira A, Blanché C, and Bosch M. 2011. *Shift towards autogamy in the extremely narrow endemic Aquilegia paui and comparison with its widespread close relative A. vulgaris (Ranunculaceae).* Plant Sistematics and Evolution, 259:73–82.

Massias M, Carbonnier J, and Molho D. 1987. *Constituants de Gentiana montserratii Vivant (Gentianaceae).* Bullettin du Museum National d'Historie Naturelle (Paris), sèrie 2, Section B:221–222.

Meusel H, Jäger E, Rauschert S, and Weinert E. 1978. *Vergleichende Chorologie der Zentraleuropäischen Flora - 2.* Veb Gustav Fischer Verlag, Jena, Printed in German Democratic Republic.

Miller MA, Holder MT, Vos R, Midford PE, Liebowitz T, Chan L, Hoover P, and Warnow T. 2009. *The CIPRES Portals. CIPRES. 2009-08-04. URL: http://www.phylo.org/sub-sections/portal. Accessed: 2009-08-04s.* Archived by WebCite(r) at http://www.webcitation.org/5imQlJeQa.

Mishiba K, Yamane K, Nakatsuka T, Nakano Y, Yamamura S, Abe J, Kawamura H, Takahata Y, and Nishihara M. 2009. *Genetic relationships in the genus Gentiana based on chloroplast DNA sequence data and nuclear DNA content.* Breeding Science, 59:119–127.

Muir G and Schlötterer C. 1999. *Which DNA marker for which purpose? - Chapter 11, Limitations to the phylogenetic use of ITS sequences in closely related species and populations: a case study in Quercus petraea (Matt.) Liebl.* E. M. Gillet, Hamburg: Wiedebusch - http://webdoc.sub.gwdg.de/ebook/y/1999/whichmarker/index.htm.

Müller G. 1982. *Contribution à la cytotaxonomie de la section Cyclostigma Griseb. du genre Gentiana.* Feddes Repertorium, 93:625–722.

Müller-Schneider P. 1986. *Verbreitungsbiologie der Blütenpflanzen Graubündens.* Veröff. des Geobot. Inst. der ETH, Stiftung Rübel , 85 - Zürich. Heft 263 S.

Navarro L. 1999. *Pollination ecology and effect of nectar removal in Macleania bullata (Ericaceae).* Biotropica, 31(4):618–625.

Nicolson SW and Thornburg RW. 2007. *Nectar chemistry. In Nicolson SW, Nepi M, Pacini E (eds.) Nectaries and nectar.* Springer, Dordrecht, Netherlands pp 215-263.

Nordal I. 1987. *Tabula rasa after all? Botanical evidence for ice-free refugia in Scandinavian reviewed.* Journal of Biogeography, 14:377–388.

Ohashi K and Yahara T. 1998. *Effects of variation in flower number on pollinator visits in Cirsium purpuratum (Asteraceae).* American Journal of Botany, 85:219–224.

Oostermeijer JGB, Altenburg RGM, and den Nijs HCM. 1982. *Effects of outcrossing distance and selfing on fitness components in the rare Gentiana pneumonanthe (Gentianaceae).* Acta botanica Neerlandica, 44 (3):257–268.

Oostermeijer JGB, den Nijs JCM, Raijmann LEL, and Menken SBJ. 1992. *Popuation biology and menagement of the marsh gentian (Gentiana pneumonanthe L.) a rare species in The Netherlands.* Botanical Journal of the Linnean Society, 108:117–130.

Oostermeijer JGB, Luijten SH, Křenová ZV, and den Nijs HCM. 1998. *Relationships between population and habitat characteristics and reproduction of the rare Gentiana pneumonanthe L.* Conservation Biology, 12 (5):1042–1053.

Pacini E and Hesse M. 2005. *Pollenkitt - its composition, forms and functions.* Flora, 200:399–415.

Petanidou T. 2007. *Ecological and evolutionary aspects of floral nectars in Mediterranean habitats. In Nicolson SW, Nepi M, Pacini E (eds.) Nectaries and nectar.* Springer, Dordrecht, Netherlands pp 343-377.

Petanidou T, den Nijs JCM, Oostermejier JGB, and Ellis-Adam AC. 1995. *Pollination ecology and patch dependent reproductive success of the rare perennial Gentiana pneumonanthe L.* New Phytologist, 129:155–163.

Pignatti S. 1982. *Flora d'Italia - vol. 3.* Edagricole, Bologna.

Polidori JL. 2008. *Une nouvelle sous-espece de Gentiana burseri Lapeyrouse, dans les Alpes Maritmes et Ligures (France et Italie).* Biocosme Mèsogèen, 25(1):1–28.

Potts SG. 2005. *Plant pollinatore interface - Recording polinator behaviour in flowers. In: Dafni A, Kevan PG, Husband BC (Eds) Practical pollination biology.* Enviroquest, Ltd., Cambridge, Cambridge, Ontario, Canada, pp 330-339.

Ramsey M and Vaughton G. 1996. *Inbreeding depression and pollinator availability in a partially self-fertile perennial herb Blandfordia grandiflora (Liliaceae).* Oikos, 76(3):465–474.

Rathman ES, Lanza J, and Wilson J. 1990. *Feeding preferences of flesh flies Sarcophaga bullata for sugar-only vs sugar-amino acid nectars.* American Midland Naturalist, 124:379–389.

Reisch C, Schurm S, and Posghlod P. 2007. *Spatial genetic structure and clonal diversity in an alpine population of Salix herbacea (Salicaceae).* Annals of Botany, 99:647–651.

Rosenbauer A. 1996. *Gentianaceae Juss. In Sebald O, Seybold S, Philippi G, Wörz A (Eds.)- DieFarn-und Blütenpflanzen Baden-Württembergs.* Ulmer, Stuttgart, pp.72–110.

Sage TL, Husband BC, and Routley MB. 2005. *Intrinsic attributes of the breeding system. In: Dafni A, Kevan PG, Husband BC (Eds) Practical pollination biology.* Enviroquest, Ltd., Cambridge, Ontario, Canada, pp 27-55.

Sargent RD, Mandegar MA, and SP Otto. 2006. *A model of the evolution of dichogamy incorporating sex-ratio selection, anther-stigma interference and, inbreeding depression.* Evolution, 60(5):934–944.

Seavey S and Bawa K. 1986. *Late-acting self-incompatibility in angiosperms.* Botanical Review, 52:195–219.

Shaw J and Small RL. 2005. *Chloroplast DNA phylogeny and phylogeography of the North American plums (Prunus subgenus Prunus section Prunocerasus, Rosaceae).* American Journal of Botany, 92(12):2011–2030.

Shaw J, Lickey EB, Schilling EE, and Small RL. 2007. *Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in Angiosperms: the tortoise and the hare III.* American Journal of Botany, 94(3):275–288.

Simmons MP and Ochoterena H. 2000. *Gaps as characters in sequence-based phylogenetic analyses.* Systematic Botany, 49 (2):369–381.

Stamatakis A. 2006. *RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models.* Bioinformatics, 22:2688–2690.

Stamatakis A, Hoover P, and Rougemont J. 2008. *A rapid bootstrapping algorithm for the RAxML web-servers.* Systematic Biology, 57:758–771.

Staphenson AG. 1981. *Toxic nectar deters nectar thieves of Catalpa Speciosa.* American Midland Naturalist, 105:381–383.

Stephenson AG. 1981. *Flower and fruit abortion: proximate causes and ultimate functions.* Annual Review of Ecology and Systematics, 12:253–279.

Struwe L and Albert VA. 2002. *Gentianaceae: Systematics and Natural History.* Cambridge University Press, Cambridge.

Swofford DL. 2002. *PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4.10b.* P. Sinauer, Sunderland, Massachusetts, USA.

Taberlet P, Gielly L, Pautou G, and Bouvet J. 1991. *Universal primers for amplification of three non-coding regions of chloroplast DNA.* Plant Molecular Biology, 17:1105–1109.

Templeton AR. 1986. *Coadaptation and outbreeding depression. In: Soulè, M. (ed.) Conservation Biology: The Science of Scarcity and Diversity, pp. 105–116.* Sinauer Associates, Sunderland, MA.

Templeton AR, Crandall KA, and Sing CF. 1992. *A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation.* Genetics, 132:619–633.

Timme RE, Simpson BB, and Linder CR. 2007. *High-resolution phylogeny for Helianthus (Asteraceae) using the 18S-26S ribosomal DNA external transcribed spacer.* American Journal of Botany, 94:1837–1852.

Traveset A. 1993. *Deceptive fruits reduce insect seed predation in Pistacia terebinthus L.* Evolutionary Ecology, 7:357–361.

Turner ME, Stephens JC, and Anderson WW. 1982. *Homozygosity and patch structure in plant population as a result of nearest-neighbor pollination.* Proceedings of the National Academy of Sciences, USA, 79:203–207.

Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, and Webb DA. 1972. *Flora Europaea, vol. 3.* Cambridge University Press, Cambridge.

Vender C, Aiello N, and Piovesana S. 2010. *Survey of yellow gentian populations of the central alps and record of their main morphological and qualitative characteristics.* Acta Horticulturae, 860:101–104.

Vivant J. 1975. *Gentiana montserratii, species nova, dans les Pyrènèes occidentales de la province de Huesca (Espagne).* Bull. Soc. Bot. France, 122(7-8):331–337.

Štorchovà H and Olson MS. 2007. *The architecture of the chloroplast psbA-trnH non-coding region in angiosperms.* Plant Systematics and Evolution, 268:235–256.

Wagenius S and Lyon SP. 2010. *Reproduction of Echinacea angustifolia in fragmented prairie is pollen-limited but not pollinator-limited.* Ecology, 91: 733–742.

Webb CJ and Lloyd DG. 1986. *The avoidance of interference between the presentation of pollen and stigmas in angiosperms II. Herkogamy.* New Zealand Journal of Botany, 24:163–178.

Westerkamp C. 1996. *Pollen in bee-flowers relations: some consideration on melittophily.* Botanica Acta, 109:325–332.

Westphal C, Bommarco R, Carrè G, Lamborn E, Morison N, Petanidou T, Potts S, Roberts S, Szentgyorgyi H, Tscheulin T, Vaissiere B, Woyciechowski M, Biesmeijer J, Kunin W, Settele J, and Steffan-Dewenter I. 2008. *Measuring bee diversity in different European habitats and biogeographical regions.* Ecological Monograph, 78:653–671.

White TJ, Bruns T, Lee S, and Taylor J. 1990. *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In Innis MA, Gelfand DH, Sninsky JJ, White T (eds.) - PCR protocols a guide to methods and applications.* Academic Press, San Diego.

Williams PH. 1994. *Phylogenetic relationships among bumblebees (Bombus Latr.):a reappraisal of morphological evidence.* Systematic Entomology, 19: 327–344.

Wraber T. 1986. *Košutnik, in to Vardjanov. Gentiana lutea ssp. vardjanii.* Proteus, 48:341–346.

Xu DH, Abe J, Sakai M, Kanazawa A, and Shimamoto Y. 2000. *Sequence variation of non-coding regions of chloroplast DNA of Soybean and related wild species and its implication for the evolution of different chloroplast haplotypes.* Theoretical and Applied Genetics, 101:724–732.

Yankova E, Baldzhiev G, Petrova M, Zayova E, and Yurukova P. 2010. *Analysis on pollen and seed productivity and effectiveness in Gentiana lutea L.* Biotechnology & Biotechnological Equipment - Second Balkan Conference on Biology, 21-23 May, Plovdiv, 50 years University of Plovdiv:45–4.

Yuan YM and Küpfer PH. 1995. *Molecular phylogenetics of the subtribe Gentianinae (Gentianaceae) inferred from the sequences of internal transcribed spacers (ITS) of nuclear ribosomal DNA.* Plant Systematics and Evolution, 196:207–226.

Yuan YM and Küpfer PH. 2005. *Geographic structure of molecular variation of Gentiana ligustica (Gentianaceae) in the Maritime and Ligurian regional hotspot, inferred from ITS sequence.* Taxon, 54:887–894.

Yuan YM, Kupfer P, and Doyle JJ. 1996. *Infrageneric phylogeny of the genus Gentiana (Gentianaceae) inferred from nucleotide sequences of the internal transcribed spacers (ITS) of nuclear ribosomal DNA.* American Journal of Botany, 83(5):641–652.

Zangerl AR, Berenbaum MR, and Nitao JK. 1991. *Parthenocarpic fruits in wild parsnip: decoy defence against a specialist herbivore.* Evolutionary Ecology, 5:136–145.

Zapata TR and Arroyo MTK. 1978. *Plant reproductive ecology of a secondary deciduos tropical forest in Venezuele.* Biotropica, 10(3):221–230.

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