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PROTECTION OF NORWEGIAN ORCHIDS – A REVIEW OF ACHIEVEMENTS AND CHALLENGES

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ABSTRACT

Norway has a rich and diverse orchid flora consisting of 36 species. Orchids are found throughout the country, but most of the species are confined to calcareous or base-rich substrates. Important orchid-rich types of vegetation include rich pine and spruce forests, rich deciduous forests, open calcareous meadows, rocky outcrops and screes, hay meadows and calcareous mires and fens. Many species are rare, and 17 species and 3 subspecies are red listed. 13 species are generally protected. Both the orchids and their habitats are susceptible to various disturbances such as e.g. building activities, road construction, quarrying, drainage, forestry and changes in agricultural practices (less intense grazing, termination of mowing), which has resulted in the continuation of the previously inhibited succession. Most of the types of habitat mentioned are important conservation sites and thus many orchid occurrences (e.g. of *Cypripedium calceolus*, *Epipogium aphyllum*, *Epipactis palustris* and *Ophrys insectifera*) are protected by a network of nature reserves designated for these habitats. However, there is an urgent need to secure species with small populations in some of the mire reserves, and succession is also a problem in many of the reserves. Protection of the few existing localities for some species is also needed. A more detailed discussion of the status of the red listed species is presented.

Keywords: orchid conservation, nature reserves, management, orchid habitats, Norway

Introduction

Orchids are an important group of organisms associated with nature conservation in Norway, and especially spectacular and "charismatic" species such as e.g. *Cypripedium calceolus, Ophrys insectifera* and *Cephalanthera rubra,* are actively used for promoting nature conservation in various contexts (see e.g. Bjørndalen 2006). Many of the orchid-rich types of vegetation or nature described in the present paper have high conservation values because they are generally also habitats of high biodiversity (see e.g. Direktoratet for naturforvaltning 1999; Fremstad and Moen 2001; Lindgaard and Henriksen 2011).

Norway has a relative rich and diverse orchid flora, with occurrences all the way north to the northernmost county, Finnmark, and even in alpine areas. Most of the species are confined to calcareous or base-rich substrates and many species appear to be rare despite being widely distributed. The current edition of the Norwegian flora (Lid and Lid 2005) presents 36 species, including 2 subspecies. Eighteen hybrids are listed, some of them between different genera. One species is extinct, *Liparis loeselii*. Three additional subspecies have recently been distinguished and are on the current red list (Kålås et al. 2010). However, it is uncertain whether or not one of them, *Gymnadenia conopsea* ssp. *densiflora,* is a separate species, *G. densiflora* (Bjerke and Strann 2009). *Dactylorhiza pseudocordigera* was earlier regarded as a separate species, but is now included in *D. lapponica* (Wischmann and Norddal 1987; Lid and Lid 2005). A further discussion of the nomenclature of Orchidaceae presented in the latest edition of the Norwegian flora (Lid and Lid 2005) is given by Elven (2007). Hedrén et al. (2012) present a new classification of the taxa of Norwegian *Dactylorhiza*, which greatly differs from Lid and Lid (2005), but this discussion is beyond the scope of the present paper.

For comparison, 55 species of orchid are found in the entire Fennoscandian area (Mossberg and Stenberg 2003). Most of the additional species are confined to the limestone islands Öland and Gotland in the Baltic Sea, and *Calypso bulbosa* in northern Sweden occurs close to the Norwegian border.

Many of the orchids and their habitats are susceptible to a variety of negative effects such as e.g. urban development and building activities, road construction, quarrying, drainage of mires and wetland, forestry, changes in agricultural practices, such as e.g. less grazing and mowing, as a result of which succession occurs. Thirteen species are generally protected in Norway, and four additional species and three subspecies are red listed (Table 1).

The habitats became the most important issue for conservation in Norway after 1970 and the conservation of the important orchid-rich types of nature (e.g. mires, conifer forests and rich deciduous forests) has resulted in a comprehensive network of nature reserves and other conservation areas, which are also beneficial for both rare and more common species of orchids.

A more thorough treatment of the conservation status and assessment of how representative the protection is have been carried out only for a few species (e.g. through action plans for or special investigations of selected species, see below). This paper is the first attempt to present an overall review of orchid protection in Norway. This material was originally presented at the International Conference on Temperate Orchids (TORC'15) in Samos, Greece, in April 2015.

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Table 1 Protected and red listed orchid taxa in Norway, with IUCN annotation of their red list categories and rarity criteria based on the current red list (Kålås et al. 2010). Estimated fractions of the total European population of the taxa are taken from the fact sheets provided by the Norwegian Biodiversity Information Centre (http://www.artsdatabanken.no).

Species/subspecies	Cat	Crit	Eur pop
Liparis loeselii	RE	–	–
Coeloglossum viride ssp. *islandicum*	CR	B	<1%
*Herminium monorchis**	CR	B	<1%
*Cephalanthera rubra**	EN	B; D	<1%
*Dactylorhiza purpurella**	EN	B; C	1–5%
*Epipactis palustris**	EN	A; B	<1%
Epipactis helleborine ssp. *neerlandica*	EN	B; C	1–5%
*Lysiella oligantha**	EN	D	>50%
*Microstylis monophyllos**	EN	B; C	1–5%
*Nigritella nigra**	EN	B	>50%
*Dactylorhiza sambucina**	VU	A	<1%
Dactylorhiza traunsteineri	VU	A	1–5%
Gymnadenia conopsea ssp. *densiflora*	VU	D	<1%
*Anacamptis morio**	NT	–	<1%
*Cephalanthera longifolia**	NT	–	1–5%
*Cypripedium calceolus**	NT	–	1–5%
*Epipogium aphyllum**	NT	–	1–5%
Neottia nidus-avis	NT	–	1–5%
*Ophrys insectifera**	NT	–	<1%
Pseudorchis albida	NT	–	1–5%

* protected species
Cat (category): RE regionally extinct; CR critically endangered; EN endangered; VU vulnerable; NT near threatened
Crit (criterium): A severe population reduction B limited area in decline C small population in decline D very small population/area
Eur pop: % of European population

Material and Methods

The basis for the present paper is primarily the author's own work and nearly 45 years of experience of studying different aspects of the Nordic limestone landscape, including plant sociological studies, documentation and assessment of conservation values, floristic and phytogeographical studies, vegetation mapping, etc. which has resulted in numerous papers and conservation reports (some of which will be listed in the text). The most important contributions are conservation programs for basiphilous pine forests (one of the most orchid-rich of all habitats) in Norway and Sweden (see e.g. Bjørndalen 1985, 1986, 1987, 2006; Brandrud and Bjørndalen 1985; Bjørndalen and Brandrud 1989).

I have used different means to assess the extent to which the different species of orchids are protected. One important source has been the fact sheets provided by the Norwegian Environmental Agency for all of the protected areas in Norway ('the Nature Base'), available on http://www.miljodirektoratet.no. Information in the fact sheets on red listed species provided by Artsdatabanken, the Norwegian Biodiversity Information Centre. In addition, distribution maps from Artsdatabanken and occurrences within conservation areas of some of the species included in this paper were examined.

Additional information can be found in a large number of conservation reports, which document areas of conservation interest, and also in many papers published in national botanical journals. However, it is difficult to combine this information with the actual distribution of the respective species and their representation in the network of nature reserves, or at least it is a time consuming task that is not yet complete. Thus, the present paper is a preliminary report, but I hope to work further with this material. The nomenclature for vascular plants follows Lid and Lid (2005).

Vegetation (Nature) Types with Orchids

Orchids can occur in a variety of types of vegetation and in different microhabitats within them. However, some of them are more important than others, and since the type of vegetation (or in a broader sense type of nature) is the most important criterion in most conservation contexts I will give a short presentation of the most important types. Most of them are rare and often have a high value as habitats for rare species and for high biodiversity in general. Classification of the types of nature in Norway (including also the especially valuable types) and more comprehensive information about them can be found in e.g. Fremstad (1997), Direktoratet for naturforvaltning (1999), Fremstad and Moen (2001), Halvorsen et al. (2009, 2015) and Lindgaard and Henriksen (2011). Most of the species of orchids in Norway occur in various types of forest, mostly rich communities in both conifer and deciduous forests. Many species (including also some of the forest species) occur in different types of open calcareous vegetation, e.g. rocky outcrops, dry meadows, screes and alpine heaths. Rich-fens and calcareous mires can be rich in orchids, and many of the rarest species are found in such habitats. Some species are favoured by scything both dry hay meadows and mires. Orchids also occur in sea shore habitats.

Conifer Forests

Orchids are mostly confined to calcareous or otherwise base-rich conifer forests. One of the most orchid-rich types of forest in Norway is *basiphilous pine forest*, i.e. light-open pine forests on very shallow calcareous soils (Bjørndalen 1985, 2006; Bjørndalen and Brandrud 1989). Such forests are rare, but occur scattered throughout the country north to Finnmark in N Norway. These basiphilous pine forests with their many microhabitats are generally rich in species of vascular plants, many of them rare. There is a great variation between different geographical regions, and e.g. species of dry meadows, steppes and rocky areas with a south eastern distribution are important in SE Norway, while calcicolous alpine species can be important in N Norway. There is also a great diversity of calcicolous fungi in this community. Orchids like *Ophrys insectifera*, *Cypripedium calceolus* and *Cephalanthera rubra* occur mainly in basiphilous pine forests and their associated communities. Many species also occur in ancient deciduous forests (e.g. *Orchis mascula, Neottia nidus-avis* and *Cephalanthera longifolia*), on calcareous rocks and screes (e.g. *Epipactis atrorubens*) and in dry to mesic base-rich meadows (e.g. *Gymnadenia conopsea*, *Coeloglossum viride*, *Platanthera bifolia* and *P. montana*). Many of the same species can also occur in rich spruce forests (low and tall types of herbaceous plants) on deeper brown earth. Of the other orchids in rich conifer forests the following should be mentioned: *Dactylorhiza fuchsii, Listera ovata, Epipactis helleborine* and *Goodyera repens*. *Epipogium aphyllum* requires conditions to remain stable over a long period of time and is confined to old-growth conifer forests, often richer stands on base-rich soil. Coniferous swamp forests can also contain orchids, richer types e.g. *Listera ovata* and poor types e.g. the non-calcicolous species *Dactylorhiza maculata*, *Listera cordata* and *Corallorhiza trifida*.

Deciduous Forests

The mixed thermophilous deciduous forests of Querco-Fagetea character in Norway are outposts of the Central European deciduous forest zone, but *ancient deciduous forests* occur along the coast and fjord districts north to Helgeland just south of the Arctic Circle, although *Ulmus glabra* occurs at Beiarn, which is even further north (Rønning 1954). The ancient deciduous forests can occupy extensive areas on south- and west-facing slopes in the western Norwegian fjord landscape. There is a high diversity of microhabitats in these forests, with many rare species. Orchids like *Orchis mascula*, *Cephalanthera longifolia* and *Neottia nidus-avis* have their core habitats in such forests, partly also e.g. *Epipactis helleborine* and *Listera ovata*. However, these species can even be found in rich conifer forests. Meadow species can occur in more open patches. In northern parts of Norway the thermophilous deciduous forests are replaced by birch forests or mixed deciduous forests with more boreal species such as e.g. *Betula pubescens*, *Alnus incana*, *Populus tremula* and *Salix caprea/coaetanea*. The rich boreal deciduous forests can also contain orchids such as e.g. *Cypripedium calceolus*, *Neottia nidus-avis*, *Epipactis helleborine* and *Orchis mascula*. Calcareous birch forests (mostly on screes) in N Norway are floristically and ecologically related to the basiphilous pine forests, and contain many of the same species (e.g. large populations of *Cypripedium calceolus*). Rich swamp forests with e.g. *Alnus glutinosa* and *Salix* spp. can occasionally contain rare orchids, and e.g. *Epipactis palustris* (and other rich-fen species) can occur in such forests.

Open Calcareous Vegetation

Many species of orchids occur in a variety of open communities on limestone, marble and dolomite. The open limestone vegetation in SE Norway consists mostly of rocky outcrops with a sparse cover of vegetation (Sedo-Scleranthetea communities) and more densely covered dry meadows on shallow soil (Trifolio-Geranietea communities), see e.g. Marker (1969) and Fremstad (1997). Dry meadows dominated by *Geranium sanguineum* can also occur on deeper sea shell deposits where some orchids can be found, e.g. *Epipactis atrorubens* and in rare cases even *Ophrys insectifera*. A special case is dry meadows with the rare *Anacamptis morio* on some small islands in the southernmost parts of Norway, but these habitats are not especially lime-rich (Andreassen and Åsen 1980; Baugen 2003). Open calcareous vegetation is a more important habitat of orchids in N Norway, and most of the *Ophrys insectifera* occurrences at Salten, Nordland, are on marble outcrops, in dry meadows on shallow soil, heaths, sea shell meadows, sand dunes and fine-textured screes with *Dryas octopetala* the dominant species (Bjørndalen 2006). Species such as e.g. *Epipactis atrorubens*, *Coeloglossum viride* and *Gymnadenia conopsea* can also be abundant in such vegetation. Orchids can also occur in alpine areas, most often in *Dryas* heaths, e.g. *Chamorchis alpina*, *Coeloglossum viride*, *Pseudorchis straminea* and *Epipactis atrorubens*. *Lysiella oligantha* is extremely rare, and occurs only at a few localities in the counties of Troms and Finnmark in N Norway.

Rich-fens and Calcareous Mires

Many orchids are more or less confined to sloping mires and fens with calcium-rich ground water, habitats which are vulnerable to drainage and other disturbances. Many localities have been destroyed. Such mires were earlier mowed, and many of the rich-fen orchids are favoured by scything (see e.g. Moen 1990). Succession and/or less intense grazing has affected some of these mires. The strong decline and local or regional extinction of many species of orchids is due to these negative effects, and some of the species are now endangered. Examples are *Microstylis monophyllos*, *Nigritella nigra*, *Epipactis palustris, Dactylorhiza traunsteineri* and *Herminium monorchis*. *Dactylorhiza incarnata* (including both ssp. *incarnata* and ssp. *cruenta*), *D. lapponica* and *Hammarbya paludosa* occur more widespread.

Hay Meadows and Culturally Influenced Sites

Many species of orchids occur in mowed, often dry base-rich meadows and are favoured by scything. The most prominent example is *Dactylorhiza sambucina*, which has its core area in hay meadows in the interior of Telemark (Nordal and Wischmann 1996; Norderhaug et al. 1997). *Pseudorchis albida* is another rare species that prefers mowed or grazed habitats. Many other species of orchids can occur in dry and mesic hay meadows, e.g. *Platanthera bifolia*, *P. montana*, *Gymnadenia conopsea*, *Coeloglossum viride* and *Listera ovata*. Rich hay meadows are rapidly disappearing because they are not mowed any longer or affected by the application of fertilizer, and proper management is necessary to preserve the occurrences of e.g. *Dactylorhiza sambucina* (see below).

Sea Shore Meadows, Salt Marshes and Other Sea Shore Habitats

Only a few of the Norwegian orchids are confined to sea shores. In particular the rare and endangered *Dactylorhiza purpurella* and the disputed species *D. praetermissa* (see e.g. Skrede 2001 and below). Some species can occur in sand dunes with calcareous material, e.g. *Epipactis atrorubens* and the red listed taxa *Coeloglossum viride* ssp. *islandicum* and *Epipactis helleborine* ssp. *neerlandica*.

Threats and Their Effects on Orchids and Orchid Habitats

Rare orchids are vulnerable to wide range of disturbances, ranging from drainage, succession and clear-cutting of forests, etc to complete destruction of large parts of the landscape, which have negative effects on their viability and lead to a reduction in population size. Areas in the interior Oslofjord and the limestone areas from the Cambro-Silurian period in SE Norway are densely populated and have for a long time been affected by urban development, infrastructure and industry, and much of the original natural and agricultural landscape has disappeared. These lowland areas are a climatically favourable region with the highest total biodiversity in Norway. The intensity of building was especially high after the Second World War, and e.g. half of the valuable basiphilous pine forests in the Cambro-Silurian district Skien-Langesund was destroyed between 1972 and 1986 (Bjørndalen 1988). Many orchid localities, especially those of rare xerothermic species confined to open calcareous rocks and meadows in the SE Norwegian lowlands were destroyed. In addition, the building of holiday cabins along the Oslofjord and the Skagerak coast also destroyed many valuable areas, and e.g. affected the basiphilous pine forests and localities where rare species such as e.g. *Ophrys insectifera* occurred (Bjørndalen and Brandrud 1989). Even small-scale house building in remote areas adversely affected the localities of e.g. *O. insectifera* in its core area in N Norway (Bjørndalen 2006). Limestone quarrying completely changes the landscape and basiphilous pine forests are particularly susceptible to such activities, which also destroyed areas with rich orchid floras (Bjørndalen and Brandrud 1989). However, there are also examples of abandoned quarries being secondary habitats for rare orchids such as *Ophrys insectifera* and *Cephalanthera rubra* (Økland 1984; Hanssen 2006). Some of the few remaining localities for *Herminium monorchis* in Østfold, which were protected, were affected and partly destroyed by the building of boat depots on land and the transformation of sea shore meadows for recreational use (cf. fact sheet 156 from Artsdatabanken).

The paradox is that more subtle disturbances have been more harmful to orchids (and many other rare species) than the disturbances described above. In particular, drainage, cultivation and afforestation of mires and wetlands, but also succession in such habitats due to changes in agricultural practices that resulted in less intense grazing, cessation of mowing (including mires) and an increase in the application of fertilizer. *Nigritella nigra*, *Epipactis palustris*, *Microstylis monophyllos* and *Herminium monorchis* are examples of rich-fen species that were seriously affected. Those species that prefer base-rich hay meadows and other sites in the cultural landscape are adversely affected when these habitats are no longer mowed or are over fertilized. Examples are *Dactylorhiza sambucina* and *Pseudorchis albida*. Many other species are also negatively affected by succession and growths of dense thickets in rich conifer and deciduous forests (see examples below). Intensive use of recreation areas can affect some localities with orchids, e.g. *Ophrys insectifera* (Bjørndalen 2006). Flower picking and digging orchids up to plant in gardens is a problem as was in earlier days the collection of herbarium specimens by botanists (an activity which contributed to the extinction of *Liparis loeselii*).

Protection of Norwegian Orchids and Their Habitats

Strict protection based on environmental laws is essential for safeguarding biological occurrences, both for the organisms themselves and their habitats. Conservation work in Norway became more systematic after 1970 with the passing of the Law of Nature Conservation and the establishment of the Ministry of the Environment. A large number of nature reserves and other conservation areas were established (around 2900 at the moment), reflecting a representative selection of the great variety of types of nature in Norway. The new Law of Nature Diversity passed in 2009 (http://lovdata.no/dokument/NL/lov/2009-06-19-1) is an even better tool for nature conservation. National parks and some reserves and landscape protection areas can include extensive parts of undisturbed or little disturbed nature.

The official responsibility for nature conservation in Norway has three levels. The Ministry of Climate and Environment and their scientific unit Norwegian Agency of Environment, which is located in Trondheim have the national and international responsibilities, and the establishment of all conservation areas are approved by the King and his council (the Government). The regional level consists of the Section for Environment as a division under the County Governor in each of the 18 counties (Oslo and Akershus have a joint County Governor). These units have the daily responsibility for their counties and have a good competence in environmental sciences. Finally, the 428 local municipalities have responsibility for safeguarding valuable types of nature and red listed species by planning and carefully controlling activities that might adversely affect such occurrences. However, the local level of nature conservation does not function optimally and political signals that the responsibility of municipalities for nature conservation are to be increased could present problems (see discussion below).

Orchids were early recognized as conservation objects and some botanists worked hard to get some special occurrences protected. For instance, the small *Cypripedium calceolus* locality Versvik mentioned below was protected in 1928. The large plant protection area Junkerdalen-Balvatn in N Norway with one of the largest populations of *C. calceolus* and numerous rare alpine species was established in 1935 (recently transferred to a national park and a large nature reserve).

The most important contributions to nature conservation in Norway are the thematic conservation programs for selected types of nature, namely mires, wetlands, conifer forests, ancient deciduous forests and other rich deciduous forests and coastal environments. Most of the network of nature reserves is associated with these types of nature. This was extremely beneficial for orchid conservation since most of the species occur in these types of nature. Good examples are forest species such as e.g. *Cypripedium calceolus*, *Ophrys insectifera*, *Cephalanthera rubra*, *C. longifolia*, *Epipogium aphyllum* and *Neottia nidus-avis* and rich-fen species such as e.g. *Nigritella nigra*, *Epipactis palustris*, *Dactylorhiza traunsteineri* and *Microstylis monophyllos*.

Protection Status of Selected Orchid Species

General information about distribution is taken from Lid and Lid (2005), but other references are included if the respective species is treated in more detail in papers, reports or in the series 'Maps of distribution of Norwegian vascular plant' (Fægri 1960; Gjærevoll 1990; Fægri and Danielsen 1996; Elven et al. 2013). The on-line distribution maps provided by the Norwegian Biodiversity Information Centre (Artsdatabanken) were also consulted (http://www.artsdatabanken.no) for some of the species.

Anacamptis morio

A. morio has one of the most limited distribution of all Norwegian orchids and occurs only on some small islands in the two municipalities Grimstad and Lillesand in Aust-Agder in the southernmost parts of Norway (Fægri 1960; Andreassen and Åsen 1980; Pedersen and Åsen 1994; Baugen 2003). This species occurs in grass-rich meadows and on rocks near the sea. The status of *A. morio* (up to 2012) is treated in http://botanikk.no/Narrmarihand/htm. A slight decline was recorded during the last decade, and this species is susceptible to ongoing succession on small islands. As a paradox, sheep grazing which was supposed to open up the island landscape seems to have negative effects as many plants are eaten by the sheep. No protection areas for this species have been established so far. *A. morio* has the red list status near threatened and it is questioned whether the status should be adjusted to endangered.

Cephalanthera longifolia

C. longifolia has mainly a coastal distribution in Norway and occurs north to the Trondheimsfjord area (Fægri 1960; Nordal and Wischmann 1986). This species is mostly found in rich deciduous forests, but can occasionally be important even in basiphilous pine forests (Nordfjord in Sogn og Fjordane, cf. Bjørndalen 2005). Viable populations occur in many of the nature reserves with ancient deciduous forests (especially in W Norway), but the representativeness of the overall protection has not been evaluated. The most important inland occurrence (Kleppefjell in Hjartdal, Telemark, cf. Nordal and Wischmann 1986) is situated in a basiphilous pine forest reserve. It is supposed that *C. longifolia* can endure succession better than many other species of orchids since it is often found in dense stands in deciduous forests.

Cephalanthera rubra

C. rubra is one of the most vulnerable of the forest orchids, and an action plan for this species has been presented (Direktoratet for naturforvaltning 2006). This species has a strict south eastern distribution and is mostly confined to a few localities east of Oslofjord (Fægri and Danielsen 1996; Hanssen 1996). This species is primarily found in basiphilous pine forests and associated communities (Bjørndalen and Brandrud 1989; Hanssen 1996), and destruction of such forests for building houses has been extensive in some of the Cambro-Silurian areas in the Oslo region, e.g. Eiker (Hanssen 1996) and

Skien-Langesund (Bjørndalen 1988). *C. rubra* is also susceptible to clear-cutting and succession and even flower picking has been documented (Direktoratet for naturforvaltning 2006). This species is recorded in nine nature reserves (mostly basiphilous pine forests), but some other localities are not protected other than in terms of the general protection of this species (Direktoratet for naturforvaltning 2006). Management is practised in some areas, especially the thinning of thickets in forests. Hanssen (2006) and Hoell (2013) give status reports on this work along with the action plan for this species.

Cypripedium calceolus

Most of the occurrences of *C. calceolus* in Norway are associated with basiphilous pine forests (Fig. 1), but it also occurs in calcareous birch forests on screes in N Norway (Bjørndalen and Brandrud 1989). It can also be found in calcareous spruce forests on shallow marble soil at Hattfjelldal in county Nordland (Bjørndalen 2003) and in other rich spruce and deciduous forests on deep brown earth. In rare cases this species can be found in alpine areas on calcareous screes (e.g. in Bøverdalen in the Jotunheimen area). *C. calceolus* and *Ophrys insectifera* are important icons ("charismatic" species according to Bjørndalen 2006) in the conservation plans for basiphilous pine forests (Bjørndalen and Brandrud 1989). The most important threat is clear-cutting, but also general succession resulting in thickets developing in e.g. basiphilous pine forests, which can reduce the viability of this species. Flower picking and digging up plants with roots for planting in gardens are also implicated. The implementation of the conservation plans for conifer forests and rich deciduous forests has resulted in numerous nature reserves where *C. calceolus* is abundant. These reserves are situated within the distribution of this species, which is mainly in the east (Elven et al. 2013), i.e. from Porsgrunn in Telemark in SE Norway and north to Alta in Finnmark. The largest populations are in N Norway and there are perhaps several thousand clones at each of the localities at Junkerdalsura in Nordland and Brennfjell in Troms (see e.g. Elvebakk and Sortland 1985). However, even if the representation of *C. calceolus* in protected areas can be considered as good there are still localities that need to be protected. Most of the occurrences do not need any special management, but the problem of succession resulting in dense shrubberies in basiphilous and deciduous forests can be a problem in certain areas. A good example is the tiny plant protection area established in 1928 at Versvik in Telemark, SE Norway. The locality was fenced and as a consequence a dense shrub layer developed. In 1972 there was only one small group of *C. calceolus* (Bjørndalen 1972), but after the area was made a nature reserve the shrubbery was thinned in the 1980s and *C. calceolus* responded quickly and already in 1996 (personal observations) new clones had spread throughout the reserve from the original group.

Dactylorhiza praetermissa

D. praetermissa is known only from one locality (Stad in Sogn og Fjordane, W Norway), where it occurs in brackish sea shore meadows together with *D. purpurella*. Its existence in Norway was controversal and disputed (Nordhagen 1972; Wischmann 1989; Skrede 2001), and the locality was supposedly destroyed by the lowering of the water table for cultivation purposes (Skogen and Odland 1991). However, later on new plants were re-established (Skrede 2001) and this occurrence is cited in the latest edition of the Norwegian flora (Lid and Lid 2005). As a consequence, *D. praetermissa* should be protected. Skrede (2001) mentions the need for management. This species has been given the red list category critically endangered in the fact sheet from Artsdatabanken, but has not been included in the current red list (Kålås et al. 2010).

Dactylorhiza purpurella

D. purpurella and *D. praetermissa* are the only species of orchids that exclusively occur in sea shore meadows and salt marshes, *D. purpurella* also occurs in depressions in sand dunes. *D. purpurella* occurs at a few localities on the outer parts of the western Norwegian coast between Jæren in Rogaland and Ørlandet in Sør-Trøndelag (Lid and Lid 2005), and has the status endangered in the red list (Kålås et al. 2010). This species occurs in some nature reserves, e.g. in Hå, Rogaland and Giske, Møre og Romsdal. It is now only known to be present at 4–5 of the almost 20 previously recorded localities according to the fact sheet from Artsdatabanken and the decline seems to be primarily due to succession. It is not known whether all the localities where it is currently recorded are protected (distribution map not available on-line).

Dactylorhiza sambucina

D. sambucina has a strictly limited distribution in Norway occurring in interior parts of Telemark and adjacent areas (Nordal and Wischmann 1987; Fægri and Danielsen 1996). This species, of which only the yellow form occurs in Norway, seems to be favoured by mowing and is mostly found in base-rich hay meadows and more rarely in pastures (Norderhaug et al. 1997; Bratli and Myhre 1999). A more natural occurrence is recorded in an area of basiphilous pine forest with patches of open calcareous vegetation at Kleppefjell in Hjartdal (Nordal and Wichsmann 1987), which is now a nature reserve. *D. sambucina* is not recorded in other conservation areas, which poses a challenge for the conservation of this species since it is dependent on long term management. However, local farmers and organizations are aware of this species (which is also designated as the official county flower of Telemark) and voluntarily manage the localities by scything. Such activities should be agreed with the County Environmental Section.

Epipactis palustris

E. palustris is restricted to some localities around Oslofjord with some isolated occurrences at Jæren and Karmøy in Rogaland (Fægri and Danielsen 1996; Hanssen 1998). This species is confined to rich-fens, rich swamp forests and other wetlands, and is vulnerable to habitat destruction, drainage and changes in hydrological regimes, cultivation, water and soil pollution and successional development of reed beds in wetlands. It has disappeared from more than half of the earlier known 42 localities and an action plan is now in place (Hanssen 2011; Hoell 2011a). The red list status is endangered (fact sheet 157 from Artsdatabanken), but *E. palustris* occurs at nine of the remaining localities within nature reserves and landscape protection areas (Hanssen 2011). There are relatively large populations at Ultvedttjern in Ringerike, Buskerud (Bjørndalen 1999), and viable populations in other conservation areas. A voluntary agreement with the farmer who owns the ground where *E. palustris* occurs at Karmøy, Rogaland, about fencing out grazing animals has so far been successful (Lundberg 2013). An interesting experiment in which the remaining plants in the nature reserve Oppsjømyrene in Akershus were transferred to the Botanical Garden in Oslo for later reintroduction was recently carried out, opens up the possibility of managing endangered species with small populations in this way (Røsok et al. 2013).

Epipogium aphyllum

The Ghost Orchid (*E. aphyllum*) is a fascinating saprophytic species, which can remain dormant for several years. It has a marked eastern distribution in Norway and occurs scattered north to Porsanger in Finnmark (Wischmann 1965; Elven et al. 2013). It is one of the few vascular plants confined to old-growth conifer forests, a type of nature otherwise characterized by a high diversity of fungi, lichens, mosses and wood-living insects (especially beetles) associated with rotting trees and dependent on it remaining undisturbed for a long time. These organisms are susceptible to clear-cutting and modern forestry is a threat to almost 50% of the species on the Norwegian red list (Kålås et al. 2010). *E. aphyllum* prefers moss-rich patches in rich spruce and pine forests, but occasionally occurs in old-growth birch forests and other boreal deciduous forests. The conservation plans for conifer forests has over the last two to three decades been implemented to a high degree and this species occurs in nature reserves throughout its distribution. However, there is no proper evaluation of how representative the occurrences in protected areas are compared with the known localities of the species (see discussion below). There are also still some old-growth conifer forests left to be protected.

Herminium monorchis

H. monorchis has suffered a dramatic reduction in its distribution from earlier occurring scattered throughout SE Norway to occurring currently only in the outer Oslofjord (Fægri and Danielsen 1996; Lid and Lid 2005), and its status on the red list is critically endangered (Kålås et al. 2010). The decline of *H. monorchis* started early, and only seven localities were known after 1955. This species is confined to rich-fens and calcareous wet meadows, types of habitat that are strongly susceptible to drainage and other types of destruction. The three remaining populations, with an estimated 500 individuals, occur at Asmaløy in Hvaler, Østfold (according to fact sheet 156 from Artsdatabanken), both inside the coastal and marine national park Ytre Hvaler and in a nearby nature reserve. The habitat of this species in the national park is currently being managed. An action plan for this species is being prepared according to a web note from the Ministry of Environment in 2011, but it has not been possible to verify this.

Lysiella oligantha

L. oligantha (former names *Platanthera oligantha; P. obtusata* ssp. *oligantha*) is one of the rarest orchids in Norway. It has an extremely restricted distribution in Europe, where the only European populations are in the counties Troms and Finnmark in northernmost Norway and Abisko National Park in N Sweden (Gjærevoll 1990; Høiland 1990; Alm 2012). The nearest occurrences are in the Jenisei area in eastern Siberia from where it spreads further east to Kamchatka (Hultén and Fries 1986). This species was earlier over-collected by botanists (Alm 2012) and suffered from habitat destruction even after it was protected. A sad example was road construction work in 2011–12 at Kåfjord in Alta, Finnmark, which destroyed a classic locality of *L. oligantha* (Alm 2012, 2013). Norway together with Sweden has a special international responsibility to protect this species. The largest viable populations in Norway are found in the Reisa National Park. This species occurs also in the conifer forest reserve Skoganvarre at Porsanger. An earlier record from Skibottsdalen in Troms (Benum 1958) has not been confirmed in recent years (Høiland 1990), but this occurrence is probably situated within the nature reserve Brennfjell. At least, there are viable populations on the mountain Sáhkkobátni at Kåfjord (Alm 2012), an area which is not yet protected.

Microstylis monophyllos

The rich-fen species *M. monophyllos* (former name *Malaxis monophylla*) has undergone a similar reduction in distribution to *Herminium monorchis*, as it earlier occurred scattered throughout SE Norway (Schumacher et al. 1992; Fægri and Danielsen 1996; Lid and Lid 2005). Most of its former localities were destroyed by ditching and drainage of mires and wetlands. Some of the few remaining localities occur within nature reserves such as e.g. the “classic” locality Karusputten and the adjacent Holbekken at Nordmarka in Oslo (see e.g. Moen and

Wischmann 1972; Schumacher et al. 1982). *M. monophyllos* is included in a surveillance program for selected species in the county of Oslo, and the total number of individuals in the years 2010–14 varied between 71 and 113 (http://www.naturarv.no). The species occurs also in the nature reserves Arekilen at Hvaler, Østfold, and Gullerudtjern at Ringerike, Buskerud.

Nigritella nigra

N. nigra is confined to hay meadows and traditionally mowed mires, and is now extremely rare in both Norway and Sweden. The taxonomy of the *Nigritella* complex is uncertain, but the Scandinavian population seems to be the endemic ssp. *nigra* (Hedrén 1999; Hedrén et al. 2000) although the plants on serpentine are given the name *Gymnigritella runei*, which is regarded as an endemic (Teppner and Klein 1989). The current Norwegian flora (Lid and Lid 2005) accepts only *Nigritella nigra* whereas the Nordic flora (Mossberg and Stenberg 2003) lists both taxa as *Gymnadenia nigra* and *G. runei*, respectively. Anyway, the restricted distribution of this complex in the interior parts of Sør-Trøndelag and adjacent areas in Norway and Jämtland and Härjedalen in Sweden has decreased markedly and this species is given endangered status in both the Norwegian and Swedish red lists (Gärdenfors 2005; Kålås et al. 2010). In addition, there are some isolated occurrences at Troms in northernmost Norway (Johansen 1981; Engelskjøn and Skifte 1984; Sætra 1987). The number of occurrences recorded in Norway is 44, and an action plan for *N. nigra* in Norway has recently been presented (Miljødirektoratet 2013). This report gives a good summary of the biology and ecology of this species, with a comprehensive reference list including international literature. The three decade long investigation at the mire reserve Sølendet at Røros, Sør-Trøndelag has given a wealth of information on *N. nigra* and other plants favoured by the scything of mires and on mire ecology in general (Moen 1990; Moen and Øien 2003, 2009, 2012; Øien and Moen 2001, 2005, 2009). Primary localities for *N. nigra* are probably naturally open areas rich in herbaceous plants along rivers, creeks and at the edge of mires and of water filled depressions in sloping mires, habitats that are grazed by small rodents such as lemmings (Moen and Øien 2012). The most serious threats to this species are succession and the application of fertilizers. The thorough studies of *N. nigra* at Sølendet have provided important information for management of this species and of mires and rich-fens in general. Thinning of trees and shrubs and mowing using scything machines are used to restore mires, and more or less regular mowing is recommended as a management procedure for the reserve (Miljødirektoratet 2013). Similar management of the *N. nigra* localities in Sweden was successful (Björkbäck and Lundquist 2005). There are also viable populations of *N. nigra* at Kvikne, Hedmark, within and in contact with the Knutshø landscape protection area where it occurs in more primary habitats subject to less cultural influence (Hoell et al. 2011), and a status report for implementation of the national action plan for this species has been presented for the occurrences in the counties of Hedmark and Oppland (Hoell 2011b). *N. nigra* was given the status of priority species by the Ministry of Climate and Environment in May 2015, which brings this species under the protection of the Law of Nature Diversity. Only 13 species have been given this status so far.

Fig. 1 *Cypripedium calceolus* occurs abundantly in the basiphilous pine forests in the nature reserve Bergsåsen in Snåsa, Nord-Trøndelag.

Ophrys insectifera

Bjørndalen (2006) discusses the conservation status of *O. insectifera* in Norway and presents an overview of the localities where it is protected. Most of the Norwegian occurrences are in basiphilous pine forests, and *O. insectifera* together with *Cypripedium calceolus* are explicitly used as “iconic” species for promoting the conservation of this type of vegetation (Bjørndalen and Brandrud 1989). This species is especially vulnerable to habitat destruction caused by building of houses and holiday cabins, but clear-cutting and mechanical damage caused by logging machines, growth of shrubs in basiphilous pine forests and intensive use of recreation areas also adversely affect this species (Bjørndalen 2006). *O. insectifera* has three distinct distribution centres in Norway (Elven et al. 2013). The SE Norwegian sub region is the most extensive, and almost all localities were protected in 2005 (Bjørndalen 2006). Some additional reserves were established since then. The same is the case for the sub region north of Trondheimsfjord in C Norway, were large populations occur in the outstanding botanical locality and nature reserve Bergsåsen at Snåsa, Nord-Trøndelag. The third sub region occurs just north of the Arctic Circle in the Salten district in Nordland, where locally large populations occur in both basiphilous pine forests and in particular in open calcareous vegetation such as dry meadows, rocky outcrops, sea shell meadows and screes. Most of the localities where *O. insectifera* occurs in basiphilous pine forests are protected as nature reserves and a few of the occurrences in open calcareous vegetation were protected in 2005 (Bjørndalen 2006). However, many of the valuable localities with such vegetation were recently made nature reserves. A notable exception is Ausvika north of Bodø where the large population in sea shell meadows in a municipal recreation area is being adversely affected (see discussion below).

Other Species

Characteristic species of ancient deciduous forests and other rich deciduous forests such as e.g. *Orchis mascula*, *Neottia nidus-avis* and *Epipactis helleborine* seem to be well represented in nature reserves. These species, in addition to *Dactylorhiza fuchsii*, *Goodyera repens* and the more mesic *Listera ovata* do also occur in many reserves with basiphilous pine forests and rich spruce forests. Rich-fen species such as e.g. *Dactylorhiza traunsteineri*, *D. incarnata* (both subspecies *incarnata* and *cruenta*), *D. lapponica* and *Hammarbya paludosa* are found in many mire reserves. *Epipactis atrorubens* occurs in a variety of dry calcareous communities (included basiphilous pine forests) and occurs in reserves throughout its distribution area from south to north. Widespread meadow and rich forest species such as e.g. *Gymnadenia conopsea*, *Platanthera bifolia*, *P. montana* and *Coeloglossum viride* obviously occur in a variety of reserves, but there are no studies on whether they are abundant there. The same is the case for species with a more alpine affinity such as *Pseudorchis albida*, *P. straminea* and *Chamorchis alpina*. *P. albida* is red listed and considered as threatened in the cultural landscape, but seems to be more common in primary habitats in certain areas such as e.g. mountain areas at Sunnmøre, W Norway (Holtan and Grimstad 2001). Widespread and non-calcicolous species such as *Corallorhiza trifida*, *Listera cordata* and *Dactylorhiza maculata* are well represented throughout the country in reserves with moist forests, wet meadows and poor to intermediate bogs and mires.

Discussion and Concluding Remarks

Conservation and management practices are complicated matters and should be based on good science and good collaboration between the environmental authorities and scientists. Protection in the form of a nature reserve (or other protection area categories) enshrined in the Law of Nature Diversity as well as IUCN standards, both for organisms and ecosystems (see e.g. IUCN 2008; general discussion in Kålås et al. 2010; Rodriguez et al. 2011), is the best way to sustain current levels of biodiversity and protect valuable habitats. Strict regulations for a reserve and its proper management can then be implemented. Landscape protection areas and national parks can also protect rare species, although these conservation categories are generally weaker than nature reserves.

A good network of nature reserves with rare orchids and orchids in general has been achieved since most of the valuable habitats with orchids are important conservation areas, such as e.g. rich conifer forests, rich ancient and boreal deciduous forests and rich-fens. Action plans for some orchid species are being implemented. I will discuss some aspects of orchid conservation such as whether there is a good representation of orchids in the network of protected areas and the need for further conservation and management and the challenges and limitation that lay ahead before we can claim that the protection of Norwegian orchids and their habitats is adequate.

Assessment of the Representation in Terms of Species of Orchids in the Network of Protected Areas

Artsdatabanken (the Norwegian Biodiversity Information Centre) has published standardized fact sheets for many of the red listed species, including the orchid species presented in the present paper (http://www.artsdatabanken.no/publikasjoner/faktaark). These fact sheets are also part of my background material for assessing the representation of orchids in protected areas. However, the quality of the descriptions in the fact sheets is variable, but they are currently being updated and the presentation for some of the species improved.

The relationship between threats, registration of their status in the field and the conservation and management needs of some species, i.e. the species for which there are special action plans, such as *Epipactis palustris* (Hanssen 2011), *Cephalanthera rubra* (Direktoratet for naturforvaltning 2006; Hanssen 2006) and *Nigritella nigra* (Miljødirektoratet 2013) are being given more attention. The Norwegian Botanical Society is also involved in the implementation of various aspects of the actions plans (see Hoell 2011a, 2011b, 2013). Some species are dealt with in more detail in terms of the list of localities, field status and recommendations for further protection. Here I can mention e.g. *Lysiella oligantha* (Høiland 1990) and *Ophrys insectifera* (Bjørndalen 2006). *L. oligantha* needs special attention, because even though there are viable populations in the Reisa National Park (and 1–2 nature reserves) the situation is nevertheless alarming as several localities have recently been destroyed (Alm 2012). *Dactylorhiza purpurella* is another species that urgently needs an action plan. The only locality with *D. praetermissa* at Ervik in Stad should be protected and managed properly. This species should also be included in the next edition of the Norwegian red list. The status of *Anacamptis morio* on small islands at Aust-Agder is alarming because of the threat both from succession and from grazing by sheep currently being used to stop succession occurring (!), and its red list status should be changed to endangered. *Herminium monorchis* with only three localities needs special attention and steps need to be taken to improve the habitat for this species in the Ytre Hvaler National Park. There are very few localities for *Microstylis monophyllos*, but they are protected within some mire reserves.

However, a more detailed assessment of the protection is needed. *Dactylorhiza sambucina* with its preference for hay meadows is dependent on management and the continuation of mowing (Norderhaug et al. 1997), and formal agreements between the environmental authorities and local farmers and organizations should be agreed.

Finally, a more detailed assessment of the conservation status of the more widespread red listed species, such as *Cypripedium calceolus, Epipogium aphyllum, Pseudorchis albida, Neottia nidus-avis* and *Dactylorhiza traunsteineri*, and protection of their localities is desirable. Such an assessment is time-consuming and requires the study of distribution maps, herbarium data, published and unpublished papers and reports as well as checking the Nature Base. I have carried out a quick check for *Epipogium aphyllum* and *Cypripedium calceolus* using the distribution maps provided by Artsdatabanken, by enlarging the dots to determine the exact positions and whether they are situated inside protection areas. There are important occurrences of these two species in nature reserves and other conservation areas, but it is difficult to judge whether they are representative. Only around 20 of the 221 dots for *E. aphyllum* (around half of them, those with coordinated positions, were checked) are currently inside nature reserves and national parks. A high number of dots (almost 1100) were for *C. calceolus*. The majority of them were not coordinate based and only attributed to a municipality as a whole, and therefore impossible to use. However, *C. calceolus* is protected in more than 30 conservation areas, and most of them include important types of habitats for species such as e.g. basiphilous pine forests and other rich conifer forests and in N Norway also rich deciduous forests. To conclude, these distribution maps must be used with caution and are not suitable on their own for making a proper assessment. There is no information on whether this species still occurs at a particular locality or if it is based on old and dubious collections or records. Another problem with *E. aphyllum* is that this species can be dormant for many years at a locality and it is therefore difficult to confirm its presence. But like *C. calceolus*, many of the important localities for *E. aphyllum* are included in the national conifer forest conservation program. I want to carry out a more detailed analysis of the distribution of *C. calceolus*.

The Need for Management

Management connected with the protection of orchids and their habitats are repeatedly mentioned throughout this paper, and it is obvious that many species are dependent on some kind of management (e.g. *Dactylorhiza sambucina*, *Nigritella nigra*, *Epipactis palustris* and *Herminium monorchis*). Management of many of the nature reserves with red listed orchids is needed. The reasons for such management are manifold and complex, and thorough research is often necessary to develop a proper management regime. In other cases just the thinning of thickets can improve a locality for some species. There are also more fundamental questions regarding management and restoration, e.g. *ex situ* rescue as in the case of *Epipactis palustris* (Røsok et al. 2013).

The network of nature reserves has increased steadily over the last few decades, but following up with management plans and proper funding has generally not been given the highest priority. The most common problem is succession in both forested and open parts of the reserves, and the negative effects of e.g. the earlier draining of mires and wetlands still affects the quality of these sites. In the most serious cases (like *E. palustris*) the loss of a rare species one wants to protect can result. The Governmental Revision Agency has focused on this problem, and has criticized the environmental authorities for not giving priority and funding the management of the reserves (Riksrevisjonen 2006).

The complexity of the management of orchids indicates that it needs to be based on good and detailed scientific research (as e.g. in cf. Moen 1990; Moen and Øien 2003, 2009) on for e.g. their population ecology, pollination, mycorrhizal relationships, demography and effects of land use. Also comparisons with management and its implementation in other European countries (e.g. Fennoscandia, N Europe, The British Isles) can be informative.

Habitat Protection vs. Species Protection

The Law of Nature Diversity makes it possible to protect species and 13 red listed species of orchids are protected in Norway. This protection is meant to secure the localities of these species against flower picking, activities that negatively affect these species and the destruction of their habitats and provide a signal to local authorities, developers and the public that these localities are in need of special care. But is the formal protection of law enough to secure the populations of these rare species without following up with special initiatives? And if the latter are necessary who is responsible for carrying them out?

A good example of this problem are the rich occurrences of *Ophrys insectifera* at Ausvika north of Bodø, Nordland. There are several large populations of this species at Ausvika (own material) and in good flowering years there can be several hundred individuals. The vegetation is a mixture of sea shell meadows and marble outcrops with shallow soil dominated by *Dryas octopetala*, partly also calcareous sand dunes and birch thickets with tall herbaceous plants. The calcareous meadows are extremely species-rich and contain rare alpine species such as e.g. *Kobresia myusuroides* and *Oxytropis lapponica*, and I have recorded there almost 70 species of vascular plants and mosses (many calciphilous) in 1 m^2 plots. Ausvika is also a popular recreation area and designated as an official area for recreational use by the municipality of Bodø. The resulting intensive use, trampling and erosion in many places has had a strong negative effect on the vegetation. There are also marked signs of illegal off-road driving. In addition, the development of birch thickets has had an adverse effect on the rich open calcareous vegetation, and thinning and management is needed. There is also a need to revegetate the eroded areas. The municipality is only concerned with the recreational aspects, not the safeguarding of *O. insectifera*. It should at least have provided information to the public about the vulnerable nature of the area and how it should be treated. The protection of *O. insectifera* under the present conditions at this locality has little value.

The Ausvika example is not unique, and many of the non-protected areas where red listed species occur are potentially likely to be destroyed. Protection of the entire habitat is crucial for safeguarding these species so only a vague species protection without any specific actions makes the future of many of these populations of rare species quite uncertain. More subtle effects associated with succession and forestry practices are also part of the problem and need to be addressed if proper management is to be adopted.

Local vs. Governmental Conservation

If the locality at Ausvika had been designated a nature reserve or landscape protection area the environmental authorities could have carried out proper management, given clearer rules and restrictions for the use of certain parts of the area and provided better control. The county environmental authorities are generally competent and have the necessary legal tools to safeguard biodiversity. They have the daily responsibility for the conservation areas in their respective counties and are the keepers of an outstanding network of nature reserves, which includes examples of the entire range of different types of nature with high species diversities present in Norway. At the national level the Norwegian Environmental Agency provides the scientific basis for the quality of the security at all conservation areas and is a guarantor that Norway will adhere to international conventions and agreements.

The 428 local municipalities protect nature in different ways. They are responsible for taking care of valuable nature and species within their borders, which are not protected by the Law of Nature Diversity, and for integrating this into their planning and other activities. All of the municipalities are responsible for elaborating plans for registering biodiversity. Direktoratet for naturforvaltning (1999) has specified 56 types of nature and numerous subtypes, which are of special value for biodiversity and red listed species, and provided guidelines on how this work should be carried out. Over the last 20 years all over Norway there have been projects on nature conservation and incentives for municipalities to employ environmental advisors. However, as a result of the decline in municipal economies these advisors were quickly dispensed with when governmental incentives ceased.

Political signals and priorities influence all parts of society and can have serious consequences for nature conservation both at national and regional/local levels. Although it is not easy to document in a scientific paper it is highly relevant to safeguarding rare species. The present conservative government in Norway shows signs of changing from favouring governmental control of the protection of nature to giving municipalities increased freedom to manage their natural resources. This is a part of the process to weaken the regional environmental authorities by reducing their control over the municipalities and ability to object to a municipality authorizing the building, e.g., of marinas, apartments, office complexes, etc. in the littoral zone, which they are currently allowed to do.

As mentioned above, the municipalities have little competence in biological sciences and there are often local pressure groups and developers with economic resources that influence and lobby the municipal administration and politicians. Volunteers working in local organisations interested in, e.g., botany, ornithology, entomology and nature conservation, everyday face problems, which local authorities are asked to adjudicate on, however, these local organisations often feel they lose out to strong economic interests. A problem is that e.g. private entrepreneurs and even municipal workers do not know where the valuable habitats and rare species occur, and there are many reports of destruction of nature due to lack of information. Even if valuable areas are safeguarded and regulated as nature areas a new municipal board after an election can signal other priorities for future planning. The conclusion is that protection based on government legislation and maintaining the competence and power of the environmental sections of the county councils to guide and correct the municipalities is the best way to sustain biodiversity.

This discussion is relevant also for orchid protection in various ways. The biodiversity plans of the municipalities are supposed to locate sites with valuable types of nature and red listed species on maps, which are coordinated with other thematical maps and actively used in the planning process and different activities. Since many of the localities of the generally protected and red listed species of orchid are outside nature reserves the municipalities are responsible for safeguarding them. Also many of the valuable types of nature with high biodiversity as defined by the Direktoratet for naturforvaltning (1999) are rich in orchids, e.g. basiphilous pine forests, ancient deciduous forests, open calcareous vegetation, rich-fens and dry hay meadows. The latter habitat needs management like scything, and such procedures are a challenge for a local municipality to fund, administrate and implement.

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Addendum

The new Norwegian red list for species was published in November 2015 (Henriksen and Hilmo 2015), with the following changes in status: *Anacamptis morio* is upgraded to endangered (as recommended in the paper), *Epipogium aphyllum* upgraded to vulnerable and *Coeloglossum viride* spp. *islandicum* downgraded to endangered. *Hammarbya paludosa* is new with status near threatened. *Neottia nidus-avis* and *Gymnadenia conopsea* ssp. *densiflora* are omitted.

RESULTS OF A STUDY OF *CYPRIPEDIUM* IN SEVERAL REGIONS OF SIBERIA (RUSSIA)

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ABSTRACT

This study of populations of *Cypripedium calceolus* L., *Cypripedium macranthon* Sw. and their hybrid *Cypripedium ventricosum* Sw. in several regions of Siberia was carried out in 2009–2014. Isolated and mixed populations of the two species and their hybrid were surveyed. In sympatric populations, *C. macranthon* is most common in the central part and those of *C. calceolus* prevail in the western part of the range. Mature vegetative and/or reproductive shoots were present but vegetative reproduction was clearly most important in most of the populations investigated. Five colour variations were recorded for *C. ventricosum*. All species benefit from the increase in the intensity of light reaching ground level in forests after ground fires in spring destroy young trees and undergrowth. Vegetative reproduction and the size of the plants increases in burnt areas. Important factors determining the size and the ontogenetic structure of *Cypripedium* populations are: the duration of the growing season, the ecological conditions, the activities of pollinators and the relationships among the three species in sympatric populations.

Keywords: *Cypripedium*, orchids, Siberia

Introduction

The life history of rare species of plants in plant communities must be studied in detail in order to understand how their populations are regulated. *Cypripedium calceolus* L., *Cypripedium macranthon* Sw. and their interspecific hybrid *Cypripedium ventricosum* Sw. are included in the Red Data Book of the Russian Federation (2008) and in many regional Red Data books. These species are listed in Appendix II of CITES. *C. calceolus* is also listed in various international conventions and directives (Terschuren 1999). Isolated and mixed populations of the two species and the hybrid occur in the east of the European part, in the Middle of the Urals, in the south of Siberia and the Far East of Russia.

In Russia, *C. calceolus* occurs in the forest zone in the European part of the country, in southern Siberia and the Far East. *C. macranthon* occurs in the southwest of Central Russia, in the Volga-Kama region, the Ural region, the south of Siberia and in the Far East. The hybrid *C. ventricosum* occurs in the territory between the Urals and Vladivostok where the ranges of the two species overlap (Averyanov 1999, Fig. 1). The sizes of populations of these three rare species vary from solitary plants to thousands of individuals in different parts of their ranges. The most abundant populations occur on calcareous soils, in spring fens and on limestone slopes (Vakhrameeva et al. 2008). *C. calceolus* does not thrive if subject to competition from other herbaceous plants and low levels of illumination (Zaugolnova et al. 1992). Adult vegetative and/or generative plants are dominant in most of the populations studied in coniferous, coniferous-broad-leaved, broad-leaved, small-leaved, coniferous-small-leaved forests and on transitional bogs (Varlygina and Matsenko 1987; Tatarenko 1996; Ishmuratova et al. 2003). The age spectrum of *Cypripedium* populations is 0:8:44:48 (juvenile : immature : vegetative : reproductive) (Tatarenko 1996). This ratio of ontogenetic groups indicates the importance of vegetative reproduction in populations of this species. The ratio of ontogenetic groups differs from the basic spectrum only in populations of *Cypripedium calceolus* growing on slopes in river valleys, with a sparse moss and grass cover (Puchnina 1999), in pine forests with *Sphagnum* cover (Ishmuratova et al. 2003) and in wetland habitats and on limestone outcrops (Teteryuk 2003). A significant number of juvenile (58%) and immature (52%) plants were recorded in those populations that reproduced sexually as well as vegetatively. Apparently, the favourable conditions for the development of seedlings include sufficient moisture and light and the absence of competition from other species. The objectives of this study were: 1) to determine the number of sympatric and isolated populations of *C. calceolus* L., *C. macranthon* Sw. and *C. ventricosum* Sw.; 2) to identify common and specific features of the ontogenetic structure and reproduction in *Cypripedium* populations; 3) to study the spatial structure of *Cypripedium* populations; 4) to make detailed measurements of the morphometric traits in order to identify intraspecific and interspecific variation.

Materials and Methods

Research was carried out in (1) the south of the Krasnoyarsk Territory in 2009 and 2011, (2) in the Irkutsk region and the Republic of Buryatia in 2008, 2010, 2013 and 2014 and (3) in the Altai Republic in 2012 (Fig. 1). In total, 65 populations were studied.

1. Botanical surveys of plant communities with orchids were carried out on 3–10 plots (100 m²). A list plus

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the abundance of each species (Braun-Blanquet 1964) was compiled for each site. The classification of plant communities was based on the dominant species.

2. Above-ground shoot was the accounting unit for the rhizomatous species *C. calceolus, C. macranthon* and *C. ventricosum* (Zaugolnova et al. 1988). The ontogenetic stage was identified for each shoot based on the "concept of discrete ontogeny" (Gatzuk et al. 1980) developed in Russia. According to this concept, ontogeny of an individual plant is divided into 4 periods and 9 stages: I. Latent period (seed); II. Pre-reproductive period (seedling, juvenile (j), immature (im), mature vegetative (v)); III. Reproductive period (young, mature and old reproductive (g)); IV. Post-reproductive period (senescent (s)) (Rabotnov 1950; Uranov 1975; Zaugolnova et al. 1988). The post-reproductive period is rarely observed in orchids (Vakhrameeva and Tatarenko 1998).

3. For each species, 3–30 flowering shoots were measured in each population. Twenty traits were recorded: 1) number of leaves, 2) shoot height, 3 and 4) the length and width of the second leaf, 5) the number of ribs on the second leaf, 6 and 7) the length and width of the dorsal outer petal (sepal), 8 and 9) the length and width of the lateral petal (sepal), 10 and 11) the length and width of the inner petal, 12 and 13) the length and width of the labellum, 14) the depth of labellum, 15) the length of the hole in the labellum from the staminodium to forefront, 16) the width of the wrapped inside edge of the hole in the middle of the labellum, 17) the number of half-turns in the spiral inner petal, 18 and 19) the length and width of the bracts and 20) the length of the free parts of the lateral petal.

For statistical analyses, Excel 2003 and Statistica-6 were used.

Results and Discussion

The regions studied differed in their climates, in particular, the duration of the growing season and amount of annual precipitation. In the south of the Krasnoyarsk region and the Altai Nature Reserve, the growing season is the longest: 140–150 days. On the northeastern coast of Lake Baikal (Barguzinsky Nature Reserve) it is the shortest, 90 days. The highest rainfall was recorded in the mountains on the northeastern and southern coasts of Lake Baikal (about 1000 mm per year). The lowest annual rainfall was recorded in the south of the Krasnoyarsk Territory (about 300 mm per year).

Sympatric populations of *Cypripedium* grow in forests of pine, birch, larch or poplar, in both wet (margins of marshes) and dry habitats (limestone slopes). However, in all these habitats the canopy cover of the trees was between 0.3–0.5, that of the undergrowth between 0.5–0.7 or absent and that of grass between 5–60%. Periodic surface fires regularly occur in Siberia in spring and destroy young trees and undergrowth, which increases the level of illumination in these forests. This promotes active veg-

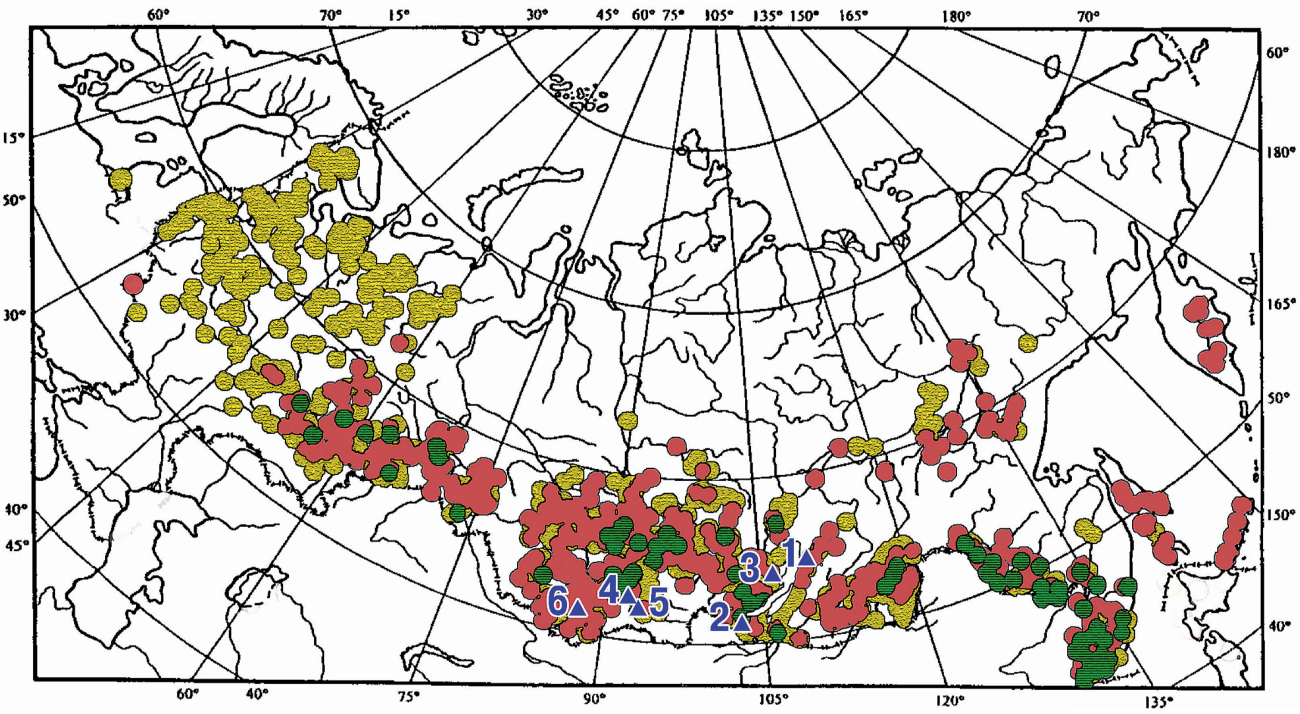

Fig. 1 Map showing the distributions of *Cypripedium calceolus, C. macranthon* and *C. ventricosum* (redrawn with additions from Averyanov 1999) and the locations of the areas surveyed in Russia. *Cypripedium calceolus* (yellow), *Cypripedium macranthon* (red), *Cypripedium ventricosum* (green). The locations of the areas surveyed: 1. Northeastern Baikal (the Barguzin Nature Reserve), 2. Southern Baikal, 3. Western Baikal (the Baikal-Lensky Nature Reserve), 4. Minusinsk Basin (the Shushensky Pine Forest National Park), 5. Usinsk Basin, 6. Lake Teletskoye (the Altai Nature Reserve).

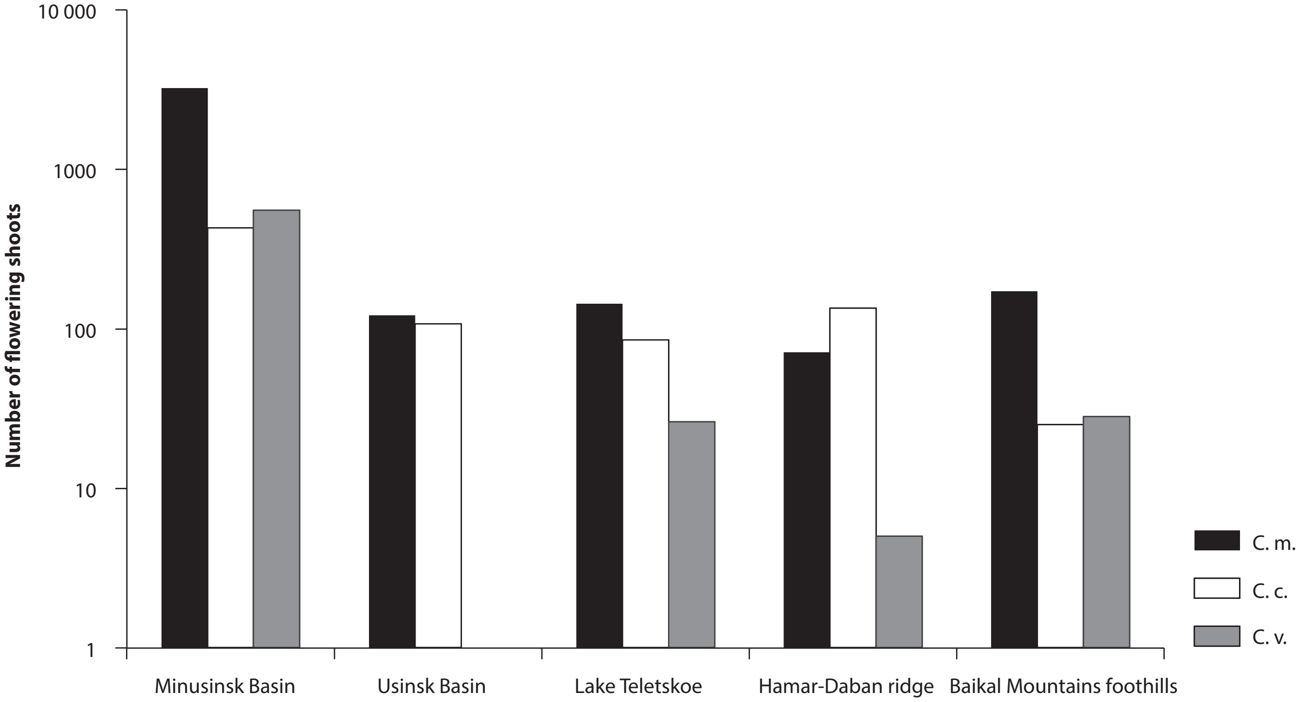

Fig. 2 The numbers of flowering shoots recorded in sympatric populations of *Cypripedium macranthon*, *C. ventricosum* and *C. calceolus* in different areas in Siberia.

etative reproduction and growth of *Cypripedium*, as described for similar conditions in other regions (Smirnov 1969; Dementieva 1985). The largest population of *Cypripedium* (about 5,000 shoots), occupying an area of about 56,620 m², was recorded in the Minusinsk Basin (Fig. 2). There are populations consisting of a few plants in the western foothills of the Baikal Ridge scattered over a 530 m length along the right bank of the Lena River. The average density was 0.04–6.00 shoots/m².

C. macranthon plants prevailed in all sympatric populations of *Cypripedium*, except those on the Khamar-Daban Ridge (Fig. 2). This is typical of populations in the central part of the range, but not the more westerly habitats, where *C. calceolus* was more abundant (Mamaev et al. 2004). The sympatric populations of *Cypripedium* on the Khamar-Daban Ridge are under significant anthropogenic pressure. The *C. macranthon* population has suffered the most serious damage. It is possible that the prevalence of *C. calceolus* is due to removal of *C. macranthon* from natural populations. For example, about 130 plants of *C. macranthon*, 15 of *C. ventricosum* and 3 of *C. calceolus* had been collected from natural habitats near the Sludynka River and planted in private gardens. Another 50 plants, mainly of *C. macranthon* were transplanted to the botanic garden in Irkutsk in 2002, 2004 and 2007 (The gene pool of plants included in the Red Book of the Russian Federation 2012).

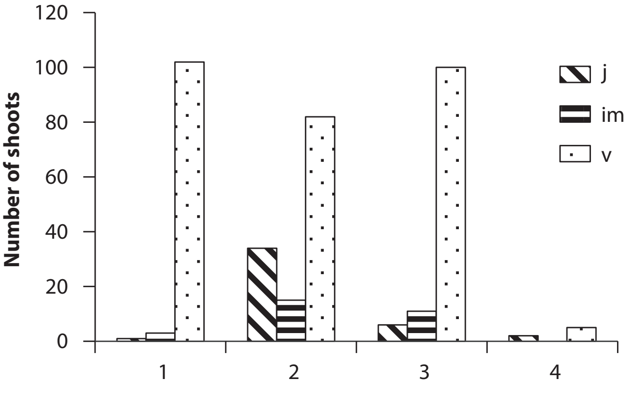

Fig. 3 Numbers of non-flowering shoots recorded in sympatric populations of *Cypripedium* in the Altai Nature Reserve in 2012; j – juvenile plants, im – immature plants, v – vegetative plants.

C. macranthon outnumbered *C. calceolus* by more than seven times, however, hybrid plants occur in sympatric populations of *Cypripedium* in the Shushensky Pine Forest National Park and the Baikal-Lena Reserve. The sizes of the populations of *C. calceolus* and *C. ventricosum* recorded there were similar. However, the number of flowering shoots of *Cypripedium* can vary significantly from year to year: e.g. in 2014, the number of flowering shoots of *C. macranthon* was only half of that recorded in 2013, while for *C. calceolus* and *C. ventricosum* it was the same.

Only one individual of *C. ventricosum* was found in a sympatric population *C. calceolus* and *C. macranthon* in the Usinsk Basin. This could be due to the smaller overlap in the flowering periods of *C. calceolus* and *C. macranthon* there than that recorded in the Minusinsk Basin and the area surrounding Lake Teletskoye. A similar situation was recorded in sympatric populations of

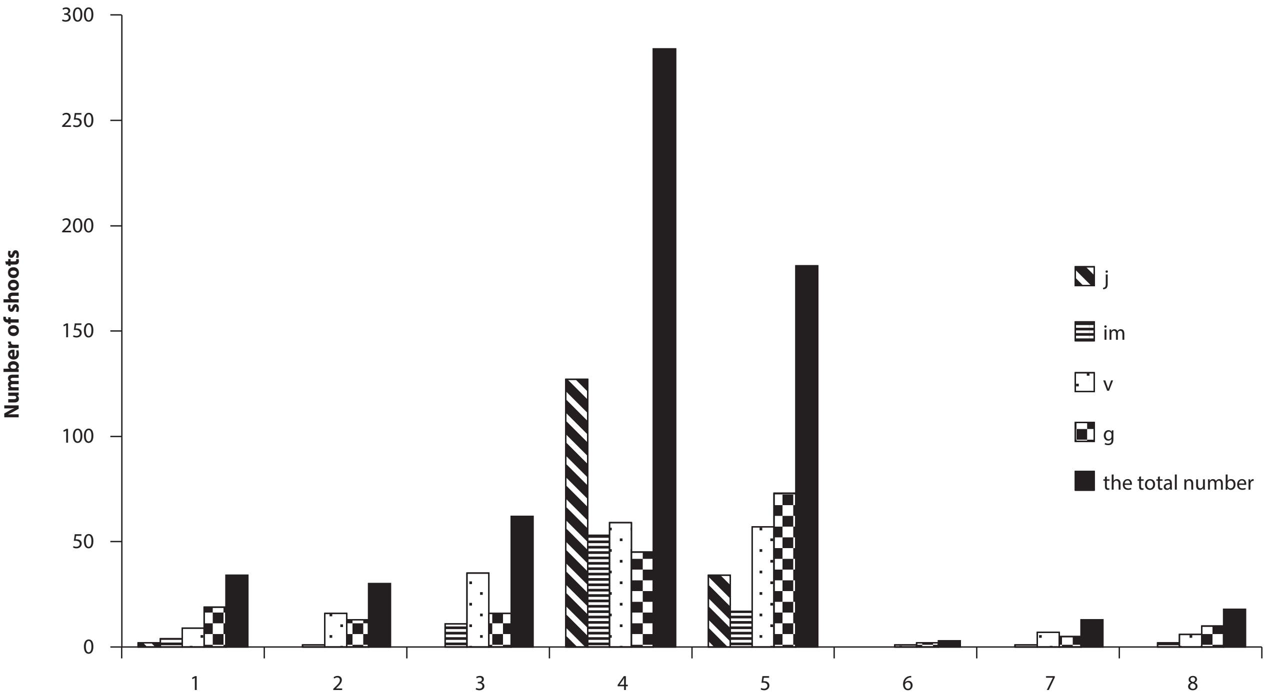

Fig. 4 Ontogenetic spectra recorded for isolated populations of *C. macranthon* and *C. calceolus* in the Barguzinskiy reserve in 2010; 1–5: Populations of *C. macranthon* in: 1) a pine-spruce forest with *Pinus sibirica* and herbaceous plants, 2) a pine forest with *Pteridium aquilinum* and *Rubus saxatilis*, 3) a pine forest with birch and herbaceous plants, 4) a pine forest with herbaceous plants, 5) a pine forest with *Juniperus sibirica*; 6–8: Populations of *C. calceolus* in: 6) a pine forest with *Rubus saxatilis* and *Pteridium aquilinum*, 7) a mixed (*Pinus sibirica* and *P. sylvestris*) forest with herbaceous plants, 8) a spruce forest with aspen and herbaceous plants; j – juvenile plants, im – immature plants, v – vegetative plants, g – reproductive plants.

Table 1 Significant and non-significant parameters of flowering shoots of *C. macranthon, C. calceolus* and *C. ventricosum* revealed by the factor analysis (principal components).

Parameters	***C. macranthon***	***C. calceolus***	***C. ventricosum***	***C.m. – C.c. –C.v.***
L shoot	+	+	+	+
L leaf	+	+	+	–
H leaf	+	–	+	–
Lbr	+	–	+	+
Hbr	+	–	+	+
L dt	+	+	+	–
H dt	+	+	+	+
L lt	+	+	+	+
H lt	–	+	+	+
L it	+	+	+	+
H it	+	–	–	+
L l	+	+	+	+
H l	+	+	+	+
D l	–	–	–	+
H lh	–	–	+	–
N ht	–	–	–	+

L shoot – shoot height; L leaf – the length of the second leaf; H leaf – the width of the second leaf; L br – the length of the bract; H br – the width of the bract; L dt – the length of the dorsal outer petal (sepal); H dt – the width of the dorsal outer petal; L lt – the length of the lateral petal (sepal); Hlt – the width of the lateral petal; L it – the length of the inner petal; H it – the width of the inner petal; L l – the length of the labellum; H l – the width of the labellum; D l – the depth of labellum; H lh – the width of the wrapped inside edge of the middle of the hole in the labellum; N ht – the number of half-turns in the spiral inner petal; + significant parameters; – non-significant parameters.

Table 2 Mean measurements of some of the morphometric parameters of flowering shoots of *C. macranthon*, *C. calceolus* and *C. ventricosum*.

	Species	**mean ± SD**	**mean ± SD**	**mean ± SD**	**F**	***p***
		Total	**Min**	**Max**		
C. macranthon	L shoot	30.94 ± 6.18	25.28 ± 4.71(BLR)	33.94 ± 5.9(AR)	**18.74**	**0**
	L leaf	14.38 ± 2.71	11.93 ± 2.01 (BLR)	16.32 ± 2.29 (AR)	**27.99**	**0**
	H leaf	7.11 ± 1.77	5.51 ± 1.11 (BLR)	8.25 ± 1.57 (AR)	**22.70**	**0**
	L dt	4.62 ± 0.67	4.21 ± 0.59 (BLR)	4.98 ± 0.65 (AR)	**9.74**	**0**
	H dt	3.38 ± 0.5	3.13 ± 0.47 (BLR)	3.57 ± 0.49 (AR)	**5.22**	**0**
	L lt	3.51 ± 0.62	3.13 ± 0.67 (BLR)	3.78 ± 0.57 (AR)	**8.06**	**0**
	L it	5.37 ± 0.69	5.07 ± 0.62 (BLR)	5.73 ± 0.67 (AR)	**7.48**	**0**
	H it	1.96 ± 0.36	1.87 ± 0.57 (Sl)	2.04 ± 0.37 (AR)	**3.32**	**0.02**
	L l	4.52 ± 0.64	4.3 ± 0.64 (BLR)	4.75 ± 0.65 (AR)	**4.04**	**0.01**
	H l	3.55 ± 0.53	3.35 ± 0.5 (BLR)	3.76 ± 0.56 (AR)	1.26	0.29
	D l	2.44 ± 0.30	2.37 ± 0.21 (Shb)	2.5 ± 0.32 (Sl)	**13.88**	**0**
C. calceolus	L shoot	34.72 ± 7.16	29.53 ± 5.61 (BLR)	38.49 ± 5.62 (Shb)	**7.74**	**0**
	L leaf	14.56 ± 2.82	12.21 ± 2.43 (Sl)	16.11 ± 2.44 (Shb)	**12.79**	**0**
	H leaf	7.20 ± 1.70	6.20 ± 1.45 (Sl)	8.31 ± 1.44 (AR)	**4.24**	**0.01**
	L dt	4.84 ± 0.67	4.23 ± 0.58 (BLR)	5.56 ± 0.43 (AR)	**17.48**	**0**
	H dt	1.90 ± 0.38	1.59 ± 0.31 (BLR)	2.20 ± 0.22 (AR)	**9.44**	**0**
	L lt	4.40 ± 0.71	3.74 ± 0.69 (BLR)	5.01 ± 0.51 (AR)	**13.21**	**0**
	Hlt	1.64 ± 0.26	1.48 ± 0.21 (BLR)	1.76 ± 0.26 (Shb)	**5.16**	**0**
	L it	4.9 ± 0.81	4.39 ± 0.68 (BLR)	5.4 ± 1.13 (AR)	**3.93**	**0.01**
	H it	0.60 ± 0.16	0.50 ± 0.10	0.66 ± 0.14 (AR)	2.39	0.08
	L l	2.98 ± 0.36	2.77 ± 0.32 (BLR)	3.23 ± 0.24 (AR)	**3.91**	**0.01**
	H l	1.94 ± 0.32	1.62 ± 0.20 (BLR)	2.13 ± 0.18 (AR)	**7.26**	**0**
	D l	1.56 ± 0.20	1.54 ± 0.16 (AR	1.58 ± 0.23 (Shb)	0.05	0.98
C. ventricosum	L shoot	36.14 ± 8.18	27.35 ± 7.77 (BLR)	41.68 ± 7.86 (AR)	**21.08**	**0**
	L leaf	14.30 ± 3.16	12.05 ± 2.70 (BLR)	16.66 ± 2.56 (AR)	**8.94**	**0**
	H leaf	7.26 ± 1.80	5.79 ± 1.76 (BLR)	8.45 ± 2.06 (AR)	**10.25**	**0**
	L dt	5.02 ± 0.68	4.52 ± 0.38 (BLR)	5.82 ± 0.46 (AR)	**19.65**	**0**
	H dt	2.69 ± 0.47	2.34 ± 0.42 (BLR)	3.10 ± 0.42 (AR)	**11.39**	**0**
	L lt	4.26 ± 0.58	3.86 ± 0.43 (BLR)	4.76 ± 0.42 (AR)	**10.32**	**0**
	Hlt	2.33 ± 0.34	2.20 ± 0.30 (BLR)	2.47 ± 0.34 (AR)	2.14	0.13
	L it	5.40 ± 0.69	4.9 ± 0.35 (BLR)	6.24 ± 0.60 (AR)	**19.55**	**0**
	H it	1.12 ± 0.20	1.07 ± 0.21 (BLR)	1.19 ± 0.19 (AR)	1.24	0.30
	L l	3.87 ± 0.49	3.65 ± 0.46 (BLR)	4.05 ± 0.49 (AR)	2.44	0.10
	H l	2.72 ± 0.38	2.52 ± 0.43 (BLR)	2.79 ± 0.25 (Shb)	2.99	0.06
	D l	2.06 ± 0.19	2.01 ± 0.22 (BLR)	2.22 ± 0.13 (AR)	**5.76**	**0**

L shoot – shoot height; L leaf – the length of the second leaf; H leaf – the width of the second leaf; L dt – the length of the dorsal outer petal (sepal); H dt – the width of the dorsal outer petal; L lt – the length of the lateral petal (sepal); Hlt – the width of the lateral petal; L it – the length of the inner petal (petal); H it – the width of the inner petal; L l – the length of the labellum; H l – the width of the labellum; D l – the depth of labellum; N ht – the number of half-turns in the spiral inner petal; AR – Altai Nature Reserve; Shb – Shushensky Pine Forest National Park; BLR – Baikal-Lensky Nature Reserve; Sl – Southern Baikal (Sludyanka); significant differences are in bold.

C. calceolus and *C. macranthon* growing on the shores of Lake Baikal in the southern Baikal area in 2014. Sympatric populations of *C. macranthon* and *C. ventricosum* in which there are no *C. calceolus* and isolated populations of *C. ventricosum* were recorded in the Altai Nature Reserve. Hybrid plants could have been a result of pollinators' transferring *C. calceolus* pollen over a long distance.

The non-flowering stages of these three species of *Cypripedium* are indistinguishable from one another. The non-flowering shoots of *Cypripedium* were mainly mature in all the populations studied and illustrated in Fig. 3 for the Altai Nature Reserve. Success of reproduction by seed in *Cypripedium* populations was estimated based on the number of juvenile plants. The percentage abundance of juvenile plants in the populations studied was 1.1–8.0%.

Isolated populations of *Cypripedium calceolus* occurred in pine, larch, spruce and birch forests in the south of the Krasnoyarsk region (Usinsk Basin) and on the northeastern coast of Lake Baikal (Barguzinsky Nature Reserve). *C. calceolus* was most abundant in a birch forest in the Usinsk Basin (246 shoots), where the average number of shoots was 0.001–13.0 m^{-2}.

Isolated populations of *Cypripedium macranthon* occurred in both pine and pine with birch and aspen forests. *C. macranthon* was most abundant in the mixed pine with birch forest in the Barguzinsky Nature Reserve (527 shoots), where the average number of shoots was 0.1–5.6 m^{-2}. Maximum sizes of isolated populations of *C. calceolus* and *C. macranthon* were significantly smaller than those of sympatric *Cypripedium* populations in the Minusinsk Basin.

In most isolated populations, mature vegetative and reproductive plants clearly dominated, which corresponds to the basic spectrum for this species, which indicates that vegetative reproduction is most successful (Tatarenko 1996). Juvenile plants made up 44.7% of one of the *C. macranthon* populations in the Barguzinsky Nature Reserve (Fig. 4). This was probably a result of favourable weather combined with optimal illumination and the fact that plants were growing on limestone outcrops. Juvenile plants were not recorded in small populations of *C. calceolus* in the Usinsk Basin and Barguzinsky Nature Reserve.

Factor analysis (principal components) revealed which parameters are significant for the differences between populations and species (Table 1).

One-way ANOVA revealed significant differences for most of parameters studied, except the number of leaves, number of ribs, the length of the hole in the labellum measured from the staminode to the forefront, the width of the wrapped inside edge of the hole in the labellum measured in the middle and the length of the free parts of the lateral petal (Table 2). The parameters of the *C. ventricosum* flowers are intermediate between its parental species, *C. calceolus* and *C. macranthon*. Similar peculiarities of these three species have been recorded in the Urals (Knyazev et al. 2000). *C. macranthon, C. calceolus* and *C. ventricosum* plants were bigger in populations in wet forests in the warmer western regions: the Altai Nature Reserve (AR) and the southern part of the Krasnoyarsk Territory (Shushensky Pine Forest National Park (Shb)). The plants growing on dry limestone slopes in the Baikal-Lensky Nature Reserve were smaller (Table 2). Two habitat-dependent forms of *C. calceolus* were also recorded growing in the Komi Republic: plants growing in wet forests and swamps were taller than those growing on dry limestone slopes (Martynenko et al. 2003).

Five colour variations of *C. ventricosum* were recorded. Hybrid plants with typically coloured perianth (dark pink with white) dominate in *C. ventricosum* populations; hybrid plants with pink petals and a white labellum or with dark red petals and a yellow labellum with red or orange veins were rare.

Typical colour of the perianth was recorded more frequently than the atypical colour, which was rare. In the Shushensky Pine Forest National Park in 2011 the colour of the perianth of between 21.4 and 34.9% of the *C. ventricosum* plants was atypical (Fig. 5). Such plants (mainly with dark red petals and yellow labellum with with red or orange veins) were likely to be the result of backcrossing of *C. ventricosum* with *C. calceolus*. Similar coloured flowers were recorded in the Altai Nature Reserve and on the Khamar-Daban Ridge. Plants with a white labellum were extremely rare.

The percentage of plants fruiting in sympatric populations of *Cypripedium* was measured in the Shushensky Pine Forest National Park in 2011. The highest percentage was recorded for *C. macranthon* and the lowest for *C. ventricosum* (Fig. 6). Pollen of *C. ventricosum* can be fully or partially sterile (Andronova 2011). The induction of fruit in *C. ventricosum* is most often a consequence backcrossing with one of the parental forms. In the habitats surveyed in the current study and in the Urals (Knyazev et al. 2000), the low percentage fruiting

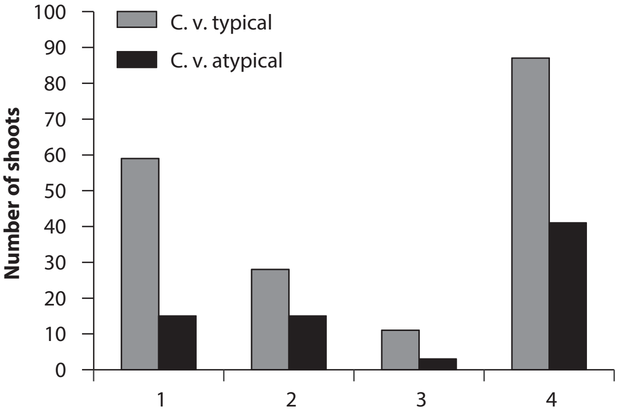

Fig. 5 The number of flowering shoots with typically and atypically coloured perianths recorded in *C. ventricosum* populations in the Shushensky Pine Forest National Park in 2011.

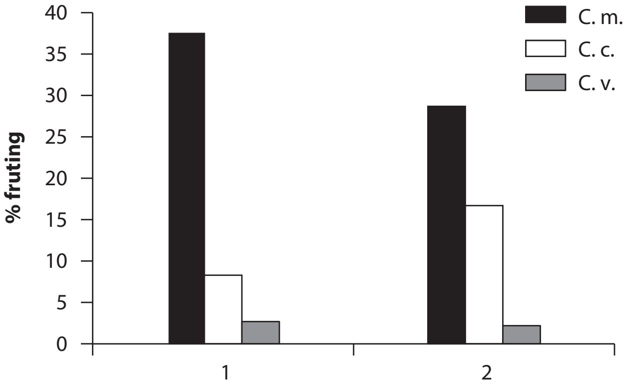

Fig. 6 Percentage of plants recorded fruiting in populations of *Cypripedium* in the Shushensky Pine Forest National Park in 2011.

plants of *C. calceolus* was possibly due to competition with *C. macranthon* for pollinators, the latter has larger and brighter flowers.

Conclusions

1. The largest sympatric populations of *Cypripedium* were recorded in the Minusinsk Basin in the south of the Krasnoyarsk territory.

2. The plants of *C. macranthon* dominated in most sympatric populations of *Cypripedium*, which is apparently typical of populations of these species in the central part of the area studied but not the more western parts, where *C. calceolus* plants dominated.

3. Mature vegetative and/or reproductive shoots and vegetative reproduction clearly dominated the ontogenetic structure of most of the populations investigated, which is the basic spectrum for these species.

4. Important interspecific differences were recorded in shoot height, bract length, bract width, width of dorsal outer petal, length of lateral petal (sepal), width of lateral petal, length of inner petal, width of inner petal, length of labellum, width of labellum, depth of labellum and number of half-turns in the spiral inner petal.

5. Intraspecific (inter-population) differences were recorded in the length of the shoot, leaf parameters, length of dorsal outer petal (sepal), width of dorsal outer petal, length of lateral petal (sepal), width of lateral petal, length of inner petal, length of labellum, and width of the labellum. Plants of *C. macranthon*, *C. calceolus* and *C. ventricosum* growing in wet forests in the warmer western regions of the Altyi and the southern part of the Krasnoyarsk Territory were the biggest and the smallest were those growing on dry limestone slopes in the Baikal-Lensky Nature Reserve.

6. Hybrid plants with a typically coloured perianth were dominant in *C. ventricosum* populations.

7. The highest percentage of fruiting plants was recorded for *C. macranthon*, possibly because it wins in competition for pollinators with *C. calceolus*. *C. ventricosum*, for which lowest percentage fruiting was recorded in sympatric populations in the Shushensky Pine Forest National park, where it fruits only when it backcross with one of the parental forms.

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EXTENT AND REASONS FOR MEADOWS IN SOUTH BOHEMIA BECOMING UNSUITABLE FOR ORCHIDS

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ABSTRACT

Decline in wet grasslands, which in the past resulted particularly from the intensification of agriculture, was accompanied by the loss of a lot of populations of organisms, including protected and endangered species of plants and animals associated with these habitats e.g. terrestrial orchids. The survival of populations of many species of European orchids is strongly dependent on appropriate site management, especially regular mowing or grazing. In addition, humans can negatively affect the persistence of orchid populations in various ways, such as conversion of orchid meadows into building areas, dams, roads etc. or the intensive use of fertilizers and contamination of areas by fertilisers from nearby fields. Comparison of historical data with the present distribution of orchids can reveal a lot about the main reasons for the decline in this endangered group of plants. Here we present an extensive study of the persistence of 192 historical orchid sites in South Bohemia, with particular reference to the 5 commonest species of orchids, *Anacamptis morio*, *Dactylorhiza majalis*, *Epipactis helleborine*, *Epipactis palustris* and *Platanthera bifolia*. We show that the most abundant species at the sites studied was *Dactylorhiza majalis*. *E. palustris*, *A. morio* and *P. bifolia* are currently not present at any of the historical localities for these species. Considering more recent history, the situation regarding orchid localities in South Bohemia is not critical, but the fate of these species should be closely monitored. The majority of this loss is due to the cessation of mowing of the sites. Thus more attention should be paid to the management of the existing sites. During this study, some new sites were discovered.

Keywords: extinction, meadows, terrestrial orchids, South Bohemia

Introduction

The diversity of life on Earth is in rapid decline (Dirzo and Raven 2003; Possingham and Wilson 2005). Thus understanding the main factors determining species diversity is crucial for their survival (Possingham and Wilson 2005) and identifying important areas for their conservation (Tsiftsis et al. 2011). This is especially the case for threatened groups such as orchids (Efimov 2011; Feldman and Prat 2011).

Extensively cultivated, species-rich wet meadows have until recently been a common part of the central European landscape. These, from the viewpoint of a farmer, are frequently regarded as secondary habitats and their value is still not fully appreciated, not only from a landscape viewpoint (in particular the retention of water in the countryside with all its consequences for the local microclimate), but also because of their high and valuable contribution to the biodiversity of the area. Decline in wet grasslands, which started in the past and was particularly associated with the intensification of agriculture, was accompanied by the extinction of a lot of populations of organisms, including those of protected and endangered species of plants and animals associated with these habitats, e.g., terrestrial orchids (Wotavová et al. 2004).

The survival of populations of many European orchids is very dependent on appropriate site management, especially regular mowing or grazing (Waite and Hutchings 1991; Lind 1992; Kull 2002). Mowing is generally considered to result in conditions favourable for orchids (Kull 2002), but present methods of mowing does not necessarily result in the persistence of orchid populations (Tamm 1991). Effect of management on orchid populations is best assessed by long-term monitoring (Inghe and Tamm 1988; Wells and Cox 1989, 1991; Vanhecke 1991; Willems and Bik 1991; Falb and Leopold 1993; Sieg and King 1995; Gill 1996; Wells et al. 1998; Kindlmann and Balounová 2001; Brzosko 2002; Janečková and Kindlmann 2002; Jersáková et al. 2002; Øien and Moen 2002; Tali 2002 etc.).

Humans, however, can negatively affect the persistence of orchid populations in many other ways. One is direct conversion of orchid meadows into building areas, dams, roads or other products of civilisation. The other is intensification of agriculture: negative side-effects of modern agricultural management (intensive use of fertilizers or contamination by fertilisers washed out from nearby fields) on the occurrence of orchid populations are confirmed by the results of several manipulative experiments (Dijk and Olff 1994; Silvertown et al. 1994; McKendrick 1996).

Comparison of historical data with the present distribution of orchids can reveal a lot about the main reasons for the decline in this endangered group of plants. This was previously done for the common orchid species *Dactylorhiza majalis* at 50 historical sites in South Bohemia by Wotavová et al. (2004). They found that the main reasons for its extinction were cessation of mowing, intensive use of fertilizers and contamination by fertilisers washed out from nearby fields.

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Here we present a much more extensive study of the persistence of 192 historical orchid sites in South Bohemia, with particular reference to the 5 commonest orchid species, *Dactylorhiza majalis*, *Epipactis helleborine*, *Epipactis palustris*, *Anacamptis morio* and *Platanthera bifolia*. Four of them live in meadows and *E. helleborine* is a forest species, which is included for comparison. Our results indicate that the most abundant species at the sites studied is *Dactylorhiza majalis*. No plants of *E. palustris*, *A. morio* and *P. bifolia* were found at any of the historical localities. Considering the more recent history, the situation regarding orchid localities in South Bohemia is not critical, but these species should be closely monitored. The majority of the extinctions were recorded at sites that are no longer mowed, which is consistent with the results of Wotavová et al. (2004). Thus more attention should be paid to the management of existing sites. During this study, some new sites were discovered.

Methods

For our analyses, we selected the following five most abundant species in the study area:

- *Dactylorhiza majalis*, also known as the western-marsh orchid (Reichenb., Hunt and Summerh), has a relatively wide ecological tolerance occurring in lowlands and mountains, in wet to damp meadows, wetlands and peatlands and is less sensitive to high levels of nitrogen than other orchid species (Jatiová and Šmiták 1996; Wotavová et al. 2004; Janečková et al. 2006). Its leaves appear usually during April and last until July and it flowers mainly in May and June (Wotavová et al. 2004; Janečková et al. 2006). This species is almost extinct in lowland basins, where it survives mainly in protected areas (Jatiová and Šmiták 1996). Generally speaking, it is classified among the most vulnerable species by the IUCN (Wotavová et al. 2004).
- Broad-leaved helleborine (*Epipactis helleborine* (L.) Crantz) is the commonest and most widely distributed species in this genus and occurs from the Mediterranean area to boreal zones up to 2000 m (Ehlers et al. 2002; Delforge 2006). It grows in forests, at forest edges and also in anthropogenic habitats such as rural and urban road verges, tracks, parks and lawns (Hollingsworth and Dickson 1997; Stefaniak et al. 2011) and flowers mainly from June to September (Rewicz et al. 2015). In this study, it is the only representative of a forest species.
- *Epipactis palustris* (marsh helleborine) is widely distributed occurring throughout most of Europe, but totally absent from the southern Mediterranean region. This species grows on moist to wet substrates, which are poor in nutrients. It has a long flowering period; however, the peak of flowering occurs between mid-July and mid-August (Jacquemyn et al. 2014).
- *Anacamptis morio* is a perennial herb, which has a broad ecological range and occurs in various habitats: in semi-wet meadows, pastures and on shrubby slopes in lowland areas and the submontane zone (Delforge 1995; Jersáková et al. 2002). This species flowers in early May and after seed dissemination all above ground parts die off and the first leaves appear again in September to October. *A. morio* belongs to the orchid group with an above ground rosette, which persists over winter (Jersáková et al. 2002).
- *Platanthera bifolia* (bog orchid), is found in moist habitats in temperate and subtropical regions (Patt et al. 1989). It is a terrestrial orchid with a Eurasian distribution (Hultén and Fries 1986), growing in open forests and meadows (Stpiczyńska 1997) and flowering from May to July (www.botany.cz).

We surveyed all the available historical and recent databases of terrestrial orchid localities in South Bohemia. We selected at random a total of 192 of these orchid localities, which according to the databases hosted some of the above five species in the past and revisited all of them in the flowering periods of the orchid species indicated as present there in the databases. We distinguished "historical sites", those reported before 2000, and "recent sites", those reported after 2000. The choice of the year 2000 as a border line between "historical" and "recent" sites was chosen arbitrarily.

At each of the sites where there were no longer any orchids we determined the reason for this as either "overgrown", lack of mowing or grazing, or "human pressure", conversion of orchid meadows into building areas like dams, roads or other products of civilisation, or effects of intensive use of fertilizers or contamination by fertilizers washed out from nearby fields. Some sites were labelled as "not found" or "undiscovered". This was the cases when a site corresponding to the description in the database was not found in the area indicated or in the vicinity of up to about 1 km. These may be cases where man has changed the landscape so dramatically during recent decades (e.g., by merging fields, building roads or other buildings) that the original site was no longer recognizable.

Results

During 2014 we visited a total of 192 localities. Numbers of localities visited where the five most abundant species were recorded are shown in Table 1. Fig. 1 presents numbers of sites where there were living orchids, where there were currently no orchids and those that could not be found. The most abundant species was *D. majalis*, most likely because it is less demanding in terms of environmental conditions than the other species.

The commonest causes of localities becoming unsuitable for orchids are given in Fig. 2: overgrowing of the site by other more dominant species. Even some forest sites of *E. helleborine* were overgrown by undergrowth. Other causes include human activities like converting meadows into fields or deforestation in the case of *E. helleborine*.

Figs. 3 and 4 show the effect of differences in management on populations of *E. palustris* and *A. morio*. Both of the sites for *A. morio* hosted a viable population of this species 15 years ago. The population at the unmown site went extinct, while that at the mown site is thriving and consists of tens of thousands of flowering plants.

Figs. 5 and 6 show the percentage of localities that became unsuitable for particular orchids (Fig. 5) and at historical vs. recently recorded localities (Fig. 6). There were no records of historical localities in the databases for this species, which explains the lack of corresponding data in Fig. 6.

For orchid dynamics it is very important that even during the first year we found a number of previously unrecorded localities (Fig. 7): 11 for *D. majalis*, 12 for *E. helleborine* and 1 each for *O. morio* and *P. bifolia*. No new localities were recorded for *E. palustris*.

Discussion

The small decline in the number of localities with *D. majalis* may be due to its typical habitat, wet meadows, being unsuitable for farming and therefore its localities were usually not as much affected by people as other kinds of habitats. The decline in this species was therefore caused mainly by the absence of mowing and intensive use fertilizer (Wotavová et al. 2004). Jatiová and Šmiták (1996) in contrast, report that only 202 out of 1141 sites for *D. majalis* in Moravia (eastern part of the Czech Republic) still hosted this species in 1995. It may be that agricultural intensification was more marked in Moravia. It is a general trend that wet meadows, which are rich in biodiversity, are currently rapidly disappearing (Wotavová et al. 2004).

E. helleborine is a forest species. Forests have not been disturbed as much as open localities and therefore there has been less of a decline in the number of suitable localities for *E. helleborine*. According to Rewicz et al. (2015), there are big differences between natural and anthropogenic populations of *E. hellebrine* in the amount of seed produced and the size of the plants, which can affect its population dynamics and therefore its persistence in the countryside.

E. palustris has similar ecological demands to *D. majalis*, so the decline in the number of suitable localities for this species is similar, except for historical sites. However, the databases contain only 2 historical sites, neither of which are now suitable and therefore this estimate is not

Table 1 Number of localities with the five most abundant species visited in South Bohemia.

Species	Number of localities visited
Dactylorhiza majalis	121
Epipactis helleborine	28
Epipactis palustris	6
Anacamptis morio	12
Platanthera bifolia	25

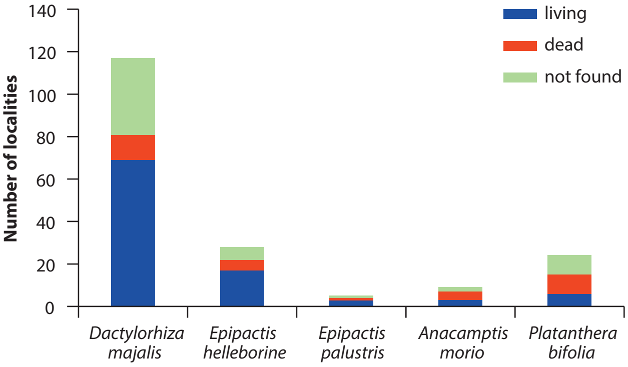

Fig. 1 Number of localities in South Bohemia where the different orchids are currently present or extinct, and the number of localities that could not be found.

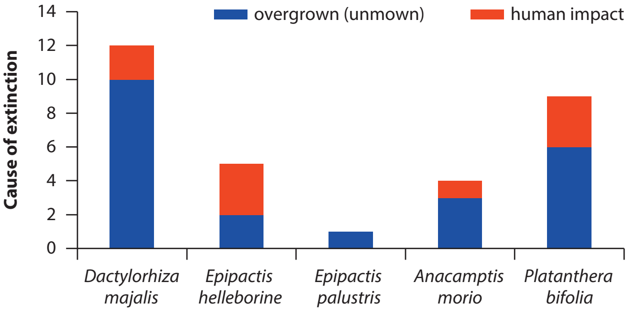

Fig. 2 Main causes of localities becoming unsuitable for orchids in South Bohemia.

Fig. 3 Photograph showing the overgrowing of a locality for *Epipactis palustris* by *Phragmites australis*.

Fig. 4 Comparison of an unmown (A) and mown (B) site for *A. morio*. Both sites hosted a viable population of this species 15 years ago. The population at the unmown site went extinct, while that at the mown site is thriving and hosts ten of thousands of flowering plants.

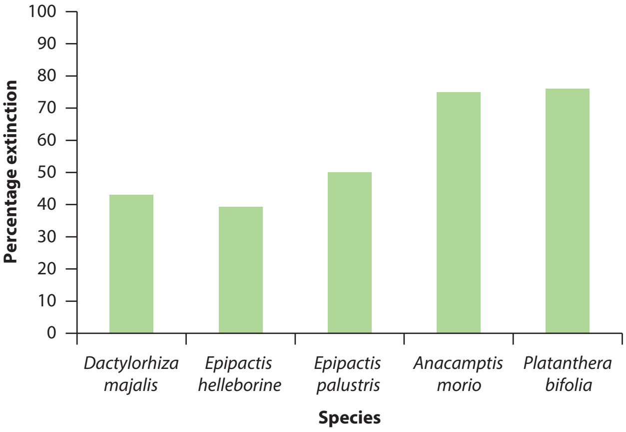

Fig. 5 Percentage extinctions at all localities for the five species of orchids.

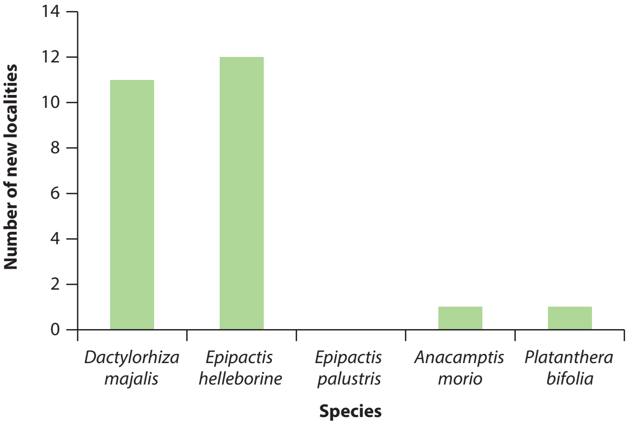

Fig. 7 Number of new localities recorded during summer 2014.

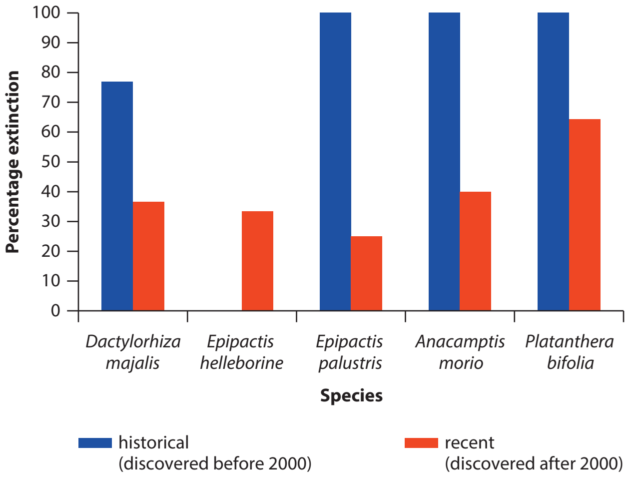

Fig. 6 Percentage extinctions at historical vs. recent localities for the five species of orchids.

reliable. In fact, there has been a marked decline in the distribution of *E. palustris* in Britain (Kull and Hutchings 2006). The number of populations declined by about 24.6%, most of which disappeared before 1930. The major cause of its decline is believed to be drainage of marshes together with agricultural improvements and inadequate management (Jacquemyn et al. 2014).

A. morio grows in dry meadows and pastures in the study area, which were very often converted into agricultural fields and because of this the number of localities for this species declined (especially in the case of the historical localities). Another reason for the rapid decline might be the cessation of mowing or management in general (Fig. 4; see also Kindlmann and Balounová 1999, 2000; Jersáková et al. 2002).

The situation for *P. bifolia* is more complicated. This species seems to be affected by habitat conditions, as they differ in size, proportion of flowering individuals and the effectiveness of reproduction, which is connected with their ability to store resources before flowering (Calvo 1990; Brzosko 2003). Shade can also play a role. For example, Brzosko (2003) found that plants growing in full light had longer inflorescence and produced more flowers and fruits than those growing in shade. We are not quite sure, why the decline was so dramatic in the populations studied. More research is therefore needed on this topic.

Conclusions

Our results indicate that the most abundant species at the sites studied was *Dactylorhiza majalis*. There are currently no plants of *E. palustris*, *A. morio* and *P. bifolia* at any of the historical localities for these species. Considering more recent history, the situation regarding orchid localities in South Bohemia is not critical, but these species should be closely monitored. The majority of extinctions was due to cessation of mowing, which is consistent with the results of Wotavová et al. (2004). Thus more attention should be paid to the management of existing sites. During this study, some new localities for these orchids were recorded.

Acknowledgements

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IN VITRO POLLEN GERMINATION OF ORCHIDS TRADITIONALLY USED TO PRODUCE SALEP

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ABSTRACT

In Turkey the tubers of about 120 orchid species are widely collected for manufacturing the traditional drink salep. In this study, we focused on the *in vitro* germination of the pollen of the salep orchid species *Ophrys mammosa*, *Orchis provincialis*, *Anacamptis morio* subsp. *morio*, *Orchis simia* and *Neotinea tridentata* and discussed the potential effects this might have on the conservation of these orchids by reducing the need to collect them in the field. Pollen was sown on different media; Knudson, Orchimax and the medium described by Malmgren, and then incubated at 24 ± 1 °C in darkness for 24 h. Germinated pollen was stained with Brilliant Blue and examined under a stereoscopic microscope. Results of Tukey and Dunnett T3 statistical tests indicated that in terms of percentage germination, the best germination was observed on *O. mammosa* by 55% and Orchimax was the most successful medium by 50.5%. For pollinaria germination, the best rate was observed on *O. mammosa* by 69%. The medium Malmgren was the best germinative by 61.3%. It is clearly seen that difference in germination rates among studied species are achieved using different media. The development of such a method of studied species in this research points to the fact that this is possible and should serve as encouragement for others to devise procedures for other species. These kinds of researches on propagation of orchids would be useful to reintroducing some of the rarer, endangered and endemic species in Turkey such previously succeed for *Orchis militaris* and *Liparis loeselii* in Great Britain.

Keywords: pollinium, pollinarium, orchid, *in vitro* germination, salep, conservation

Introduction

Orchids are cosmopolitan and occur in almost every habitat except in the Polar Regions (Edwards 2007). With more than 1000 genera and at least 25,000 species, the family Orchidaceae is the largest and the most diverse family of flowering plants (Harrap and Harrap 2009). While nearly 70% of orchids live on other plants as epiphytes, they can also grow in soil (25%), and live on rocks and decaying plants (5%) (Arditti 1979; Renz and Taubenheim 1984). In Turkey there are 170 terrestrial orchids belonging to 24 genera (Kreutz 2009).

In addition to their aesthetical and medicinal importance, orchids are ecological indicators (Joshi et al. 2009). Moreover, orchids are used to manufacture a nutritious drink, called salep, which has been very popular for centuries in Anatolian and Arabian cultures and used as an additive in the production of "Maras" ice cream in Turkey (Sezik 1984; Baytop 1999; Kreutz 2009).

Table 1 Tuber weights and tuber counts of orchids per 1000 kg (Sezik 1984).

Commercial name	Average tuber weight (g)	Number of tubers per kg
Muğla Salebi	0.23	4348
Kastamonu Salebi	0.50	2000
Silifke Salebi	0.35	2857
Antalya Salebi	0.21	4762
Maraş Salebi	1.60	625
Van Salebi	1.00	1000

Around 120 orchid species, including the genera; *Aceras, Anacamptis, Balia, Dactylorhiza, Himantoglossum*, *Neotinea*, *Ophrys*, *Orchis* and *Serapias*, are used to make salep in different places in Anatolia. Therefore, orchids belonging to these genera are widely and intensively collected from nature. In this study, we focused on the orchids *Ophrys mammosa* (Desf.), *Orchis provincialis* (Balb.), *Anacamptis morio* (L.) R. M. Bateman, Pridgeon and M. W. Chase subsp. *morio*, *Orchis simia* (Lam.) and *Neotinea tridentata* (Scop.) R. M. Bateman, Pridgeon and M. W. Chase, which have been collected since ancient times in Anatolia and used to make salep (Özhatay et al. 1997; Baytop 1999).

Although orchids are protected worldwide from overexploitation by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (UNEP-WCMC 2013), they are still threatened by the illegal trade in orchids and the collecting of them from nature to produce salep and "Maras" ice cream (Sezik et al. 2007; Kreutz 2009). It is estimated that 20–30 million orchids weighing 20 tons are collected from nature annually for producing salep (See Table 1).

Because of the threat from logging, mining, urbanisation as a consequence of the increase in the human population, agricultural activities and the collecting of orchids from nature for the manufacture salep it is important to develop new and advanced techniques for their sustainable usage (Kreutz 2009; Cribb 2011). This has resulted in improvements in the *in vitro* propagation of orchids in order to reduce the need to collect orchids from nature and make it possible to conserve the most threatened species (Magrini et al. 2011). Thus, in this

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Table 2 List of the species studied, locations, dates of collection and numbers of pollinaria collected.

Species	Collection location (Çanakkale, Turkey)	Collection of pollinaria	
		Date (2014)	Number of pollinaria
Anacamptis morio subsp. *morio*	Kilitbahir	April 15	210
Neotinea tridentata	Onsekiz Mart University Terzioğlu Campus	April 20	211
Ophrys mammosa	Kilitbahir	March 31	201
Orchis provincialis	Denizgöründü village	April 21	328
Orchis simia	Denizgöründü village	April 21	199

study, we developed a new method for the *in vitro* propagation using the pollen of orchids used in the manufacture of salep.

Although there are studies on the evolution (Bradshaw and Schemske 2003), ecology (Desrochers and Rieseberg 1998), pollen ovule aging (Proctor 1998; Bellusci and Musacchio 2010), morphology and ultra-structure of orchids (Feijo and Pais 1989; Pacini and Michael 2002) the germination pollen has not been previously studied (Pritchard and Prendergast 1989; Aybeke 2002; Pacini and Michael 2002).

While many orchid genera have pollinia, which is an adhesive mass of pollen, there are a few genera that produce single pollen grains. Therefore, orchids differ greatly in terms of whether they produce a mass of sticky pollen or individual grains of pollen (Aybeke 2002). While the pollen of *Pterostylis plumosa*, *Pterostylis concinna*, *Neottioids*, *Neuwiedia*, *Cypripedium acaule*, *Cypripedium calceolus*, *Apostasia wallachii* consists of individual grains, that of *Epipactis microphylla*, *Bletilla striata*, *Neottia*, *Cleistes divaricata*, *Neottia nidus-avis, Epidendrum scutella*, *Epidendrum ibaguense*, *Loroglossum hircinum*, *Pleurothallis eumecocaulon* and *Calypso bulbosa* consists of groups of four pollen grains (Pacini and Michael 2002). Using light-microscopy we observed that *O. mammosa*, *O. provincialis*, *A. morio* subsp. *morio*, *O. simia* and *N. tridentata* produce tetrad groups of pollens contained in pollinia.

The aims of this study are to: (1) determine whether it is possible to germinate the pollen of *O. mammosa*, *O. provincialis*, *A. morio* subsp. *morio*, *O. simia* and *N. tridentata*; (2) whether there differences in percentage germination between species and (3) the effects of KN, ORC and SV media on percentage germination.

Material and Methods

Collection of Study Materials

Pollinia were collected from 5 species of orchids: *Ophrys mammosa* (Desf.), *Orchis provincialis* (Balb.), *Anacamptis morio* (L.) R. M. Bateman, Pridgeon and M. W. Chase subsp. *morio*, *Orchis simia* (Lam.) and *Neotinea tridentata* (Scop.) R. M. Bateman, Pridgeon and M. W. Chase, growing wild in the centre of Çanakkale city and its surroundings (Turkey) (See Table 2 and Fig. 1).

Plants were identified using "Flora of Turkey and the East Aegean Island" vol. 8 (Renz and Taubenheim 1984) and vol. 11 (Kreutz 2000), "Orkidelerimiz" (Sezik 1984) and "Türkiye Orkideleri" (Kreutz 2009).

In vitro Germination of Pollen

Germination tests were started in March and April 2014. The surfaces of pollinia were sterilized by placing them in a 0.5% solution of NaOC1 for 5 minutes after which they were rinsed three times in sterile distilled water and between rinses they were broken into pieces in a vortex mixer.

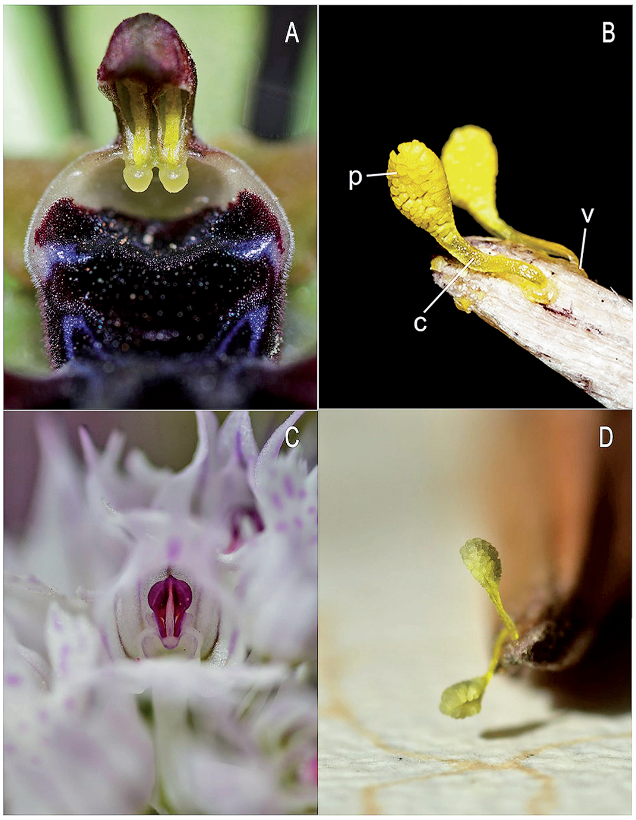

Fig. 1 Detailed images of flowers and pollinaria of *N. tridentata* and *O. mammosa*. Reproductive parts of *Ophrys mammosa* (A) and *Neotinea tridentata* (C). Pollinarium of *Ophrys mammosa* (B) and *Neotinea tridentata* (D); p: pollinium, c: caudicle, v: viscidium.

Table 3 Species studied, their codes used in subsequent tables and numbers of sown pollinia and pollinaria on individual media.

Species	Code	Number of sown pollinia			Number of sown pollinaria		
		KN	ORC	SV	KN	ORC	SV
Anacamptis morio subsp. *morio*	A MOR	71	107	111	79	76	55
Neotinea tridentata	N TRI	82	65	72	76	76	59
Ophrys mammosa	O MAM	165	237	738	67	62	71
Orchis provincialis	O PRO	119	84	121	113	123	92
Orchis simia	O SIM	72	75	81	50	67	82

Two different commercial media (Orchimax Orchid Medium "ORC", O0257 and Knudson C Orchid Medium "KN", K0215, Duchefa Biochemie BV, Haarlem, the Netherlands) and one special medium (SV) (Malmgren 2006) were used. KN and ORC media were supplemented with 20 g/l sucrose, 1 g/l activated charcoal and 6 g/l agar. SV was prepared with; 90 mg/l $Ca_3(PO_4)_2$, 90 mg/l KH_2PO_4, 90 mg/l $MgSO_4$, 20 g/l sucrose, 1 g/l activated charcoal, 6 g/l agar and 30 ml/l pineapple juice. The pH of the medium was adjusted to 5.75 ± 0.1 before autoclaving at 121 °C and 101 kPa for 20 min. Approximately 50–200 pollinia (Table 3) were sown under axenic conditions in each Petri dish (10 cm in diam.), containing 25 ml of either KN, ORC or SV medium. There were five replicates for each accession of pollinia. The Petri dishes were sealed with stretch film, wrapped in aluminium foil to exclude light and incubated at 24 ± 1 °C in darkness for 24 h.

The pollen was stained with Brilliant Blue R (Sigma-Aldrich B7920) and the number that had germinated counted under a stereoscopic microscope (Olympus SZ-51).

Statistical Analyses

Results were analyzed using one-way ANOVA (IBM SPSS Statistics ver. 22), followed by Tukey's HSD and Dunnet T3 tests to determine whether the results differed significantly for the different orchids and media.

Results

We observed that germination occurred solely between connecting surfaces of pollinia to pollinaria and pollen tubes were elongated such fringes from pollen grains while there was no germination observed on outer surfaces of pollinia (Fig. 2). We also investigated Species + Media interaction effects on germination rate and performed statistical analyses. Statistical analyses showed that Species + Media interaction was significant at $p < 0.05$.

The best germination rates of pollinia for A MOR and N TRI were on the medium KN, for O MAM and O PRO on the medium ORC and for O SIM on the medium SV (Table 4). The germination rates, however, did not statistically differ between A MOR and O PRO, but there were significant differences between N TRI, O MAM and O SIM (Table 4). Overall germination rates of individual media for pollinia are shown in Table 5. There were no significant differences in germination rates of pollinia among media (Table 5). Overall pollinia germination rates for individual species are shown in Table 6. The differences in germination rates between species were not statistically significant (Table 6).

We observed germination within the inner sides of pollinaria where pollinia were connected. Pollen tubes increasingly elongated from inside to outside (Fig. 3). Statistical analyses confirmed significant differences of species-media composition and species-media interaction on germination rates ($p < 0.05$).

The best germination rates of pollinaria for O PRO and O SIM were on the medium KN, for N TRI and O MAM on the medium SV, for A MOR on the medium ORC (Table 7). There were statistically significant differences between individual media for O MAM, A MOR and N TRI, but there was none between O PRO and O SIM (Table 7). Overall germination success rates of pollinaria for individual media are shown in Table 8; there was no significant difference between the media. The overall germination rates of pollinaria for individual species are shown in Table 9. There were no statistically significant differences between these.

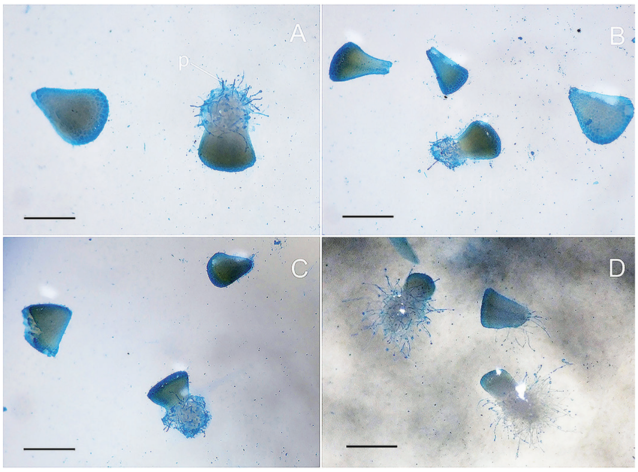

Fig. 2 Pollen tube (p) development on pollinia of *Ophrys mammosa* (A), *Orchis simia* (B), *Neotinea tridentata* (C) and *Orchis provincialis* (D). Scale bars: 1 mm.

Table 4 Germination rates of pollinia (mean ± SD) of individual species on each medium.

	KN	ORC	SV	p value	$F_{2,12}$
A MOR	39.6 ± 5.5	32.7 ± 5.3	36.9 ± 11.6	0.413	0.952
N TRI	51.2 ± 4.3	40.9 ± 6.3	44.4 ± 6.8	0.049	3.903
O MAM	54.4 ± 3.6	65.6 ± 8.7	52.3 ± 3.0	0.007	7.760
O PRO	42.0 ± 9.8	46.6 ± 12.1	37.1 ± 15.6	0.508	0.716
O SIM	28.2 ± 9.3	46.8 ± 7.2	50.6 ± 8.4	0.002	10.367

Table 5 Overall germination rates of pollinia (mean ± SD) on each medium.

	mean ± SD	p value	$F_{2,12}$
KN	43.4 ± 10.9	0.885	0.124
ORC	46.6 ± 11.0		
SV	44.4 ± 9.5		

Table 6 Overall pollinia germination rates (mean ± SD) for individual species.

	mean ± SD	p value	$F_{4.10}$
A MOR	36.1 ± 3.4	0.060	3.233
N TRI	46.2 ± 4.9		
O MAM	56.7 ± 5.5		
O PRO	42.0 ± 6.1		
O SIM	42.5 ± 4.9		

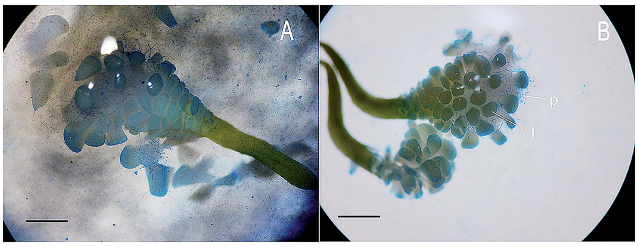

Fig. 3 Pollen tube (p) development and pollinium (l) view on pollinarium of *Ophrys mammosa* (A) and *Anacamptis morio* subsp. *morio* (B). Scale bars: 1 mm.

Table 7 Germination rates of pollinaria (mean ± SD) for each medium and species.

	KN	ORC	SV	p value	$F_{2,12}$
A MOR	38.4 ± 8.2	66.4 ± 12.9	59.9 ± 17.6	0.016	5.973
N TRI	52.1 ± 6.0	54.6 ± 8.2	69.2 ± 8.6	0.009	7.135
O MAM	51.6 ± 19.8	49.9 ± 6.9	81.7 ± 14.7	0.015	6.041
O PRO	57.5 ± 13.7	52.5 ± 15.9	48.4 ± 4.7	0.524	0.683
O SIM	61.9 ± 11.2	56.7 ± 13.1	51.2 ± 5.9	0.309	1.297

Table 8 Overall pollinaria germination rates (mean ± SD) on each medium.

	mean ± SD	p value	$F_{2,12}$
KN	57.2 ± 13.0	0.661	0.428
ORC	56.8 ± 6.1		
SV	62.7 ± 13.3		

Table 9 Overall pollinaria germination rates (mean ± SD) for individual species.

	mean ± SD	p value	$F_{4.10}$
A MOR	59.3 ± 10.4	0.588	0.737
N TRI	46.2 ± 4.9		
O MAM	68.3 ± 14.9		
O PRO	54.5 ± 5.6		
O SIM	56.6 ± 5.4		

Discussion

Although *in vitro* symbiotic and asymbiotic seed germination has become a favoured and useful technique for orchid propagation and use in plant reintroduction to nature, *in vitro* pollinium or pollinarium germination has not been studied widely in terrestrial orchids. This is especially important for species used for commercial salep production. Therefore, here we provide the first successful *in vitro* pollinia and pollinaria germination and comparison report of salep orchids *Ophrys mammosa*, *Orchis provincialis*, *Orchis simia* and *Neotinea tridentata*.

All plants, which have pollinium instead of pollen grains, need water for hydration of complex pollen clusters (Marginson et al. 1985). During pollinium development of *Epidendrum ibaguense*, plasmodesmata and thin cytoplasmic channel formations enclose tetrads, and consequently proximal sides of each tetrad have thinner walls (Yeung 1987). This structural differentiation helps water to be absorbed easier, which explains germination success of inner parts of pollinaria. As observed recently (Yeung 1987; Feijo and Pais 1989; Aybeke 2002), for both pollinia and pollinaria, only inner surfaces where pollinia were attached to pollinaria, germinated and pollen tubes were developed. It can be explained by hydration advantages of pollen tetrads at broken parts of pollinium during the breaking processes of pollinaria, and by thinner walls of proximal sides of tetrads. Therefore, before sowing process, breaking pollinaria into pollinia and even smaller pieces can increase the success of germination significantly. These results are parallel with other *in vivo* results regarding germination differences between inner and outer walls of tetrads (Swamy 1947; Poddubanya-Arnoldi 1976). Our comparison of pollinia and pollinaria germination supports those results.

It is known that sugar level of germination medium is very important for germination as well. While Pfundt

(1909) recommends sucrose level by 5–20% (w/v), another researcher found orchid pollen germinates best on the medium with a range of 3–10% (w/v) sucrose level (Miwa 1937). Relatively recently, 1–10% (w/v) sugar level was suggested (Pritchard and Prendergast 1989). Considering this and according to our pilot experiments we decided 2% (w/v) sucrose level as the optimum for each germinating medium in our study.

We show that Svante medium can be used as a basic germinating medium for orchids *Ophrys mammosa*, *Orchis provincialis*, *Anacamptis morio* subsp. *morio*, *Orchis simia* and *Neotinea tridentata*. Future studies can develop germination medium with different combinations of supplements and investigating medium success on more orchid species. Future studies must be aimed to ensure further development of the plants until adulthood for further reintroduction to nature and manufacturing salep from their tubers.

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WAITING FOR INTEGRATIVE TAXONOMY: MORPHOSPECIES AS AN OPERATIONAL PROXY FOR THE RADIATIVE AND RETICULATE GENUS *OPHRYS* L. (*ORCHIDACEAE*)?

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ABSTRACT

Recently published botanical floras provided an opportunity to develop operational systems for identifying in the field in France of species of the difficult genus *Ophrys*. Its specific and infra-specific taxonomy is extremely complex because of conflicting points of view and/or insufficient knowledge about specific biological features. In order to produce an identification key based on observable morphological criteria we developed a pragmatic taxonomy suitable for non-expert botanists, which includes "morphospecies" that are unambiguously identifiable based on a number of unique or a combination of diagnostic criteria and "subspecies" that are sets of populations sharing the same geographical and ecological adaptations but not distinctly differing morphologically. The taxonomic scheme reviewed here for the section *Pseudophrys* is well correlated with the floral chemical signatures of all the French taxa. This intermediate position, between splitters (mainly orchidologists) and lumpers (mainly geneticists), will hopefully enable us to revise the taxonomy of this genus at the Euro-Mediterranean level.

Keywords: morphospecies, *Ophrys*, taxonomy

Introduction

Recent publication of two French floras (Tison and De Foucault 2014; Tison et al. 2014) provided an opportunity to work on French species of the taxonomically difficult genus *Ophrys* L. (*Orchidaceae*). As for all classical floras, the main objective of these floras was to develop an operational system for identifying plant species in the field, including difficult radiative and/or reticulate genera like *Hieracium* L. (Krak et al. 2013), *Rosa* L. (Ritz et al. 2005) or *Ophrys* (Breitkopf et al. 2015). The radiative genera are characterised by a long-time isolated clade and then a very quick, intense and recent diversification phase. The reticulate genera are characterised by non-linear diversification phases due to crossing between clades. Both are making the taxonomic assessments more difficult.

Specific and infra-specific taxonomy within the genus *Ophrys* is extremely complex because of conflicting points of view (splitters vs. lumpers, Table 1) and/or poor knowledge of specific features (pollination, floral chemistry, karyology, phylogeny…). For example, the specific pollinators of 80 of the 250 species are unknown (Delforge 2005), chemotaxonomy seems to be promising (Mant et al. 2005; Véla et al. 2007) but only beginning to be used for delimiting species (cf. Joffard et al. 2014), karyology and phylogenetics are still incomplete and were rarely used to identify species (Soliva et al. 2001; Devey et al. 2008; García-Barriuso 2010) until LFY genes were used (cf. Schlüter et al. 2007a, 2011). It explains why it is still difficult to develop a truly integrative taxonomy, except in a few cases at a regional level and only by considering several but not all aspect of biology (Véla 2007, 2008a; Schlüter et al. 2007b, 2009; Xu et al. 2011).

For field botanists, classical French floras (e.g. Fournier 1947) appear to be obsolete with only 2 taxa in the section *Pseudophrys* Godfery: *O. fusca* Link and *O. lutea* Cav. Currently, a lot of experienced and non-expert botanists have great difficulty identifying orchids despite the abundance of illustrated books on the orchid flora in France (Souche 2004; Bournérias and Prat 2005; Dusak et al. 2009; Delforge 2012), which generally are contradictory with from 6 to 14 *Pseudophrys* taxa.

Because of this confusion, we decided to produce an identification key based on observable morphological criteria, which is pragmatic and can be used by non-expert botanists. Of course, this provisional taxonomy, although useful, will eventually be superseded by the development of a truly integrative taxonomy (Pires and Marinoni 2010). But pending the outcome of DNA barcoding we need a taxonomy based on morphology (Dunn 2003), especially for *Ophrys* orchids.

Methodology and Taxonomical Choices

As herbaria are generally not very useful for studying the flower morphology of the genus *Ophrys*, we studied populations of living plants in the field. The method used was as follows: 1) In a given country, several popula-

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Table 1 Number of taxa in the genus *Ophrys* according to authors that can be classified to varying degrees as splitters or lumpers.

Number of units according to maximum taxonomic level considered	Delforge 2005	Baumann et al. 2006	Pedersen and Faurholdt 2007; Faurholdt and Pedersen 2009	Devey et al. 2008
Species	250	65	25	10
Subspecies	–	175	80	–

tions of each species were studied; 2) At each locality, the whole population was surveyed; 3) The variability, based on at least 10 and if possible 30 specimens, was recorded, which also included extreme values; 4) We worked in the field and took calibrated photographs in order to record and eventually improve the measurements. 5) For each flower of *Pseudophrys*, we usually measured: length and width of sepals, length and width of petals, length and width of labellum and width of yellow border, if present (cf. Barriau et al. 2011).

We then classified the specimens studied using the following definitions:

- "**morphospecies**" are phenotypical entities that can be unambiguously identified based on a number of unique or a combination of diagnostic criteria;
- "**subspecies**" is a set of populations with the same geographical and ecological adaptations (e.g. specific pollinator, flowering time, etc.) within a morphospecies, which can often be discriminated in terms its quantitative range of variation, but not by an unambiguous morphological criterion;
- unclassified variation includes both intraspecific variability and (at least provisional) taxonomical synonyms.

Cases Studied

We delimited species and produced an identification key for French and Algerian-Tunisian *Ophrys*, with particular emphasis on the section *Pseudophrys*. In France, including Corsica, (Tison and de Foucault 2014) we identified 6 morphospecies of *Pseudophrys* and considered other specific names to be undistinguished infraspecific variations and/or taxonomic synonyms (at least provisionally). In Algeria (Rebbas and Véla 2013) we identified 12 morphospecies, without considering subspecies (at least at this stage). In Tunisia (Martin et al. 2015) we identified a total of 14 elementary taxa consisting of 12 morphospecies and 2 subspecies.

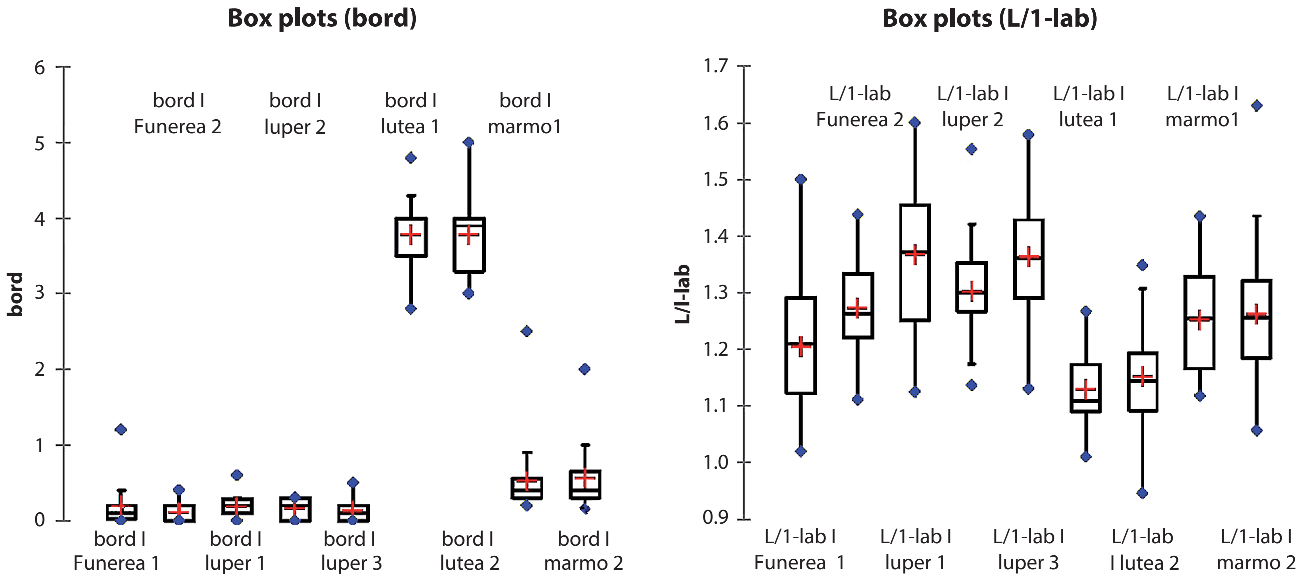
For the 4 main taxa in continental Mediterranean France (Tison et al. 2014): *O. funerea* Viv., *O. fusca*, *O. lutea* and *O. marmorata* G. and W. Foelsche, we provided an *a posteriori* calibration of the morphometric results (Barriau et al. 2011). Box-plot graphs or principal component analyses enabled us to distinguish between discriminant vs. diagnostic criteria: a lot of partially overlapping averages and variances of statistical values can be used to statistically discriminate between taxa but are not diagnostic for their identification and therefore not useful in keys, while in some cases a single criterion or combination of criteria is sufficient to distinguish between taxa, which we call "morphospecies" (Fig. 1):

- a wide yellow border is diagnostic for *O. lutea* vs. the other three species;
- the length / width ratio of the lip is a discriminant criterion for *O. lutea* and *O. fusca* but not a diagnostic one;
- the relative length of the petals is a discriminant criterion for *O. funerea* and diagnostic if combined with the shape and colour of the labellum;
- the combined criterion « yellow border + labellum length » is diagnostic for *O. marmorata* vs. *O. fusca*.

A comparison of our results with species delimitations based on the floral chemical signature of the main taxa in Mediterranean France and Corsica (Joffard et al. 2014) surprisingly revealed almost the same taxonomic scheme as that deduced from our morphometric study:

- For *O. fusca* (sensu lato) vs. *O. marmorata* (sensu lato), the chemical signature is diagnostic, confirming our morphospecies delimitations.
- Within *O. marmorata* sensu lato (incuding *O. bilunulata* Risso, *O. peraiolae* G. Foelche et al. and *O. delforgei* J and P Devillers T), the discriminant but not diagnostic chemical signatures suggest possible subspecies, as expected for continental populations (Véla et al. 2007; Fig. 2).
- For *O. lutea* vs. *O. corsica* G. and W. Foelsche, the chemical signature is discriminant but not diagnostic, suggesting a subspecies level, not yet formalized in the current floras.

Northwestern Africa (particularly Algeria and Tunisia) is a regional hotspot for the section *Pseudophrys*. The classical flora (e.g. Maire 1960) already includes 6 species or 9 taxonomic units when infraspecific ranks are considered, plus 3 poorly known taxonomic units, considered to be hybrids by the author. The modern revision of Faurholdt and Pedersen (2009), who are considered to be lumpers, recognize 4 elementary taxa at the species level and 9 if subspecies are included. According to our methodology, there are 14 morphospecies and 19 taxonomic units including subspecies in both Algeria and Tunisia. Five of the six morphospecies in France are also present in north western Africa, but are different sub-species.



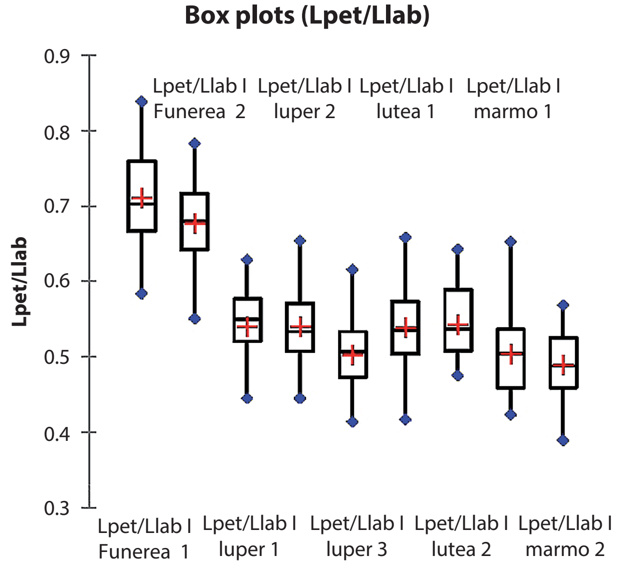


Fig. 1 Box plots of the comparative morphometrics of the labellum of the four main continental French *Pseudophrys* taxa. Two populations by taxon, respectively *O. funerea* (funerea), *O. fusca* p.p. = *O. lupercalis* (luper), *O. lutea* s.s. (lutea), *O. marmorata* s.s. (marmo). Variables presented here are: width of the yellow margin (bord), length to width ratio of the labellum (L/l-lab), petalum length to labellum length ratio (Lpet/Llab). According to Barriau et al. 2011 (+ unpublished data).

Taxonomic Debate

The evolutionary biology of genus *Ophrys* is so complex that several interpretations of its taxonomy are possible (Bateman et al. 2010) depending on 1) which criterion is given priority (morphology, pollination biology, genetics …), and 2) where the limit between species is placed (splitter vs lumper), i.e. the analytical level and accuracy of our knowledge. Over the last few decades advances were made thanks to studies on pollination biology, which resulted in ethological species delimitations based on species-specific interactions between *Ophrys* and pollinators (Paulus and Gack 1983, 1990; Paulus 2006). This liberal, pollinator-centred and ethological taxonomy, however, was recently criticised and still remains controversial (cf. Bateman et al. 2011; Vereecken et al. 2011).

The point of view presented here is clearly intermediate between those of the most extreme splitters and lumpers, respectively Delforge (2005) and Devey et al. (2008). From a methodological point of view, our taxonomic delimitations are similar to those of Sundermann, which were formally approved by Pedersen and Faurholdt (Pedersen and Faurholdt 2007; Faurholdt and Pedersen 2009), but with a tendency to split more than lump (cf. Véla 2008b). Simplifying, our subspecies rank corresponds to their variety rank, our species to their subspecies and their species to our non-formal rank of aggregate. Their hybrid complexes, which they treated as species, were carefully studied, and we limited the hybrid appellation to the recently hybridogenic populations that are still morphologically unstable. Using this slightly modified threshold, we provide a more operational proxy, acceptable by both moderate orchidologists and rational botanists. Our analytical level is quite similar to that of Baumann et al. (2006) but gave different results, mainly because they did not have an objective methodology. Depending on the opinion of taxonomists, i.e. if they are more of a splitter or lumper than us, it is possible to use the same framework but change the cursor's position.

Conclusion

This methodological framework can be used as the starting point of a debate and offers an alternative to the subjective and empirical approaches traditionally developed by orchidologists. As a practical test, the analytical keys for identification can be evaluated by users and improvements incorporated.

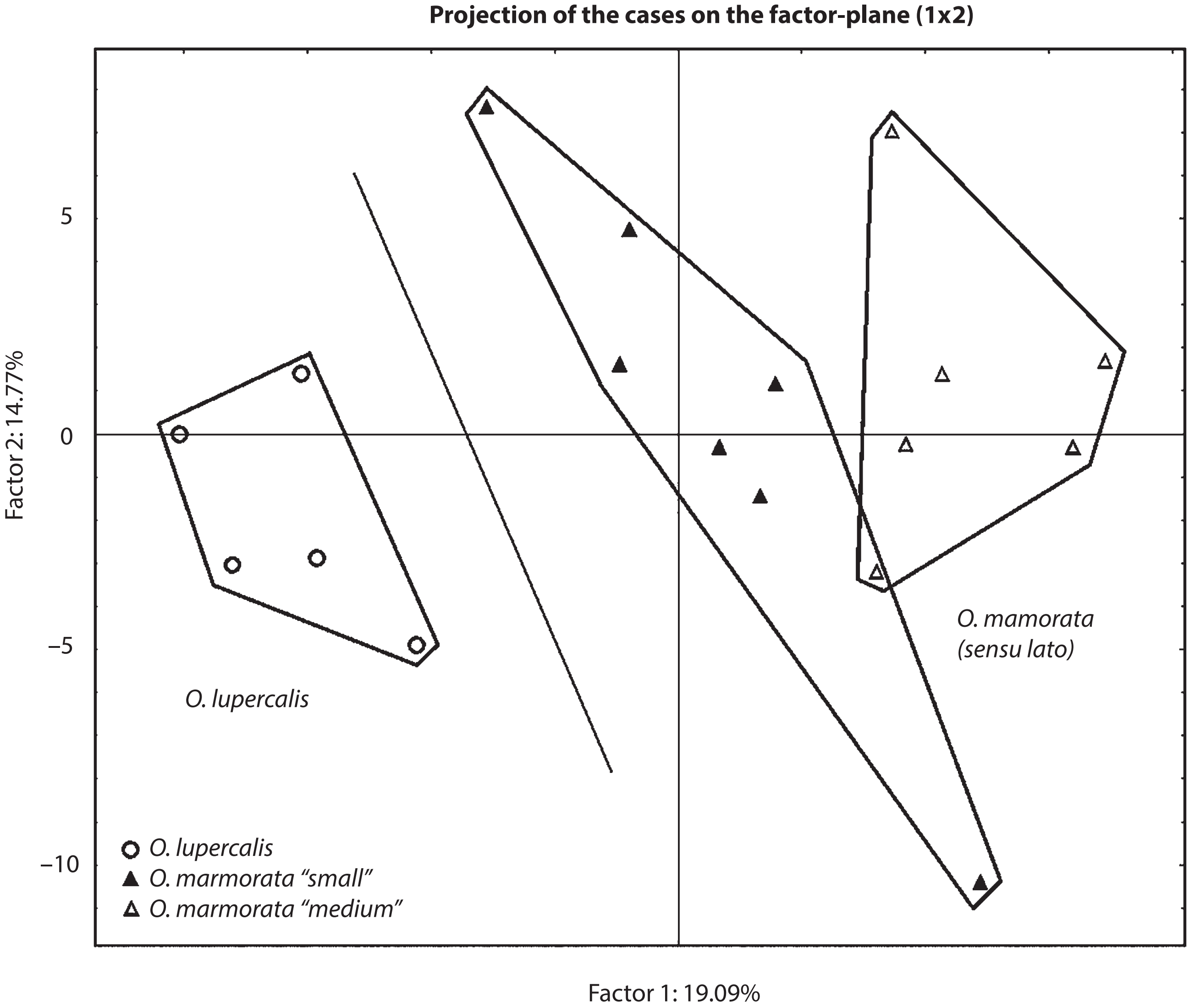


Fig. 2 Principal component analysis summarizing variation in floral chemical signature based on 120 cuticular lipidic compounds detected using gas chromatography of labella collected from 18 samples of *Ophrys lupercalis*, *O. marmorata* 'medium-sized labellum' and *O. marmorata* 'small labellum' collected in continental South-eastern France (Bouches-du-Rhône). Modified from Véla et al. (2007).

From now on, it will be possible to revise *Ophrys* taxonomy for the whole Euro-Mediterranean area on a sound and scientific basis, including at least morphology, phenology and biogeography. At each stage in the geographic expansion of the analysis, morphospecies and subspecies are distinguished respectively by diagnostic and discriminating criteria, which implies a continual revision of the general taxonomic scheme and identification keys for this genus.

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IDENTIFICATION OF TWO ENTOMOPATHOGENIC FUNGI NATURALLY INFECTING *PTEROCHLOROIDES PERSICAE* (CHOLODKOVSKY 1899) (HEMIPTERA, APHIDIDAE) IN PEACH ORCHARDS IN TUNISIA

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ABSTRACT

Symptoms of mycosis of *Pterochloroides persicae* (Cholodkovsky 1899) (Hemiptera, Aphididae) in a peach and almond orchard were recorded in April 2011. The causal agent was morphologically identified. Polymerase chain reaction (PCR) of the internal transcribed spacer region (ITS1 and ITS4) was used for molecular identification and verification of the morphological determination. Both methods gave consistent results and we recorded for the first time the natural occurrence of two fungal species belonging to the order Hypocreales (Phylum Asocomycota); *Beauveria bassiana* Viull (Hypocreales, Cordycipitacae) and *Metacordyceps liangshanensis* (Hypocreales, Clavicipitaceae).

Keywords: *Beauveria bassiana*, *Metacordyceps liangshanensis, Pterochloroides persicae*, Tunisia

Introduction

The giant brown peach aphid *Pterochloroides periscae* (Cholodkovsky 1899) (Hemiptera, Aphididae) is a worldwide pest of peach, almond, plum, apricot, apple and citrus (Stoetzel and Miller 1998; Ateyyat and Abu-Darwish 2009; Mdellel et al. 2011). It occurs in Europe, Asia, North America and some Mediterranean countries (Kairo and Poswal 1995; Blackman and Eastop 2000). In Tunisia, it was first recorded in 1987 at Sfax (Southern of Tunisia) on almond trees (El-Trigui and El-Shérif 1989) and is considered to be a serious pest of stone fruit trees, especially peach, almond, plum and apricot (Ben Halima-Kamel and Ben Hamouda 2004, 2005; Mdellel et al. 2011). This aphid causes severe damage to its host plants at all growth stages and often kills trees (Kairo and Poswal 1995). In order to control this pest, many of its natural enemies have been identified and used as biological control agents, of which *Pauesia antennata* Mujerji (specific parasitoid of *P. persicae*) and *Coccinella algerica* Kovar are considered to be the most important (Rakshani et al. 2005, Mdellel et al. 2012). Similarly, other natural's enemies can be so used, such as the entomopathogenic fungi. Indeed, fungal diseases of insects are common and widespread and many of them are considered to be important factor regulating pest insect populations (Carruthers and Soper 1987).

Entomopathogenic fungi are recorded as important causes of aphid mortality all over the world (Latge and Papierok 1988). Currently, at least 90 genera and more than 700 species of entomopathogenic fungi infecting invertebrates have been identified, most of which are associated with insects, but only 100 of these have been or are currently being developed for insect control (Wraight et al. 2001). Intensive studies have increased our understanding of the distribution and epidemiology of fungi in insect populations and their use as biological control agents of pests of agricultural crops (Pell et al. 2001). In fact, in temperate regions, Hyphomycetes are important fungal pathogens of aphids (Barta and Cagan 2006). Thus sampling of host individuals can reveal information about the prevalence of fungal species as pathogens in natural host population. The use of entomopathogenic fungi in biological control is a new field in Tunisia. The objective of the present study is to identify isolates of entomopathogenic fungi from *P. persicae* cadavers naturally infected with fungi.

Materials and Methods

Sampling

This investigation of mycoses in *P. persicae* populations was done in three regions of Tunisia, in the north: at Ariana (SidiThabet: 36°54′31″ N, 10°02′33″ E) an arid area and at two regions on the coast of Tunisia: the first at the High Agronomic Institute at Chott Mariem (35°52′31″ N, 10°34′16″ E) and second at Jammel (35°38′24″ N, 10°45′36″ E). The last two regions have an arid climate. Aphid cadavers showing symptoms of infection with fungi (Figs. 1 and 2) were placed in ventilated plastic boxes and carried to the laboratory.

Fungal Identification

Fungal cultures were obtained from aphid cadavers by isolating them on potato dextrose agar (PDA) af-

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Fig.1 *Pterochloroides persicae* population on peach showing symptoms of infection with entomopathogenic fungi.

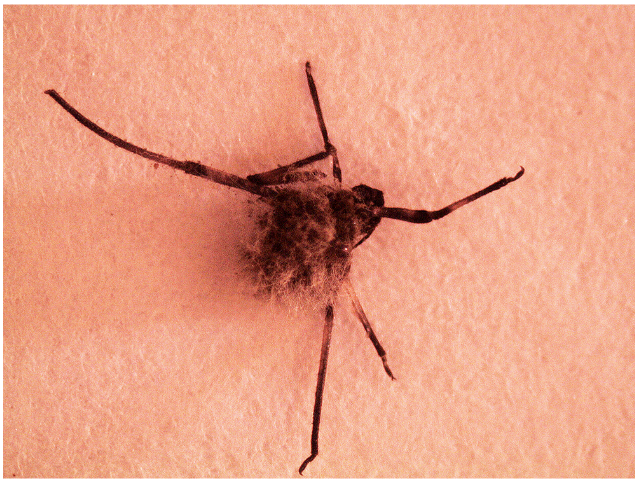

Fig. 2 *Pterochloroides persicae* cadaver showing symptoms of having been killed by a fungal infection.

ter surface-sterilization following the procedures used by Goettel et al. (2000). Microscopic descriptions were made using cultures on PDA and MA (Malt agar). Mycelia were mounted in lacto phenol cotton blue (0.01%) and observed under phase contrast using a Leitz DMRB optical microscope (40×/0.65 PH2 and 100×/1.25 OIL PH3). Fungal preparations were photographed using a Coolpix 5000 5.0 MP digital camera. The fungi were identified (based on morphological characteristics) under a stereoscopic microscope (Nikon SMZ 800 with light source Intralux 4000-1) with the aid of the taxonomic keys of Lawrence (1997), Humber (1997) and Barnett and Hunter (2006).

Genomic DNA extraction from mycelia was done using the method described by Reader and Broda (1985). DNA was amplified by PCR using the complementary primers ITS1F and ITS4 and a MyCycler TM thermocycler System (Bio-Rad, Madrid, Spain) according to the manufacturer's instructions (Promega, Madison, WI, USA). The sequences were compared with those available in the GenBank database using BLAST search analysis (sequences were initially aligned using DNASTAR Inc. (Madison, WI, USA).

Results and Discussion

Two entomopathogenic fungi naturally infecting *P. persicae* were identified: *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales, Cordycipitaceae) and *Metacordyceps liangshanensis* (Ascomycota: Hypocreales, Clavicipitaceae). These two species are reported for the first time infecting *P. persicae*. Nevertheless, Tsinovskii and Egina (1972), cited by Cross and Poswal (1996), report *Entomophthora thaxteriana* Petch infecting *P. persicae*, which is now named *Conidiobolis obscuris*, and is the cause of substantial mortality in several species of aphids. In addition, in Israel, Ben Zev (1988) report another species of entomopathogen attacking this aphid called: *Taxterosporium tubinatum* Kenneth.

Several studies have demonstrated that *B. bassiana* infects hundreds of species of hosts belonging to many insect orders (Nicolai et al. 2006). Gurulingappa et al. (2011) demonstrate the efficiency of this fungus against *Aphis gossypii* Glover. These authors report that it reduces the longevity and fecundity of aphids, and is thus potentially an excellent biological control agent. Similarly, Sevim et al. (2012) note that much effort has been put into research on the development of *B. bassiana* as a biological control agent (for inundative and inoculative biological control) for use in agriculture and forestry in temperate regions. In South Korea, Kim et al. (2013) have demonstrated also that spraying green peach aphids with a filtrate of cultures of *B. bassiana* results in a high mortality (78%) of the aphid three days after the treatment. Akmal et al. (2013) tested the efficiency of *B. bassiana* against several species of aphids (*Schizaphis graminum* (Rondani 1852), *Rhopalosiphum padi* (L., 1758), *Brevicoryne brassicae* (L., 1758) and *Lipaphis erysimi* (Kaltenbach 1843) in the field and showed it can be used as a biocontrol agent for managing these aphids. *M. liangshanensis*, Kepler et al. (2013) infects species of Lepidoptera in USA. Currently, the relative virulence of infections by *B. bassiana* and *M. liangshanensis* in *P. persicae* is unknown, but future pathogenicity assays will clarify whether they are both highly virulent biological control agents of *P. persicae*.

In conclusion, the present study provides the first report of *B. bassiana* and *M. liangshanensis* infecting *P. persicae* populations infesting peach and almond trees in Tunisia, but further research is needed on the relative efficiency of these two entomopathogenic fungi against *P. persicae* and their potential as biological control agents.

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ANTHROPIC EFFECTS ON THE MEIOFAUNA AND PHYSICOCHEMICAL CHARACTERISTICS OF THE HYPORHEIC ZONE IN A MEDITERRANEAN STREAM

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ABSTRACT

We examined the effects of the discharge from a wastewater treatment plant (WWTP) on the interstitial water chemistry and structure of the hyporheos in two sections of the La Tordera stream, one upstream and one downstream of the effluent from the treatment plant. Interstitial water samples were collected to determine the vertical hydraulic gradient (VHG) and hydraulic conductivity (K) at six sites in each of the two sections on six occasions between November 2006 and September 2007. Samples of the interstitial meiofauna were collected in upwelling zones in both sections of the river. There were no patterns in the hydraulic gradient either in its direction or its magnitude along the longitudinal axis. There were no differences in VHG recorded at the sites sampled indicating that the two sections were similar in terms of geomorphology. The hydraulic conductivity was greatest in the control section (upstream of the effluent) of the stream. There were no significant differences in specific conductance, phosphates, nitrates or ammonia in the upwelling and downwelling zones. In the upwelling zones in the section of the river downstream from the effluent the concentrations of nitrates were greater than in the downwelling zones, but levels of phosphates and ammonia were lower. There was a decrease in the interstitial nutrient concentrations along a longitudinal gradient downstream of the effluent. A total of 24 taxa were collected from the hyporheos, with Chironomids and Oligochaetes being the most abundant groups. A higher Shannon diversity index (2.86) was recorded in the downstream section of the river even though a greater taxa richness (18) was recorded in the control section. This is probably due to the fact that a larger number of rare or unusual species was found in this section. According to the BMWP biological index the water quality in the control and downstream sections was polluted and heavily polluted, respectively. The shifts in hydraulic exchange processes and the bio-physicochemical environment of the hyporheic zone are the main effects of the discharge from the Santa Maria Palautordera wastewater treatment plant on the two sections of the river studied.

Keywords: point source, invertebrates, hyporheic zone, hydraulic gradient, BMWP index

Introduction

Fluvial ecosystems in developed regions are subject to high environmental stress due to eutrophication (Fisher et al. 1998). Human activities may cause land use changes in catchment areas or modifications of the landscape that increase the transport of nutrients towards aquifers and surface water. Water courses (rivers and streams) may also be directly affected by discharges of waste water (point sources) and other modifications, which reduce their capacity to respond to an increase in nutrient concentrations. These problems are accentuated in Mediterranean rivers due to the persistent scarcity of water (Martí and Sabater 1996; Cushing and Allan 2001). Point sources of pollution of surface water bodies, for example, discharges from water treatment plants (sewage), industrial installations or high intensity precipitations, which frequently contribute high loads of pollution that affect the quality of river water, are an expected or derived consequence of urban and industrial development. In addition, and depending on the relative volumes of the effluent and the river, the discharge from a sewage works can represent an important percentage of the river volume downstream of the effluent. These factors act at different scales ranging from a particular stretch of a river to the whole catchment area. Contaminants in fluvial ecosystems may affect the quality of the groundwater in several ways: firstly, in those areas where the river loses water to the aquifer (downwelling); secondly, along sections where the aquifer is very deep, favouring the infiltration of surface water, and thirdly, during periods of flooding when the river water spreads over the floodplain. The concentrations of these contaminants may, however, be reduced by biogeochemical processes, which take place in shallow aquifers and the hyporheic zone (Boulton et al. 1997; Fuller and Harvey 1999; USGS 2001).

Since 1950 aquatic ecologists have emphasized the importance of the interaction between surface water and groundwater in the functioning of aquatic ecosystems. The region where the water in the aquifer and the river mix is known as the hyporheos and is important in that in its sediments there are diverse, metabolically active microbial communities, which retain and transform essential biological elements. There are few studies on the influence of the hyporheos on fluvial ecosystem metabolism, but some conceptual models propose that this depends on the type and metabolic rate of the microbial communities, the proportion of the river volume that flows through them and their influence on hydrological residence times (Triska et al. 1989a; Fellows et al. 2001). The hyporheic zone can have a very dynamic flow regimen. At maximum volume or *flooding peaks*, water flows from the surface to the subsurface, recharging the aquifer. At low volumes, however, water flows from

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the aquifer into the river, providing the main contribution to the basal volume of most rivers. The recharge and discharge zones are more abundant where the bottoms of rivers consist of coarse sediments rather than sand. Studies of the changes in fluvial geomorphology along a longitudinal profile have produced a model in which areas of recharge (*downwelling)* alternate with areas of discharge (*upwelling*). When the depth of the water channel decreases, high pressure zones are produced where the river bed starts to rise (*riffle*), into which water flows, displacing the interstitial water and flowing under the river bed to the opposite end of the riffle where the increase in depth produces a low pressure zone (*pool*) and the water in the hyporheos comes to the surface (*upwelling* zone). The alternating flow model describes a gradient of physicochemical conditions between the river and groundwater, where biochemical processes, together with the hydraulic residence time in the zone, also interact. In general, the concentration of nutrients in the hyporheic water is significantly higher than in the surface water. Given the importance of nutrients in production processes, a reserve of these in the hyporheos could be of fundamental importance in the metabolic processes of fluvial ecosystems (Sabater and Vila 1991; Franken et al. 2001).

Nutrient retention is a function of the fluvial ecosystems that could contribute to the recovery of water quality in contaminated rivers. This is governed by the interaction between physicochemical and biological factors, among the most important being the topography of the river bed, the thickness of the hyporheic layer, hydrological factors, etc. Depending on prevailing conditions, however, some of these factors will take precedence over others. The exchange of water and nutrients in the hyporheic zone results in a reduction in the transport of certain elements, whether due to physicochemical transformations or microbiological absorption, making this exchange zone, or surface/subsurface transition zone of vital importance for the optimization of the auto-regenerating capacity of those rivers considered to be pristine. Some years ago, Jones and Holmes (1996) reported that the underlying hyporheic zone acted as a source of nitrates in nitrogen deficient streams, but as a sink in streams with high nitrogen concentrations. In addition, Hill et al. (1998) suggest that in streams with high nitrogen concentrations the retention of nitrates is particularly affected by the size of the surface water retention zone. A larger retention zone has a longer residence time, resulting in increased contact with those sites adjacent to the surface/subsurface interphase where the exhaustion of nitrates occurs. This capacity for physical and biological nutrient retention can be modified by changes in the morphology of the river channel or the permeability of the sediment, or by increases in the nutrient load from point sources of contamination, such as effluents from wastewater treatment plants. This increase in the availability of macronutrients, such as nitrogen and phosphorus, produces an increase in the primary productivity of the system and may accelerate the obstruction or clogging of the interstitial spaces in the sediment, consequently reducing the exchange of elements in the surface / aquifer interphase. Taking into consideration these ideas the aim of this study was to evaluate the effects of the effluent from an urban wastewater treatment plant on the meiofauna and physicochemical characteristics of the hyporheic zone along the La Tordera River.

Material and Methods

Study Area

The catchment area of the La Tordera river is located in Cataluña, Northeast Spain, and extends over 868.5 km^2 (Fig. 1). Owing to the Mediterranean climate it has a low average annual precipitation of about 805.6 mm irregularly distributed throughout the year with spring and autumn being the wettest seasons, and an average annual discharge at its mouth of 3.17 m^3 s^{-1}. The hydric balance of the catchment is positive with a net annual gain of between 70 and 80 hm^3 distributed in zones tending towards a deficit and others tending towards an excess. Due to the proximity of the catchment basin to the coast it has been intensively settled and developed, with a population higher than the average for the country. This has resulted in a series of environmental disturbances, such as water pollution, the occupation of the alluvial plain, the destabilization of the natural fluvial dynamics and the overexploitation of surface and underground watercourses (Albiol 2002). The experimental sub-catchment within the La Tordera drainage basin was selected because of the location of a WWTP (Santa María de Palautordera), which discharges polluted water into the river, dividing it into two sub-reaches, upstream (control section) and downstream (altered section) of the effluent, thus permitting us to investigate the effect of the effluent on the functioning of the surface and subsurface ecosystems (Fig. 1). In La Tordera, land use within the sub-catchment located upstream of the section of the river studied (7024 inhabitants and 80.4 km^2) there is 87.4% forest and open land, 10.8% arable and grassland and 1.8% urban, of which the latter is concentrated in the lower part of the basin. Each experimental section was approximately 100 m long, with a substrate mainly of cobbles (34%), pebbles (22%) and boulders (22%). The WWTP discharges into La Tordera stream the result of treating 5808 inhabitant-equivalents, where 1 inhabitant-equivalent is the biodegradable organic matter load equivalent to a BOD$_5$ of 60 g O$_2$ day^{-1}. This particular WWTP uses the activated sludge method to treat the waste water but lacks the technology to actively remove nitrogen or phosphorus. As regards the biological communities present, Diatoms dominate the microbiota and the most abundant macroinvertebrate families in the spring months are Baetidae, Ephemerellidae, Chironomidae, Simuliidae, Oligochaeta and Ancylidae (Prat 2001).

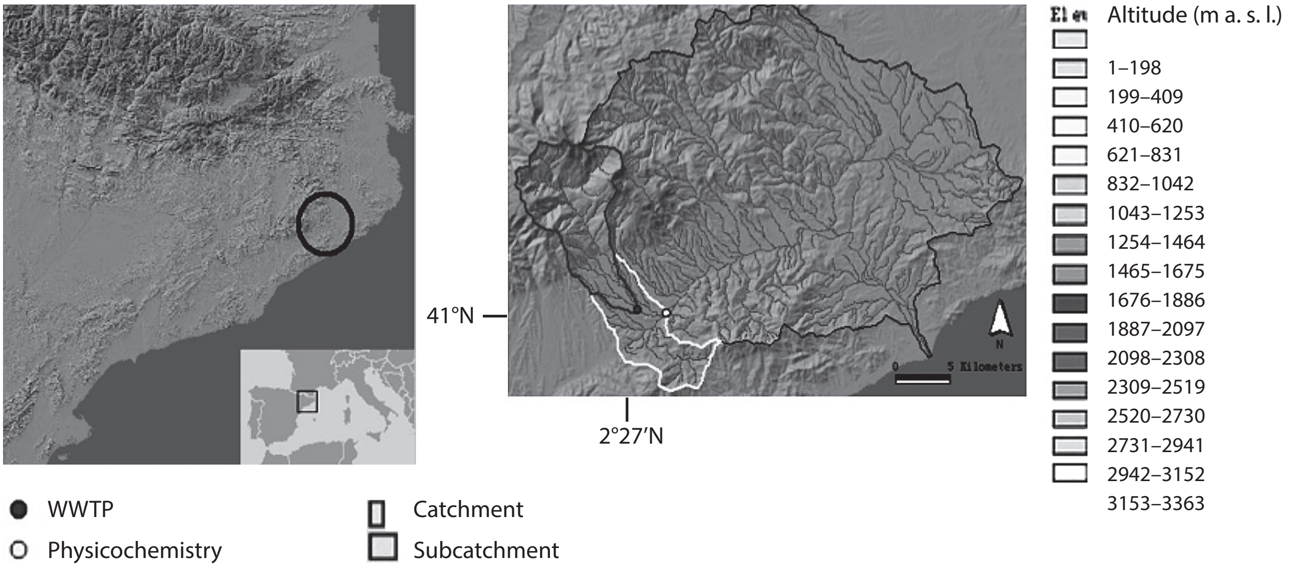

Fig. 1 Location of the La Tordera catchment area and the sub catchment (in grey) associated with the presence of the wastewater treatment plant. The area inside the white line is monitored by a physicochemical monitoring station.

Water samples were collected in triplicate from the hyporheic zone in the center of six transects located at intervals of 10 m along the two experimental sections during a hydrological year. Samples were collected using a drive-in metallic tube, 16 mm wide and 50 cm long, driven into the river bed to a depth of approximately 25 cm. A narrower tube of transparent plastic with a series of holes in the end pointing towards the river bed was then introduced inside the metal tube and interstitial water was pumped into it to a predetermined level (mini-well) using a peristaltic pump, discarding the first lot of excessively turbid samples. Once a volume of relatively transparent water was obtained, the temperature and specific conductivity (K25) of both it and the surface water adjacent to it, were determined. The samples were then immediately filtered through Whatman GF/F fiber glass filters, with a 0.7 μm pore size, and refrigerated until their analyzed in the laboratory. The dissolved reactive phosphorus was analyzed using a colorimetric method using molybdenum blue (APHA 1989) and the NO_3-N, NO_2-N and NH_4-N were analyzed automatically using a model II Technicon.

The Vertical Hydraulic Gradient (VHG) and Hydraulic Conductivity (K) were calculated for all transects in both sections. VHG was calculated by measuring the water levels inside the mini-well (piezometer) and the river surface, and then using the VHG equation to give both positive or negative values and thus the direction of surface/subsurface exchange. Hydraulic conductivity was calculated by filling the piezometer with river water using a plastic jug, and then measuring the time it took to empty to a certain level within the tube. This measurement was repeated three times and the data were then used in the respective equation.

The hyporheic fauna were collected from the intermediate zone of three transects (1, 2 and 3) in both experimental sections of the river in July. For this, a hydraulic metal tube with a mechanical water pump attached to the end of it was driven into the river bed to a depth of approximately 30–40 cm. Then, 50 l of hyporheic water was pumped into the tube through a 100 μm mesh sieve. The sieve was then washed in a plastic tray and the invertebrate sample collected placed in a plastic recipient where it was preserved and stained with rose Bengal. Samples were fully analyzed in the laboratory and organisms were identified to the taxonomic family level using the keys of Thorp and Covich (1991) and Merritt and Cummins (1978) which has been accepted as an excellent taxonomic category in most studies of biological indices of water quality (Barbour et al. 1992; Chesman 1995; Zamora and Alba-Tecedor 1996).

Formulas and Calculations

Vertical Hydraulic Gradient (VHG) (cm)

This parameter was obtained by dividing the difference between the water level inside the piezometer and the level of the river surface (Δl) between the depths of insertion of the piezometer in the sediment (h_{sed}) (Baxter et al. 2003):

$$VHG = \Delta l \, h_{sed}^{-1}$$

Hydraulic Conductivity (K) (cm s^{-1})

This parameter was calculated using Darcy's equation, which is based on the change in the position of the meniscus in the piezometer over a certain time and distance (Landon et al. 2001):

$$K = [L(t_1 - t_0)^{-1}] \ln[(H_0 \, H_1^{-1})]$$

where L is the segment of sediment under study and t is the time taken by the falling meniscus to cover the specified distance H. The data were analyzed using routine statistics (mean and variance). Paired t-tests were used to compare the means of the respective variables.

Data Analysis

All data were log-transformed prior to statistical analyses in order to stabilize variances and normalize the data sets. For the two sections studied we evaluated the effect of the effluent and the vertical hydraulic gradient (*VHG*) on the hyporheic habitat (nutrient concentrations, conductivity, temperature, hydraulic conductivity (K) and invertebrate assemblages) by comparing these parameters in the two sections sampled and upwelling – downwelling zones located upstream and downstream of the WWTP input using paired t-tests. The significance level used for all statistical tests was $p \leq 0.05$. The statistical analyses were performed using Sigma Plot 2000 for Windows statistical package. Hyporheic community parameters were used to compare invertebrate assemblages by higher groups (taxa richness (S), Shannon-Wiener diversity (H'), Jaccard's index) since there is evidence of a high correlation between the number of families and the number of species in different groups of organisms (Williams et al. 1997) and hyporheic BMWP index to compare water quality between the two sections. These analyses were performed using the statistical package InfoStat 1.2 for Windows, 2008. A between-groups principal component analysis (PCA) was used to assess spatial variation in hyporheic composition (Datry et al. 2007). Between-groups PCAs generate axes corresponding to the center of gravity for all the groups, which provides a representation of the spatial discrimination of sections. Taxa for which individuals comprised ≥ 2% of the entire dataset were included in the analysis. Invertebrate abundances were $\log_{10}(x + 1)$ transformed prior to analysis. These analyses were carried out using InfoStat 1.2 for Windows.

Results

Comparison of Wastewater Treatment Plant Effluent and Stream Flow in Terms of Nutrient Loads

The values for the WWTP outflow were lower than for the discharge of the stream (0.02 and 0.065 up / 0.083 down $m^3 s^{-1}$, respectively) (Table 1). In La Tordera the WWTP effluent represented 100% of the stream discharge in August, September and October because the stream was completely dry upstream of the input. In contrast, the input only represented 3% of the stream flow when the stream discharge was at its highest. On average, the effluent made up 38% of the stream flow. Mean concentrations of NO_3-N and PO_4-P in the effluent were higher than in the stream (10.0, 3.0 and 4.11, 0.54 mg l^{-1} respectively). The QOD and BOD (35.0 and 14.0 mg O_2 l^{-1}, respectively) of the effluent indicated a significant input of organic matter into the stream (Table 1).

Hydrology and Chemistry of the Hyporheic Zone

There were successive zones in the vertical hydraulic gradients (VHG) of the upwelling and downwelling zones in both sections of the river (Fig. 2), with the mean values of the control being greater (0.192 and −0.101 cm cm^{-1}) than those of the altered section (0.101 and −0.096 cm cm^{-1}) (Table 2). There were no significant differences in VHG values for the six transects (spatial variation) or the two sections of the river ($p > 0.05$). The average value of the hydraulic conductivity (K) recorded in the altered section was lower than recorded in the control section (Fig. 3), but it could not be measured in three transects of the control section due to the high permeability of the substrate, which was composed mainly of gravel and rock. When these extreme values for the control were removed, no significant differences between the two sections of the river were recorded and K varied between 46.33 and 51.18 cm s^{-1}.

Table 1 Water temperature, hydromorphological features and indices of water quality recorded in La Tordera. Table compares data from upstream and downstream of the WWTP, with that of the WWTP effluent.

Parameter	**La Tordera**		
	(upstream of WWTP)	**WWTP effluent**	**(downstream of WWTP)**
Temperature (°C)	17.9	–[b]	20.5
Width (m)[a]	6.75	–	6.33
Depth (m)[a]	0.09	–	0.11
Velocity (m s^{-1})[a]	0.13	–	0.14
Discharge (m^3 s^{-1})[a]	0.065	0.02	0.083
QOD (mg O_2 l^{-1})	–	35	–
BOD (mg O_2 l^{-1})	–	14	–
NO_3-N (mg N l^{-1})	2.71	10	2.57
PO_4-P (mg P l^{-1})	0.08	3	0.33

[a] According to Ortiz et al. 2006.

[b] No data available.

Table 2 Hydraulic and physicochemical characteristics of the water at the surface and the discharge (upwelling) and recharge (downwelling) zones in the two sections of the river studied. Mean values (+/– standard deviation).

Parameter	**Control section (upstream of WWTP)**		
	Surface	**Upwelling**	**Downwelling**
K (cm s^{-1})	46.33 (35.27)		
VHG (cm cm^{-1})		0.192 (0.192)	−0.101 (0.126)
Temperature (°C)	14.16 (3.96)	15.58 (4.15)	13.82 (3.78)
Conductivity (µS cm^{-1})	155.74 (25.42)	157.43 (26.49)	162.21 (25.84)
PO4 (mgl l^{-1})	0.08 (0.02)[a]	0.07 (0.06)	0.08 (0.06)
NO3 (mg l^{-1})	2.71 (0.27)[a]	6.17 (2.32)	6.20 (2.7)
NO2 (mg l^{-1})	0.21[a]	0.02 (0.01)	0.03 (0.04)
NH4 (mg l^{-1})	0.28 (0.14)[a]	0.05 (0.02)	0.04 (0.01)
	Altered Section (downstream of WWTP)		
K (cm s^{-1})	51.18 (43.41)		
VHG (cm cm^{-1})		0.101 (0.079)	−0.096 (0.131)
Temperature (°C)	15.82 (3.99)	14.79 (4.35)	16.44 (3.82)
Conductivity (µS cm^{-1})	215.0 (65.75)	215.48 (54.67)	208.08 (68.11)
PO4 (mg l^{-1})	0.33 (0.12)[a]	0.37 (0.37)	0.57 (0.47)
NO3 (mg l^{-1})	2.57 (0.3)[a]	11.44 (2.7)	9.5 (3.3)
NO2 (mg l^{-1})	0.05[a]	0.1 (0.1)	0.1 (0.1)
NH4(mg l^{-1})	1.59 (0.4)[a]	0.2 (0.2)	0.2 (0.2)

[a] According to Merseburger et al. 2005.

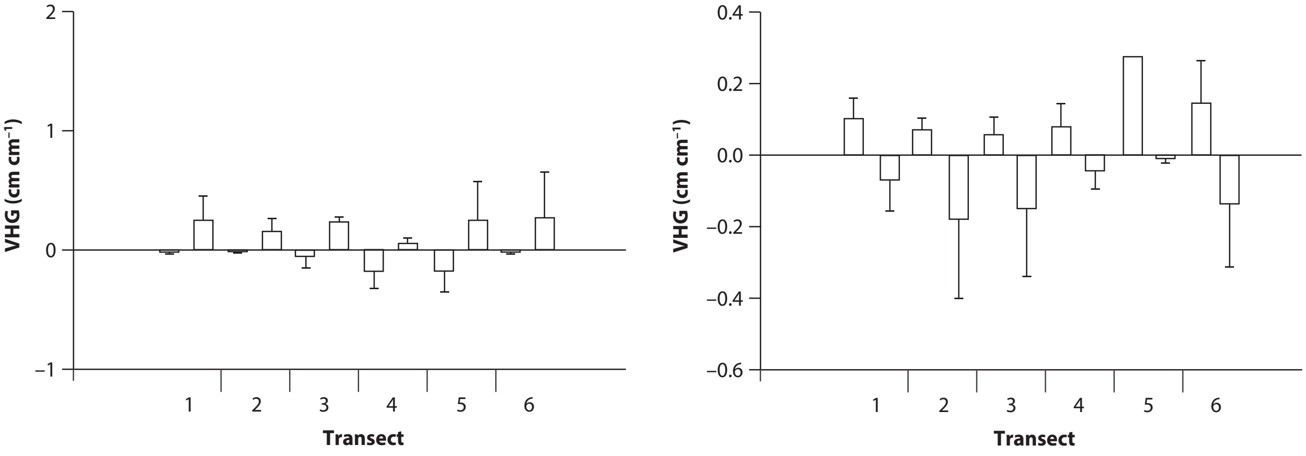

Fig. 2 Longitudinal variation in the hydraulic gradient (VHG) in up/down welling zones in the control (a) and altered (b) sections of the river.

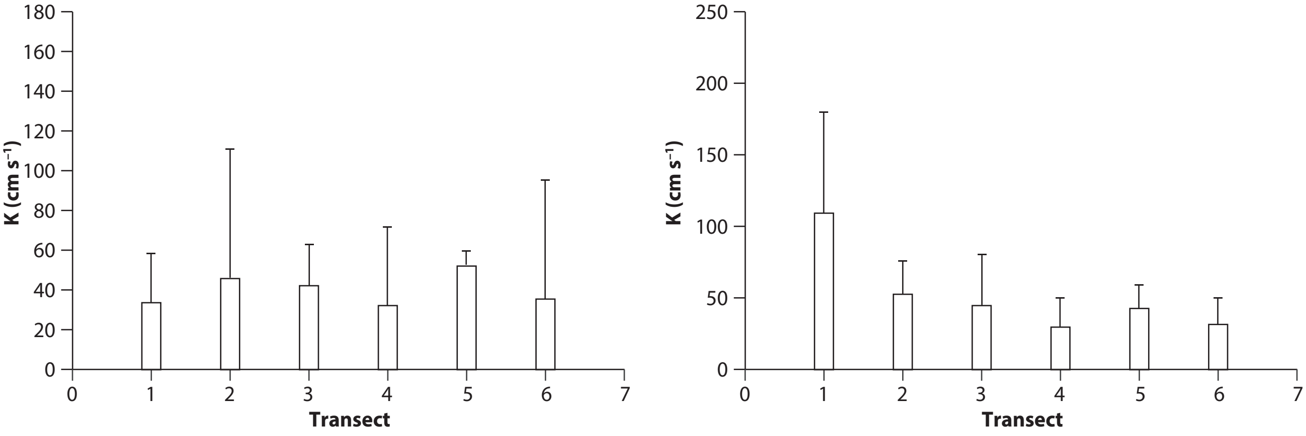

Fig. 3 Longitudinal variation in the hydraulic conductivity (K) recorded in the control (a) and altered (b) sections of the river.

The value of the specific conductivity was on average higher in the altered (215.0 µS cm^{-1}) than in the control section of the river (155.74 µS cm^{-1}). Similar conductivity means were recorded in upwelling and downwelling zones in both sections of the river probably because the values were very variable (Table 2). In general terms, there was a tendency towards higher conductivity values in the hyporheic zone compared to the surface water, especially in the control section of the river (Fig. 4). This tendency was not, however, significant ($p > 0.05$). On the other hand, a reduction in the conductivity in both sections was recorded as the warmer months approached. The water in the hyporheic zone was significantly warmer than that of the surface layer in the control section (paired *t*-test, $p < 0.05$) with a mean temperature of between 9.7 °C in January and 19.9 °C in July (Fig. 5). The altered section of the river was generally warmer than the control section with mean temperatures of between 10.5 °C in January and 21.2 °C in July. The hyporheic water in this section again tended to be warmer than that of the surface layer, although these differences were not significant (Fig. 5).

Spatial Variation in Nutrient Concentrations

There were no significant differences in the mean concentrations of PO_4-P, NO_3-N and NH_4-N in the upwelling and downwelling zones in the control section of the river. In the altered section, however, the concentrations of NO_3-N in the upwelling zones were greater than in the downwelling zones, and the mean concentrations of PO_4-P were lower in the upwelling zones than the downwelling zones (Table 2). In all of the zones, the concentrations of NO_3-N and NH_4-N were generally more variable than the mean concentrations of PO_4-P. The interstitial water was richer in NO_3-N and NH_4-N than the surface water in the altered section of the river. During the course of this study the concentrations of NO_3-N in the interstitial water in the altered section of the river were close to 10 mg l^{-1}, three times greater than the concentrations of NO_3-N in the surface water. The hyporheic

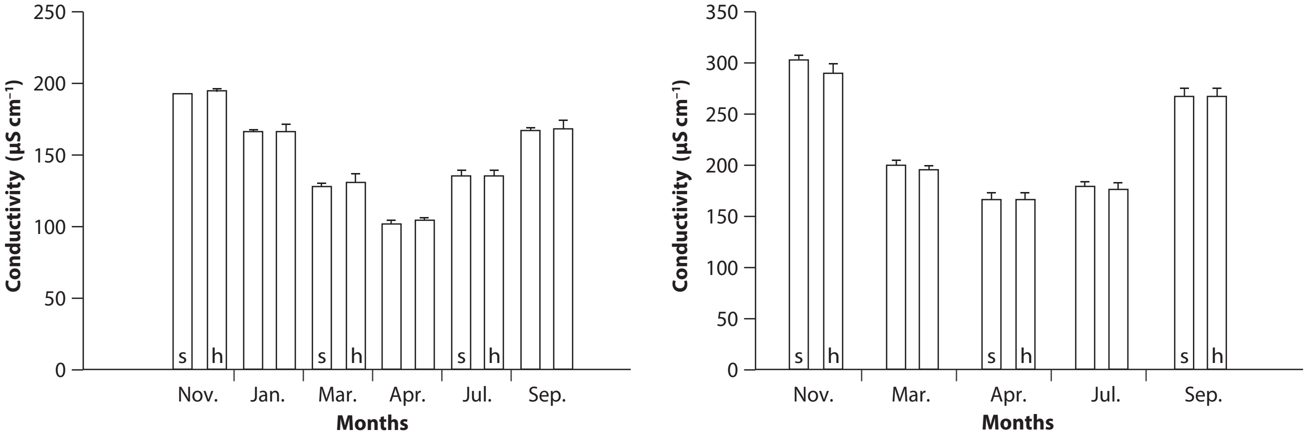

Fig. 4 Seasonal variation in the specific conductivity recorded at the surface and in the hyporheos in the control (a) and altered (b) sections of the river. Differences are not significant (s = surface, h = hyporheos).

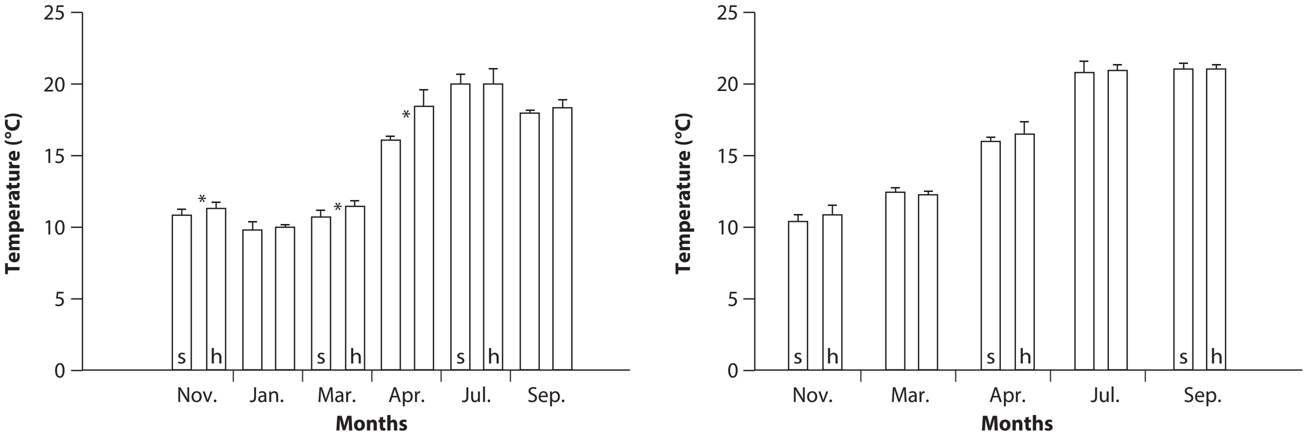

Fig. 5 Mean seasonal variation in the temperature recorded at the surface and in the hyporheos in the control (a) and altered (b) sections of the river (* = significant differences) (s = surface, h = hyporheos).

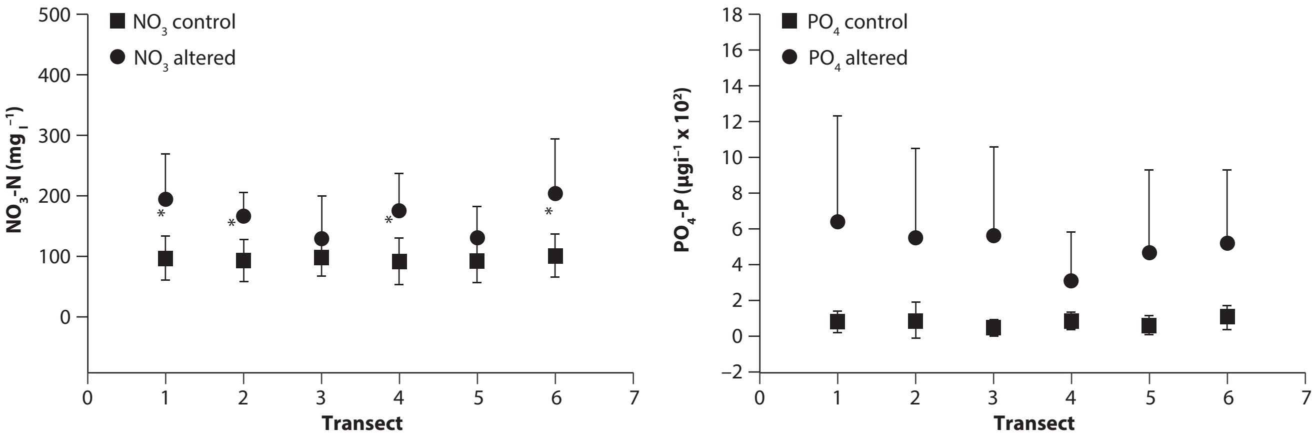

Fig. 6 Longitudinal variation in the mean hyporheic nitrate (a) and phosphate (b) concentrations recorded in the two sections river during the course of this study (* = significant differences).

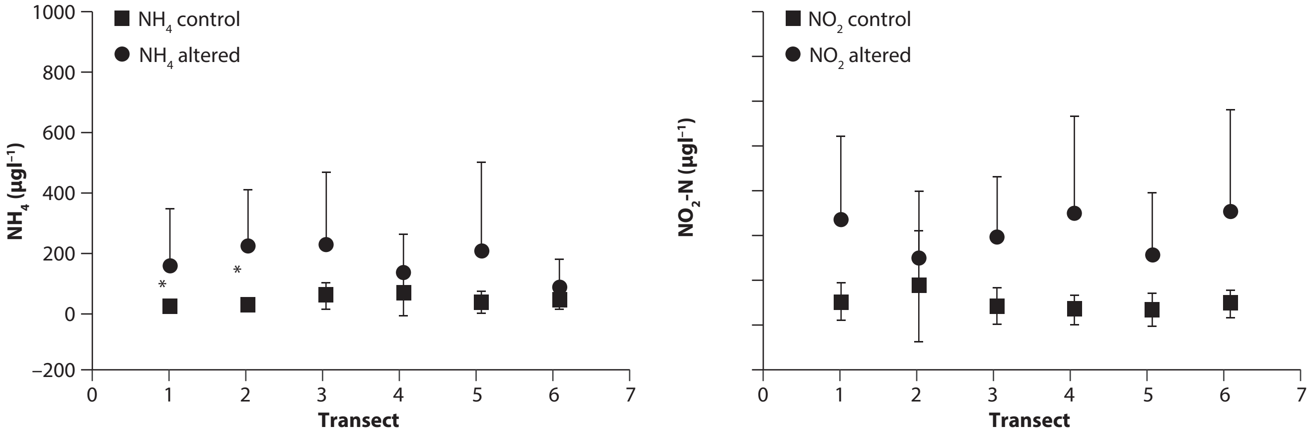

Fig. 7 Longitudinal variation in the mean hyporheic ammonium (a) and nitrite (b) concentrations recorded in the two sections during the course of this study (* = significant differences).

concentrations of NH_4-N were close to 45 and 200 µg l^{-1} for the control and altered sections, respectively, again much greater than those recorded for the surface water. The longitudinal variation in the interstitial concentrations of NO_3-N, PO_4-P and NO_2-N in the altered section of the river were similar, with a slight reduction along the lengths of the upper transects and a slight increase towards the lower transects (Figs. 6 and 7). In contrast, the pattern in the concentrations of interstitial ammonium was the reverse (Fig. 7), i.e., a tendency to increase along the upper transects and decrease towards the lower transects. In the control section of the river, there were no variations in the concentrations of these ions recorded along the longitudinal gradient. There were significant differences ($p < 0.05$, paired t-test) for nearly all of the comparisons of ionic concentrations in the two sections of the river.

Composition of the Meiofauna (*Hyporheos*)

A total of 24 taxa of hyporheic organisms caught in the three transects in each of the two sections of the river were identified. The most abundant groups of organisms were Oligochaeta, Chironomidae, Nematoda, Cyclopoida and Ephemerellidae (Table 3). The Dipterans were represented by four families: Chironomidae, Ceratopogonidae, Empididae and Simuliidae. The Ephemeropterans by two families: Ephemerellidae and Leptophlebiidae and the Syncarids by an extremely rare family: Parabathynellidae. Most of the Copepods belonged to the genus *Acanthocyclops* sp.

More groups of organisms were recorded in the control section of the river than the altered section with a maximum of 18 and a minimum of 12 taxa in the three transects studied. The lower transect (No. 6) in the altered section had the highest Shannon-Weaver diversity index (2.86), whilst in the upper transects the highest diversity index was recorded in the control section of the river, which is similar to the pattern recorded for group richness. Jaccard's index of similarity revealed a greater resemblance in community composition in the two sections in transect six (0.59) than in the other transects (0.50 and 0.36) (Table 3).

Table 3 Abundance of hyporheic organisms and community parameters recorded in each transect in the control and altered sections of the river.

Transect		**Abundance range**[a]					
		1 (upper)		3 (middle)		6 (lower)	
		Section					
Taxa		Contr.	Alter.	Contr.	Alter.	Contr.	Alter.
Platyhelminthes	Platy				1		2
Nematoda	Nema	3	3	3	3	3	3
Rotatoria	Rot	2	2	2	1		1
Tardigrada	Tard				1		
Oligochaeta	Olig	3	3	4	4	3	4
Hirudínea	Hir	1			2		
Gastropoda:							
Planorbidae	Plano	2					
Ostracoda.							
Candoninae	Cand	1	1		1	1	2
Cladocera:							
Alona sp.	Alo	2		3	1	2	2
Amphipoda:							
Niphargus sp.	Niph			2		2	
Copepoda:							
Cyclopoida:							
Acanthocyclops sp.	Acan	3	2	4	2	4	3
Harpacticoida:							
Bryocamptus sp.	Bryo	2		1		3	3
Syncarida:							
Parabathynellidae	Parab					3	
Acari	Acar	1	1	2		2	2
Collembola	Coll						3
Coleoptera:							
Haliplidae	Hali	1		3			
Diptera:							
Chironomidae	Chiro	4	4	4	3	4	4
Ceratopogonidae	Cera	1	1			2	
Empididae	Empi	1					
Simuliidae	Sim	1					
Ephemeroptera:							
Ephemerellidae	Eph	3	2	2		3	1
Leptophlebiidae	Lep		2	3	3	2	3
Heteroptera:							
Pleidae	Ple					1	
Plecoptera:							
Chloroperlidae	Chlo	1					
Community parameters							
Taxa richness (S)		18	10	12	11	14	13
BMWP [b]		46	29	35	28	37	34
Diversity index (H’)		2.59	2.23	2.54	2.29	2.45	2.86
Jaccard’s index (contr. vs alter.)		0.50		0.36		0.59	

Table 3 Legend

ᵃ Abundance of hyporheic organisms

Range	N° indiv.
0	0
1	1 to 3
2	4 to 10
3	11 to 100
4	> 100

ᵇ Water quality in terms of the BMWP index

Water quality	BMWP
Pristine water	>100
Clean, but slightly polluted water	61–100
Polluted water	36–60
Very polluted water	16–35
Extremely polluted water	<15

The rarest or scarcest groups amongst the aquatic organisms in the hyporheic fauna were the Tardigrades collected in transect No. 3 in the altered section of the river; Gastropoda (Planorbidae) in transect No. 1 in the control section; Syncarids (Parabathynellidae) in transect No. 6 in the control section; Collembola in transect No. 6 in the altered section; Diptera (Empididae and Simuliidae) in transect No. 1 in the control section and Heteroptera (Pleidae) in transect No. 6 in the control section.

In terms of the BMWP (Biological Monitoring Working Party) biological index for water quality, the control section of the river had the highest values (Table 3) with transect No. 1 having the highest index (46). The lowest values were recorded in transect No. 1 in the altered section of the river with an index of 29 and transect No. 3 with an index of 28. According to the BMWP scoring system for the evaluation of water quality the control and altered sections of the La Tordera River are classified as *polluted* and *heavily polluted*, respectively. The decrease in water quality down river of the discharge point of the sewage plant was determined on the basis of the absence of the following families of aquatic insects: Ceratopogonidae, Empididae and Haliplidae.

The highest Shannon Weaver diversity index recorded in transect No. 6 in the altered section of the river (2.86) corresponded to the highest values of the Vertical Hydraulic Gradient (0.273 cm cm^{-1}), specific conductivity (185.2 µS cm^{-1}), temperature (22.2 °C), PO_4-P (5.35×10^2 µg l^{-1}) and NO_3-N (23.3 mg l^{-1}). Nevertheless, transect No. 1 in the control section of the river had the second highest diversity index (2.59) and was the highest in hyporheic organism richness (18 groups). In contrast to transect No. 6 in the altered section, however, these values corresponded to the lowest for PO_4-P concentrations (0.9×10^2 µg l^{-1}), NO_3-N (12.63 mg l^{-1}), NH_4-N (0.3 µg l^{-1}) and NO_2-N (70.52 µg l^{-1}). It is important to note that the values given in Table 3 are the results for only one sample collected in July, which precludes more consistent and robust correlations. Thus, we can only speak in terms of tendencies and a preliminary characterization of the meiofauna in these two sections of the river. In general, the altered section of the river had higher nutrient concentrations and lower biological indices of water quality than the control section.

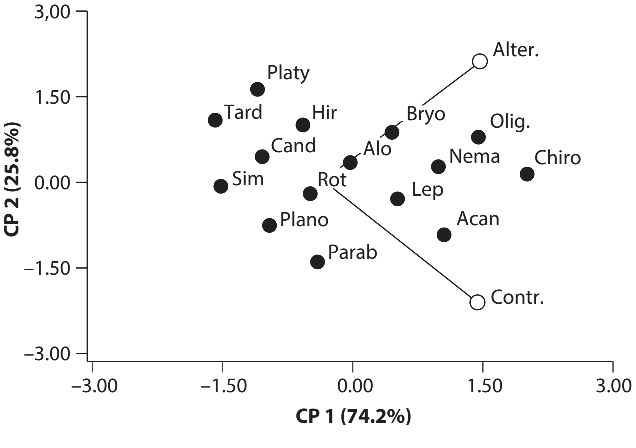

Fig. 8 Between-groups principal component analysis (PCA) of hyporheic meiofauna using log(x + 1) transformed data (Alter = altered section; Contr. = control section).

Ordination Analyses

The between-sections PCA was significant ($p = 0.019$) and indicated that 99% of the total variance in the taxonomic data was explained by spatial variation (Fig. 8) (axis 1 = 74.2%; axis 2 = 25.8%). Axis 1 was best explained by a positive relationship with Chironomidae, Oligochaeta, Nematoda, *Acanthocyclops* sp., Leptophhebiidae, *Bryocamptus* sp. and *Alona* sp. and a negative relationship with Planorbidae and Rotifera. Axis 2 was positively related to Platyhelminthes, Tardigrada and Candoninae, and negatively to Parabathyneliidae and *Acanthocyclops* sp. There was a clear separation between taxa recorded in the control section (Parabathyneliidae and *Acanthocyclops*) and those recorded in the altered section (Chironomidae, Oligochaeta, Nematoda).

Discussion

Surface – Hyporheos Interactions

The exchange processes between the surface and the hyporheos in the La Tordera River are clearly related to the spatial-temporal variations in the vertical hydraulic gradient and hydraulic conductivity. The spatial alternation of upwelling and downwelling zones in both sections of the river indicate the geomorphology is similar in both sections of the river; the river bed is mainly made up of coarse sediments (pebbles, stones, gravel) resulting in high values of hydraulic conductivity, especially in the control section. The changes recorded in the magnitude and direction of the hydraulic gradient could be the result of changes in water levels and surface water discharge (Valett et al. 1994). An increase in the surface water discharge could increase the extent of the upwelling zones, whilst in dry periods the downwelling zones would increase in size (Valett et al. 1990).

The importance of the topography of the river bed in hydrological exchange processes has been demonstrated for longitudinal sections along riffle – pool sequences. Reductions in depth result in high pressure zones at the end of a pool where surface water infiltrates the sediment, displacing the interstitial water (downwelling). Later, at the extreme end of the riffle, the increase in depth results in a low pressure zone, causing the discharge or upwelling of interstitial water (Triska et al. 1989b; Brunke and Gonser 1997; Woessner 2000). Experiments done with tracers by Harvey and Bencala (1993) indicate that these convex and concave sequences along the topography of the river bed control surface – hyporheic interactions. Surface water infiltrates in downwelling zones and flows interstitially along well defined channels. After travelling up to 10 m downstream it returns to the surface independently of contributions from the river aquifer. The obstacles present on the river bed, such as gravel bars, rocks, pebbles, tree trunks act as dykes, and aggregations of macrophytes and even lamprey nests, can result in small scale differences in hydraulic pressure modifying interstitial water flow (Hendricks and White 1991). The lack of important topographic differences in the two sections of the La Tordera River used in this study could be the reason why the hydraulic gradient does not show a defined pattern of upwelling and downwelling zones. In contrast, the higher hydraulic conductivity recorded in the control section of the river compared to the altered section demonstrates the effect of the effluent from the sewage treatment plant. This point source of pollution results in the clogging of the hyporheic interstitial spaces causing a clear reduction in the hydraulic conductivity in the altered section of the river. The discharge of residual water, or water inefficiently or improperly treated encourages the external clogging of the river bed by favouring the growth of benthic algae or the sedimentation of fine or very fine organic particles on the river bed. Internal clogging is caused by the intrusion of organic or very fine inorganic material into the interstitial spaces in the sediment. Unsustainable activities as regards soil use in the catchment basin directly enhance this process by increasing the amount of suspended particles (seston) and sediment load (Brunke and Gonser 1997; Treese et al. 2009).

The small difference in the specific conductivity of the surface water and the water in the hyporheos zone is similar to that reported by other authors such as Hendricks and White (1991) for the Michigan River, where the hyporheic conductance was significantly higher than that of the surface water. Although the specific conductance of the hyporheos also tended to be higher than that of the surface water in this study, the differences were not significant due to the large variability between replicates. Surface – aquifer exchange processes determine the temperature in the hyporheic zone in that the hyporheic temperature in general, varies according to the mean annual air temperature, whilst that of the river shows large daily and seasonal fluctuations. The temperatures recorded in this study clearly tended to be higher in the hyporheic zone than at the surface and these differences were significant in the control section of the river. These results agree with those reported by Valett et al. (1990) and Valett (1993) for rivers in the Sonora Desert with highly porous sediments. The temperature regimen in the hyporheic zone is important for both surface and underground aquatic systems due to the fact that fluvial microbial metabolic processes and the development of many invertebrates are thermo-dependant (Brunke and Gonser 1997).

Hydrology and Nutrient Content

The hyporheic concentrations of total inorganic nitrogen (NH_4-N + NO_3-N + NO_2-N) and phosphate (PO_4-P) were higher than those recorded at the surface. There were no significant differences between upwelling and downwelling zones. A large number of publications report the richness of the nutrients in the hyporheic zone (Valett et al. 1990, 1994 and 1996; Stanley and Boulton 1995; Jones Jr and Holmes 1996). There is a strong correlation between surface nitrate concentrations and the magnitude of the vertical hydraulic gradient (VHG) in upwelling zones. The results from this study indicate that contributions from nitrogen-rich interstitial waters may increase surface nitrate concentrations in upwelling zones, thus making the hyporheic zone a source of nitrates in the control section of the La Tordera River. In contrast, in the altered section of the river there is a high concentration of surface NO_3-N, NH_4-N, DIN and DOC as a consequence of the discharge from the wastewater treatment plant (Merseburger et al. 2005). Similar studies indicate that in these situations the hyporheic zone acts as a nutrient sink or trap (Triska et al. 1989; Jones Jr and Holmes 1996; Taleb et al. 2008). In the hyporheic zone, especially in the aerobic – anaerobic interphase, an intense metabolism of nitrogen takes place during the nitrification – denitrification processes. Both processes are related to the existing gradients of dissolved oxygen, dissolved organic carbon (DOC), ammonium and nitrates as well as flows between the surface and aquifer compartments. Allochthonous sources of nitrate entering the river may be assimilated by the epiphyton, which stimulates the growth of benthic algae. This constitutes one of the most important effects of point sources of pollution by nutrients in oligotrophic water bodies (Valett et al. 1992; Jones Jr and Holmes 1996). There were no significant differences in the phosphate concentrations between the upwelling and downwelling zones in most of the samples, although there was a tendency for them to be higher in the downwelling zone in the altered section of the river. Valett et al. (1994) report similar results for Sycamore Creek, Arizona and also establish that the hyporheic phosphate concentrations are not affected by the magnitude of the surface – aquifer exchange in the upwelling zones, although they were very close to being significant. The high concentrations of phosphate recorded in the hyporheic zone and their relation to the VHG indicate that the dynamics of this anion are controlled by physicochemical processes such as adsorption. Biological processes may also affect the concentrations of this anion, but in this study area are probably insignificant due to the fact that there is an excess of this nutrient, especially in the altered section of the river (Valett et al. 1990).

Composition of the Meiofauna and its Relation to Hyporheic Physicochemistry

Chironomids were the most abundant taxon in the hyporheos in the area studied, followed by Oligochaetes. These results are similar to those reported by Fraser and Williams (1998) and Franken et al. (2001) in Ontario, Canada. The number of taxa identified by these authors was also similar to that recorded in this study. Nevertheless, Rotifers, Cladocera and Syncarids recorded in the La Tordera River in this study have not been reported previously in this region (Boulton and Stanley 1995; Fraser and Williams 1998; Bartoszek 2001; Danielopol and Pospisil 2001; Franken et al. 2001). The Rotifera and Cladocera recorded in the hyporheic samples form part of the fluvial benthos, which indicates that these organisms also occasionally visit the interstitial habitat in search of food or refuge (Williams 1993). Amongst the groups identified, the Syncarids are rare and a most important find.

The multivariate analysis indicated that the species composition of the invertebrates recorded in the two sections differed. We suggest that this reflects differences in the taxa in their tolerance of pollution. Species richness and diversity did not vary greatly, possibly reflecting the rapidity with which most hyporheic sensitive taxa, such as Cyclopoida, Syncarids, Ephemeroptera and Gastropoda are replaced. The Amphipoda, Syncarida, Coleoptera, Heteroptera and Gastropoda were absent from the altered section. Belaidi et al. 2004 report that Crustacea do not occur in heavily polluted subterranean water in the Tafna stream in Algeria, which demonstrates the vulnerability of these organism to pollution and their value as indicator organism in subterranean environments. The highest richness of meiofauna was recorded in the control section of the river, where the interstitial nutrient concentrations were lowest. The meiofauna richness recorded in the aquifer discharge (upwelling) zones in all transects in both sections of the river was high. This distribution can be attributed to several factors: habitat differences, response to changes in sediment size and underflow dynamics, and to increasing hypoxia and pollution (Belaidi et al. 2004). Franken et al. 2001 and Febria et al. 2010 report a large number of groups of organisms in aquifer recharge (downwelling) zones as well as in upwelling zones. As regards the preferences of a group of organisms for a particular section of a hydraulic gradient, the results of this study are not conclusive owing to the lack of samples of the hyporheic fauna in downwelling zones.

In an attempt to determine the quality of the interstitial water in the two sections of the La Tordera River we used the BMWP (Biological Monitoring Working Party) biological index specially adapted for Mediterranean rivers (Armitage 1983; Alba-Tercedor and Sánchez-Ortega 1988), which takes into consideration the presence/absence of aquatic benthic invertebrates in the hyporheic zone. We considered it appropriate to apply this index to the hyporheos in this study because many benthic larvae occupy the hyporheic zone during part of their lifecycle, as well as those groups that permanently occupy this habitat. According to the BMWP index the control section of the river is *polluted* and the altered section *heavily polluted*. The development and application of a standardized sampling technique in this study is analogous to the establishment of sampling methods for assessing the biological quality of rivers using the BMWP/ASPT scoring system (Hawkes 1997). Although the range of BMWP scores generated from the samples collected is roughly comparable to that expected from standard benthic kick sampling, the calculated scores for the hyporheic zone are much lower. The BMWP scoring system was designed to highlight biological differences in a wide spectrum of different types of river (from high altitude torrential streams dominated by Ephemeroptera, Plecoptera and Trichoptera to sluggish, lowland rivers dominated by Chironomids and Oligochaetes). In contrast, the hyporheic zone, although not actually a natural part of that spectrum, is analogous to a highly truncated portion of it, with all samples being taken from essentially the same microhabitat, almost regardless of the character of the overlying river. The range of back-calculated values for benthic BMWP (121–171) (Prat et al. 2001) is thus much narrower than the hyporheic BMWP values recorded in this study (28–46). Within the hyporheic zone samples, therefore, it is much more likely that high BMWP scoring taxa will occur alongside low scoring taxa than would be the case in the benthos.

When the taxa richness of the benthic samples (Ortiz et al. 2006) is analyzed the BMWP index is much higher than that recorded for the hyporheic samples. This reflects the relative scarcity of benthic macroinvertebrates in the hyporheic zone, the truncated functional biodiversity for these organisms and also the dominance of meiofauna in the sample that was not collected using a standard benthos net (the mesh being too coarse). When assessing the conservation value of a site the taxonomic richness of the fauna is one of the primary measures used (Pryce et al. 2010). Of the additional (non-BMWP) taxa recovered using hyporheic techniques the majority were ubiquitous (e.g. Nematoda, Cyclopoida and Harpacticoida Copepoda, Rotatoria) (Table 3). Other groups were present in very low numbers (Tardigrada, Planorbidae, Empididae were collected in only one section). Six taxa are potential bioindicators (present in most sections): Cladocera, Ostracoda, Ceratopogonidae, Acari, Platyhelminhes, Hirudinea. However, little is known about their relative tolerance of pollution and disturbance and more research would be required before they could be used as bioindicators. It is also possible that they are routinely present in kick samples but either pass through the mesh or are simply too small to be recorded in the normal sample sorting procedure. Hyporheic assemblages are located at the interface between ground and surface waters and are therefore able to give an earlier warning of the ecological response of lotic ecosystems to groundwater borne pollution. Groundwater species provide the potential for biomonitoring aquifers and assessing their response to pollution (e.g. diffuse nitrogen pollution). Monitoring of hyporheic and groundwater assemblages would provide information on the health of these compartments of the lotic ecosystem and provide a more comprehensive assessment of a river's ecological status (Robertson et al. 2008). This monitoring would be particularly timely in the light of the Groundwater Directive 2006, which encourages member states to conduct research in order to provide better criteria for ensuring groundwater ecosystem quality and protection.

The effluent from the Santa María de Palautordera wastewater treatment plant contributes a high nutrient load. When N induces a change in the dominant form of transport (NO_3-N) to the reduced form (NH_4-N), this has a negative effect on stream biota, especially fish communities (Marti et al. 2004). The WWTP effluent is an important source of contamination resulting directly or indirectly in external clogging of the interstitial spaces in the sediment, reducing the available physical space for the development of the hyporheic fauna and producing an increase in benthic algal cover. The fauna in the interstitial biotope appears to be highly dependent on sediment porosity and composition. The presence of a potentially highly biodegradable biomass favours detritivors and microbial organisms over filter feeders (Table 3), resulting in a change in the composition and structure of the hyporheos (Brunke and Gonser 1997; Palmer et al. 1997; Stepenuck et al. 2002; Morais et al. 2004). In general, this community is extremely sensitive to external perturbations, and is categorized as having *low resistance* (Fowler and Death 2001; Sliva and Williams 2005). In addition, subterranean, lenthic and oligotrophic lotic ecosystems have a long recovery time, that is, *low resilience* (Stepenuck et al. 2002; Febria et al. 2009). Thus, these perturbations, principally of anthropic origin, are damaging both to the ecological equilibrium and the autoregenerating capacity of the hyporheic zone, the main link in the process of integral recovery of fluvial ecosystems.

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