Philipps Universität Marburg

Population genetics on anthropogenic and natural sites, subspecies differentiation and fungal community of *Gymnadenia conopsea s.l.* (Orchidaceae)

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“There are no places left on Earth that don’t fall under humanity’s shadow”.

(Gallagher & Carpenter 1997)

List of papers

This thesis is based on the following publications and manuscripts. They will be referred to in the text by the term "chapter" and their roman numerals. The author's and co-author's contributions to the manuscripts are listed below:

Paper I: Fungi from the roots of the common terrestrial orchid *Gymnadenia conopsea*

Together with Wiesław Babik and Walter Durka
Mycological Research 113 (2009): 952- 959

The study was designed by Walter Durka and me. I did the laboratory work, analyzed the data and wrote the manuscript. Wiesław Babik gave methodical advice in the laboratory and commented on earlier versions of the manuscript.

Paper II: Strong genetic differentiation between *Gymnadenia conopsea* and *G. densiflora* despite morphological similarity

Together with Wiesław Babik, Stefan G. Michalski, Grit Winterfeld and Walter Durka
Manuscript submitted to Plant Systematics and Evolution.

The study was designed by Walter Durka and me. I did the laboratory work, analyzed the data and wrote the manuscript. Walter Durka took part in the analysis of the data and preparation of the manuscript. Wiesław Babik did the PHASE reconstruction. Stefan Michalski helped with the phylogenetic analysis and discussed the data. Grit Winterfeld counted the chromosomes.

Paper III: The value of anthropogenic habitats for conservation: A case study on *Gymnadenia conopsea* and *G. densiflora*

Together with Walter Durka
Manuscript

The study was designed by Walter Durka and me. I did the laboratory work, analyzed the data and wrote the manuscript. Walter Durka gave statistical advice and commented on earlier versions of the manuscript.

Table of contents

Zusammenfassung

Eingriffe des Menschen in die Natur sind heute die Hauptursache für viele globale Veränderungen wie z.B. den Klimawandel oder das Aussterben von Arten (Rockström et al. 2009). Eine der schwerwiegendsten anthropogenen Eingriffe ist die Umwandlung von naturnahem Land in vom Menschen intensiv genutzte Flächen. Auf diese Weise wurden bisher ca. 40-50% der Erdoberfläche in industriell oder landwirtschaftlich genutzte Flächen überführt (Chapin et al. 2000), auf 1 % der Fläche werden Bodenschätze abgebaut (Walker 1992). Der Verlust bzw. die Degeneration oder Fragmentierung von Lebensraum sind direkte Folgen solcher Umwandlungen und daher eine der Hauptursachen für das Aussterben von Arten. Indirekte Folgen sind beispielsweise der Verlust an genetischer Diversität und von lokal angepassten Populationen, zunehmende Inzucht durch kleiner werdende Populationen und die Störung ökologischer Interaktionen (z.B. Mykorrhiza, Bestäubung) (Vitousek et al. 1997). Diese Faktoren beeinflussen das Fortbestehen von Populationen und somit die Überlebenswahrscheinlichkeit einer Art in der Natur.
Orchideen sind besonders empfindlich gegenüber Veränderungen in der Umwelt, denn mit Keimung und Bestäubung hängen zwei der wichtigsten Abschnitte im Lebenszyklus einer Orchidee von symbiotischen Interaktionen ab (Rasmussen 1995; Swarts & Dixon 2009b). Für einen effektiven Schutz von Orchideen ist es daher wichtig, ein grundlegendes Verständnis ihrer Biologie und der Faktoren zu erlangen, die die oft sehr spezifischen Habitatansprüche und sich daraus ergebenden Verbreitungsmuster bestimmen (Swarts & Dixon 2009b).

Anhand von *Gymnadenia conopsea s.l.*, einer noch relativ weit verbreiteten Orchideenart, die in vielen verschiedenen Habitattypen vorkommt, untersucht diese Arbeit verschieden Aspekte der Orchideenbiologie, die für die Rekrutierung junger Individuen und das langfristige Fortbestehen von Orchideen-Populationen in der Natur eine wichtige Rolle spielen, nämlich die Spezifizität der Orchideen-Pilz-Symbiose, intraspezifische Differenzierung und die Populationsgenetik dieser Art.

Mit Gymnadenia conopsea assoziierte Pilzgemeinschaft

Ein wichtiger Aspekt für das Fortbestehen und die Etablierung neuer Orchideen-Populationen ist die Rekrutierung von Jungpflanzen. Für Orchideen ist das Vorhandensein kompatibler Mykorrhizapilze dafür eine Grundvoraussetzung, da diese die für die Keimung und das anfängliche Wachstum notwendigen Kohlehydrate zur Verfügung stellen (Leake 1994; Rasmussen 1995). Für Arten, die nur mit ganz bestimmten Pilzen eine solche symbiotische Verbindung eingehen können, kann das Vorhandensein dieser speziellen Pilze ein limitierender ökologischer Faktor sein, der ihre Verbreitung bestimmt. Die Spezifizität zwischen Orchidee und Pilz hat also einen maßgeblichen Einfluss auf die Rekrutierungschancen von Jungpflanzen (Bidartondo & Read 2008) und ist somit auch ein wichtiger Faktor, der die Kolonisierungsfähigkeit einer Art mitbestimmt.

In einer ersten Studie (Kapitel 1) wurden daher in zwei Untersuchungsgebieten in Ost- und Norddeutschland die Pilzgemeinschaften von insgesamt sechs *G. conopsea s.str.* Populationen untersucht. Die 28 identifizierten Pilztaxa lassen eine hohe Diversität an Pilzen erkennen, die mit *G. conopsea* assoziieren, was auf eine geringe Spezifizität der Orchideen-Pilz-Symbiose schließen lässt (Timms & Read 1999). Für Arten mit einem breiten taxonomischen Spektrum an potentiellen Pilzpartnern sollte die Wahrscheinlichkeit, einen passenden Partner zu finden, relativ groß sein, weshalb man eine relativ weite Verbreitung und gute Kolonisierungsfähigkeit erwarten kann (Batty et al. 2002; Currah et al. 1997). Dies hat man z.B. für zwei australische Orchideenarten gefunden, einmal für eine sich schnell über den Kontinent ausbreitende invasive Art und auch für eine einheimische weitverbreitete Art, die in vielen verschiedenen Habitattypen vorkommt (Bonnardeaux et al. 2007). Die hohe Diversität an mit *G. conopsea* assoziierenden Pilztaxa ist daher wahrscheinlich auch ein wesentlicher Grund für ihre noch relativ weite Verbreitung und Fähigkeit, in sehr verschiedenen Habitaten wachsen zu können.

Die meisten bisher gefundenen Mykorrhizapilze von Orchideen (OM) sind Basidiomyceten der *Rhizoctonia*-Gruppe (Warcup & Talbot 1967; Warcup & Talbot 1971). In allen untersuchten *G. conopsea* Populationen wurden Vertreter der bekannten OM-Gattungen *Tulasnella*, *Ceratobasidium*, *Thanatephorus* und *Sebacina* (Moore 1987; Warcup & Talbot 1967) gefunden. Dies deutet darauf hin, dass *G. conopsea* typische OM-Pilze als Mykorrhiza nutzt. Es wurden jedoch auch zu den Ascomyceten gehörende Taxa der Pezizales und Helotiales gefunden, die erwiesenermaßen Ektomykorrhiza bilden können. Diese morphologisch sehr feinen Pilze sind bisher weniger untersucht als ihre robusteren „Gegenstücke“ der Basidiomyceten, weshalb man noch sehr wenig über ihre Taxonomie oder Ökologie weiß (Tedersoo et al. 2006). Es ist daher wahrscheinlich, dass auch ihre Bedeutung als Mykorrhizapilze ernsthaft unterschätzt wird (Egger 2006). Die durchgängige Präsenz solcher Taxa in allen Populationen von *G. conopsea* könnte ein Hinweis sein, dass *G. conopsea* auch eigentlich Ektomykorrhiza bildende Taxa als Mykorrhiza nutzen kann. Dies könnte eine Strategie sein, sich Kohlehydrate zu sichern auch in Habitaten, wo *Rhizoctonias* entweder nicht verfügbar sind oder die Fotosyntheseleistung zu gering ist, wie z.B. in Waldhabitaten (Selosse et al. 2004). Typischerweise kommt *G. conopsea* in offenen Graslandhabitaten vor, ist aber auch in Waldhabitaten zu finden (Gustafsson 2000; Scacchi & de Angelis 1989). Das Nutzen von Ektomykorrhiza würde also eine stabilere Kohlehydratquelle bedeuten und könnte ein weiterer Aspekt sein, der zu ihrer weiten ökologischen Verbreitung beiträgt.

Starke genetische Differenzierung zwischen Gymnadenia conopsea und G. densiflora, trotz morphologischer Ähnlichkeit

Die Taxonomie von *Gymnadenia conopsea* (L.) R.BR. *s.l.*. ist viel diskutiert. Die zwei am häufigsten unterschiedenen Taxa sind *G. conopsea* (L.) R.BR. ssp. *conopsea* und *G. conopsea* ssp. *densiflora* (WAHLENB.) K. RICHT., für die auch schon der Status getrennter Arten vorgeschlagen wurde (Bateman et al. 1997; Campbell et al. 2007; Marhold et al. 2005). Die Taxa werden beschrieben, sich hinsichtlich Morphologie, Phänologie, Duft und Habitatansprüchen zu unterscheiden (z.B. Jersáková et al. 2010; Marhold et al. 2005). Genetische Differenzierung wurde zwischen Blühvarianten (Gustafsson & Lönn 2003) und Ökotypen (Scacchi & de Angelis 1989) gefunden. Für die Zuordnung der in Ost- und Norddeutschland untersuchten *Gymnadenia*-Populationen zu den beiden Taxa wurden diese morphologisch und genetisch analysiert. Um die Fragestellung in einem größeren geographischen Maßstab zu untersuchen, wurden außerdem Proben aus anderen europäischen Regionen untersucht (Kapitel 2).

Die Analyse der ITS Region hat eine 2%ige Nukleotid-Divergenz ergeben, ähnlich der von anderen *Gymnadenia*-Arten. Dies, zusammen mit sich größtenteils nicht überschneidenden Mikrosatellitenallelen, unterstützt den Status verschiedener Arten von *Gymnadenia conopsea* (L.) R.BR. *s.str*. und *Gymnadenia densiflora* (WAHLENB.) DIETRICH. Die beiden Arten sind jedoch keine Schwesterarten, da die Sequenzen von *G. densiflora* eine hoch unterstützte monophyletische Gruppe bilden, die sich gemeinsame Vorfahren mit *G. nigra* und *G. austriaca* teilen. Die evolutionäre Geschichte von *G. conopsea s.str.* bleibt jedoch unklar, da sich auf Basis der ITS Daten die eigentlich morphologisch klar differenzierte Art *G. odoratissima* nicht von *G. conopsea s.str*. unterscheiden lässt. Hinsichtlich der Ploidie war *G. conopsea s.str.* entweder diploid oder tetraploid, wohingegen *G. densiflora* ausschließlich diploid war. Da sich die Mikrosatellitenallele der diploiden und tetraploiden *G. conopsea s.str.* kaum unterschieden und die häufigsten ITS Sequenzen in beiden vorkamen, ist eine autopolyploide Entstehung der tetraploiden aus der diploiden *G. conopsea s.str.* wahrscheinlich. Dies deutet darauf hin, dass *G. conopsea s.str.* und *G. densiflora* sich getrennt haben, bevor es zur Abtrennung anderer Gruppen kam, wahrscheinlich vor Entstehung der Polyploidie innerhalb von *G. conopsea s.str.*

Die morphologische Differenzierung war weniger eindeutig. Obwohl einige Parameter (z.B. Blütenanzahl, Blütendichte) eine relativ gute Trennung der Taxa erlaubt, erschwert eine erhebliche morphologische Variabilität eine eindeutige Bestimmung. Orchideen sind ein Paradebeispiel der Selektion durch Insektenbestäuber (Thompson 1994). Sowohl *G. densiflora* als auch *G. conopsea s.str*. haben relativ spezialisierte Blüten und werden von den gleichen Lepidoptera Taxa bestäubt. Es könnte also sein, dass konvergente Selektion durch Bestäuber zu einer ähnlichen Blütenmorphologie geführt hat. Darüber hinaus wird *G. densiflora* als stark, *G. conopsea s.str.*

jedoch nur als schwach duftend beschrieben (Schmeil 1996). Dies könnte für *G. conopsea s.str.* zum Selektionsdruck geführt haben, sich morphologisch *G. densiflora* anzupassen, um die gleichen Bestäuber anzulocken.

Ein weiterer Aspekt, der zur Vielfalt von Orchideen beigetragen hat, ist ihre Symbiose mit Mykorrhizapilzen (Waterman & Bidartondo 2008). Für die phylogenetische Divergenz von Blütenvariationen innerhalb des *Hexalectris spicata* Komplexes z.B. werden zumindest teilweise Unterschiede hinsichtlich der assoziierten Pilztaxa verantwortlich gemacht (Taylor et al. 2003). Es wird daher vermutet, dass solche kleinräumigen Verbreitungsmuster die Diversifikation fördern. Mykorrhizapilze können einen Effekt auf das Pflanzenwachstum haben und verschiedene Typen des gleichen Pilztaxons können sich hinsichtlich ihrer Wirkung unterscheiden (Lee 2002). Die für *G. conopsea s.str*. gefundenen Verbreitungsmuster der assoziierten Pilztaxa deuten auf eine ‚nicht-zufällige' Verteilung hin. Sowohl die morphologische Ähnlichkeit zwischen den beiden *Gymnadenia*-Arten als auch die morphologische Variabilität innerhalb dieser könnte also u.a. auch auf ihre Interaktion mit Mykorrhizapilzen zurückzuführen sein. Bisher ist noch nichts über die mit *G. densiflora* assoziierten Pilztaxa bekannt, und es sind weitere Untersuchungen notwendig, um tiefergehende Einblicke in die Rolle dieser Symbiose für die phylogenetische und ökologische Differenzierung zwischen *G. conopsea s.str.* und *G. densiflora* zu erhalten.

Der Wert von anthropogenen Standorten für den Artenschutz: Eine Fallstudie zu Gymnadenia conopsea und G. densiflora

Der Abbau von Bodenschätzen ist eine der schwerwiegendsten menschlichen Eingriffe in die Natur, da dadurch sowohl die ursprüngliche Vegetation als auch die Bodenstruktur zerstört werden. Andererseits entstehen auf diese Weise auch neue Lebensräume; denn auf diesen Flächen stellt eine Vielzahl an verschiedenen Substraten mit meistens extremen Umweltbedingungen potentielles Habitat für viele spezialisierte Arten dar (Brändle et al. 2003; Ratcliffe 1974; Varela et al. 1993). Um jedoch zum Schutz bedrohter Arten beizutragen, muss die genetische Diversität von Populationen auf anthropogenen Standorten mit der von Populationen auf natürlichen Standorten vergleichbar sein. In einer dritten Studie (Kapitel 3) wurden daher die genetische Diversität und der allgemeine Pflanzenzustand von Populationen auf anthropogenen Standorten (Braunkohletagebau in Ostdeutschland, Steinbrüche in Norddeutschland) untersucht und mit der von umgebenden natürlichen Standorten verglichen. Die Ergebnisse ergaben für die Populationen in der Bergbaufolgelandschaft eine verringerte genetische Diversität und einen reduzierten Fruchtansatz, wohingegen solche Effekte für die Populationen in den Steinbrüche nicht zu erkennen waren. Der allgemeine Pflanzenzustand war für alle untersuchten *Gymnadenia*-Populationen ähnlich.

Die Ergebnisse zeigen, dass die Größe und Intensität einer Störung einen erheblichen Einfluss auf den Kolonisierungsprozess eines neuen Habitats haben kann. Samenausbreitung und die Rekrutierung von Jungpflanzen sind zwei wichtige Faktoren, die die Besiedlung neuer oder gestörter Lebensräume beeinflussen (Vekemans & Hardy 2004). Die Ausbreitung von Samen wird u.a. durch die räumliche Verteilung reproduzierender Individuen und deren Samenproduktion bestimmt (Nathan & Muller-Landau 2000). Bergbaustandorte sind i.d.R. relativ groß und die Distanz zu den nächsten natürlichen Standorten als potentielle Wiederbesiedlungsquellen kann erheblich sein. Dies ist z.B. für die Bergbaufolgelandschaft in Ostdeutschland der Fall, wo die nächsten natürlichen *Gymnadenia*-Populationen geographisch in einer anderen Region liegen. Ein verringerter Zustrom von Samen in die Bergbaufolgelandschaft könnte daher eine Ursache für die verringerte genetische Diversität der jeweiligen Populationen sein und zur Etablierung von nur wenigen und/oder isolierten Populationen führen. Der reduzierte Fruchtansatz könnte die Folge einer veränderten Bestäuberaktivität sein, entweder aufgrund einer verringerten Abundanz oder einer veränderten Artzusammensetzung, und es gibt geringe Hinweise auf Inzuchtdepression. Für die *G. densiflora* Populationen in der Bergbaufolgelandschaft wurden zwar erhöhte Inzuchtkoeffizienten gefunden, die jedoch nicht mit dem Fruchtansatz korrelierten, wohingegen für *G. conopsea* eine Korrelation zwischen Fruchtansatz und genetischer Diversität nachzuweisen war. Langfristig könnten die kleinen Populationsgrößen, der reduzierte Fruchtansatz und die geringe Diversität daher das Fortbestehen dieser Populationen gefährden.
Einen Einfluss des Menschen auf die genetische Diversität wurde z.B. auch für *Epipactis helleborine* gefunden, für die die durchschnittliche genetische Diversität von städtischen Populationen geringer war als für ländliche (Hollingsworth & Dickson 1997). Kein Unterschied wurde zwischen *Epipactis palustris* Populationen in der gleichen Bergbaufolgelandschaft und auf natürlichen Standorten gefunden (Esfeld et al. 2008), ähnlich zu den Ergebnissen der *G. conopsea* Populationen in den Steinbrüchen. Hier fördern kleinere Abgrabungsstätten, die geographisch mit natürlichen Standorten durchmischt sind, wiederholte Besiedlungen von verschiedenen Ursprungspopulationen. Es ist daher wahrscheinlich, dass ein hohes Maß an Genfluss zwischen den Populationen, entweder durch Samenausbreitung oder Aktivität von Bestäubern, zu der hohen genetischen Diversität innerhalb und geringen genetischen Differenzierung zwischen diesen Populationen beiträgt.

Die kleinen Populationen in der Bergbaufolgelandschaft könnten auch ein Hinweis auf eine geringe Rekrutierungsrate sein, denn für eine erfolgreiche Keimung ist ein geeignetes Mikrohabitat notwendig (Nathan & Muller-Landau 2000). Bei Orchideen ist dies stark von der Verfügbarkeit kompatibler Mykorrhizapilze abhängig (Jersáková & Malinová 2007). Untersuchungen haben gezeigt, dass räumliche Verbreitungsmuster von Bodenpilzen z.B. von Kohlenstoff-Gradienten, Landnutzung oder von durch Pflanzenwachstum verursachte Mikrostrukturen im Boden abhängen

können (Ettema & Wardle 2002; Kasel et al. 2008). Anthropogene Standorte werden oft durch extreme Umweltbedingungen bestimmt. Man kann daher erwarten, dass sich aufgrund unterschiedlicher ökologischer Präferenzen von Mykorrhizapilzen die Pilzgemeinschaften von anthropogenen und natürlichen Standorten unterscheiden. Dies hat man z.B. für *Collinsia sparsiflora* gefunden, für die an Serpentin angepasste Ökotypen mit anderen arbuskulären Mykorrhizapilzen assoziierten als nicht angepasste Ökotypen (Schechter & Bruns 2008). Die regionale Differenzierung der mit *G. conopsea* assoziierenden Pilzgemeinschaft zusammen mit der hohen Variabilität auf Populationsebene könnte ein Hinweis sein, dass lokale Faktoren die Taxonvielfalt vor Ort und somit die Diversität der Region als Ganzes beeinflussen. Die Tatsache, dass von 6 Populationen in der Bergbaufolgelandschaft 5 aus *G. densiflora* bestehen, obwohl in der umgebenden Region v.a. *G. conopsea* vorherrscht, deutet auf eine komplexe Interaktion extrinsischer und intrinsischer Faktoren hin, die die Pilzgemeinschaft von *G. conopsea* und *G. densiflora* bestimmt.

Die Ergebnisse dieser Arbeit zeigen, dass anthropogene Lebensräume wie z.B. Abbaustandorte zum Erhalt der Biodiversität beitragen, und zwar nicht nur auf Ebene der Artenvielfalt, sondern auch zum Schutz der genetischen Ressourcen. Typischerweise sind solche Standorte sehr heterogen mit periodisch gestörten Abschnitten der frühen Sukzessionsphasen, die durch extreme abiotische Bedingungen und nur geringe Produktivität gekennzeichnet sind (Novák & Prach 2003; Schulz & Wiegleb 2000). Ähnliche Standorte sind in den modernen Landschaften von heute selten geworden, da der Mensch durch ständige Produktionssteigerung eher mittlere Sukzessionsphasen fördert (Hoekstra et al. 2005). In vielen Regionen sind es daher v.a. auf frühere Sukzessionsphasen angewiesene Arten, die ernsthaft bedroht sind und gerade für diese Arten stellen Abgrabungsstandorte wertvolle Habitate dar (Thomas et al. 1994; Wenzel et al. 2006). Andererseits belegen die Ergebnisse jedoch wie wichtig es ist, dass Populationen durch Genfluss miteinander in Verbindung bleiben, um Gründereffekte zu vermeiden. Es ist daher wichtig, durch geeignete Managementmaßnahmen wie z.B. Beweidung oder regelmäßige Mahd eine fortschreitende Sukzession aufzuhalten, um die Vielfältigkeit solcher Standorte zu erhalten.

G. conopsea s.l. ist eine noch immer relativ häufige Orchideenart und die geringe Spezifizität ihrer Pilz-Orchideen-Beziehung trägt wahrscheinlich zu ihrer Fähigkeit bei, in sehr unterschiedlichen Habitattypen wachsen zu können. *G. conopsea s.l.* umfasst die zwei genetisch differenzierten Arten *Gymnadenia densiflora* (WAHLENB.) DIETRICH und *Gymnadenia conopsea* (L.) R.BR. *s.str*. Bisher ist noch wenig über *G. densiflora* als eigene Art bekannt. Unterschiede hinsichtlich der Diversität oder Zusammensetzung der mit *G. densiflora* und *G. conopsea s.str*. assoziierten Pilztaxa könnten z.B. zu verschiedenen Habitatansprüchen und Kolonisierungsfähigkeiten führen. Zukünftige Studien sollten sich z.B. damit befassen, welche Rolle genetische Differenzierung auf die mit ihnen

assoziierenden Pilzataxa spielt und inwiefern dies die Verbreitung und Kolonisierungsfähigkeit von z.B. anthropogenen Standorten beeinflusst. Es ist nur wenig über die Faktoren bekannt, die die mit Orchideen assoziierenden Pilztaxa bestimmen und welche Folgen sich daraus für Habitatansprüche und Verbreitungsmuster von Orchideen ergeben. *G. conopsea s.str.*/ *G. densiflora* ist daher ein gutes Studiensystem, um ein besseres Verständnis für die Biologie von Orchideen zu erlangen und herauszufinden, welche Rolle ökologische und genetische Faktoren dabei spielen.

General Introduction

The first life on Earth developed between 3.5- 4.0 billion years ago (Campbell et al. 1999). Since then, evolutionary processes have created a biological diversity that is estimated to comprise between 2- 10 million species, whereof barely 20% (1.75 million) have been taxonomically described (Hawksworth & Kalin-Arroyo 1995). A key process of biological diversification is speciation, the emergence of new species by splitting existing lineages. Mechanisms that can lead to speciation are versatile (reviewed in Coyne & Orr 2004): It may evolve gradually by a series of allelic substitutions at several loci or it happens by a single step such as polyploidisation. Speciation can occur as a by-product of adaptation to different niches or contrasting environments, or it can take place without any adaptation whatsoever. For populations that are geographically separated for a long time speciation is inevitable, but it also can occur between populations of the same geographic regions that are connected by considerable gene flow (Butlin et al. 2009).

For plant speciation, polyploidisation and hybridization are of special importance (Rieseberg & Willis 2007). A recent study indicates that 60-70% of all flowering plants have experienced at least one episode of polyploidy in their ancestry (van de Peer et al. 2009) and polyploidisation is thought to be involved in 2-4% of all speciation events (Otto & Whitton 2000). Due to generally higher levels of genetic variation, polyploids are often considered to be ecologically more flexible and possess higher fitness, e.g. during colonization of newly available habitats (Comai 2005; Soltis & Soltis 2009). One of the main reasons for polyploidy is hybridization, the fusion of genomes from different taxa (allopolyploidy), but it can also arise by chromosome duplication within a species (autopolyploidy) (Mallet 2007; Soltis & Soltis 2000). Hybrid speciation, however, is a general term that refers to the mode of speciation in which gene flow between species plays a major role, which is not necessarily accompanied by polyploidisation. More than 25% of plant species seem to be involved in hybridization with other species (Mallet 2005), but its frequency appears to vary considerably between groups (Paun et al. 2009).

What exactly constitutes a species is a still ongoing discussion that dates back to Darwin's "On the origin of species" (Darwin 1859). Today, at least 22 different species concepts do exist, each emphasizing different aspects of what should be considered as the basis for species delineation (Mayden 1997). The most important concept for sexually reproducing species is the biological species concept that describes species as "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups" (Dobzhansky 1937; Mayr 1963), whereas the morphological species concept emphasizes phenotypic similarity (Cronquist 1978; Grant 1981). Furthermore, the increasing use of DNA data during the last 20 years allows to infer organismal relationships from sequence data, which is reflected in the phylogenetic species concept (Rosen 1978; Wheeler & Platnick 2000). However, there is no single species concept that is applicable to all questions, but the used concept may have a profound impact on the results of e.g. biodiversity assessments and our understanding of biodiversity.

Genetic diversity

Besides ecosystem and species diversity, the IUCN designates genetic diversity as one of three levels of biodiversity requiring conservation (McNeely et al. 1990). Genetic diversity means the extent of genetic variation among individuals and/or populations within a species or across a group of species (Frankham et al. 2002), and as such it is the raw material for adaptation (Geffen et al. 2007). As genetic variation is a precondition for a species' ability to respond to continuously changing environmental conditions, it is a key factor for the long-term survival of a species in the wild (Frankham et al. 2002). However, in the short-term genetic diversity is important for the maintenance of reproductive fitness on the population level (Leimu et al. 2006). Threatened species tend to have small and/or declining populations. With decreasing population size, the probability of biparental inbreeding or selfing increases, and small populations are more susceptible to inbreeding depression (Mustajärvi et al. 2001). Inbreeding has long been known to have deleterious consequences for reproduction and survival in naturally outbreeding species (Charlesworth & Willis 2009). There is now strong evidence that the loss of genetic diversity and inbreeding contribute to the extinction risk of populations in the wild (e.g. Newman & Pilson 1997; Saccheri et al. 1998). Hence, the protection and restoration of genetic diversity is of major importance for the long-term survival or the sustainable management of (endangered) species and ignoring genetic factors may lead to inappropriate conservation strategies (Frankham 2005).

Therefore, genetic analyses are an integral part of many aspects of conservation biology. For example, in order to identify populations with a potentially increased inbreeding and future genetic decline (Frankham et al. 2002), it is necessary to quantify genetic variation and to analyze genetic patterns and processes within populations (Fay & Krauss 2003). For this purpose, molecular markers provide valuable information about the allelic variation at a given locus (e.g. genes or defined, non-functional DNA-sequences). The most widely used genetic markers are microsatellites, which are short, non-coding DNA stretches that consist of tandemly repeated motifs of 1-6 nucleotides (Ellegren 2004; Oliveira et al. 2006). Their high mutation rate and abundance throughout the genome make them suitable markers for population genetics, paternity testing and mapping (Schlötterer 2004).

Furthermore, phylogenetic studies can help to identify distinct lineages of high conservation priority (Soltis & Gitzendanner 1999), such as subspecies that might require separate management measures. A standard method for the phylogenetic analysis of angiosperms is the comparison of the nuclear ribosomal RNA genes (rDNA) (Baldwin et al. 1995). Organized in long tandem repeats, they consist of the transcribed regions 18S, 5.8S and 26S rDNA and two small internal transcribed spacers (ITS1 and ITS2). While the coding regions are rather conservative, the ITS regions evolve relatively fast and may vary among populations or species of the same genus (Baldwin et al 1995).

Altogether, information provided by genetic analyses allows for a better understanding of the biological system at hand and today they are an integral part of conservation biology, used for e.g. reintroduction programs or the design of nature reserves.

The European cultural landscape

Most of Central Europe belongs to the cool temperate moist forest zone and a closed forest cover is often considered to be the potential natural vegetation (Ellenberg 1996). In contrast to this theoretical climax vegetation that would be present without human influence, Central Europe is a cultural landscape created by historic land use patterns in combination with the prevailing climatic and geomorphological conditions (Sirami et al. 2010). The result is a landscape that is characterized by a complex mosaic of different habitat types like forests, wetlands, grasslands, agricultural and urban areas (Jongman 2002). The analysis of e.g. historic pollen and invertebrate data indicates that already in pre-agricultural times wild megaherbivores have continuously created open habitats by browsing and grazing (Pärtel et al. 2005; Sutherland 2002; Svenning 2002), and during the last millennia this effect has been replaced by grazing of domestic livestock and human activities such as mowing or tillage (Pärtel et al. 2005). Due to the existence of open habitats over millennia, many species have become adapted to these conditions and now depend on the persistence of such habitats (Sletvold et al. 2010). However, as soon as extensive land use is abandoned, succession starts and the associated vegetation will be replaced by scrubland and forests (Behre 1988; Franzén & Nilsson 2008). Hence, open habitats can only be maintained by an extensive and continuous land use by humans, for which reason they are often considered as anthropogenic or semi-natural habitats (Pärtel et al. 2005).

Today, the nature of human alteration has changed drastically and the global influence of humans has transformed about 40-50% of Earth's ice-free land surface into urban and agricultural systems (Chapin et al. 2000). This raises the question of what has to be considered as "natural" and whether this can mean without any human influence. Ecosystems could be imagined along a continuum from one pole where ecosystems are totally devoid of human influence to the opposite pole of urban or agricultural systems created by humans (Hunter 1996). In a cultural landscape where virtually all habitats are anthropogenically influenced to some extent, extensively managed habitats like grasslands represent the most natural status, in contrast to e.g. excavation sites, where mining activities have destroyed the soil structure and original vegetation (e.g. quarries, post-mining areas).

The excavation of mineral resources represents one of the most severe anthropogenic land transformations as the affected areas are totally devoid of diaspores and a re-colonization has to take place from the regional species pool. As this theoretically starts with a few individuals,

founder effects may reduce the genetic diversity and fitness of the respective populations, threatening their long-term persistence. On the other hand, the special physico-chemical properties and low nutrient levels of many mining sites provide habitat for specialized and therefore endangered species (Brändle et al. 2003). In the post-mining area in East Germany, for example, 16 different orchid species are found (Baasch & Seppelt 2004), some of them having no other occurrences outside this area. However, in order to contribute to the conservation of endangered species, populations on anthropogenically disturbed sites should maintain similar levels of genetic diversity than those from (semi-)natural habitats.

The Orchidaceae

The Orchidaceae is the largest family of flowering plants, and with approximately 25.000 species (Dressler 1987) they are a prime example for diversification (Gill 1989). Orchids are famous for their extraordinary floral diversity and the elaborate systems they developed to attract specific pollinators, ranging from vertebrates to invertebrates. Many of the orchid-pollinator relationships are species specific, with 67% out of 456 species with known pollinators being pollinated by a single species (Tremblay 1992). Hence, pollinator specificity is thought to act as the main mechanism of pre-mating reproductive isolation for orchids (Cozzolino et al. 2006).

Another fascinating aspect of orchid biology is their dependence on mycorrhizal fungi for germination (Rasmussen 1995). Orchids are optimally adopted to wind dispersal as they produce a high number of minute seeds (Arditti & Ghani 2000). However, these so-called “dust seeds” do not provide any nutrient reserves and orchid seeds rely on the colonisation by a compatible fungus providing carbohydrates for germination in the wild. The developing seedling remains dependent on fungal sugars for several years, a strategy called mycoheterotrophy (Leake 1994; Rasmussen 1995). For most orchid species it is only during further development that the achlorophyllous protocorm becomes autotrophic, although some species remain mycoheterotrophic throughout the adult stage (Abadie et al. 2006; Julou et al. 2005). Molecular studies revealed a considerable specificity between some orchids and their mycorrhizal fungi (McCormick et al. 2004; Shefferson et al. 2007) and for species that require specific fungi their presence might determine habitat suitability and influence orchid distribution (McCormick et al. 2004; Swarts et al. 2010).

This specialization for pollination and germination is often considered to be the major driver for orchid diversification, but it also contributes to the high number of endangered species in this family (Swarts & Dixon 2009b). The high ecological interdependency is probably one important reason for the often unique habitat requirements of many orchid species, which makes them vulnerable to environmental changes. The major threats for populations in the wild are habitat

destruction and degradation, breakdown of ecological connections (mycorrhiza, pollinators) and changed abiotic conditions (e.g. soil and hydrology) (Swarts & Dixon 2009a), often induced by human activities such as land clearing for agriculture, mining and urban development (Swarts & Dixon 2009b). Provisions that can enhance the survival of orchid diversity are e.g. habitat protection and management, reintroduction of plants into the wild and ex situ seed banking (Cribb et al. 2003; Swarts & Dixon 2009a). However, an effective application of these methods needs a thorough understanding of the factors that determine orchid distribution and how they might influence population dynamics and genetics (Light et al. 2003). Hence, orchids are model systems to study fundamental questions of biology and apply them for the conservation of biodiversity.

The study system: Gymnadenia conopsea s.l.

Gymnadenia R. BROWN is an Eurasian genus with approximately 30 species, covering most of Europe and parts of Asia (Tutin et al. 1980). *Gymnadenia conopsea* (L.) R. BR. *s.l.* is a terrestrial orchid that occurs from Western Europe to China. It prefers calcareous soils, but also occurs on neutral or low acidic underground (Delforge 2006). In Europe it is still relatively common with a wide ecological amplitude, including habitats like wet to dry grasslands and open woodlands (Gustafsson 2000); and it is typical for anthropogenically disturbed habitats like quarries or post-mining areas (Heyde & Krug 2000).

The fragrant orchid has a slender appearance with a cylindrical inflorescence. The flowers are mostly lilac and rarely pale pink, with oval, obtuse sepals that are 4-7 mm long with rolled up margins. The petals are shorter, asymmetric and forming a hood with the dorsal sepal and the lip is deeply 3-lobed, appearing broader than long (Delforge 2006; Schmeil 1996). *G. conopsea s.l.* is rewarding and pollinated by a variety of Lepidoptera (Schmeil 1996). Although self-compatible, it depends on the pollination by insects for fruit set and spontaneous autogamy or apomixis is absent (Gustafsson 2000).

Gymnadenia conopsea (L.) R.BR. *s.l.* is a controversial taxon. A high morphological variability gave rise to various taxonomic treatments, ranging from 1 species with 3 varieties (Delforge 2006) to 5 species plus 2 subspecies (Dworschak 2002). The two most commonly distinguished taxa are *G. conopsea* (L.) R.BR. ssp. *conopsea* and *G. conopsea* ssp. *densiflora* (WAHLENB.) K. RICHT., but also a species status has been proposed for *G. conopsea* (L.) R.BR. *s.str.* and *G. densiflora* (WAHLENB.) DIETRICH (Bateman et al. 1997; Campbell et al. 2007; Marhold et al. 2005). They are described to differ in morphology, flowering phenology, scent emission and habitat preferences e.g. (Gustafsson & Lönn 2003; Jersáková et al. 2010; Marhold et al. 2005; Scacchi & de Angelis 1989) (Fig. 1, Tab. 1). Furthermore, reports on the ploidy status are controversial, with some authors stating *G. c*. ssp. *conopsea* as polyploid and *G. c*. ssp. *densiflora* as diploid (e.g. Marhold et al.

2005) or vice versa (e.g. Hagerup in Bisse 1963; Mrkvicka 1993; Vöth & Sontag 2006); genetic differentiation has been found between e.g. ecotypes (Scacchi & de Angelis 1989) and flowering-time variants (Gustafsson & Lönn 2003; Soliva & Widmer 1999).

Figure 1 The two most commonly distinguished taxa of *Gymnadenia conopsea s.l.*, for which also species status has been suggested: *G. conopsea* ssp. *conopsea* (left) and *G. conopsea* ssp. *densiflora* (right) (Fotos: Karl Heyde, Christiane Stark).

Table 1 Parameters described to differ between *G. c.* ssp. *conopsea* and *G. c.* ssp. *densiflora* (Heyde & Krug 2000).

	Gymnadenia conopsea ssp. *conopsea*	*Gymnadenia conopsea* ssp. *densiflora*
Height	23- 57 cm	28- 64 cm
Inflorescence length	13-26 cm	7-19.5 cm
Flower number	17- 64	31- 77
Flower color	pale pink	strong lilac
Scent	weak	strong
Phenology	May/ June	June/ July
Habitat	dry habitats	wet habitats

Aim and structure of this thesis

The recruitment of new individuals into a population is of crucial importance for the persistence of populations in the wild and is one of the major factors that determines population dynamics and population genetics (Lowe et al. 2004). We still lack an understanding of why so few if any of the thousands to millions seeds that are produced by an individual orchid during its life time develop into vigorous reproductive plants (Light et al. 2003) and which consequences this has for

population dynamics and genetics. However, for an effective conservation of orchid populations in the wild, a thorough understanding of the factors that determine orchid distribution is required. This is of particular importance in a cultural landscape, where natural habitats are virtually absent and anthropogenic influence is continuously growing. Using *Gymnadenia conopsea s.l.* as study system, a common orchid that occurs over a wide ecological amplitude, this thesis addresses aspects of orchid biology that might be critical for orchid recruitment and the long-term persistence of populations in the wild, namely the specificity of the mycorrhizal symbiosis, population genetic diversity and genetic differentiation (Tab. 2).

One of the most critical stages of an orchid's life cycle is germination and initial growth. Due to the obligate nature of the mycorrhizal relationship, the availability of suitable fungal partners is a precondition for germination in the wild. For orchids that require specific fungi their presence determines the suitability of a given habitat and thus constitutes an environmental factor critical for orchid recruitment (McCormick et al. 2004). A first study (chapter 1) focuses on the question whether *Gymnadenia conopsea* as a common orchid species that is found across a wide range of different habitats has the ability to associate with multiple fungi. A low fungal specificity towards certain fungal taxa is likely to increase the potential habitat availability as well as its tolerance towards disturbances.

While the availability of suitable fungal partners might be a limiting factor for seedling recruitment for species with specific requirements, this symbiotic relationship also enables orchids to thrive on the limits of plant growth. Many orchids are well known as primary colonisers and are often found in anthropogenic habitats (Adamowski 1998; Cribb et al. 2003). In order to assess whether founder effects during colonization threaten the long-term survival of the respective populations, a second study (chapter 3) compared the genetic diversity and fitness of populations from anthropogenically disturbed and surrounding natural habitats in two German regions (East Germany: lignite post-mining area; North Germany: quarries, Fig. 2 & 3).

In the study region in East Germany both taxa *G. conopsea* (L.) R.BR. ssp. *conopsea* and *G. conopsea* ssp. *densiflora* (WAHLENB.) K. RICHT. occurred, for which also a species status has been proposed previously. Due to a high morphological overlap between the taxa, identification in the field is difficult. Hence, for taxon assignment all populations were genetically and morphologically analyzed. In order to investigate the relationships on a larger geographic scale, additional samples from other European regions were included. In particular it was tested whether the differentiation between the taxa is consistent across regions and parameters and whether they share a common ancestor (chapter 2).

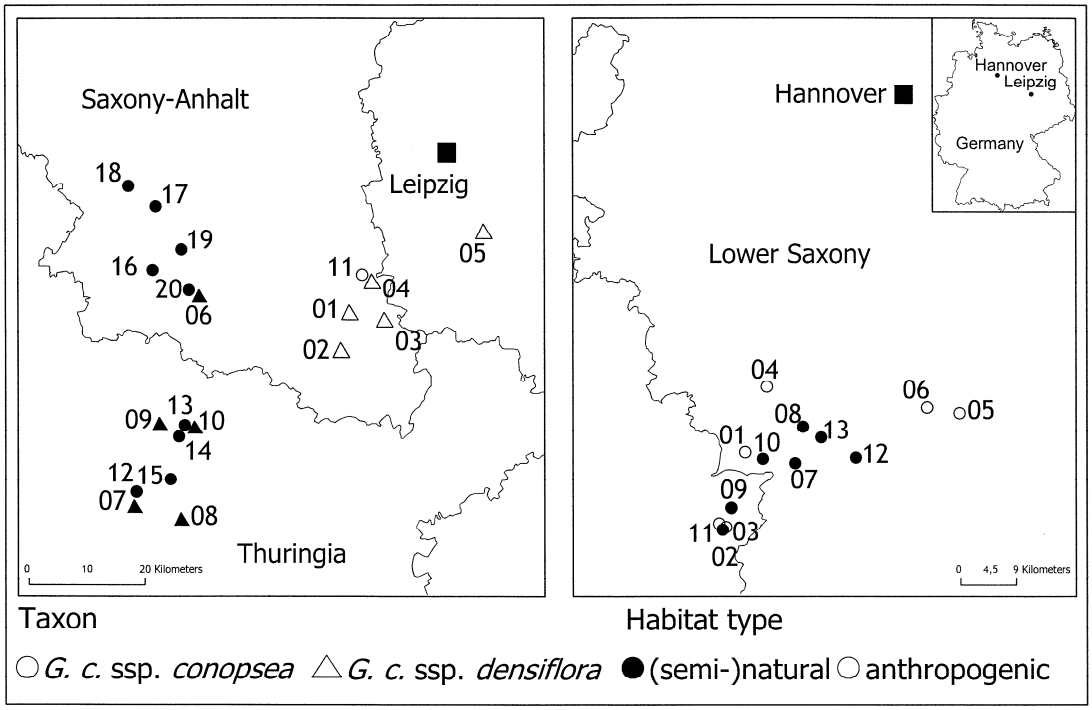

Figure 2 Locations of the sample sites in the two main study regions in East Germany (EG, left) and North Germany (NG, right). In East Germany the analyzed anthropogenic populations were located in a lignite post-mining area, in North Germany in a system of quarries, and were compared with surrounding natural sites respectively. In East Germany both taxa *G. c.* ssp. *conopsea* and *G. c.* ssp. *densiflora* were present, whereas in North Germany only *G. c.* ssp. *conopsea* was found.

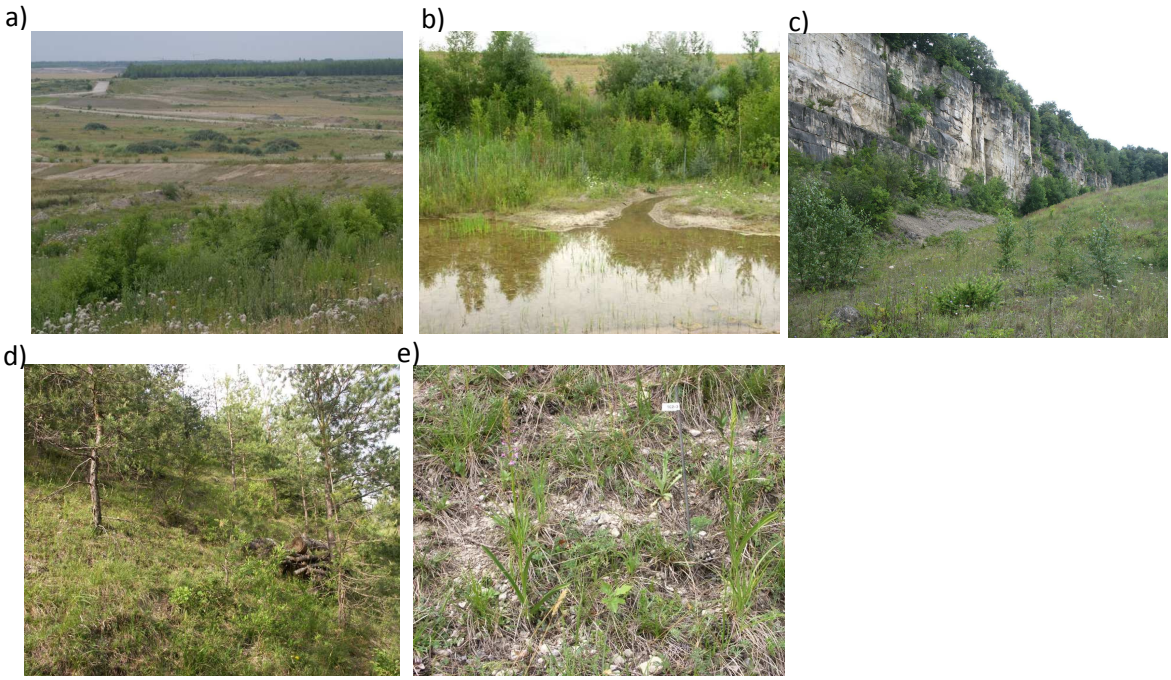

Figure 3 Habitat types of *G. c.* ssp. *conopsea* and *G. c.* ssp. *densiflora*: Anthropogenic habitat typical for the post-mining area in East Germany (a+b) and the quarries in North Germany (c); and the typical natural habitat of semi-dry grasslands (d+e) (Fotos: Karl Heyde, Christiane Stark).

Table 2 Overview of the sample sites including location, Gauss-Krüger coordinates, assigned taxon, habitat type (A = anthropogenic, SN= semi-natural), population size and the analysis conducted: characterization of the fungal community, sequencing of the ITS region (ITS), microsatellite analyses (Msats), determination of chromosome numbers (ploidy), morphometric analyses (morph), plant performance (perform) and reproductive success (fruit set).

Sample sites		Coordinates (G-K)	Taxon	Habitat Type	Population Size	Chapter 1: Fungal community	Chapter 2: Subspecies differentiation				Chapter 3: Genetic diversity		
Location	Code						ITS	Msats	Ploidy	Morph.	Msats	Perform	Fruit Set
Jaucha (Sd9)	EG01	4507726/5666773	ssp. *densiflora*	A	1000		x	x	x	x	x	x	x/14
Theißen (Sd7)	EG02	4505321/5660546	ssp. *densiflora*	A	44		x	x	x	x	x	x	x/16
Predel (Sd8)	EG03	4513552/5665317	ssp. *densiflora*	A	40		x	x	x	x	x	x	x/10
Domsen (Sd10) sterile	EG04	4511764/5671855	ssp. *densiflora*	A	16		x	x		x	x	x	
Domsen (Sc10)	EG11	4509939/5672536	ssp. *conopsea*	A	29		x	x		x	x	x	x/14
Espenhain (Sd11)	EG05	4531091/5679827	ssp. *densiflora*	A	8			x		x	x	x	
Tote Täler (Sd4)	EG06	4481617/5672809	ssp. *densiflora*	SN	1200		x	x	x	x	x	x	x/15
Tote Täler (Sc4)	EG20	4481348/5672301	ssp. *conopsea*	SN	1200	x	x	x		x	x	x	x/10
Rothenstein (Td8)	EG07	4470169/5635782	ssp. *densiflora*	SN	167			x		x	x	x	x/15
Rothenstein (Tc1)	EG12	4470581/5635734	ssp. *conopsea*	SN	136			x		x	x	x	x/15
Würze (Td6)	EG08	4478046/5633475	ssp. *densiflora*	SN	140		x	x	x	x	x	x	x/15
Klingelsteine (Td5)	EG09	4475020/5649490	ssp. *densiflora*	SN	295			x		x	x	x	x/15
Jägertalwiese (Td7)	EG10	4480907/5648635	ssp. *densiflora*	SN	182			x	x	x	x	x	x/17
Zietschkuppe (Tc4)	EG13	4479124/5648588	ssp. *conopsea*	SN	1000			x		x	x	x	x/15
Alter Gleisberg (Tc3)	EG14	4479291/5646649	ssp. *conopsea*	SN	330			x	x	x	x	x	x/15
Rabis (Tc2)	EG15	4476453/5639637	ssp. *conopsea*	SN	120		x	x	x	x	x	x	x/15
Krawinkel (Sc5)	EG16	4474926/5674814	ssp. *conopsea*	SN	490	x		x	x	x	x	x	x/15
Steigra (Sc2)	EG17	4475904/5685383	ssp. *conopsea*	SN	370	x		x	x	x	x	x	x/16
Grockstädt (Sc1)	EG18	4471519/5688938	ssp. *conopsea*	SN	320		x	x		x	x	x	x/17
Langer Berg (Sc3)	EG19	4479836/5677986	ssp. *conopsea*	SN	900		x	x		x	x	x	
Im Schießstand (Ho5)	NG02	3524581/5738674	ssp. *conopsea*	A	77			x		x	x	x	x/21
Alter Steinbruch (Ho4)	NG03	3524860/5738320	ssp. *conopsea*	A	146			x		x	x	x	x/20
Polle (Ho3)	NG01	3527498/5750383	ssp. *conopsea*	A	500	x	x	x		x	x	x	x/22
Hehlen (Ho1)	NG04	3531000/5760840	ssp. *conopsea*	A	40		x	x		x	x	x	x/18
Stb. Bärenbrink (Ho2	NG05	3560840/576600	ssp. *conopsea*	A	14		x	x		x	x	x	x/20
Stb. Delligsen (Ho17)	NG06	3555887/5757580	ssp. *conopsea*	A	300			x		x	x	x	x/20
Rauschenberg (Ho7)	NG09	3525427/5741660	ssp. *conopsea*	SN	43			x		x	x	x	
Rühle (Ho10)	NG08	3536628/5754569	ssp. *conopsea*	SN	300	x	x	x		x	x	x	x/20
Burgberg (Ho13)	NG07	3535405/5748803	ssp. *conopsea*	SN	300	x		x		x	x	x	x/20
Poppenburg (Ho8)	NG10	3530299/5749340	ssp. *conopsea*	SN	30		x	x		x	x	x	x/08
Bielenberg (Ho6)	NG11	3524167/5738332	ssp. *conopsea*	SN	750		x	x		x	x	x	x/21
Bocksberg (Ho15)	NG12	3544789/5749642	ssp. *conopsea*	SN	55			x		x	x	x	x/20
Holberg (ho14)	NG13	3539443/5752813	ssp. *conopsea*	SN	40			x		x	x	x	x/20

Chapter 1

Fungi from the roots of the common terrestrial orchid *Gymnadenia conopsea*

with Wiesław Babik and Walter Durka

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Abstract

The fungal community associated with the terrestrial photosynthetic orchid *Gymnadenia conopsea* was characterized through PCR-amplification directly from root extracted DNA and cloning of the PCR products. Six populations in two geographically distinct regions in Germany were investigated. New ITS-primers amplifying a wide taxonomic range including Basidiomycetes and Ascomycetes revealed a high taxonomic and ecological diversity of fungal associates, including typical orchid mycorrhizas of the Tulasnellaceae and Ceratobasidiaceae as well as several ectomycorrhizal taxa of the Pezizales. The wide spectrum of potential mycorrhizal partners may contribute to this orchid's ability to colonize different habitat types with their characteristic microbial communities. The fungal community of *Gymnadenia conopsea* showed a clear spatial structure. With 43% shared taxa the species composition of the two regions showed only little overlap. Regardless of regions, populations were highly variable concerning taxon richness, varying between 5 and 14 taxa per population. The spatial structure and the continuous presence of mycorrhizal taxa on the one hand and the low specificity towards certain fungal taxa on the other hand suggest that the fungal community associated with *Gymnadenia conopsea* is determined by multiple factors. In this context, germination as well as pronounced morphological and genetic differentiation within *Gymnadenia conopsea* deserve attention as potential factors affecting the composition of the fungal community.

Introduction

Eucaryotic micro-organisms such as fungi are often regarded as ubiquitously distributed due to their small size and great abundance (Finlay 2002). On the other hand clear distributional patterns have been detected, and are thought to be determined by e.g. large scale soil carbon gradients, land use or small-scale soil textures produced by plant growth (Ettema & Wardle 2002; Kasel et al. 2008). The development of plants of the Orchidaceae directly depends on the presence of fungal partners, because orchid seeds lack any nutrient reserves and germination in the wild is only possible upon colonisation by a compatible fungus providing carbohydrates. The developing seedling remains dependent on fungal sugars for several years, a strategy called mycoheterotrophy (Leake 1994; Rasmussen 1995). For most orchid species it is only during further development that the achlorophyllous protocorm becomes autotrophic, although some species remain mycoheterotrophic throughout the adult stage (Abadie et al. 2006; Julou et al. 2005). As a consequence of this symbiosis the degree of specificity between fungus and orchid is an important factor determining chances of successful seedling establishment (Bidartondo & Read 2008). For orchids that require specific fungi, their availability determines the suitability of a given habitat and thus constitutes an environmental factor critical for orchid recruitment. On the contrary for species exhibiting diverse associations this factor may not be limiting (McCormick et al. 2004).

Early studies on the specificity between orchids and their mycorrhizal fungi were mainly based on cultivation methods or germination tests under laboratory conditions and found a considerable phylogenetic breadth of associated fungi (Curtis 1939; Knudson 1922). However, physiological compatibility under laboratory conditions may be broader as it does not reflect the complexity of interactions under natural conditions (Masuhara & Katsuya 1994; Perkins et al. 1995). Furthermore the general problems of the unculturability of many mycorrhizal fungi or outgrowing contaminants could have additionally biased these early results. Modern PCR-based approaches largely eliminate these biases and allow the direct assessment of the fungal diversity present within an orchid root (Kristiansen et al. 2001; Taylor & McCormick 2008).

Indeed, more recent investigations applying molecular methods have shown a more complex picture, pointing to a considerable specificity between some orchid species and their mycorrhizal fungi. Most recorded fungi associated with photosynthetic orchids are *Rhizoctonia*-forming fungi (Roberts 1999) belonging to the Ceratobasidiaceae and Tulasnellaceae (McCormick et al. 2004; Otero et al. 2002; Rasmussen 2002), whereas mycoheterotrophic and mixotrophic orchids are rather associated with ectomycorrhizal Basidiomycetes like the Thelephoraceae and Russulaceae (Abadie et al. 2006; Girlanda et al. 2006; Julou et al. 2005). However, even some ascomycetous genera have been shown to form true orchid mycorrhizas (Currah et al. 1988; Selosse et al. 2004).

In the present study we performed a screen for fungal associates of the photosynthetic orchid species *Gymnadenia conopsea*, a still common orchid found in a wide range of different habitat types. We were interested whether a widely distributed species has the ability to associate with multiple fungi, which would likely increase its habitat availability as well as its tolerance to disturbances (McCormick et al. 2004). We set out for a comprehensive description of the fungal community of *Gymnadenia conopsea* by using new ITS-primers that amplify a broad taxonomic spectrum of Basidio- and Ascomycetes. Here we report on the fungal diversity found in the roots of *Gymnadenia conopsea* and discuss overall geographical differentiation between two study regions in Germany.

Materials and methods

Plant and fungal material

Diversity of fungal root associates was investigated of *Gymnadenia conopsea*, a terrestrial photosynthetic orchid species geographically widely distributed in Eurasia (Tutin et al. 1980).Like most other orchids, this species is declining, but is still relatively common in Central Europe and found in various habitat types, ranging from wet to dry grasslands and open woodlands (Gustafsson 2000). We analysed samples from six dry grassland sites located in two geographically distinct regions in Eastern Germany (area of Leipzig; coordinates E1: 11°64´E/ 51°21´N, E2: 11°65´E/ 51°30´N, E3: 11°73´E/ 51°19´N) and Northern Germany (area of Hannover; coordinates N1: 9°51´E/ 51°87´N, N2: 9°40´E/ 51°89´N, N3: 9°53´E/ 51°92´N), approximately 300 km apart. In spring 2006 before flowering, root material of three randomly chosen individuals per site was collected and cleaned several times with sterile water to minimize the detection of soil fungi. Samples were either processed immediately for fungal isolation or lyophilised for molecular analyses.

Fungal isolation and primer design

On the basis of ITS sequences obtained from fungal taxa isolated from the roots of *G. conopsea* new ITS -primers were designed. Fungal isolation was performed from three root pieces per individual. 1-2 cm root segments were surface sterilized with 1% hypochlorite for 2 min and then rinsed three times for 10 minutes in sterile water before placing onto nutrient agar (Laiho 1970). The plates were kept in the dark at room temperature and growing colonies were separated onto fresh media. DNA was extracted with the DNeasy 96 Plant Kit (Qiagen, Hilden, Germany). For fungal identification the ITS region of nuclear ribosomal DNA was amplified by polymerase chain

reaction (PCR) using the primers ITS1 and ITS4 (White et al. 1990). PCR products were purified with MinElute (Qiagen) and sequenced using the BigDye cycle sequencing v.3.1 kit (Applied Biosystems, Darmstadt, Germany), and run on an ABI 3100 genetic analyzer (Applied Biosystems). For taxonomic identification the sequences were compared with known sequences from GenBank using a BLASTN search (Altschul et al. 1997). The taxonomic spectrum of identified species was used for the development of new primers. These PCR primers ITS_ufz01: 5'-TGAACCTGCGGARGGATCATTA-3' and ITS_ufz02: 5'-CCGCTTATTGATATGCTTAAGT-3' amplify fungal ITS covering a broad taxonomic spectrum of Basidio- and Ascomycetes, but they do not amplify *G. conopsea* ITS. BLAST searches of the primer sequences against orchid sequences available in GenBank showed that only one orchid species perfectly matches the 3' end of both primers. All other orchid sequences showed mismatch of at least one 3' terminal nucleotide of each primer.

DNA extraction and PCR amplification

Fungal diversity was assessed directly through PCR-amplification from root extracted DNA of 18 individuals from six sites in total. For each individual DNA was extracted from 6 root pieces separately (equivalent of approximately 6 cm of the root system), using the DNeasy Plant 96 Kit (Qiagen). A separate PCR amplification was conducted for each piece. Fifty µl PCR reactions contained 5µl of 10 x HotStart Buffer (Fermentas, St. Leon-Rot, Germany), 2 mM $MgCl_2$, 0.16 mM of each dNTP (Fermentas), 1 µM of each primer (ITS_ufz01 and ITS_ufz02) and 1U of HotStart *Taq* (Fermentas). The cycling scheme was 95°C for 5 min, followed by 40 cycles at 95°C for 40 s, 57°C for 30 s, 72°C for 40 s and the final extension step at 72°C for 10 min.
In order to reduce the number of necessary cloning experiments to two per individual, two pooled reactions, each consisting of products of three individual PCRs were prepared (Renker et al. 2003). We purified 30 µl of this PCR pool with the MinElute Purification Kit (Qiagen) and eluted them with 10µl EB buffer (Qiagen). Each purified PCR pool was checked on an agarose gel.

Cloning and sequencing

PCR pools were cloned using the pGEM T-Easy vector system (Promega, Mannheim, Germany). Recombinant clones were detected by blue/white screening, colonies picked from plates were used directly as a template in PCR with the standard sequencing primers M13F (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and M13R (5'-TCACACAGGAAACAGCTATGAC-3'), 20µl PCR reactions contained 2µl 10x PCR buffer (Fermentas), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1µM of each M13 F-and R-primers and 0.5U of Taq (Fermentas). The cycling scheme

was 94°C for 3 min, followed by 30 cycles with 94°C for 30 s, 58°C for 30 s, 72°C for 1 min and the final extension step with 72°C for 3 min. PCR products of positive clones were purified with ExoSap-IT (USB, Staufen, Germany) and sequenced as described above.

As several different template sequences were present in PCR pools, formation of amplification chimaeras was possible (Judo et al. 1998; Zylstra et al. 1998). In order to detect and remove chimaeras we compared our sequences to GenBank sequences using BLAST. Those sequences with low bit scores and high E-values or with parts obviously corresponding to different species were removed from the dataset as suspected chimaeras.

Data analysis

Sequences were manually trimmed and grouped according to their similarity with Sequencher v.3.1. (Gene Codes, Ann Arbor, MI, USA). To determine taxonomic affiliation sequences were compared with known sequences in the GenBank using BLASTN. Taxonomic affiliation as well as putative ecology was inferred from the closest hits. One representative of each taxon was deposited in GenBank under the accession numbers listed (Accession No. GQ223448-GQ223476).

The fungal taxon composition between the two geographic regions was compared with the Jaccard index based on shared fungal taxa. Diversity of the fungal community was assessed and compared on the spatial levels of regions and populations. Diversity is reported as the fungal taxon richness (*R*, number of fungal taxa in the region/ population) and taking abundance of the taxa into account as Shannon diversity (*H*'). The values of the Shannon diversity have to be considered with caution, as abundance after cloning may not exactly reflect abundance in the roots.

A rarefaction analysis (Simberloff 1978) was used to determine whether clone sampling effort saturated the number of taxa, using the analytical approximation algorithm (Hurlbert 1971) embedded in the Analytic Rarefaction freeware program from Steven M. Holland http://www.uga.edu/strata/software/Software.html.

Results and discussion

Diversity of fungi associated with Gymnadenia conopsea

On the basis of BLASTN searches we assigned 330 obtained sequences to 28 different taxa belonging to the Basidiomycetes and Ascomycetes (Tab. 3). The closest BLAST hits enabled classification to different taxonomic levels, depending on the fungal group. The majority of identified taxa (57%) has previously been shown to be mycorrhizal, either orchid-mycorrhizas (OM

7%) or ectomycorrhizas (ECM 50%). However, a substantial part of taxa (43%) were unspecific plant endophytes (PE), plant pathogens (PP) or uncultured taxa (UC).

Basidiomycetous mycorrhizas. Most so far identified orchid mycorrhizas are Basidiomycetes of the *Rhizoctonia* group (Rasmussen 1995; Warcup & Talbot 1967; Warcup & Talbot 1971) a polyphyletic assemblage including teleomorphs of the genera *Tulasnella*, *Ceratobasidium*, *Thanatephorus* and *Sebacina* (Moore 1987; Warcup 1981; Warcup & Talbot 1967; Warcup & Talbot 1971). Sequences of all these families were amplified from the roots of *G. conopsea*. The Sebacinaceae have also been shown to form ectomycorrhizas on trees (Selosse et al. 2002), like the typical ectomycorrhizal families Russulaceae (Dearnaley 2007) and Thelephoraceae (Abadie et al. 2006), which were also found in the roots of *G. conopsea*.

Ascomycetous mycorrhizas. Ascomycete ectomycorrhizas differ morphologically from their more robust and well established basidiomycetous counterparts. Typically they produce only thin mantles with sparsely growing hyphae. So far they have been less studied and comparatively little is known about the taxonomy and ecology of these fungi. Hence, their importance as mycorrhizas is probably seriously underestimated, a view supported by the fact that Tedersoo et al. (2006) identified several new mycorrhizal taxa within Pezizales. The Pezizales and Helotiales are two ascomycetous orders which have been shown to include taxa interacting as mycorrhizas (Julou et al. 2005). In our study we identified five pezizalean genera *Peziza*, *Terfezia*, *Morchella*, *Geopyxis* and *Wilcoxina*, all previously shown to interact as ectomycorrhizas (Abadie et al. 2006; Buscot 1994; Dahlstrom et al. 2000; Tedersoo et al. 2006). We were not able to characterize sequences assigned as Helotiales in more detail, because taxonomic assignment of Helotiales sequences in the GenBank is poor reflecting difficulties with taxonomy and limited knowledge of this group (Wang et al. 2006b; Wang et al. 2006a). As the Helotiales are an ecologically diverse order including plant pathogens, different types of saprobes, plant endophytes and both ericoid and ectomycorrhizal fungi (Vrålstad et al. 2002; Wang et al. 2006b), an ecological function was difficult to assess. Nevertheless, because Helotiales also include ectomycorrhizal species and the only genus identified within the Helotiales was the ectomycorrhizal *Cadophora* (Vrålstad et al. 2002), we classified the Helotiales sequences as potentially ectomycorrhizal. Similar problems exist with sequences classified as 'uncultured' taxa, because due to the lack of information no ecological characterization is possible, but a potential role for plant performance cannot be ruled out.

Endophytes. 'Endophyte' is a general term referring to organisms that grow inside plant tissues without causing disease symptoms (Carroll 1988; Chanway 1996). Little is known about the role of endophytes for orchid performance, although some endophytes have been shown to confer fitness benefits to host plants, including tolerance to heat, disease and drought (Rodriguez & Redman 2008). Most of the identified endophytes of *G. conopsea* are Ascomycetes such as *Exophiala*,

Fusarium, *Leptodontidum* or *Tetracladium*, some of them possibly also representing surface contaminants. Interestingly, we detected *Tetracladium* in five out of six populations of *G. conopsea* in Germany. Only recently Selosse et al. (2008) drew attention to the presence of these aquatic asexual fungi in terrestrial ecosystems. Although they are commonly occurring in running fresh water they were reported as endophytes from healthy looking plant tissue of several species (Abadie et al. 2006; Murat et al. 2005; Russell & Bulman 2005; Tedersoo et al. 2007). Our findings support the hypothesis that some aquatic fungi spend a part of their life in plants and have a planktonic, aquatic and aerial dispersal (Selosse et al. 2008).

We found a surprisingly high diversity of fungi associated with *G. conopsea*, indicating that this orchid shows only little specificity to certain fungal clades. The basidiomycetous mycorrhizas are mostly of confirmed mycorrhizal status for orchids (seven of the eight Basidiomycetes), whereas we also identified a variety of ascomycetous taxa which are known to form ectomycorrhizas on other plants. Their detection suggests a potential role as mycorrhizas for *G. conopsea* and emphasizes the need for further investigations of the role of ascomycetous taxa as mycorrhizas in orchids. The wide taxonomic range of mycorrhizal associates found in the roots of *G. conopsea* might contribute to its ability to grow in very different habitat types with their respective fungal communities, including rather disturbed habitats in quarries and mines.

Table 3 Taxa found within the roots of *Gymnadenia conopsea* and putative ecological roles as inferred from the closest relatives (OM = orchid mycorrhiza; ECM = ectomycorrhiza; PE = plant endophyte; PP = plant pathogen or saprobes) and the number of clones of the respective fungal taxa amplified from the roots of three individuals per site of *Gymnadenia conopsea* for Eastern German (E) and Northern German (N) region (individual sites).

	Tentative identification[1] and putative ecology		Closest NCBI-Hit (Accession No.)/ taxonomic affiliation	ID (%)	Number of Clones	
					E	N
1	Tulasnellaceae (B)	OM	Unc. Tulasnellaceae (DQ925600)/ Tulasnellaceae	99	17 (4/13/0)	27 (0/4/23)
2	*Sebacina* sp. (B)	ECM/OM?	*Sebacina incrustans* (EU668266)/ Tulasnellaceae	99	1 (1/0/0)	-
3	Ceratobasidiaceae -OM (B)	OM	*Ceratobasidium* sp. (EU668239)/ Ceratobasidiaceae	99	34 (0/0/34)	7 (1/6/0)
4	Ceratobasidiaceae –ECM (B)	ECM	Uncultured ectomycorrhiza Ceratobasidiaceae (AY634129)	96	1 (0/1/0)	-
5	*Lactarius* (B)	ECM	*Lactarius pubescens* (AY336958)/ Russulaceae	99	1 (1/0/0)	-
6	Russula (B)	ECM	*Russula exalbicans* (DQ974759)/	99	3 (0/3/0)	2 (0/2/0)
			Russula maculata (AY061688)/ Russulaceae	99		
7	Thelephoraceae (B)	ECM	Unc. *Tomentella* (EU668209) / Thelephoraceae	99	1 (0/1/0)	-
8	*Terfezia* (A)	ECM	*Terfezia* sp. (DQ061109)/ Pezizales	86	19 (0/0/19)	4 (0/0/4)
9	*Peziza* (A)	ECM	*Peziza proteana (DQ491497)/* Pezizales	85	-	11 (11/0/0)
10	*Morchella* (A)	ECM	*Morchella spongiola* (AJ539478)/ Pezizales	96	1 (0/1/0)	-
11	*Geopyxis* (A)	ECM	*Geopyxis rehmii* (Z96991) / Pezizales	91	1 (1/0/0)	-
12	*Wilcoxina* (A)	ECM	*Wilcoxina rehmii* (AF266708) / Pezizales	98	-	1 (0/1/0)
13	*Cadophora* (A)	ECM	*Cadophora* sp. (DQ317329)/ Helotiales	92	-	1 (0/1/0)
14	Helotiales (A)	ECM?	Unc. Helotiales (DQ182424)/Helotiales	100	9 (6/3/0)	17 (12/3/2)
15	*Cenococcum* (A)	ECM	*Cenococcum geophilum* (DQ474346)/ Dothideomycetes	99	2 (0/2/0)	1 (0/1/0)
16	*Phialophora* sp. (A)	ECM?	*Phialophora europaea* (EF540756)/ Sordariomycetes	91	1 (1/0/0)	-
17	*Tetracladium* (A)	PE	*Tetracladium maxilliforme* (DQ068996)	100	20 (13/7/0)	14 (3/9/2)
18	*Leptodontidum* (A)	PE	*Leptodontidium orchidicola* (AF486133)	98	14 (8/4/2)	40 (13/14/13)
19	*Cryptococcus* (B)	PE	*Cryptococcus carnescens* (AB105438)/ Tremellales	99	2 (0/2/0)	-
20	*Verpa* (A)	PE	*Verpa conica* (AJ544206) / Pezizales	97	5 (0/0/5)	1 (0/1/0)
21	*Lecanora* (A)	PE	*Lecanora reuteri* (AF070026)/ Lecanorales	95	1 (1/0/0)	-
22	*Exophiala* (A)	PP	*Exophiala salmonis*(AF050274)/ Herpotrichiellaceae	96	7 (5/2/0)	11 (6/3/2)
23	*Fusarium* (A)	PE/ PP?	*Fusarium oxysporum* (EU8622401)/ Hypocreales	100	-	2 (0/0/2)
24	*Neonectria* (A)	PE/ PP?	*Neonectria radicicola* (AJ875336)/ Hypocreales	100	5 (2/3/0)	12 (0/5/7)
25	Hypocreales (A)	PP	Unc.Hypocreales (EU035406)	99	-	3 (3/0/0)
26	Herpotrichaceae (A)	PP	Uncultured Herpotrichiellaceae (EF619700)	89	6 (0/6/0)	-
27	Pezizomycotina (A)	?	Uncultured Pezizomycotina (DQ182456)	99	2 (0/0/2)	-
28	Uncultured Taxa	?	taxonomy unknown		12 (7/5/0)	11 (2/6/3)

[1] A= Ascomycetes; B = Basidiomycetes

Geographic differentiation of fungal communities

From the total of 28 taxa, seven were widespread and occurred in at least four out of six sites, five taxa occurred at 2-3 sites. However, the majority of 16 taxa were found at only one site. The total number of fungal taxa differed between regions: 23 taxa (80%) were detected in the East and 17 (60%) in the North. Taking into consideration the abundance of the taxa, the Shannon diversity was slightly higher in the East (*H'*= 2,6) than in the North (*H'*=2,4). However, for both regions rarefaction analysis showed a clear levelling off after approximately 100 and 65 sequences respectively, with a gain of only three species following additional sampling in both regions (Fig. 4). This indicates that our sequence sampling effort, while by no means exhaustive, captured a substantial proportion of the diversity of fungal taxa associated with *G. conopsea*.

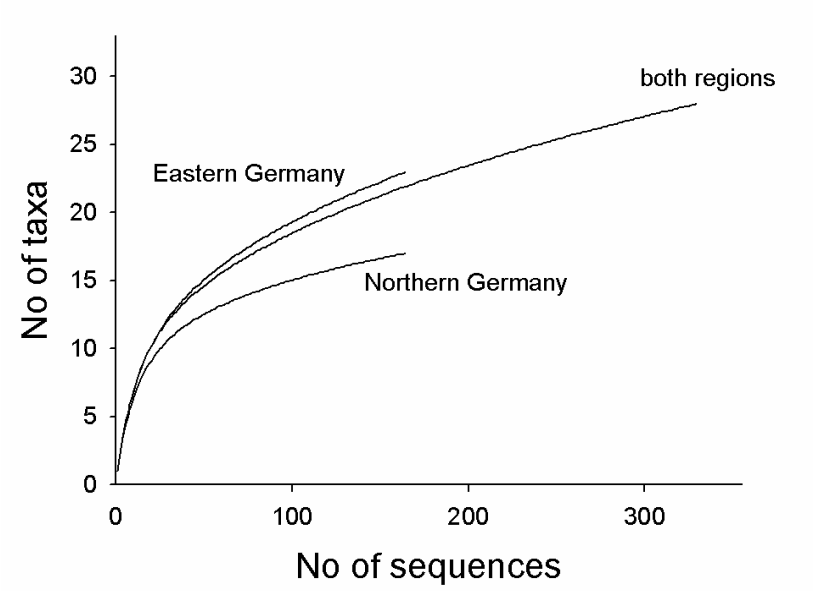

Figure 4 Rarefaction curve of the number of sequences sampled in Eastern Germany, Northern Germany and in both regions together.

Species composition of the two regions showed limited overlap, as only 43% of taxa were shared, including the most abundant ones Tulasnellaceae, Ceratobasidiaceae, *Leptodontidum* and *Tetracladium*. When only more common taxa were considered (present in at least three clones) similarity increased to 64%, indicating that inclusion of rare species may inflate the sampling error and thus underestimate similarity between regions. However, substantial differences in taxon richness were found among populations. Irrespective of region, the number of taxa per population varied between 5 (18%) and 14 (50%) (Fig.5), while the mean population taxon richness was similar in both regions (East: R = 10.3, SD = 4.7; North R = 10.0 SD = 2.6, p= 0.92 t-test).

Regardless of the observed differences in the community composition of fungal taxa common patterns can be readily recognized. Considering functional groups, in each population at least one basidiomycetous OM of the Tulasnellaceae and/or Ceratobasidiaceae was detected, suggesting that *G. conopsea* utilizes fungi from these known OM families, like most photosynthetic orchids. In addition, in all populations several ascomycetous ectomycorrhizal taxa of the Pezizales and/or Helotiales were also present. This pattern holds even when Helotiales are not considered as their ectomycorrhizal status is not confirmed. The presence of ectomycorrhizal taxa in all populations might indicate that *G. conopsea* has the ability to utilize ascomycetous ectomycorrhizal taxa as mycorrhizas.

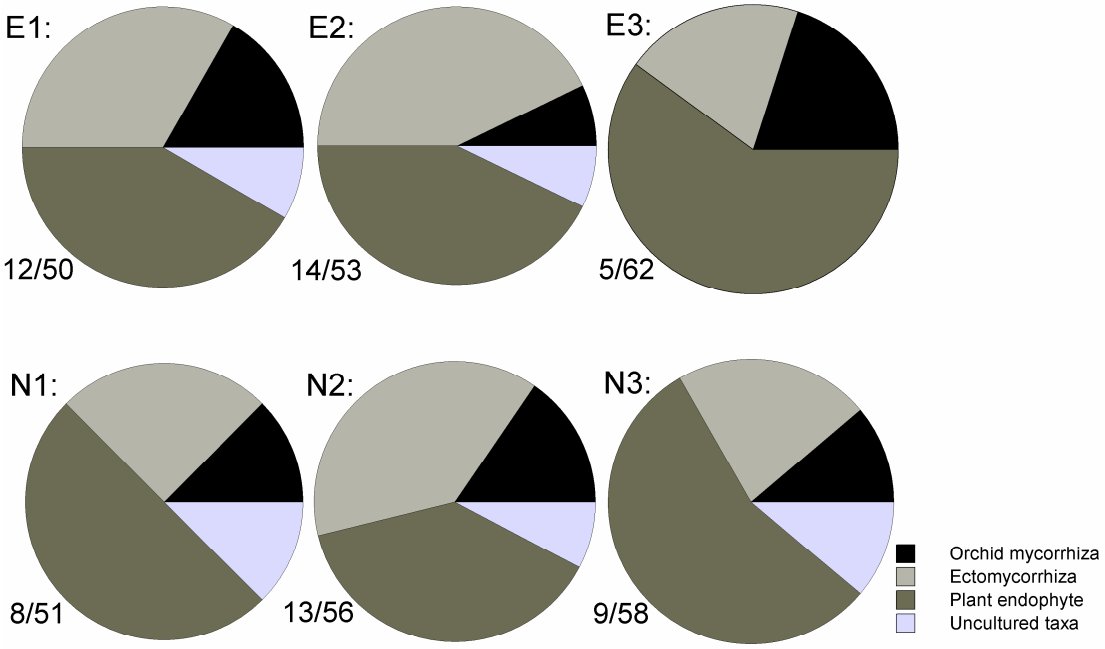

Figure 5 Distribution of putative ecological roles of the taxa found for each population in Eastern Germany (E1-E3) and Northern Germany (N1-N3). Digits present numbers of taxa found in the respective populations (left) and number of clones checked (right). Endophytes include plant endophytes, pathogens and saprobes.

Selosse et al. (2004) suggested that the replacement of the usual *Rhizoctonias* in Neottieae by ectomycorrhizas may be a strategy to secure access to fungal carbohydrates where *Rhizoctonias* are either not available or where photosynthesis rate is limited due insufficient light availability like in forest habitats. The adoption of ectomycorrhizal fungi as mycorrhizas would mean a more stable carbon resource and made light deficient habitats accessible. *G. conopsea* is generally known to

colonize a wide variety of different habitat types, typically occurring on open grassland sites, but also found in shaded forest habitats. Hence, the adoption of ectomycorrhizal taxa could have contributed to its ability to grow in such diverse habitats by expanding its potential habitat to shaded conditions. Nevertheless, at the current stage these ectomycorrhizal taxa found in the roots of *G. conopsea* have to be considered as 'potential partners', because the amplification of fungal taxa directly from root extracted DNA does not necessarily imply that these fungi interact as true mycorrhizas. The standard method to test whether a fungus is compatible with an orchid species are germination tests. Unfortunately, ectomycorrhizal taxa are known to be difficult to cultivate. However, the fact, that these fungi are obligatory symbiotic (Erland & Taylor 2002) makes us confident that they are not simply surface contaminants but that they indeed play a role for the performance of *G. conopsea*. Which role exactly, certainly needs further investigation.

In general, the diversity of compatible fungi (degree of specificity) is expected to influence the competition, survival and distribution of an orchid species. For orchids that require specific fungi, availability of appropriate symbionts may determine which habitats allow orchid growth and what environmental factors are critical for orchid recruitment, while diverse associations may be less limiting (McCormick et al. 2004). Furthermore, orchids with a broad taxonomic spectrum of potential fungal partners should be expected to be more easily distributed and colonize new habitats as the probability to find a compatible fungus after dispersal should be high. However, Irwin et al. (2007) investigated the fungal partners of the common terrestrial orchid *Pterostylis nutans* across its range in eastern Australia. He identified two fungi of the *Ceratobasidium* to be the main fungal partners and showed that specificity occurs in this species, despite its wide distribution. In contrast, Bonnardeaux et al. (2007) found that two weed-like orchid species and a widespread native, disturbance-intolerant species in Australia were associated with a diversity of fungal associates and had broad webs of mycorrhizal fungi. Most associated fungi belonged to the *Rhizoctonia* alliance with a worldwide distribution, whereas for *G. conopsea* we additionally identified several ectomycorrhizal taxa as potential fungal partners.

So far only little is known of the factors determining the diversity and composition of fungal communities associated with orchids. On the one hand micro-organisms are hypothesized to be omnipresent, at least in the form of diaspores, forming a basically common species pool. Consequently the same environmental conditions, both biotic and abiotic, should select the same microbial community in different locations (Taylor et al. 2006). On the other hand, however, there are also parameters known to influence the fungal community, e.g. extrinsic factors such as habitat type, geography or intrinsic factors like genetic differentiation (Schechter & Bruns 2008; Shefferson et al. 2008; Taylor et al. 2004; Taylor & Bruns 1999b). Such a complex interaction of

different factors was shown to influence the fungi associated with the fully mycoheterotrophic orchid *Corallorhiza maculata*. Taylor & Bruns (1999a) found that *C. maculata* associated with only one single, never fruiting *Russula* species, whereas there were also six other *Russula* taxa on the same plot. Furthermore, a strong correlation between specificity and plant community was detected as certain *Russula* species were the dominant symbionts of orchids growing in *Quercus* forests, but these ones were never found in samples from nearby coniferous forests (Taylor & Bruns 1999a; Taylor & Bruns 1997). Further studies on this orchid showed that even the genotypes of *C. maculata* individuals played an important role as different genotypes never shared the same *Russula* species, even when growing together (Taylor et al. 2004). These investigations on *C. maculata* showed that factors determining the fungi associated with an orchid species can be highly complex and are not solely driven by the absence of alternatives.

Such a complex interaction of different factors may also play a role for the determination of the fungi associated with *G. conopsea*. The taxon composition of the fungal partners associated with *G. conopsea* was not homogenous over all localities, but showed a clear spatial structure and only little overlap between regions. This regional differentiation in species composition together with the high variability on the population level suggest that factors at the local scale may strongly affect local species composition and hence diversity at the regional level. *G. conopsea* is known to show a high intraspecific morphological variability (Scacchi & de Angelis 1989; Soliva & Widmer 1999). Currently there are two differentiated subspecies, which can occur sympatrically and genetic differences as well as in the ploidy level have been reported (Gustafsson & Lönn 2003; Marhold et al. 2005). Differences in fungal diversity found between the investigated populations of *Gymnadenia conopsea* might be due genetic differences and indicate an ongoing diversification between populations. In addition environmental factors, like pH or water availability, may differentiate localities. This emphasizes the need for future investigations to integrate multiple factors such as ploidy or habitat type in order to analyse what are the main factors determining the fungal associates of *G. conopsea*. Especially in the light of such a high intraspecific variation as this is observed for *G. conopsea* a more detailed analysis of the determining parameters is certainly warranted and might contribute to the general understanding of this very unique relationship between orchids and their mycorrhizal fungi.

Chapter 2

Strong genetic differentiation between *Gymnadenia conopsea* and *G. densiflora* despite morphological similarity

with Wiesław Babik, Stefan G. Michalski, Grit Winterfeld and Walter Durka
submitted to Plant Systematics and Evolution

Abstract

Gymnadenia conopsea (L.) R.BR. *s.l.* is a controversial taxon with the two most commonly distinguished subspecies *G. conopsea* ssp. *conopsea* and *G. conopsea* ssp. *densiflora*. Despite morphological similarity, differentiation between the taxa was reported for several traits; however trait variation within taxa obviated a clear consensus. We conducted morphological analyses in 626 samples from Germany and assessed microsatellite variation, ITS sequences and chromosome numbers on a larger European scale (1420 samples).

Morphologically the taxa differed significantly in a number of diagnostic traits, but discriminant analysis showed that on the individual level an unequivocal assignment is not possible as 96% of *G. c.* ssp. *conopsea* but only 72% of *G. c.* ssp. *densiflora* could be assigned correctly. Chromosome numbers showed that *G. c.* ssp. *conopsea* was either diploid or tetraploid, while *G. c.* ssp. *densiflora* was diploid throughout. Microsatellite analysis showed a strong genetic differentiation between the taxa due to largely non-overlapping sets of alleles. This was confirmed by the ITS analysis which revealed 2% nucleotide divergence, similar to the divergence between other *Gymnadenia* species. The sequences of *G. c.* ssp. *densiflora* form a well supported monophyletic group sharing a most recent common ancestor with *G. nigra* and *G. austriaca*. Thus, the two taxa are no sister species and a species rank is supported for *G. densiflora* (WAHLENB.) DIETRICH and *G. conopsea* (L.) R.BR. *s.str.* Further studies are needed on trait variation within and among species and ploidy levels to allow for a better identification of the genetically differentiated but morphologically similar taxa.

Introduction

Orchids (Orchidaceae) is one of the largest families of flowering plants, harboring approximately 25.000 species (Dressler 1987). As such they are a prime example for diversification and with one of the highest speciation rates of all flowering plants (Gill 1989) they offer an extraordinary opportunity to study speciation (Peakall 2007). Major drivers for orchid diversification are thought to be their highly specialized pollinator mechanisms (Cozzolino & Widmer 2005; Micheneau et al. 2009) and their symbiotic interaction with fungi for germination (Otero & Flanagan 2006; Swarts & Dixon 2009b). Orchids are known for the ease with which inter-specific and inter-generic hybrids can occur as they do not have strong prezygotic reproductive barriers or incompatibilities (Delforge 2006; Mallet 2005). However, due to strong postzygotic barriers, e.g. chromosomal incompatibilities (Scopece et al. 2008), hybridization not necessarily leads to speciation. Still, in several orchid genera and most prevalent in the subtribe Orchidinae (Gill 1989) hybridization and polyploidy are common features. Thus, e.g. the high diversification of the Iberian orchid flora is partly attributed to polyploidy, with species-rich groups such as *Dactylorhiza* showing particularly high proportions of intra-generic polyploidy (83.3%) (Amich et al. 2007). While autopolyploidy does occur, e.g. in *Dactylorhiza* (Devos et al. 2005; Ståhlberg 2009) or *Vanilla* (Bory et al. 2008), allopolyploidy seems to be more common and has been reported in several genera including e.g. *Dactylorhiza* (Hedrén et al. 2001) and *Nigritella* (now included in *Gymnadenia*) (Hedrén et al. 2000). Due to the fusion of rather uniform but divergent diploid genomes during hybridization or polyploidisation, in polyploids often higher levels of genetic variation are found compared to their diploid progenitors (Pillon et al. 2006). As a consequence of the high genetic variation, polyploids are often considered to be ecologically more flexible and possess higher fitness, e.g. during colonization of newly available habitat (e.g. Comai 2005; Soltis & Soltis 2009).

Gymnadenia conopsea (L.) R.BR. *s.l.*, the fragrant orchid, is a controversial taxon. *Gymnadenia* is an Eurasian genus, covering most of Europe and parts of Asia (Tutin et al. 1980); and *G. conopsea s.l.* is distributed from Western Europe to China. In Europe it is a still relatively common orchid with a wide ecological amplitude including forest habitats and wet to dry grasslands. Morphologically, *G. conopsea s.l.* is highly variable which gave rise to various taxonomic treatments, ranging from 1 species with 3 varieties (Delforge 2006) to 5 species plus 2 subspecies (Dworschak 2002). The two most commonly distinguished taxa, particularly in local Floras, are *G. conopsea* (L.) R.BR. ssp. *conopsea* and *G. conopsea* ssp. *densiflora* (WAHLENB.) K. RICHT. Increasingly the two taxa are treated as distinct species *G. conopsea* (L.) R.BR. *s.str.* and *G. densiflora* (WAHLENB.) DIETRICH (cf. Marhold et al. 2005). They are described to differ in morphology, flowering phenology, scent emission and habitat preferences (Gustafsson 2000; Gustafsson & Lönn 2003; Jersáková et al. 2010; Marhold et al. 2005; Scacchi & de Angelis 1989; Soliva & Widmer 1999). However, due to the considerable morphological variability of *G.*

conopsea s.l., a clear assignment to either taxon is often difficult in the field. Additionally, flowering time has been shown to depend on the ploidy level and does not allow a clear distinction either (Jersáková et al. 2010). Previous investigations revealed genetic differentiation at allozyme loci (Scacchi & de Angelis 1989), at the DNA-sequence level (Gustafsson & Lönn 2003) and between flowering-time variants in Sweden (Gustafsson and Lönn 2003) and Switzerland (Soliva & Widmer 1999). Reports on the ploidy status are complex, with authors stating ssp. *conopsea* as polyploid (e.g. Marhold et al. (2005): tetraploid, Jersáková et al. (2010): octoploid) or diploid (Marhold et al. 2005; Vöth & Sontag 2006) and ssp. *densiflora* as diploid (Marhold et al. 2005) or tetraploid (Hagerup in Bisse (1963); Jersáková et al. 2010; Mrkvicka 1993). Autopolyploidy has been postulated within *G. conopsea* (Jersáková et al. 2010); however no clear evidence has been presented yet.

So far previous studies inherently assumed that the two taxa are sister groups and compared among others, morphology, chromosome counts, genetic variation and phylogenetic analyses on samples from various geographic regions. However, a comprehensive analysis of these parameters on one sample is missing. We analyzed 32 sites of ssp. *conopsea* and ssp. *densiflora* in East and North Germany based on morphological and genetic parameters, including chromosome counts, ITS sequences and microsatellite analyses. We found a strong genetic, but only moderate morphological differentiation between the taxa. In order to further analyze the relationships on a larger geographic scale, we included additional samples from other European regions. In particular we tested if the differentiation between the taxa is consistent across regions and parameters analyzed and whether they in fact share a most recent common ancestor.

Materials and Methods

Sampling

Detailed morphometric and population genetic analyses were conducted in East Germany (EG) and North Germany (NG) (see Tab. 6). Here, individuals from a total of 32 sites were characterized for morphology (626 samples), microsatellites (843 samples) and ploidy, which was assessed by direct counting (31 samples, 10 sites) and based on microsatellite phenotypes. Prior to analyses all investigated populations were assigned to either of the subspecies *conopsea* or *densiflora* based on a synthesis of the morphological characters described.

Additionally, phylogenetic and microsatellite analyses were conducted on a larger geographic scale, including samples from the above two study regions in East and North Germany and additional German samples from the Eifel (WG: 5 sites), Saarland (SWG: 4 sites), Bavarian Alps (SEG: 8 morphological varieties *sensu* Dworschak (2002) and from Sweden (SE, Gotland, Öland: 5), France (FR, French Alps: 6 sites) and Austria (AT, Austrian Alps: 5) (Tab. 6).

Morphometric analyses

For morphometric analyses, 32 locations in the main study regions in East and North Germany were sampled. If possible, on 20 individuals per location (in total 626) the following parameters were measured, which are traditionally used to identify the subspecies: plant height (ph), inflorescence length (il), number of flowers (nf), flower density (fd, number of flowers / inflorescence length), number of leaves (nl), length of the second lowermost leaf (ll), maximum width of the second lowermost leaf (lw), spur length (sl) and ovary length (ol) of a central flower (flower traits only measured for 30 locations). We tested for significant difference between the subspecies as well as the two study regions using a mixed effect model with region/subspecies as fixed factors and population and individuals as random factors. If not normally distributed, data was either log- or sqrt-transformed to meet assumptions.

In order to assess the level of morphological differentiation between the subspecies, we first performed principal component analysis (PCA) on individuals and on populations, the latter using the mean values of nine morphological variables (30 populations, 408 individuals). Thereafter a linear discriminant analysis was performed with individuals grouped to subspecies. The morphological variables were correlated with the posterior probabilities, indicating the contribution of each variable to the discrimination between subspecies. Morphometric data analysis was performed in R 2.10.1 (R Development Core Team 2009) using the MASS library (Venables & Ripley 2002).

Determination of ploidy level

Chromosomes were counted for 31 representative individuals from 10 sites in East Germany. Young growing buds were placed into 8-hydroxyquinoline for two hours to synchronize cell division. Fixation was for 3 hours at room temperature with a 3:1 mixture of ethanol (99%) and pure acetic acid. Chromosome preparations of mitotic and meiotic states were made from enzyme-treated buds, as described by Schwarzacher et al. (1980). Tissue was squashed on a slide in a drop of 45% propionic acid with 2% carmine according to the protocol described by Winterfeld & Röser (2007). Chromosome numbers in orchids are notoriously difficult to determine due to the occurrence of aneuploidy (e.g. Bernardos et al. 2003; Bianco et al. 1991; Greilhuber & Ehrendorfer 1975), which results in variable numbers that often depart from multiples of the basic chromosome number of x= 20. Thus ploidy levels were deduced as diploid if n= 18-20 or 2n= 31-41, triploid if 2n was around 60 and tetraploid if 2n= 60-80. Additionally we used microsatellite data (see below) to infer chromosome numbers. Based on the number of alleles detected per individual they were characterized as either polyploid or diploid with a diploid individual showing a maximum of two peaks per locus and a polyploid showing up to four alleles. These polyploids are most probably

tetraploids; however, the presence of triploids cannot be excluded, as three alleles can occur in both of them. Populations were classified as either diploid if all plants had less than 3 alleles at both loci, polyploid, if at least 50% of plants had more than 2 alleles and of mixed ploidy when 0 - 50 % of plants had more than 2 alleles.

Microsatellite analysis

Microsatellite data was obtained for a total of 1414 individuals from 62 sites (main study regions EG and NG: 843 samples, 32 sites; additional regions: 571 samples, 30 sites, for details see Tab. 6). Total genomic DNA was extracted with the DNeasy 96 Plant Kit (Qiagen, Hilden, Germany). Ten microsatellite loci have been described for *G. conopsea s.l.* (Campbell et al. 2002; Gustafsson & Thorén 2001). However, only five loci gave repeatable and interpretable PCR products and were used for further analysis: Loci Gc17 (fluorescent label PET), Gc42 (FAM), Gc77 (VIC) were amplified in a multiplex reaction, whereas Gc49 (PET) and Gc51 (PET) where amplified separately. A 10 µl PCR reaction contained 5 µl Multiplex PCR Kit (Qiagen, Hilden, Germany), 1 pmol of each primer and 1-10 ng DNA. The cycling scheme was 3 min at 94°C for initial denaturation, followed by 35 cycles of 30 sec at 94°, 30 sec at respective annealing temperature, 45 sec at 72°C and ended by a final elongation time of 10 min. Samples were run on an ABI 3100 genetic analyser (Applied Biosystems, Darmstadt, Germany) and genotyped manually using GeneMapper 3.7 (Applied Biosystems, Darmstadt, Germany). We used principal component analysis (PCA) to display the relationship among populations based on allele frequencies, using loci Gc42 and Gc51 which were consistently amplified in all individuals. Two analyses were conducted, first with the 32 sites from East and North Germany, and second for all samples. The PCA was performed with R (R Development Core Team 2009).

Molecular phylogenetic analysis

DNA sequences were obtained for the nuclear ITS region including ITS1, the 5.8S rDNA gene and ITS2 using the primers ITS1 and ITS4 (White et al. 1990). A 50 µl PCR reaction contained 5 µl 10x PCR buffer (Fermentas, St. Leon-Rot, Germany), 2 mM $MgCl_2$, 0.16 mM of each dNTP, 1 µM of each primer, 1 U of *Taq* (Fermentas) and 1 -10 ng DNA. The cycling scheme was 95°C for 5 min, followed by 40 cycles at 95°C for 40 s, 57°C for 30 s, 72°C for 40 s and the final extension step at 72°C for 10 min. PCR products were purified with MinElute (Qiagen) and 40 µl were concentrated into 10 µl. PCR products were sequenced using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems), using the primer ITS4 (White et al. 1990). A 10 µl reaction contained 1.75 µl SB buffer, 3.2 pmol Primer, 0.5 µl BDT and 1 µl template. The cycling scheme

was 96°C for 1 min, followed by 40 cycles at 96°C for 10 s, 50°C for 5 s, 60°C for 2 min. Samples were run on an ABI 3100 genetic analyser. Sequences were aligned using the software SeqScape v2.6 (Applied Biosystems). In case of sequences containing ambiguous sites, haplotypes were resolved using the algorithms provided by PHASE (Stephens et al. 2001) as implemented in DnaSP (Librado & Rozas 2009). A list of unique haplotypes was created with the program TCS (Clement et al. 2000). Sequences are deposited at GenBank (accession numbers JF414017-JF414052).

Genetic divergence between taxa was based on *p*-distances, which is the proportion of total base pair differences between two sequences (Nei & Kumar 2000), with the computer software MEGA4 2003 (Tamura et al. 2007). For tree inference the alignment included all unique haplotypes (Tab. 5), published sequences of both subspecies as well as published sequences of other congeneric species and a sequence of *G. odoratissima* obtained from a sample from the Swiss Alps. A *Dactylorhiza incarnata* sequence was added as outgroup. Phylogenetic trees were obtained applying two criteria, maximum parsimony and maximum likelihood. Most parsimonious trees were inferred using MEGA4 with a heuristic search and initial trees produced by the random addition option. Alignment gaps were removed from the data set before analysis. Preceding the maximum likelihood estimation we selected the best-fit model of nucleotide substitution by running jModelTest (Posada 2008) with default options and using the Akaike Information Criterion. Using the alignment and the estimated model of nucleotide substitution (a simple Jukes and Cantor model) and a BIONJ starting tree, we inferred a phylogeny using PhyML version 3 (Guindon & Gascuel 2003). For both methods, reliability of the branching pattern was tested by bootstrapping the dataset 500 times.

Results

Morphology

Altogether 626 samples were investigated, with 372 individuals in East Germany (ssp. *densiflora*: 173 samples, ssp. *conopsea*: 199 samples) and 254 in North Germany (all ssp. *conopsea*). Significant differences between the subspecies were found for seven out of nine morphological parameters. Subspecies *densiflora* was in general larger and showed higher levels than ssp. *conopsea* for plant height ($p < 0.01$), leaf number ($p < 0.001$), leaf length ($p < 0.01$), leaf width ($p < 0.001$), flower number ($p < 0.001$) and flower density ($p < 0.001$), but had a shorter spur ($p < 0.01$) (Fig. 6, Tab. 7 Appendix). The largest relative differences were found for the number and density of flowers and for the number and width of the leaves. For ssp. *conopsea* only small differences were found between the regions for plant height ($p = 0.05$) and leaf length ($p = 0.02$).

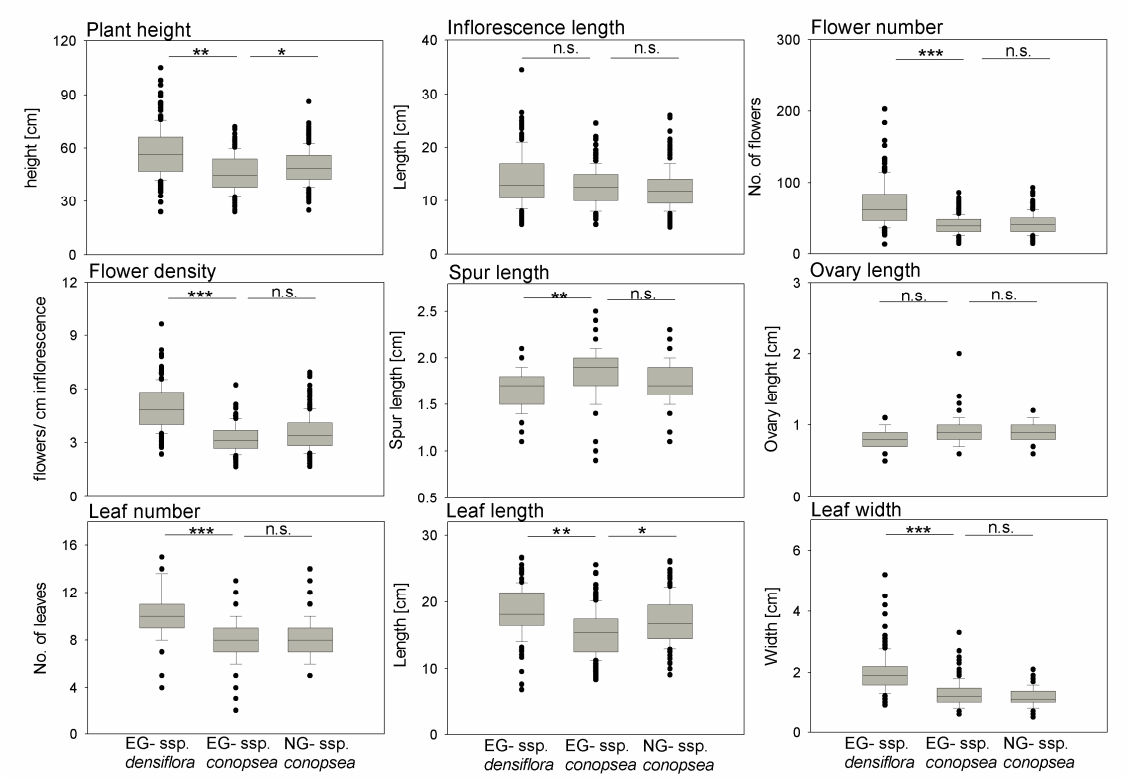

Figure 6: Morphometric analysis for 32 sites in the study regions in East Germany (EG) and North Germany (NG), using the parameters that are traditionally used to identify the two subspecies *G. c. ssp. densiflora* and *G. c. ssp. conopsea*.

In the PCA, 65.6% and 75.5 % of the morphological variation were explained by the first two axes for individuals and populations, respectively (Figure 7). When subspecies were mapped on the PCA scores, they were separated mainly along PC 1, however with substantial overlap. Visual inspection of the scores of the morphological variables indicates that flower density, flower number and number of leaves were most distinct between subspecies. Subspecies *densiflora* showed a higher morphological variability than ssp. *conopsea*. In accordance with PCA the discriminant analysis was not fully successful to separate the subspecies, as 96% of ssp. *conopsea* individuals, but only 73% of ssp. *densiflora* were assigned correctly. Variables most highly correlated with the posterior probabilities were flower density (r = 0.76), number of flowers (r = 0.73), leaf width (r = 0.72) and number of leaves (r = 0.70). Note that in this data set, ssp. *densiflora* was found to be diploid and ssp. *conopsea* tetraploid (see below).

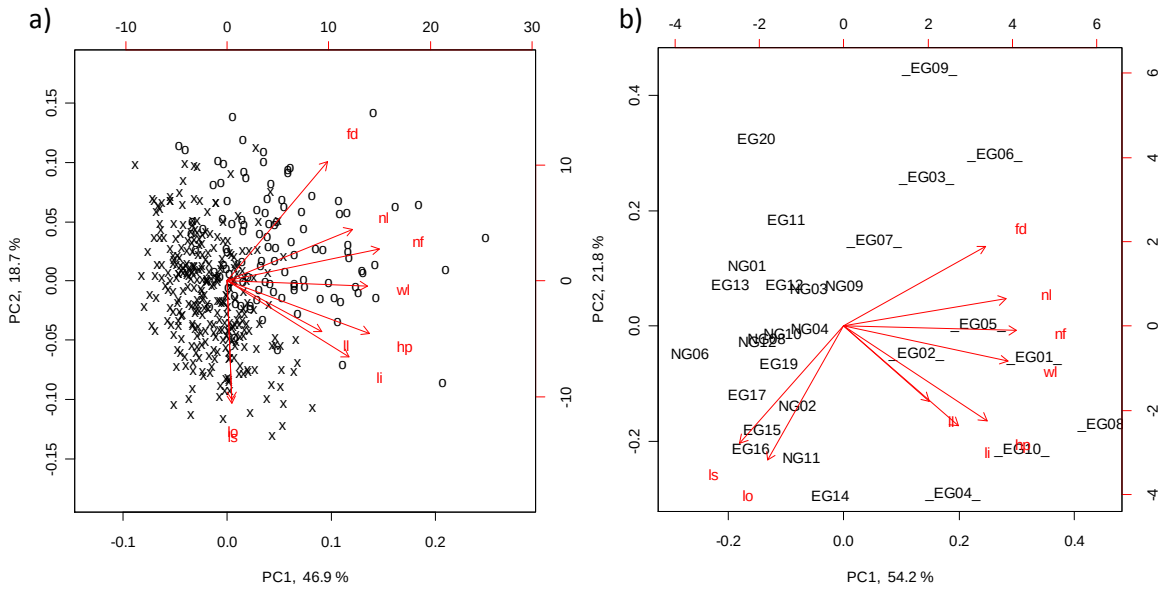

Figure 7 Biplot of principal component scores PC1 vs. PC2 of 9 morphometric variables for (a) individuals and (b) populations of *G. c.* ssp. *conopsea* (x) and *G. c.* ssp. *densiflora* (o, underlined site codes) and of underlying morphological traits (for explanation see text).

Ploidy level

Chromosome numbers were directly counted in 4 populations from East Germany morphologically classified as ssp. *conopsea* and 6 populations as ssp. *densiflora* (Tab. 4). All individuals assigned to ssp. *conopsea* were either tetraploid (12 individuals) or triploid (4 individuals). In contrast, all individuals assigned to ssp. *densiflora* proved to be diploid (15 individuals).

Microsatellite markers showed a maximum of two alleles per individual for ssp. *densiflora*, indicating diploidy in accordance with the chromosome counts. All ssp. *conopsea* had a re-occurring polyploid pattern, with more than two alleles for at least one microsatellite locus, indicative of polyploidy. In the whole data set, populations assigned as ssp. *densiflora* were diploid throughout. In contrast, the additional ssp. *conopsea* populations were either diploid (Eifel, Bavaria, Sweden, France), tetraploid (Saarland) or of mixed ploidy (Eifel, Alps) (Tab. 6).

Table 4 Chromosome counts and ploidy levels of *Gymnadenia conopsea s.l.* for 31 individuals (N) from 10 sites in East Germany (D= diploid, P= polyploid).

Study site	Code	N	Individual chromosome counts	Ploidy level (2n)	Ploidy type
Gymnadenia conopsea ssp. *densiflora*					
Tote Täler	EG06	2	2n= 39; 2n= 40	2x	D
Theißen	EG02	1	n= 19-20	2x	D
Predel	EG03	1	2n= 35-38	2x	D
Jaucha	EG01	5	2n=36-40; 2n=32-40; n=19-20/ 2n= 36-40; 2n=35-36; n=18/ 2n= 33-38	2x	D
Würze	EG08	3	2n= 37-41; 2n= 31-37; 2n=40	2x	D
Jägertalwiese	EG10	3	n=20; 2n=38-40; 2n= 36-38	2x	D
Gymnadenia conopsea ssp. *conopsea*					
Steigra	EG17	1	2n= 63	3x	P
		5	2n= 71-79; 2n=72-78; 2n= 75; 2n= 60-74; 2n= 78	4x	P
Krawinkel	EG16	1	2n= 40-60	3x	P
		4	2n= 63-80; 2n= 80; 2n= 75-80; 2n= 80	4x	P
Rabis	EG15	1	2n= 65-66	3x?	P
		1	2n= 69-77	4x	P
Alter Gleisberg	EG14	1	2n= 62-64	3x	P
		2	2n= 73-77; 2n= 78-80	4x	P

Microsatellite analysis

Three of the microsatellite markers (Gc17, Gc49, Gc77) did hardly produce any bands in samples assigned to ssp. *densiflora*, but two loci (Gc42, Gc51) worked well in all samples and were used for the subsequent analyses. The total number of alleles detected in the two loci was three times larger in ssp. *conopsea* than in ssp. *densiflora* (data no shown). The PCA analysis of populations from East and North Germany separated the populations in two major clusters, resembling the predefined subspecific affiliation (Fig. 8a). This clear grouping is due to strongly deviating allele frequencies at both loci. Subspecies *conopsea* had a wider distribution along both axes, indicating more heterogeneous genotypes than ssp. *densiflora*. One population morphologically assigned as ssp. *conopsea* showed an intermediate position (EG11), which was due to low allelic diversity compared to other ssp. *conopsea* populations, possibly as a result of bottleneck effects. Mapping of ploidy onto the PCA showed that all ssp. *conopsea* where tetraploid and all ssp. *densiflora* were diploid.

The PCA analysis of the whole data set resulted in a differentiated pattern with the two major clusters and a few intermediate populations (Fig. 8b). All populations predefined as ssp. *densiflora* again formed a dense cluster separated along Axis 1. This cluster also included a number of populations with unclear subspecific affiliation and alpine *G. c.* ssp. *serotina sensu* Dworschak (2002), which thus can be subsumed as ssp. *densiflora.* All ssp. *densiflora* were diploid. Most other

populations clustered to ssp. *conopsea*. These populations were again more strongly separated along Axis 2 with the alpine populations somewhat separated. The ssp. *conopsea* cluster comprised diploid, tetraploid and mixed ploidy populations which however were not differentiated. Three populations took intermediate positions (WG3, SE1, AT1) in the PCA and contained individuals from both subspecies, when individual genotypes are inspected (data not shown).

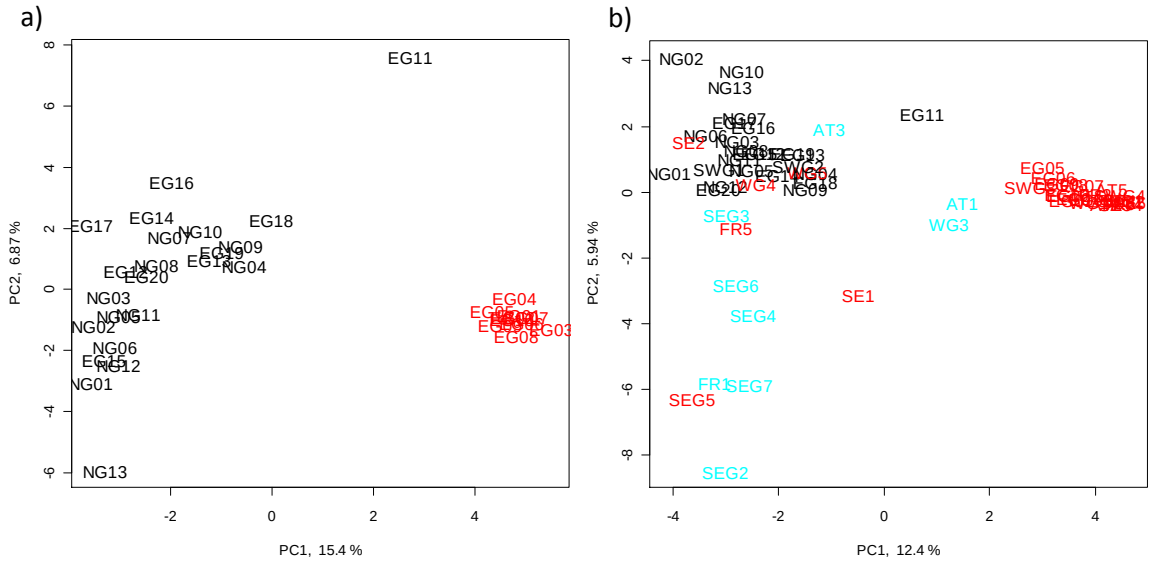

Figure 8 Principal components analysis of *G. conopsea s.l.* based on allele frequencies of microsatellite loci Gc42 and Gc51. Colors indicate ploidy as determined from microsatellite genotypes and direct counting (red = 2x, black = 4x, blue = mixed ploidy), for site codes see Table 6. **a)** 32 sites from East and North Germany **b)** whole data set.

Phylogenetic analysis

ITS sequences were obtained from 100 samples from 49 sites. These resulted in a total of 140 haplotypes as 39 sequences had ambiguous nucleotide sites that were resolved applying the PHASE algorithm. We detected 35 unique haplotypes (Tab. 5), 27 of which were ssp. *conopsea* (Gc9- Gc35) and 8 were ssp. *densiflora* (Gd1- Gd8) haplotypes. In Germany 29 of the 35 haplotypes were found, whereas three were restricted to France and Austria, respectively. In most sampling locations only one subspecies was detected, but one site in the Eifel (WG3) and France (FR4) harbored both subspecies (Tab. 6).

The alignment including sequences of both subspecies only, had a length of 599 base pairs with 26 variable sites which were located in the ITS 1 (14 sites) and ITS 2 (12 sites) regions (Tab. 5). The total alignment used for phylogenetic inference, including other congeneric species, had a length of 589 base pairs with 65 variable nucleotide sites out of which 36 were parsimony informative.

Table 5 Unique haplotypes, overall frequency, and variable nucleotide positions found for *G. conopsea s.l.* Altogether 35 haplotypes were obtained, 8 haplotypes assigned to *G. c.* ssp. *densiflora* (Gd1 to Gd8) and 27 haplotypes assigned to *G. c.* ssp. *conopsea* (Gc9 to Gc35).

		ITS 1															ITS 2											
		42	53	56	58	75	86	94	106	119	189	198	213	240	241	426	438	448	458	466	478	487	489	552	568	592	598
Haplotype	n																										
Gd1	35	C	C	C	T	C	C	C	C	G	G	C	C	C	T	A	G	G	C	T	C	T	T	T	A	A	T
Gd2	21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
Gd3	5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
Gd4	5	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
Gd5	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
Gd6[a]	1	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gd7[a]	1	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
Gd8[a]	1	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	A	.	.	.	.	.	.	.	.	.
Gc9	22	.	.	.	.	T	.	.	.	A	A	A	G	.	.	.	.	.	.	C	.	C	G	A	.	T	A
Gc10	10	.	.	.	.	T	T	.	.	A	A	A	G	.	.	.	.	.	.	C	.	C	G	A	.	T	A
Gc11	3	.	.	T	.	T	.	.	.	A	A	A	G	.	.	.	.	.	.	C	.	C	G	A	.	T	A
Gc12	3	.	.	T	.	T	.	.	.	A	A	A	G	.	.	.	.	.	T	C	.	C	G	A	.	T	A
Gc13	3	.	.	.	.	T	.	.	.	A	A	A	G	.	.	.	.	.	A	C	.	C	G	A	.	T	A
Gc14	3	T	.	.	.	T	T	.	.	A	A	A	G	.	.	.	.	.	.	C	.	C	G	A	.	T	A
Gc15	2	.	.	T	.	T	.	.	.	A	A	A	G	.	.	T	.	.	T	C	.	C	G	A	.	T	A
Gc16	1	.	.	.	.	T	.	.	.	A	A	A	G	.	.	.	.	.	.	C	.	C	G	A	T	T	A
Gc17	1	.	.	.	.	T	.	.	.	A	A	A	G	.	.	.	.	.	.	C	.	.	G	A	.	T	A
Gc18	2	.	.	.	.	T	.	.	.	A	A	A	G	.	.	.	.	.	A	C	T	C	G	A	.	T	A
Gc19	2	.	.	.	.	T	T	.	.	A	A	A	G	.	.	.	.	.	A	C	.	C	G	A	.	T	A
Gc20	1	.	.	.	C	T	.	.	.	A	A	A	G	.	.	.	.	.	.	C	.	C	G	A	.	T	A
Gc21	2	.	.	T	.	T	T	.	.	A	A	A	G	.	.	.	.	.	.	C	.	C	G	A	.	T	A
Gc22	2	.	.	T	.	T	T	.	.	A	A	A	G	.	.	T	.	.	T	C	.	C	G	A	.	T	A
Gc23	1	.	.	.	.	T	.	.	.	A	A	A	G	.	.	.	.	.	T	C	.	C	G	A	.	T	A
Gc24[a]	1	.	.	.	.	T	.	.	.	A	A	A	G	.	.	.	A	.	.	C	.	C	G	A	.	T	A
Gc25[a]	1	T	.	.	.	T	.	.	.	A	A	A	G	.	.	.	A	.	.	C	.	C	G	A	.	T	A
Gc26[a]	1	.	.	T	.	T	T	.	.	A	A	A	G	.	.	.	.	.	T	C	.	C	G	A	.	T	A
Gc27[a]	1	.	.	T	.	T	.	.	G	A	A	A	G	.	.	.	.	.	T	C	.	C	G	A	.	T	A
Gc28[a]	1	.	.	.	.	T	.	T	.	A	A	A	G	.	.	.	.	.	A	C	.	C	G	A	.	T	A
Gc29[a]	1	.	.	.	.	T	.	T	.	A	A	A	G	.	.	.	.	.	A	C	T	C	G	A	.	T	A
Gc30[a]	1	.	.	.	.	T	.	T	.	A	A	A	G	.	.	.	.	.	.	C	.	C	G	A	.	T	A
Gc31[a]	1	.	.	.	.	T	T	T	.	A	A	A	G	.	.	.	.	.	.	C	.	C	G	A	.	T	A
Gc32[a]	1	.	.	.	.	T	T	.	.	A	A	A	G	.	G	.	.	.	.	C	.	C	G	A	.	T	A
Gc33[a]	1	.	.	T	.	T	.	.	.	A	A	A	G	.	.	.	.	.	A	C	.	C	G	A	.	T	A
Gc34[a]	1	.	.	T	.	T	.	.	.	A	A	A	G	.	.	.	.	.	A	C	T	C	G	A	.	T	A
Gc35[a]	1	.	.	.	.	T	.	.	.	A	A	A	G	.	.	.	.	.	.	C	T	C	G	A	.	T	A

[a]solely as reconstructed haplotype

Both, the maximum parsimony (Fig. 9) and the maximum likelihood method (Fig. 10 Appendix) yielded very similar results. In both trees the samples separated into two major clades, but with only low bootstrap support (50 / 66 % and 72 / 77 % for parsimony and maximum likelihood, respectively). The first one comprised most *G. conopsea s.l.* sequences from GenBank, our ssp. *conopsea* haplotypes Gc9- Gc35 as well as *G. odoratissima*, but revealed no further structuring. The second clade consisted of six congeners, but only few groupings within the clade received support > 80 %: *G. crassinervis* clustering with a sample of "*G. conopsea"*, both from China; two *G. frivaldii* sequences, *G. austriaca* together with *G. nigra*, both formerly *Nigritella*; and lastly the clade including published *densiflora*-sequences, as well as a late-flowering Swedish "*G. conopsea"* and our ssp. *densiflora* sequences Gd1- Gd8, originating from nearly all sampled regions. Both phylogenies strongly suggest that ssp. *conopsea* and ssp. *densiflora* are no sister species. Rather, the former *Nigritella* is the sister group of ssp. *densiflora*. However, the phylogenetic status of ssp. *conopsea* remains unclear.

Disregarding the published sequence from China and the late-flowering Swedish "*G. conopsea"*, which seem not to be *G. conopsea*, the mean genetic divergence between the *Gymnadenia* species was 2% (p= 0.02 SD 0.007), ranging from p_{min}= 0.002 between *G. odoratissima* and ssp. *conopsea* to p_{max}= 0.032 between *G. crassinervis* and *G. austriaca*. The mean divergence between sequences assigned as ssp. *conopsea* and ssp. *densiflora* from our study was considerable with 2% (p= 0.02 SD 0.002), whereas within the subspecies divergence was low (ssp. *conopsea*: p= 0.003 SD 0.002; ssp. *densiflora*: p= 0.003 SD 0.002). The divergence between the two subspecies was due to 11 nucleotide sites, whereof 8 differences were fixed and three sites showed variability within one of the clades (Tab. 5).

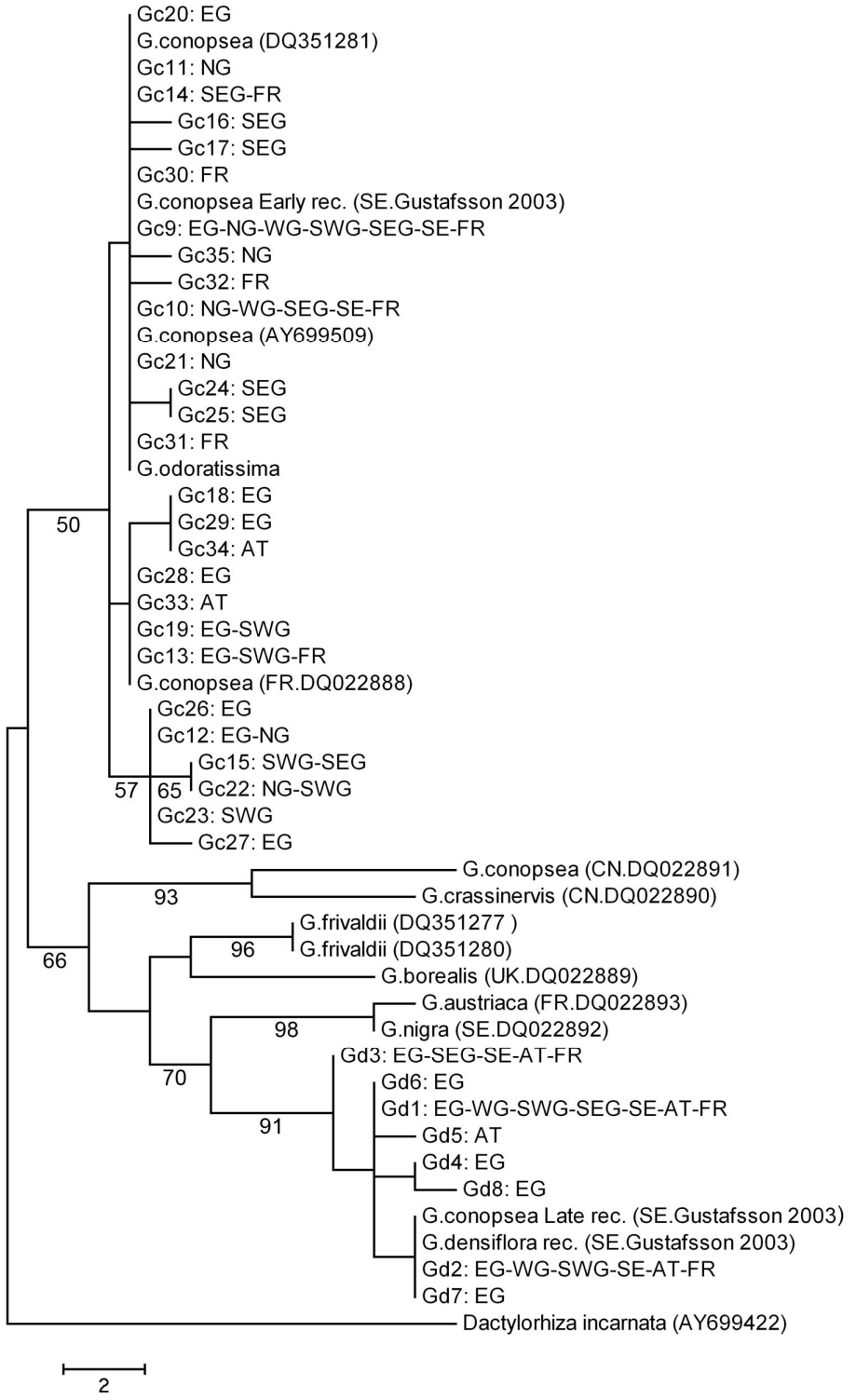

Figure 9 One tree out of 441 most parsimonious trees (length = 62) for unique *G. conopsea s.l.* sequences of the ITS region (ITS1, 5.8s, ITS2) from this study (Gd1-Gd8, Gc9-Gc35) and published *Gymnadenia* data. The consistency index of the tree is 0.725, the retention index is 0.931, and the composite index is 0.766 for parsimony-informative sites. *Dactylorhiza incarnata* has been set as outgroup. Bootstrap support based on 500 replicates and >50% is given. Localities where the sequence types occur are presented as EG = East Germany, NG= North Germany, WG= West Germany, SWG= South-West Germany, SEG= South-East Germany, SE= Sweden, FR= France, AT= Austria or by international abbreviation codes for GenBank samples.

Table 6 Study sites, prior field classification, number of samples analyzed for morphology (N_{morph}) and ITS (N_{ITS}), ITS haplotypes detected, ploidy level inferred from microsatellites (D: diploid, P: polyploid, * indicates confirmation by chromosome counts (Table 4)), number of samples analyzed for microsatellites (N_{micro}) and total number of alleles detected at loci Gc42 and Gc51 (A), and the taxon to which the populations were assigned.

Study site	Code	E	N	Prior field classification	N_{morph}	N_{ITS}	Haplotypes	Ploidy	N_{micro}	A	Taxon
East Germany (EG)											
Jaucha	EG01	12°11‘	51°14’	ssp. *densiflora*	20	2	Gd1	D*	28	3	Gd
Theißen	EG02	12°07’	51°08‘	ssp. *densiflora*	20	3	Gd1	D*	30	5	Gd
Predel	EG03	12°19‘	51°12‘	ssp. *densiflora*	20	2	Gd1, Gd3	D*	19	6	Gd
Domsen	EG04	12°17‘	51°18‘	ssp. *densiflora*	5	3	Gd1, Gd2	D	31	3	Gd
Espenhain	EG05	12°44‘	51°25‘	ssp. *densiflora*	8	-		D	7	5	Gd
Tote Täler	EG06	11°74‘	51°19‘	ssp. *densiflora*	20	3	Gd1, Gd2	D*	27	8	Gd
Rothenstein	EG07	11°57‘	50°86‘	ssp. *densiflora*	20	-		D	27	9	Gd
Würze	EG08	11°69‘	50°84‘	ssp. *densiflora*	20	7	Gd1, Gd2, Gd4, Gd6, Gd7, Gd8	D*	29	8	Gd
Klingelsteine	EG09	11°64‘	50°98‘	ssp. *densiflora*	20	-		D*	27	8	Gd
Jägertalwiese	EG10	11°73‘	50°97‘	ssp. *densiflora*	20	-		D*	30	6	Gd
Domsen	EG11	12°14‘	51°19‘	ssp. *conopsea*	20	1	Gc9	P	26	9	Gc
Rothenstein	EG12	11°58‘	50°86‘	ssp. *conopsea*	20	-		P	26	37	Gc
Zietschkuppe	EG13	11°70‘	50°97‘	ssp. *conopsea*	20	-		P	23	30	Gc
Alter Gleisberg	EG14	11°70‘	50°95‘	ssp. *conopsea*	20	-		P	25	39	Gc
Rabis	EG15	11°66‘	50°89‘	ssp. *conopsea*	20	1	Gc20	P*	25	33	Gc
Krawinkel	EG16[a]	11°64‘	51°21‘	ssp. *conopsea*	20	-		P*	29	34	Gc
Steigra	EG17[a]	11°65‘	51°30‘	ssp. *conopsea*	20	-		P*	26	35	Gc
Grockstädt	EG18	11.59‘	51°33‘	ssp. *conopsea*	20	2	Gc12, Gc26, Gc27	P	19	24	Gc
Langer Berg	EG19	11.71‘	51°24‘	ssp. *conopsea*	20	2	Gc13, Gc18, Gc19	P	27	22	Gc
Tote Täler	EG20[a]	11°73‘	51°19‘	ssp. *conopsea*	19	1	Gc28, Gc29	P	29	33	Gc

Table 6- *continued*

Study site	Code	E	N	Prior field classification	N_{morph}	N_{ITS}	Haplotypes	Ploidy	N_{micro}	A	Taxon
North Germany (NG)											
Stb. Polle	NG01[a]	9°40‘	51°89‘	ssp. *conopsea*	20	1	Gc21, Gc22	P	25	35	Gc
Stb. „Im Schießstand“	NG02	9°36‘	51°78‘	ssp. *conopsea*	20	-		P	27	38	Gc
Stb. „Alter Steinbruch“	NG03	9°36‘	51°78‘	ssp. *conopsea*	20	-		P	25	35	Gc
Stb. Hehlen	NG04	9°45‘	51°98‘	ssp. *conopsea*	20	2	Gc11, Gc21	P	25	25	Gc
Stb. Bärenbrink	NG05	9°88‘	51°94‘	ssp. *conopsea*	14	1	Gc9, Gc10	P	26	33	Gc
Stb. Delligsen	NG06	9°81‘	51°95‘	ssp. *conopsea*	20	-		P	27	33	Gc
Burgberg	NG07[a]	9°51‘	51°87‘	ssp. *conopsea*	20	-		P	25	31	Gc
Rühle	NG08[a]	9°53‘	51°92‘	ssp. *conopsea*	20	1	Gc12	P	24	37	Gc
Räuschenberg	NG09	9°37‘	51°81‘	ssp. *conopsea*	20	-		P	30	18	Gc
Poppenburg	NG10	9°44’	51°88‘	ssp. *conopsea*	20	1	Gc11	P	25	28	Gc
Bielenberg	NG11	9°35’	51°78‘	ssp. *conopsea*	20	1	Gc9	P	27	37	Gc
Bocksberg	NG12	9°65’	51°88‘	ssp. *conopsea*	20	-		P	25	35	Gc
Holberg	NG13	9°57’	51°91‘	ssp. *conopsea*	20	-		P	22	32	Gc
Eifel (WG)											
Alenberg	WG1	6°38’	50°22‘	ssp. *densiflora*	-	3	Gd1, Gd2	D	35	3	Gd
Ripsdorfer Moor	WG2	6°39’	50°23‘	ssp. *densiflora*	-	1	Gd1	D	40	5	Gd
Hillesheim	WG3	6°40’	50°17‘	ssp. *densiflora*	-	3	Gd1, Gc9, Gc10	D+P	39	21	mixed
Eierberg	WG4	6°38’	50°22‘	ssp. *conopsea*	-	1	Gc9	D	20	21	Gc
Höneberg	WG5	6°40’	50°23‘	ssp. *conopsea*	-	1	Gc9, Gc10	D	20	18	Gc
Saarland (SWG)											
Niedergailbach	SWG1	7°12’	49°08‘	ssp. *conopsea*	-	3	Gc9	P	40	24	Gc
Bliesransbach	SWG2	7°05’	49°09‘	ssp. *conopsea*	-	3	Gc13, Gc15, Gc19, Gc22, Gc23	P	40	26	Gc
Nachtweide	SWG3	7°04’	49°12‘	ssp. *densiflora*	-	3	Gd1, Gd2	D	40	10	Gd
Ensheim	SWG4	7°07’	49°12‘	ssp. *densiflora*	-	2	Gd1, Gd2	D	39	8	Gd

Table 6- *continued*

Study site	Code	E	N	Prior field classification	N_{morph}	N_{ITS}	Haplotypes	Ploidy	N_{micro}	A	Taxon
Bavarian Alps (SEG)											
Bavaria-South	SEG1	*12°00'*	*47°40'*	*G. conopsea* x *serotina*[b]	-	2	Gd1, Gd3	D	9	7	Gd
Bavaria-South	SEG2	*12°00'*	*47°40'*	*G. vernalis*[b]	-	1	Gc9, Gc10	D+P	10	17	Gc
Bavaria-South	SEG3	*12°00'*	*47°40'*	*G. splendida*[b]	-	2	Gc15, Gc17	D+P	12	24	Gc
Bavaria-South	SEG4	*12°00'*	*47°40'*	*G. splendida* x *odoratissima*[b]	-	1	Gc9, Gc10	D+P	13	28	Gc
Bavaria-South	SEG5	*12°00'*	*47°40'*	*G. graminea*[b]	-	2	Gc9, Gc10, Gc14	D	10	24	Gc
Bavaria-South	SEG6	*12°00'*	*47°40'*	*G. conopsea*[b]	-	2	Gc9, Gc14, Gc9, Gc10,	D+P	5	18	Gc
Bavaria-South	SEG7	*12°00'*	*47°40'*	*G. alpina*[b]	-	2	Gc16	D+P	12	25	Gc
Bavaria-South	SEG8	*12°00'*	*47°40'*	*G. alpina* x *conopsea*[b]	-	2	Gc9, Gc24, Gc25	-	-	-	Gc
Sweden (SE)											
Öland	SE1	16°38'	56°40'	ssp. *conopsea*	-	1	Gc9, Gc10	D	5	9	Gc
Gotland Lojsta	SE2	18°22'	57°18'	ssp. *conopsea*	-	1	Gc9, Gc10	D	40	41	Gc
Gotland, Lickershamn	SE3	18°30'	57°49'	ssp. *densiflora*	-	3	Gd1, Gd2, Gd3	D	6	6	Gd
Gotland, Häftings	SE4	18°39'	57°53'	ssp. *densiflora*	-	2	Gd1, Gd2	D	3	6	Gd
Gotland, Kallgatburg	SE5	18°40'	57°42'	ssp. *densiflora*	-	4	Gd1, Gd2	D	11	10	Gd
Austria (AT)											
Kärnten	AT1	13°25'	46°42'	unknown	-	2	Gd1, Gd2	D+P	20	27	mixed
Tweng	AT2	13°36'	47°11'	unknown	-	2	Gd3, Gd5	D	2	2	Gd
Gstatterboden	AT3	14°37'	47°35'	unknown	-	1	Gc33, Gc34	D+P	9	21	Gc
Obertauern	AT4	13°33'	47°15'	unknown	-	3	Gd1, Gd2	D	4	4	Gd
Steinplatte	AT5	12°05'	51°23'	unknown	-	-		D	31	11	Gd
France (FR)											
Col du Lautaret	FR1	6°24'	45°02'	ssp. *conopsea*	-	2	Gc9, Gc14	D+P	15	20	Gc
Col du Lautaret	FR2	6°24'	45°02'	ssp. *densiflora*	-	3	Gd1, Gd3	D	15	7	Gd
Clairvaux les lacs	FR3	5°46'	46°34'	ssp. *conopsea*	-	2	Gc9, Gc13		-		Gc
Clairvaux les lacs	FR4	5°46'	46°34'	ssp. *densiflora*	-	2	Gd2, Gc30, Gc31		-		mixed
Sailles-le-Haut	FR5	6°01'	45°21'	ssp. *conopsea*	-	2	Gc9, Gc10, Gc32	D	11	17	Gc
Sailles-le-Haut	FR6	6°01'	45°21'	ssp. *densiflora*	-	2	Gd1	D	15	4	Gd

[a] Site codes *sensu* Stark et al. (2009): EG16= E1; EG17= E2; EG20= E3; NG07= N1; NG01= N2; NG08= N3
[b] Taxa *sensu* Dworschak (2002), exact sampling sites unknown

Discussion

Our data provide unequivocal evidence for strong phylogenetic and genetic differentiation but incomplete morphological differentiation between the two taxa of *Gymnadenia conopsea s.l.*. Thus a species rank is supported for *Gymnadenia densiflora* (WAHLENB.) DIETRICH and *Gymnadenia conopsea* (L.) R.BR. *s.str.* as has been suggested previously (Bateman et al. 1997; Campbell et al. 2007; Marhold et al. 2005). Concerning ploidy our findings are concordant with Marhold et al. (2005) as *G. conopsea s.str.* was found to be either diploid or tetraploid, while *G. densiflora* was found to be diploid throughout.

Morphology

While a number of morphological traits, e.g. flower number and density, allow a fairly good distinction between the two species, they show considerable morphological variation, which sometimes does not allow unequivocal determination. Apart from polyploidy (see below), two aspects of orchid biology may contribute to the similarity between and variation within species, mycorrhization and pollination biology.

Orchids are obligatory associated with mycorrhizal fungi for germination and the availability of fungal partners might determine habitat suitability and orchid distribution. Furthermore, mycorrhizal fungi can have an effect on plant growth and different types of the same fungal taxon have been shown to differ in their effect, e.g. for plant size (Lee 2002). *G. conopsea s.str.* has been found to associate with a large number of fungal taxa and the spatial structure of the fungal community suggests a non-random distribution (Stark et al. 2009). Such fine-scale distribution patterns are thought to contribute to orchid diversification, for example the phylogenetic divergence of floral variants within the *Hexalectris spicata* complex is partly attributed to differences in the fungal associates (Taylor et al. 2003) or a correlation between *Corallorhiza maculata* genotypes and certain fungal associates has been found (Taylor et al. 2004). Therefore, both the overall similarity between, and the morphological variation within the two species may be related to association with mycorrhizal fungi that may either differ within or may be shared among taxa.

Orchids are prime examples of selection on flower morphology by insect pollinators (Thompson 1994). Both, *G. densiflora* and *G. conopsea s.str.* have fairly specialized flowers, which provide nectar and are pollinated by the same taxa of moths. Thus, it may be hypothesized whether convergent selection of pollinators has led to similar flower morphology, although it has been shown that the two species differ in their scent bouquet (Jersáková et al. 2010). Furthermore, as *G. densiflora* is described to be strongly scented and *G. conopsea s.str.* appears to be less scented (Schmeil 1996) the latter may be under selection to morphologically resemble *G. densiflora* to attract the same pollinators.

Genetic differentiation

ITS sequence divergence has been suggested to be indicative for differentiation on the species level in orchids (Bateman et al. 2003). Based on substantial ITS divergence, Bateman et al. (1997) recognized *G. c.* ssp. *borealis* as full species and suggested the same status for *G. c.* ssp. *densiflora,* which was further substantiated later (Bateman et al. 2006). Our results for ITS on a large sample of both *G. conopsea* and *G. densiflora* support the view that they in fact deserve species status, as the genetic divergence of 2% between the two clades was similar to the average genetic divergence between other species of the genus *Gymnadenia*. Also, sequences of *G. densiflora* form a well supported monophyletic group sharing a most recent common ancestor with *G. nigra* and *G. austriaca*. Hence, *G. densiflora* and *G. conopsea s.str.* are not even sister species. Based on ITS data, *G. conopsea s.str.* cannot be separated from *G. odoratissima*, a morphologically well distinguished species, which is in line with Gustafsson & Lönn (2003). Hence, the evolutionary history of the latter needs to be assessed with additional genetic markers.

The phylogenetic divergence between *G. conopsea s.str.* and *G. densiflora* is fully supported by microsatellite presented here and previously (Campbell et al. 2007) and by allozyme data (Scacchi & de Angelis 1989). Using a different set of microsatellite loci Campbell et al. (2007) similarly found strong differentiation of allele frequencies on the British Isles, and suggested to consider them as distinct species. In our study, in addition to differences in alleles, some microsatellite markers did not produce any bands in *G. densiflora*, while all markers were amplified in *G. conopsea s.str.*, which underlines the genetic differentiation between the taxa. The strong differentiation between the taxa also contrasts with the low to moderate differentiation among populations within the two taxa shown here and elsewhere for *Gymnadenia* (Chung 2009; Soliva & Widmer 1999).

Ploidy

Recently, the formerly unclear ploidy relationships of *G. conopsea s.str.* and *G. densiflora* were resolved by Marhold et al. (2005), who concluded that *G. densiflora* is diploid and that other reports are probably based on taxonomic misinterpretations, whereas *G. conopsea s.str.* can be both diploid and polyploid. Here, using direct counting and microsatellite genotypes, we also found a clear pattern of diploidy for *G. densiflora* across all samples from the French Alps to Sweden. For *G. conopsea s.str.* we found both diploids and polyploids with some regional distribution patterns. Only diploids were detected in Sweden, consistent with microsatellite analyses (Gustafsson 2000). In contrast, only tetraploids were encountered in East Germany, North Germany and Saarland and both ploidy levels occurred either within or among populations in the Alps and in the Eifel. The occurrence of two ploidy levels within or among populations of *G. conopsea s.str.* has also been

found previously (Jersáková et al. 2010; Marhold et al. 2005). Surprisingly, so far none of the studies that used codominant molecular markers in *G. conopsea s.str*. have reported on problems with more than two alleles per locus, which would have indicated polyploidy (Campbell et al. 2007; Gustafsson 2000; Gustafsson & Lönn 2003; Gustafsson & Sjögren-Gulve 2002; Scacchi & de Angelis 1989; Soliva & Widmer 1999). Thus, in the respective regions, *G. conopsea s.str*. seems to be at least predominantly diploid, i.e. British Isles, Italy and Switzerland. This complex geographic distribution of ploidy levels suggests that a phylogeographic perspective is needed to achieve a more comprehensive picture (Nordström & Hedrén 2008). The two ploidy levels of *G. conopsea s.str.* were hardly differentiated in the microsatellite analysis and the most frequent ITS haplotypes occurred in both of them. This lack of genetic differentiation may indicate an autopolyploid origin of tetraploid from diploid *G. conopsea s.str*. as suggested by Jersáková et al. (2010). The presence of triploid individuals in some populations found here and elsewhere (Marhold et al. 2005) may indicate that gene flow between ploidy levels can occur via a triploid-bridge (Ramsey & Schemske 1998). However, considerable phenological separation has been reported between ploidy levels, which reduces the opportunity for gene flow and may select for alternative pollinators (Jersáková et al. 2010). We cannot assess the degree of morphological differentiation of the two ploidy levels in *G. conopsea s.str*. as in the main study regions only tetraploids were found. However, spur length varies significantly between ploidy levels within *G. conopsea s.str.* (Jersáková et al. 2010) and variation in other traits is probable. Interestingly, spur length of *G. densiflora* is intermediate between the two ploidy levels in *G. conopsea* (Jersáková et al. 2010). This shows that the polyploidy has further complicated the distinction of the species both morphologically and phenologically. Therefore, determination of ploidy levels is essential and it will be important to investigate which diagnostic traits are affected by ploidy and which ones are not.

Taking the findings together an evolutionary scenario emerges in which *G. conopsea s.str.* and *G. densiflora* have phylogenetically separated from each other prior to the split of other groups, e.g. the former *Nigritella*. The two taxa have achieved a large distribution range and diverged ecologically, phenologically and partly also morphologically, however retaining considerable variability. The phylogenetic split seems to have occurred also before polyploidy has arisen in *G. conopsea s.str.*, probably by autopolyploidy, while *G. densiflora* stayed at the diploid level. Polyploidy in turn may have led to increased morphological and phenological variability within *G. conopsea s.str.*

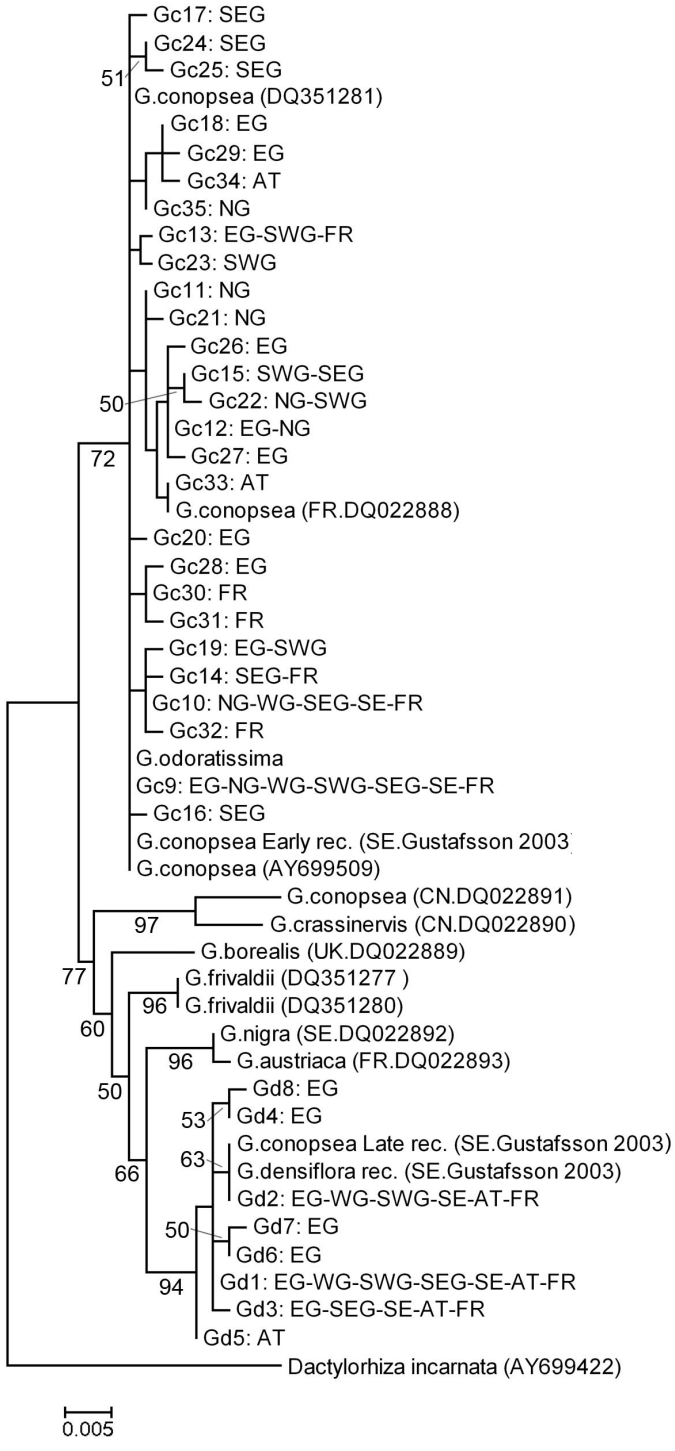

Figure 10 Maximum likelihood phylogeny for unique *G. conopsea s.l.* sequences of the ITS region (ITS1, 5.8s, ITS2) from this study (Gd1-Gd8, Gc9-Gc35) and published *Gymnadenia* data. *Dactylorhiza incarnata* has been set as outgroup. Bootstrap support based on 500 replicates and >50% is given. Localities where the sequence types occur are presented as EG = East Germany, NG= North Germany, WG= West Germany, SWG= South-West Germany, SEG= South-East Germany, SE= Sweden, FR= France, AT= Austria or by international abbreviation codes for GenBank samples.

Table 7 Morphological parameters (mean± SE) and sample size for the 32 sites of *G. conopsea s.l.* investigated in the study regions in East Germany and North Germany.

Study site	Code	N	Plant height (ph, cm)	Inflorescence length (il, cm)	Flower number (nf)	Flower density (fd, cm^{-1})	Leaf number(nl)	Leaf length (ll, cm)	Leaf width (lw, cm)	Spur length (sl, cm)	Ovary length (ol, cm)
Gymnadenia conopsea* ssp. *densiflora											
Jaucha	EG01	20	61.20 ± 1.94	16.90 ± 0.85	92.55 ± 6.53	5.49 ± 0.31	11.35 ± 0.48	17.37 ± 0.48	1.93 ± 0.08	1.80 ± 0.03	0.81 ± 0.02
Theißen	EG02	20	63.93 ± 2.10	12.78 ± 0.85	50.80 ± 3.38	4.01 ± 0.12	8.80 ± 0-31	20.31 ± 0.64	1.83 ± 0.09	1.67 ± 0.03	0.82 ± 0.01
Predel	EG03	20	48.78 ± 1.95	11.63 ± 0.71	49.90 ± 2.77	4.35 ± 0.15	10.45 ± 0.38	19.32 ± 1.37	1.85 ± 0.09	1.54 ± 0.04	0.72 ± 0.03
Domsen	EG04	5	65.20 ± 3.03	15.00 ± 1.35	60.80 ± 9.04	4.00 ± 0.37	8.20 ± 1.20	26.60 ± 0.00	2.00 ± 0.11	1.74 ± 0.08	0.88 ± 0.06
Espenhain	EG05	8	57.31 ± 9.11	16.31 ± 3.17	78.88 ± 21.64	4.31 ± 0.45	10.13 ± 0.83	13.46 ± 3.10	2.30 ± 0.40	1.64 ± 0.06	0.84 ± 0.03
Tote Täler	EG06	20	49.70 ± 2.10	14.45 ± 0.98	79.45 ± 5.41	5.56 ± 0.19	10.20 ± 0.36	17.13 ± 1.17	1.76 ± 0.15	1.49 ± 0.04	0.71 ± 0.03
Rothenstein	EG07	20	47.60 ± 1.84	10.18 ± 0.47	51.25 ± 3.38	5.04 ± 0.24	9.80 ± 0.31	18.73 ± 0.71	1.63 ± 0.06	1.81 ± 0.03	0.80 ± 0.02
Würze	EG08	20	70.58 ± 2.37	17.33 ± 0.93	95.05 ± 7.39	5.44 ± 0.24	11.90 ± 0.53	18.61 ± 0.80	2.59 ± 0.11	1.61 ± 0.03	0.94 ± 0.02
Klingelsteine	EG09	20	49.35 ± 1.93	10.63 ± 0.58	61.70 ± 2.87	5.89 ± 0.17	9.25 ± 0.32	16.07 ± 0.72	1.48 ± 0.10	1.44 ± 0.03	0.71 ± 0.02
Jägertalwiese	EG10	20	67.90 ± 3.40	15.85 ± 1.21	70.60 ± 10.29	4.22 ± 0.36	10.15 ± 0.50	21.02 ± 0.86	2.66 ± 0.27	1.71 ± 0.04	0.86 ± 0.02
Gymnadenia conopsea* ssp. *conopsea											
Domsen	EG11	20	43.85 ± 2.04	13.65 ± 0.96	37.65 ± 2.99	2.77 ± 0.10	7.90 ± 0.29	12.93 ± 0.54	1.33 ± 0.06	1.72 ± 0.03	0.70 ± 0.01
Rothenstein	EG12	20	45.45 ± 1.48	12.63 ± 0.73	41.45 ± 2.20	3.35 ± 0.16	7.33 ± 0.50	15.17 ± 0.72	1.37 ± 0.13	1.73 ± 0.05	0.84 ± 0.03
Zietschkuppe	EG13	20	42.50 ± 1.92	11.73 ± 0.57	34.90 ± 2.35	3.00 ± 0.17	6.85 ± 0.33	13.61 ± 0.77	1.16 ± 0.08	1.74 ± 0.03	0.90 ± 0.02
Alter Gleisberg	EG14	20	54.25 ± 2.04	15.58 ± 0.48	50.10 ± 3.02	3.18 ± 0.12	8.00 ± 0.34	17.86 ± 0.86	1.67 ± 0.09	1.90 ± 0.04	0.98 ± 0.03
Rabis	EG15	20	46.53 ± 1.76	13.05 ± 0.79	40.65 ± 2.66	3.13 ± 0.13	7.25 ± 0.34	17.69 ± 0.76	1.32 ± 0.09	1.80 ± 0.13	1.04 ± 0.11
Krawinkel	EG16[a]	20	49.63 ± 2.35	13.78 ± 0.77	37.10 ± 1.99	2.76 ± 0.13	7.30 ± 0.34	17.46 ± 0.85	1.17 ± 0.06	2.00 ± 0.05	0.91 ± 0.03
Steigra	EG17[a]	20	49.43 ± 2.47	12.73 ± 0.85	35.65 ± 2.50	2.84 ± 0.14	8.20 ± 0.39	13.96 ± 0.81	1.21 ± 0.08	1.98 ± 0.06	0.91 ± 0.02
Grockstädt	EG18	20	46.48 ± 2.06	12.00 ± 0.60	42.45 ± 2.62	3.54 ± 0.15	7.65 ± 0.31	16.39 ± 0.84	1.20 ± 0.08	-	-
Langer Berg	EG19	20	44.20 ± 2.16	11.58 ± 0.98	43.75 ± 3.64	3.84 ± 0.18	8.40 ± 0.28	15.37 ± 0.68	1.51 ± 0.11	1.88 ± 0.05	0.99 ± 0.03
Tote Täler	EG20[a]	19	34.95 ± 1.60	9.82 ± 0.50	37.89 ± 2.17	3.89 ± 0.15	8.63 ± 0.40	13.45 ± 0.55	1.02 ± 0.07	1.68 ± 0.04	0.80 ± 0.04
Stb. Polle	NG01[a]	20	43.98 ± 1.73	9.88 ± 0.63	39.05 ± 2.61	4.04 ± 0.21	7.20 ± 0.29	14.71 ± 0.88	1.12 ± 0.10	1.79 ± 0.04	0.91 ± 0.03
Stb. “Im Schießstand”	NG02	20	52.48 ± 2.66	13.95 ± 0.89	42.80 ± 3.22	3.11 ± 0.19	7.60 ± 0.32	16.82 ± 0.98	1.35 ± 0.07	1.79 ± 0.04	0.93 ± 0.03
Stb. “Alter Steinbruch”	NG03	20	49.68 ± 2.18	12.80 ± 0.76	46.35 ± 3.58	3.66 ± 0.24	7.35 ± 0.30	16.29 ± 0.78	1.21 ± 0.06	1.65 ± 0.06	0.86 ± 0.03
Stb. Hehlen	NG04	20	48.35 ± 1.77	11.05 ± 0.63	45.60 ± 3.58	4.11 ± 0.22	8.05 ± 0.41	18.43 ± 1.03	1.37 ± 0.06	1.75 ± 0.05	0.94 ± 0.02
Stb. Bärenbrink	NG05	14	47.36 ± 2.37	10.93 ± 0.79	35.64 ± 2.32	3.40 ± 0.26	6.71 ± 0.35	17.84 ± 1.03	1.22 ± 0.05	-	-
Stb. Delligsen	NG06	20	43.43 ± 1.51	10.08 ± 0.49	28.85 ± 1.26	2.97 ± 0.18	7.20 ± 0.28	14.64 ± 0.73	0.98 ± 0.05	1.88 ± 0.05	1.00 ± 0.03
Burgberg	NG07[a]	20	55.00 ± 2.23	15.30 ± 0.98	54.75 ± 3.94	3.59 ± 0.16	8.15 ± 0.24	19.36 ± 0.64	1.24 ± 0.07	-	-
Rühle	NG08[a]	20	49.18 ± 1.92	11.53 ± 0.71	39.50 ± 3.39	3.38 ± 0.19	7.90 ± 0.31	17.25 ± 0.92	0.95 ± 0.05	1.82 ± 0.04	0.90 ± 0.03
Räuschenberg	NG09	20	54.68 ± 2.05	11.43 ± 0.65	42.75 ± 2.56	3.89 ± 0.26	9.25 ± 0.40	17.32 ± 0.60	1.28 ± 0.05	1.65 ± 0.04	0.87 ± 0.03
Poppenburg	NG10	20	49.30 ± 1.51	10.40 ± 0.43	40.15 ± 2.17	3.91 ± 0.22	7.45 ± 0.23	19.79 ± 1.21	1.13 ± 0.06	1.81 ± 0.04	0.90 ± 0.02
Bielenberg	NG11	20	54.10 ± 2.22	15.95 ± 0.81	42.75 ± 2.78	2.67 ± 0.10	8.35 ± 0.31	17.00 ± 0.48	1.08 ± 0.06	1.81 ± 0.04	0.97 ± 0.02
Bocksberg	NG12	20	46.28 ± 1.65	12.50 ± 0.80	38.70 ± 2.56	3.13 ± 0.13	7.85 ± 0.38	15.98 ± 0.52	0.95 ± 0.05	1.74 ± 0.05	0.96 ± 0.03
Holberg	NG13	20	49.28 ± 2.73	12.25 ± 0.66	48.80 ± 2.92	4.07 ± 0.23	7.80 ± 0.44	19.30 ± 0.84	1.35 ± 0.07	-	-

[a]Site code *sensu* Stark et al. (2009): EG16= E1; EG17= E2; EG20= E3; NG07= N1; NG01= N2; NG08= N3

Chapter 3

The value of anthropogenic habitats for conservation: a case study on *Gymnadenia conopsea* and *G. densiflora*

with Walter Durka

Manuscript

Abstract

Mineral extraction is one of the most severe anthropogenic changes as it destroys the original vegetation and soil structure. In the course of time post-mining sites can develop into species-rich habitats, but in order to contribute to the conservation of endangered species, the genetic diversity of colonizing species has to be comparable to this one of natural habitats. We investigated a total of 32 populations of *Gymnadenia conopsea* and G. *densiflora* in a lignite post-mining area in East Germany and a system of quarries in North Germany and compared them with the nearest natural populations. Populations were analysed in respect of genetic diversity using microsatellite markers, fruit set and plant performance. Our results revealed that genetic diversity and fruit set were reduced for the populations in the post-mining area, whereas no such effects were found for the quarry populations. However, plant performance was similar for all investigated populations.

On the one hand our study shows that anthropogenic habitats provide valuable habitat for endangered species like orchids and that they contribute to the conservation of biological diversity and genetic resources. They provide important refugees for threatened species in intensively used landscapes. On the other hand, however, the results emphasize the important role the intensity, size and frequency of disturbance events may play for the colonization process. The quarries were relatively small and spatially intermingled with natural habitats, whereas source populations in the post-mining area were distant. While the former favours gene flow via seed dispersal and pollinator activity, the latter might influence seed arrival and pollinator activity negatively, leading to genetic depauperation and the establishment of only small and isolated populations. The combination of small population size, low genetic diversity and reduced fruit set may threaten the long-term persistence of the populations in the post-mining area. In order to prevent succession and to ensure that populations are functionally connected, appropriate management strategies like mowing or grazing are essential. Nevertheless, in this intensively used agricultural landscape bare of structural elements, the post-mining area plays an important role for the maintenance of biodiversity.

Introduction

The Central European landscape is dominated by human activity such as forestry, agriculture and various types of building activities (Jongman 2002). One of the most severe anthropogenic changes is the excavation of mineral resources as it destroys the soil structure and removes the original vegetation. Hence, excavation sites are totally devoid of diaspores and need to be re-colonized from the regional species pool. At these disturbed sites a diverse array of different substrates with special physico-chemical properties and low nutrient levels provides habitat for specialized and therefore endangered species (Brändle et al. 2003; Durka et al. 1997; Ratcliffe 1974; Varela et al. 1993). Over the years they can develop into habitats important for species conservation with complex spatial patterns of specialized communities (Brändle et al. 2000; Durka et al. 1997; Ratcliffe 1974).

Important factors that determine the colonization process of new or disturbed habitats are seed dispersal and seedling recruitment (Vekemans & Hardy 2004), with direct consequences for population dynamics and genetic structure (Jersáková & Malinová 2007). Seed dispersal is determined by the spatial arrangement of reproductive adults, their seed outputs and seed shadows, while seedling recruitment at a site mainly depends on the probability of seed arrival and the availability of suitable microsites for germination (Nathan & Muller-Landau 2000). The local environmental conditions will act as a filter, removing all species from the regional species pool that lack the traits required to survive under the prevailing physico-chemical conditions (Holdaway & Sparrow 2006; Zobel 1997). Hence, the assemblage of communities that will establish on 'new sites' depends on 1) which species arrive at a site, 2) which species are adapted to the special site conditions and 3) which species are able to establish self-maintaining populations in the long run (Bradshaw 1983).

Theoretically, a colonization process starts with a small number of individuals that carry only a portion of the overall genetic diversity of the source populations (Lowe et al. 2004). As a consequence of such genetic bottlenecks young populations may have reduced genetic diversity and different allele frequencies. However, genetic variation is the raw material for adaptation and an important precondition for a species' ability to adapt to new and permanently changing environmental conditions (Frankham & Ralls 1998; Saccheri et al. 1998). As a consequence, potentially occurring founder effects during colonization may threaten the long-term survival of young populations.

So far only a few genetic studies on plant populations compared natural versus anthropogenic habitats and the respective results are heterogeneous. There are studies that found evidence for founder effects (Hollingsworth & Dickson 1997; Liu et al. 2008; Mengoni et al. 2001; Reisch 2007), while others report similar (Esfeld et al. 2008; Krüger et al. 2002; Mengoni et al. 2000; Travis et al. 2002) or even higher (Brock et al. 2007) genetic diversity of anthropogenic habitats.

Often a decrease in genetic variation of neutral genetic markers has been correlated with a reduction in fitness (e.g. Buza et al. 2000; Lammi et al. 1999; Mavraganis & Eckert 2001; Oostermeijer et al. 1994), but others showed that the levels of genetic diversity and fitness are not always related (Podolsky 2001; Reed & Frankham 2001).

A family that is known to be severely affected by anthropogenic changes is the Orchidaceae. Orchids are particularly vulnerable to environmental changes, because with fungi for germination (Otero & Flanagan 2006) and often specific pollinators for fruit set (Cozzolino & Widmer 2005) two important life cycle stages depend on symbiotic interactions (Swarts & Dixon 2009b). Major threats for orchid populations in the wild are habitat destruction and degradation, breakdown of ecological connections (e.g. mycorrhiza, pollinators) and changed abiotic conditions (e.g. soil and hydrology) (Swarts & Dixon 2009a). However, many orchids are known to colonize anthropogenically disturbed habitats; sometimes they form populations of thousands of individuals (Adamowski 1998; Esfeld et al. 2008). In the post-mining area in East Germany, for example, 16 different orchid species are found, and some of them are known to exclusively colonize former mining areas (Esfeld et al. 2008). So far little is known about the population genetic structure and plant performance of populations colonizing such areas. In order to contribute to the conservation of species diversity, populations from anthropogenic habitats should maintain similar levels of genetic diversity like those from natural habitats to ensure their long-term persistence. Hence, orchids represent model systems to assess the value of anthropogenically disturbed habitats for the conservation of genetic diversity.

In our study we examined a total of 32 populations of *G. conopsea* and *G. densiflora* in a lignite post-mining area in East Germany and a system of quarries in North Germany and compared them with the nearest natural populations. *G. conopsea* and *G. densiflora* are relatively common orchids that are typical for anthropogenically disturbed habitats. Populations were investigated in respect to genetic diversity using microsatellite markers and plant performance in the field. The aim was to analyze whether populations on anthropogenic habitats i) have reduced genetic diversity due to founder effects, ii) are genetically more differentiated iii) have a reduced reproductive fitness and plant performance relative to natural populations.

Materials and Methods

Study species

Gymnadenia conopsea (L.) R. BROWN *s.l.* is a terrestrial orchid with a wide geographic distribution in Eurasia (Tutin et al. 1980). It prefers calcareous soils, but also occurs on neutral or low acidic underground. *G. conopsea* is a still relatively common orchid that is found in various habitat types, ranging from wet to dry grasslands and open woodlands (Delforge 2006). It is a typical species for highly disturbed habitats like quarries or post-mining areas (Heyde & Krug 2000).

G. conopsea s.l. is a controversial taxon with various taxonomic treatments. The two most commonly distinguished taxa are *G. conopsea* (L.) R.BR. ssp. *conopsea* and *G. conopsea* ssp. *densiflora* (WAHLENB.) K. RICHT. Recent investigations provide unequivocal evidence for strong phylogenetic and genetic differentiation (Stark et al. 2010 subm.). This supports a species rank for *G. densiflora* (WAHLENB.) DIETRICH and *G. conopsea* (L.) R.BR. *s.str.*, as it has been suggested previously (Bateman et al. 1997; Campbell et al. 2007; Marhold et al. 2005). The two taxa are described to differ in morphology, flowering phenology, scent emission and habitat preferences (e.g. Gustafsson & Lönn 2003; Jersáková et al. 2010; Marhold et al. 2005). Reports on the ploidy status are complex, with authors stating *G. conopsea* as polyploid (e.g. Jersáková et al. 2010; Marhold et al. 2005) or diploid (Marhold et al. 2005; Vöth & Sontag 2006) and *G. densiflora* as diploid (Marhold et al. 2005) or tetraploid (Hagerup in Bisse 1963; Jersáková et al. 2010; Mrkvicka 1993). However, in the study regions in East and North Germany *G. conopsea s.str.* was found to be polyploid and *G. densiflora* diploid throughout (Stark et al. 2010 subm.).

Study areas and sampling

In two regions in East Germany and North Germany we investigated populations from anthropogenic sites whose origin is characterised by the excavation of mineral resources, a post-mining area and a system of quarries, and compared them with the nearest natural populations.

In the East German lignite post-mining area large parts of the landscape have been excavated and no natural *Gymnadenia* populations survived. The sites are at least 30 years old and soil consists of tertiary or quaternary deposits. In this region 10 populations of *G. densiflora* (5 anthropogenic, 5 natural) and 10 populations of *G. conopsea* (1 anthropogenic, 9 natural) were investigated. In contrast, due to smaller excavation sites the quarries in North Germany represent a less intensive disturbance regime. The sites are 40- 70 years old and soils are of Triassic deposits. Here 13 populations of *G. conopsea* (6 anthropogenic; 7 natural) were analysed (Fig. 11).

For each population we determined the population size as the number of flowering plants by direct counting and randomly sampled 25- 30 individuals across the whole population, if population size allowed.

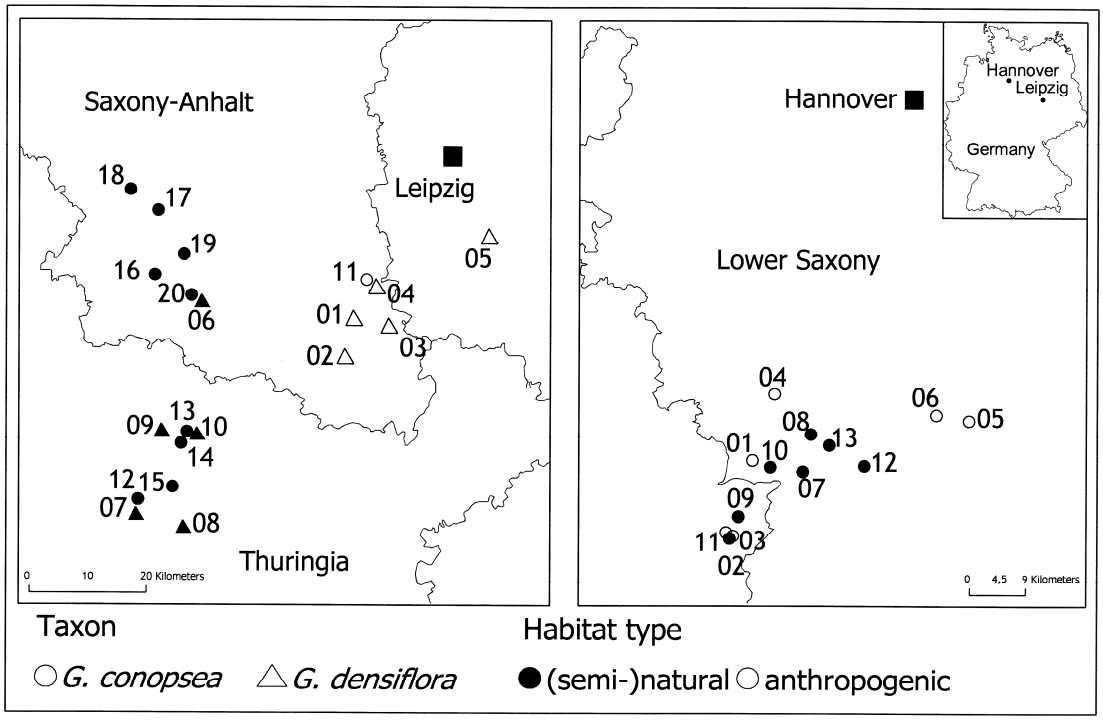

Figure 11 Study sites investigated in East Germany near the city of Leipzig (left) and in North Germany near the city of Hannover (right) (for site details see Tab. 8). *G. conopsea* (symbol: circle) occurred in both regions, whereas *G. densiflora* (symbol: triangle) was found only in East Germany. Semi-natural habitats (filled symbols) were compared with anthropogenic habitats (blank symbols). In East Germany anthropogenic habitats comprised populations in the post-mining area South of Leipzig and in North Germany populations in quarries.

Microsatellite analysis

Total genomic DNA was extracted with the DNeasy 96 Plant Kit (Qiagen, Hilden, Germany). Ten microsatellite loci have been described for *G. conopsea s.l.* (Campbell et al. 2002; Gustafsson & Thorén 2001). However, only five loci gave repeatable and interpretable PCR products and were used for further analysis: Loci Gc17 (fluorescent label PET), Gc42 (FAM), Gc77 (VIC) were amplified in a multiplex reaction, whereas Gc49 (PET) and Gc51 (PET) where amplified separately. A 10 µl PCR reaction contained 5 µl Multiplex PCR Kit (Qiagen, Hilden, Germany), 1 pmol of each primer and 1-10 ng DNA. The cycling scheme was 3 min at 94°C for initial denaturation, followed by 35 cycles of 30 sec at 94°, 30 sec at respective annealing temperature, 45 sec at 72°C and ended by a final elongation time of 10 min. Samples were run on an ABI 3100 genetic analyzer (Applied Biosystems, Darmstadt, Germany) and genotyped manually using GeneMapper 3.7 (Applied Biosystems, Darmstadt, Germany).

Data analysis

For the diploid *G. densiflora* the standard parameters for genetic variation were calculated 1) the average number of alleles per population (*A*) for the total amount of genetic variation, 2) allelic richness (A_r) as the expected number of alleles for a constant minimum sample size of seven individuals, calculated by rarefaction (El Mousadik & Petit 1996), 3) the gene diversity (H_e) as the probability that two randomly chosen alleles will be different and 4) the inbreeding coefficient (F_{IS}) as the probability that two alleles within the same individual are identical by descent (Lowe et al. 2004). In order to estimate the genetic differentiation between populations different hierarchies of fixation indexes were calculated like 5) the overall fixation index (F_{ST}) according to Weir & Cockerham (1984), and F_{SR} as part of the variation found for the subpopulations relative to the habitat and F_{RT} as variation in the habitat types relative to total amount of variation, were calculated by hand (Hartl & Clark 2007). A correlation between geographic and genetic distance was analyzed by mantel test (Mantel 1967) and deviation from Hardy-Weinberg equilibrium was tested using a randomisation test with 1000 permutations. All analyzes were conducted with the computer program FSTAT (Goudet 1995).

For the polyploid *G. conopsea* it is not possible to assign alleles to specific loci or to determine how many copies of each allele an individual possessed (dosage). Hence, we used an approach based on allelic phenotypes (Obbard et al. 2006) and calculated for the within-population diversity 1) the average number of alleles (*A*') and 2) the average number of phenotypes (A'_{phenot}) per population, 3) the phenotypic diversity (H'_S) which is the average number of unshared alleles between pairs of individuals within populations and 4) the average Shannon-Weaver diversity of phenotypes within populations (H^{SW}). As differentiation statistic 5) F'_{ST} was calculated as the proportion of total diversity that is found between populations, calculated using the two 'unshared alleles' diversity estimates H'_T (over-all diversity) and H'_S (mean within-population diversity): $F'_{ST} = H'_T - H'_S / H'_T$, similarly the other hierarchies of variation portioning F_{SR} and F_{RT} were calculated by hand. All other genetic analyzes were calculated with the program F-DASH (Obbard et al. 2006). Note that Obbards F'_{ST} is different from Hedricks (2005) $F'_{ST.}$. The effect of population size on the genetic diversity (A_r / *A*' and H_e / H'_S, depending on species) was tested by linear regression analysis with the program R 2.10.1 (R Development Core Team 2009).

Reproductive success and plant performance

As indicator for reproductive success the fruit set was assessed for 29 out of 32 populations. After flowering the total number of flowers per plant and the number of pollinated capsules were counted for 15-20 individuals per population, if possible. Fruit set was calculated as the proportion of

pollinated capsules per inflorescence. Indicative for habitat quality and plant performance the parameters plant height, flower number and leaf number were determined.

We tested for differences between habitat types using a mixed effect model with habitat type defined as fixed factor and population and individuals as random factor. If not normally distributed, data was either log- or sqrt-transformed prior to statistical testing, and percentage values were arcsin-transformed. The effect of population size and genetic diversity (A_r / A' and H_e / H'_S, depending on species) on the fruit set was tested by linear regression and by multiple regression analysis [multiple regression model selection based on Akaikes criterion using function step from the MASS library], with the program R 2.10.1 (R Development Core Team 2009).

Results

All five primer pairs amplified polymorphic loci for *G. conopsea*, but only two loci (Gc42, Gc51) worked reliably in *G. densiflora* (Stark et al. 2010 subm.). For 255 diploid *G. densiflora* individuals 18 alleles across 2 loci were obtained, and for 588 polyploid *G. conopsea* individuals 155 alleles across five loci. All alleles identified for *G. densiflora* were also found for *G. conopsea*.

Genetic diversity

The genetic diversity of the diploid *G. densiflora* populations in East Germany was low (mean A= 3.05, range 1.5- 4.5; mean A_r= 2.4, range: 1.3- 3.3) (Tab. 8). However, despite a high expected heterozygosity (H_e= 0.34, range: 0.06- 0.58), the inbreeding coefficient was high (mean F_{IS}= 0.24, range: -0.05- 0.49), but only three out of ten populations showed a significant deviation from Hardy-Weinberg equilibrium. There was no correlation between population size and genetic diversity (A_r: p> 0.4, H_e: p> 0.4). The genetic diversity of the populations from the post-mining area was significantly reduced (A_r: p= 0.018; H_e: p= 0.018), and an increased inbreeding coefficient indicates a higher selfing rate (F_{IS}: anthropogenic= 0.35; natural= 0.2, p> 0.4) (Fig. 12).

The genetic diversity of the polyploid *G. conopsea* populations in East Germany was high (A'= 12.7, range: 4.2- 16.4; A'_{phenot}= 17.9, range: 7.4- 21.8; H'_S= 3.0, range: 1.4- 3.5; H^{SW}= 2.62, range: 1.5- 2.9). The single polyploid population in the post-mining area had a lower genetic diversity than the mean genetic diversity of the nine natural populations in the same region (A'_{phenot} anthropogenic: 3.8, natural: 21.4; H'_S anthropogenic: 5.6, natural: 70.6; H^{SW} anthropogenic: 1.23, natural: 2.15; p-values not evaluated, Fig. 12). The genetic diversity of polyploid *G. conopsea* in North Germany was similarly high (A'= 13.2 range: 9- 15.2; A'_{phenot} = 19.1 range: 15.6- 22.6;

H'_S =3.1 range: 2.2- 3.6; H^{SW} =2.7 range: 2.4- 3.0). The populations in the quarries had no reduced genetic diversity (*A*': $p > 0.5$; H'_S: $p > 0.9$; H^{SW}: $p > 0.8$). For polyploid *G. conopsea* there was no correlation between population size and genetic variation (*A*: $p > 0.2$, H'_S: $p > 0.2$) and no regional differences between the natural populations ($p > 0.8$).

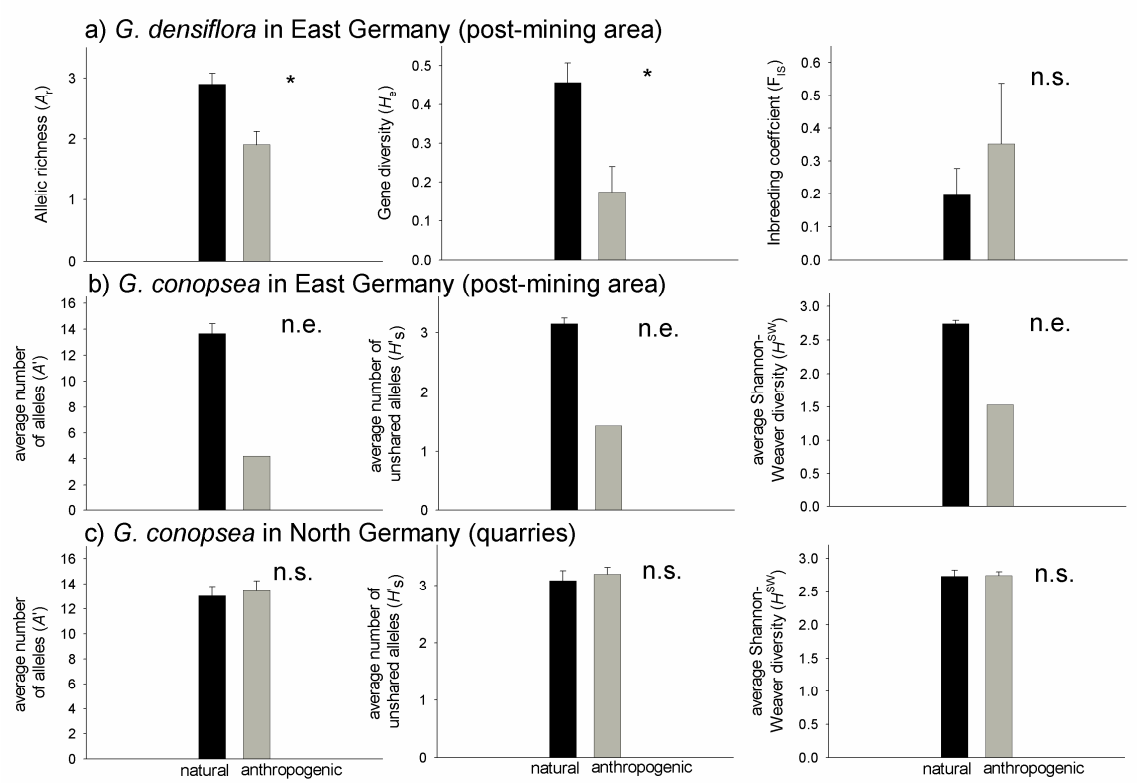

Figure 12 Estimation of genetic diversity of a) *G. densiflora* populations in East Germany, b) *G. conopsea* populations in East Germany and c) *G. conopsea* populations in North Germany. Due to only one anthropogenic *G. conopsea* population in East Germany a statistical evaluation was not possible (n.e.).

Genetic differentiation and population structure

The genetic differentiation was moderate for the diploid *G. densiflora* populations in East Germany (F_{ST} = 0.08) and the polyploid *G. conopsea* in North Germany (F'_{ST}= 0.09), but high for polyploid *G. conopsea* in East Germany (F'_{ST}= 0.14). This indicates that 8%, 9% and 14% of the variation resided among populations respectively. However, differentiation of the polyploid *G. conopsea* populations within each regions was similar (East Germany: F_{ST}= 0.08, North Germany: F_{ST}= 0.07; $p > 0.7$).

Considering the partitioning of variation by hierarchical *F*-statistics, for *G. densiflora* in East Germany 3% of the variation resided among habitat types and 5% among populations within

populations of the respective habitat types; for *G. conopsea* in North Germany 1% and 8% respectively. Comparing the differentiation among populations of the habitat types within each region, there were no differences whatsoever (East Germany, anthropogenic: F_{ST}= 0.07 natural: F_{ST}= 0.06; p> 0.9; North Germany, anthropogenic: F'_{ST}= 0.06; natural: F'_{ST}= 0.1; p> 0.4).

An isolation by distance effect was found only for the polyploid *G. conopsea* populations in East Germany (R^2= 0.12, p= 0.03, Fig. 13). The pairwise F'_{ST}-values showed that this was mainly due to the single *G. conopsea* population in the post-mining area that was strongly differentiated from the natural ones (range F'_{ST}= 0.17- 0.28), whereas the natural populations were genetically similar (mean F'_{ST}= 0.06).

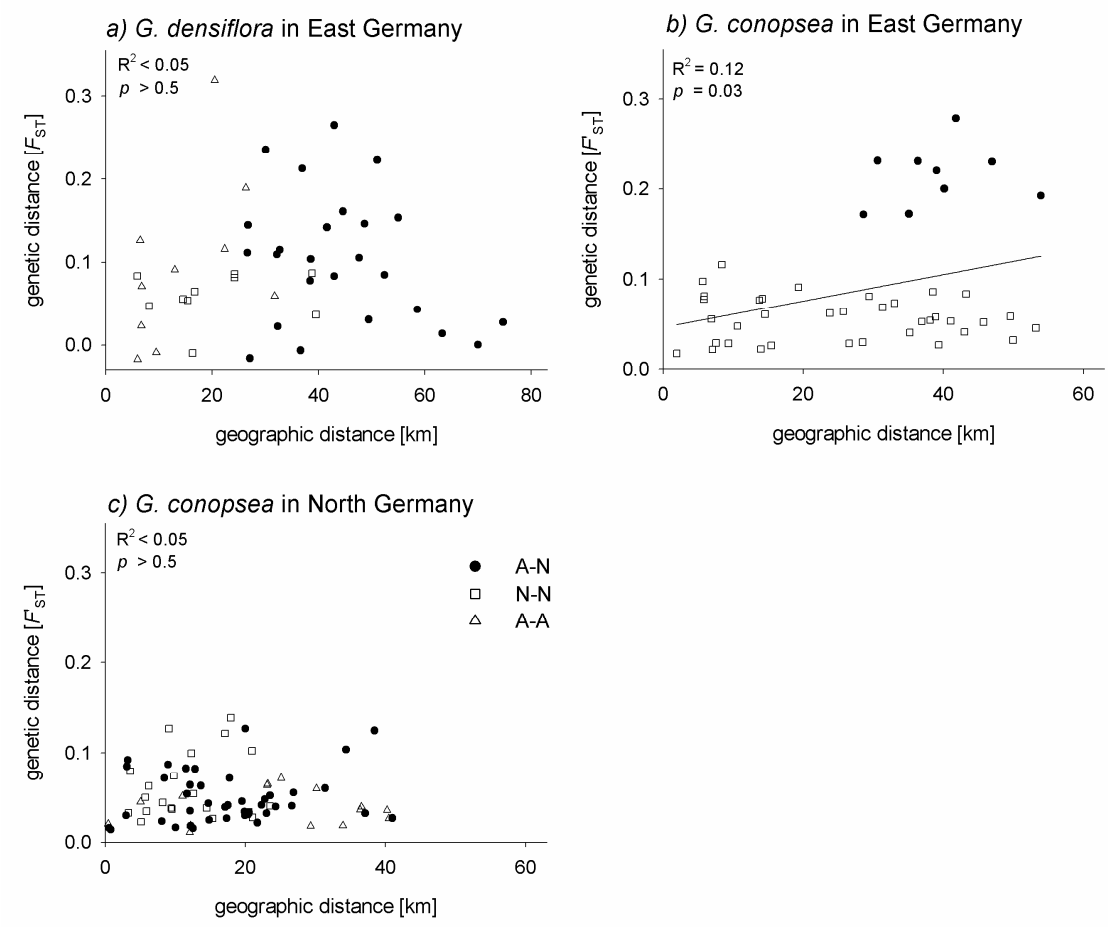

Figure 13 Isolation by distance effects of a) *G. densiflora* populations in East Germany, b) *G. conopsea* populations in East Germany and c) *G. conopsea* populations in North Germany. Compared were genetic distances between anthropogenic and natural habitats (A-N), natural and natural habitats (N-N) and anthropogenic and anthropogenic habitats (A-A) respectively. Due to only one anthropogenic *G. conopsea* population in East Germany no A-A comparison was possible.

Reproductive fitness and plant performance

Fruit set was generally high, with an average of 82% for the natural populations (Tab. 8). However, variation among populations was considerably high (East Germany, min: 50 %, max: 91 %; North Germany, min: 67 %, max: 94 %). For *G. densiflora* fruit set was not correlated to population size and only a marginal relation to genetic diversity was found (population size: $p > 0.4$; A_r: $p = 0.086$). In *G. conopsea* fruit set was affected by both population size and genetic diversity (population size: $p = 0.025$; *A*: $p < 0.001$). As population size and genetic diversity measures were not correlated, we performed a multiple regression analysis with genetic diversity (*A*') and population size, in which both were retained, but fruit set of *G. conopsea* was mainly affected by genetic diversity (overall adjusted $r^2 = 0.54$; $p < 0.001$; *A*': $p = 0.001$, population size: $p = 0.087$).

In East Germany fruit set of the diploid *G. densiflora* populations in the post-mining area was significantly reduced relative to the natural sites (anthropogenic= 81 %, natural= 62 %, $p = 0.035$, Fig. 14) and a similar trend was found for the single polyploid *G. conopsea* population in the post-mining area when compared to nine natural ones (anthropogenic= 50 %, natural= 83 %, *p*= not evaluated). In contrast, for polyploid *G. conopsea* in North Germany no such differences were found between populations from quarries and natural habitats (anthropogenic: 80 %, natural: 85 %, $p > 0.4$).

Plant performance was similar on anthropogenic and natural sites, regardless of region or species (East Germany: a) *G.d.*: plant height $p > 0.7$, flower number $p > 0.5$, leaf number $p > 0.6$; b) *G.c.*: *p*-values not evaluated; North Germany: c) *G.c.*: plant height $p = 0.085$., flower number $p > 0.2$), except for leaf number which was reduced for the quarries ($p = 0.023$). There was no correlation between population size and plant height (*G. densiflora*: $p > 0.6$; *G. conopsea*: $p > 0.2$).

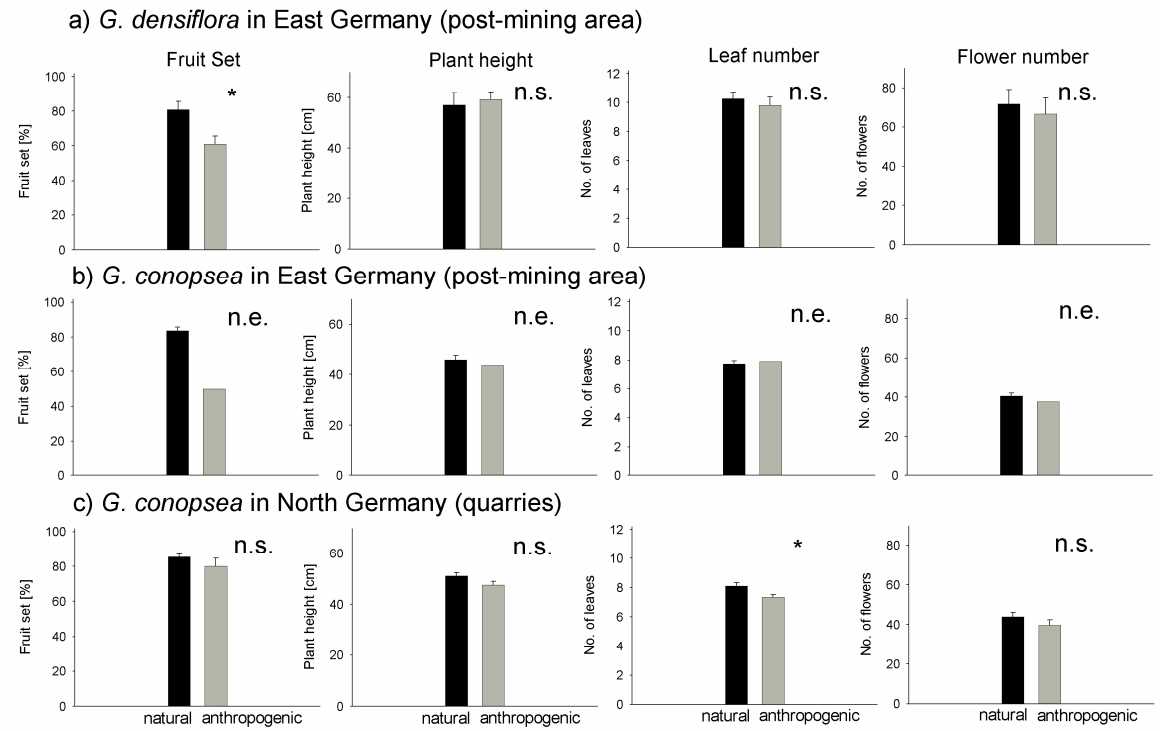

Figure 14: Estimation of fruit set (indicative for reproductive success) and plant performance (indicative for habitat quality) for a) *G. densiflora* populations in East Germany, b) *G. conopsea* populations in East Germany and c) *G. conopsea* populations in North Germany. Due to only one anthropogenic *G. conopsea* population in East Germany a statistical evaluation was not possible (n.e.).

Table 8 Study sites with population (N_{Pop}) and sample size (N_{Sample}), the parameters fruit set, plant height, flower and leaf number as well as estimators of genetic variation, for the diploid *G. densiflora* with the total number of alleles (A_{tot}), average number of alleles per population (A), allelic richness (A_r), gene diversity (H_e) and inbreeding coefficient (F_{IS}) and for the polyploid *G. conopsea* the average number of alleles (A'), average number of phenotypes per population (A'_{phenot}), phenotypic diversity (H'_S) and the Shannon-Weaver phenotype diversity within populations (H^{SW}).

Study sites				**Genetic diversity**							**Reproductive fitness and plant performance**		
***G. densiflora* in East Germany**													
Location	**Code**	**East**	**North**	**N_{Pop}**	**N_{Sample}**	**A_{tot}**	***A***	**A_r**	**H_e**	**F_{IS}**	**Fruit Set [%]**	**Plant Height**	**Flower number**	**Leaf number**
anthropogenic habitats														
Jaucha	EG01	12°11‘	51°14’	1000	28	3	1.5	1.5	0.15	1	70	61.2 ± 1.9	92.6 ± 6.5	11.4 ± 0.5
Theißen	EG02	12°07’	51°08‘	44	30	5	2.5	2.1	0.24	0.29	58	63.9 ± 2.1	50.8 ± 3.4	8.8 ± 0.3
Predel	EG03	12°19‘	51°12‘	40	19	6	3	2.1	0.2	0.19	55	48.8 ± 1.9	49.9 ± 2.8	10.5 ± 0.4
Domsen	EG04	12°17‘	51°18‘	31[1]	31	3	1.5	1.3	0.06	-0.05	n.d.	65.2 ± 3.0	60.8 ± 9.0	8.2 ± 1.2
Espenhain	EG05	12°44‘	51°25‘	8	7	5	2.5	2.5	0.46	0.07	n.d.	57.3 ± 9.1	78.9 ± 21.6	10.1 ± 0.8
natural habitats														
Tote Täler	EG06	11°74‘	51°19‘	1200[2]	27	8	4	3.3	0.58	0.49	77	49.7 ± 2.1	79.5 ± 5.4	10.2 ± 0.4
Rothenstein	EG07	11°57‘	50°86‘	167	27	9	4.5	3.2	0.42	0.02	89	47.6 ± 1.8	51.3 ± 3.4	9.8 ± 0.3
Würze	EG08	11°69‘	50°84‘	140	29	8	4	3.1	0.52	0.08	90	70.6 ± 2.4	95.1 ± 7.4	11.9 ± 0.5
Klingelsteine	EG09	11°64‘	50°98‘	295	27	8	4	2.6	0.5	0.14	64	49.4 ± 1.9	61.7 ± 2.9	9.3 ± 0.3
Jägertalwiese	EG10	11°73‘	50°97‘	182	30	6	3	2.4	0.29	0.2	84	67.9 ± 3.4	70.6 ± 10.3	10.2 ± 0.5
***G. conopsea* in East Germany**														
Location	**Code**	**East**	**North**	**N**	**N_{Sample}**	**A_{tot}**	***A***	**A_{phenot}**	**H'_S**	**H^{SW}**	**Fruit Set [%]**	**Plant Height**	**Flower number**	**Leaf number**
anthropogenic habitats														
Domsen	EG11	12°14‘	51°19‘	29	26	21	4.2	7.4	1.43	1.53	50	43.9 ± 2.0	37.7 ± 3	7.9 ± 0.3
natural habitats														
Rothenstein	EG12	11°58‘	50°86‘	136	26	82	16.4	20.2	3.36	2.76	75	45.5 ± 1.5	41.5 ± 2.2	7.3 ± 0.5
Zietschkuppe	EG13	11°70‘	50°97‘	1000	23	72	14.4	18.8	3.37	2.80	84	42.5 ± 1.9	34.9 ± 2.3	6.9 ± 0.3
Alter Gleisberg	EG14	11°70‘	50°95‘	330	25	78	15.6	20	3.47	2.81	89	54.3 ± 2.0	50.1 ± 3.0	8.0 ± 0.3
Rabis	EG15	11°66‘	50°89‘	120	25	73	14.6	20.4	3.48	2.91	91	46.5 ± 1.8	40.7 ± 2.7	7.3 ± 0.3
Krawinkel	EG16	11°64‘	51°21‘	490	29	72	14.4	21.8	3.13	2.88	88	49.6 ± 2.3	37.1 ± 2.0	7.3 ± 0.3
Steigra	EG17	11°65‘	51°30‘	370	26	65	13	18.2	2.91	2.71	82	49.4 ± 2.5	35.7 ± 2.5	8.2 ± 0.4
Grockstädt	EG18	11.59‘	51°33‘	320	19	50	10	14	2.92	2.44	75	46.5 ± 2.1	42.5 ± 2.6	7.7 ± 0.3
Langer Berg	EG19	11.71‘	51°24‘	900	27	50	10	16.8	2.61	2.55	n.d.	44.2 ± 2.2	43.8 ± 3.6	8.4 ± 0.3
Tote Täler	EG20	11°73‘	51°19‘	1200[2]	29	69	13.8	20.4	3.09	2.75	85	34.9 ± 1.6	37.9 ± 2.2	8.6 ± 0.4

Table 8- *continued*

Study sites				**Genetic diversity**						**Reproductive fitness and plant performance**			
***G. conopsea* in North Germany**													
Location	**Code**	**East**	**North**	**N**	**N_{Sample}**	**A_{tot}**	***A***	**A_{phenot}**	***H'*$_S$**	H^{SW}	**Fruit Set [%]**	**Plant Height**	**Flower number**	**Leaf number**
anthropogenic habitats														
Stb. Polle	NG01	9°40‘	51°89‘	300	25	70	14	19.8	3.31	2.78	94	44.0 ± 1.7	39.1 ± 2.6	7.2 ± 0.3
Stb. ’Schießstand‘	NG02	9°36‘	51°78‘	77	27	76	15.19	21.37	3.41	2.91	93	52.5 ± 2.7	42.8 ± 3.2	7.60 ± 0.3
Alter Steinbruch	NG03	9°36‘	51°78‘	146	25	66	13.2	18.6	3.30	2.68	76	49.7 ± 2.2	46.4 ± 3.6	7.4 ± 0.3
Stb. Hehlen	NG04	9°45‘	51°98‘	40	25	50	10	16.8	2.67	2.55	67	48.4 ± 1.8	45.6 ± 3.6	8.1 ± 0.4
Stb. Bärenbrink	NG05	9°88‘	51°94‘	14	26	76	15.2	20.4	3.39	2.88	70	47.4 ± 2.4	35.6 ± 2.3	6.7 ± 0.4
Stb. Delligsen	NG06	9°81‘	51°95‘	300	27	65	13	17.8	3.09	2.67	81	43.4 ± 1.5	28.9 ± 1.3	7.2 ± 0.3
natural habitats														
Burgberg	NG07	9°51‘	51°87‘	300	25	70	14	19.4	3.24	2.81	87	55.0 ± 2.2	54.8 ± 3.9	8.2 ± 0.2
Rühle	NG08	9°53‘	51°92‘	300	24	74	14.8	20.2	3.64	2.94	88	49.2 ± 1.9	39.5 ± 3.4	7.90 ± 0.3
Räuschenberg	NG09	9°37‘	51°81‘	43	30	45	9	16	2.22	2.39	n.d.	54.7 ± 2.0	42.8 ± 2.6	9.3 ± 0.4
Poppenburg	NG10	9°44’	51°88‘	30	25	64	12.8	19.6	3.33	2.79	88	49.3 ± 1.5	40.2 ± 2.2	7.5 ± 0.2
Bielenberg	NG11	9°35’	51°78‘	750	27	73	14.6	22.6	3.29	2.96	90	54.1 ± 2.2	42.8 ± 2.8	8.4 ± 0.3
Bocksberg	NG12	9°65’	51°88‘	55	25	70	14	20	3.28	2.86	81	46.3 ± 1.6	38.7 ± 2.6	7.9 ± 0.4
Holberg	NG13	9°57’	51°91‘	40	22	64	12.8	15.6	2.94	2.41	80	49.3 ± 2.7	48.8 ± 2.9	7.8 ± 0.4

[1]16 flowering and 15 vegetative individuals; [2] mixed populations with *Gymnadenia densiflora* and *Gymnadenia conopsea*.

Discussion

Genetic diversity and differentiation

We found reduced genetic diversity for the populations in the post-mining area in East Germany, for both *G. conopsea* and *G. densiflora*, but no such effects were detected for the quarry populations in North Germany. Our results indicate that during the colonization of the post-mining area, which was distant from potential source populations, founder effects may occur, whereas this problem seems to be negligible for *G. conopsea* populations in the quarries, which were spatially intermixed with source populations.

Orchids are optimally adapted to wind dispersal due to their minute seeds that are produced in high numbers (Arditti & Ghani 2000). As a consequence, many orchid populations are characterized by a high genetic variation within and low genetic differentiation between populations. Long-distance dispersal events have been documented (Currah et al. 1997), contributing to their well known ability to colonize new or disturbed habitats (Adamowski 1998; Adamowski 2006; Cribb et al. 2003). However, new populations may be founded by only a few individuals and are expected to exhibit lower levels of genetic diversity than long-term established populations (Amsellem et al. 2000; Muluvi et al. 1999; Slatkin 1977). An anthropogenic influence on the genetic diversity was found for e.g. *Epipactis helleborine*, for which the average level of genetic variation was lower for urban than for rural populations (Hollingsworth & Dickson 1997) and for *Saxifraga tridactylites* genetic differentiation between populations on railways and natural habitats indicates reduced gene flow and/or habitat specific selection (Reisch 2007).

A precondition for the colonization of new or highly disturbed habitats is seed arrival. Seed dispersal patterns are determined by the spatial distribution of reproductive adults and their seed outputs (Nathan & Muller-Landau 2000). Mining sites are generally fairly large and in the post-mining area in East Germany large parts of the landscape have been excavated. No natural *Gymnadenia* populations survived in this area and potential source populations were located 25-75 km westwards in another region. Hence, limited seed arrival might be one reason for the reduced genetic diversity of the populations in the post-mining area. This may lead to the establishment of only a few and/or small populations, because except of one all populations in the post-mining area had less than 50 individuals. Small populations tend to have lower levels of genetic diversity than large populations, due to an enhanced effect of genetic drift and an increased probability of inbreeding (Frankham 2005). Although such a correlation between genetic diversity and population size has been reported for many species (e.g. Fischer & Matthies 1998; Hamrick & Godt 1990), it was not found for *Gymnadenia*. This might be due to the fact that all populations of *G. conopsea* are comparatively large, whereas the overall genetic diversity of the diploid *G. densiflora* might be too low to reveal any spatial structure.

Limited seed dispersal reduces gene flow between populations and is expected to lead to genetic differentiation (Loveless & Hamrick 1984). Genetic differentiation as a result of fragmentation and reduced gene flow is well documented in a large number of studies on fragmented and declining species (Galeuchet et al. 2005; Imbert & Lefèvre 2003) and has been reported for orchids too. There are several studies that found a significant spatial genetic structure for some orchid species, e.g. *Caladenia tentaculata* (Peakall & Beattie 1996), *Spiranthes spiralis* (Machon et al. 2003), *Cephalanthera longibracteata* (Chung et al. 2004), *Liparis makinoana* (Chung et al. 2005; Sun & Wong 2001), in most cases due to limited seed dispersal. For *Gymnadenia* an isolation by distance effect was found only for polyploid *G. conopsea*, which is mainly due the genetically differentiated single population in the post-mining area, suggesting restricted gene flow.

On the other hand, however, the number of colonization events and genetic bottlenecks occurring during colonization has been shown to have a large impact on the genetic diversity and structure (Esfeld et al. 2008; Reisch 2007). Esfeld et al. (2008) for example, investigated *Epipactis palustris* in the same post-mining area in East Germany and found no difference between the genetic diversity of populations from disturbed and natural habitats, which is likely due to repeated colonisations from different source populations. We found a high genetic diversity within and low genetic differentiation between populations for *G. conopsea* in North Germany, indicative for high gene flow between populations (Loveless & Hamrick 1984). Compared to the landscape character of a post-mining area, the quarries represent a less intensive disturbance regime and the smaller excavation sites are spatially intermingled with natural habitats. Hence, high levels of gene flow and repeated, independent colonizations from different populations are likely to contribute to the high genetic diversity and low differentiation of these populations. In addition, the generally high diversity of the polyploid *G. conopsea* will further attenuate effects of genetic drift.

However, after seed arrival at a site, colonization depends on the availability of suitable microsites for germination and seedling recruitment (Jersáková & Malinová 2007; Nathan & Muller-Landau 2000). The small populations in the post-mining area in combination with their low genetic diversity indicate low recruitment rates. In the orchid family seedling recruitment is inevitably connected with the availability of compatible mycorrhizal fungi providing nutrition for germination and initial growth (Leake 1994; Rasmussen 1995). Spatial distribution patterns of soil fungi have been found to be determined by e.g. large scale carbon gradients, land use or small-scale soil textures produced by plant growth (Ettema & Wardle 2002; Kasel et al. 2008). As the environmental conditions of anthropogenic habitats are often characterised by extreme conditions (low pH-values, lack of nutrients) (Wiegleb & Felinks 2001), ecological and nutritional differences of mycorrhizal fungi may lead to differentiation of fungal communities between anthropogenic and natural habitats. This has been found for e.g. *Collinsia sparsilfora*, for which serpentine and non-

serpentine ecotypes associated with distinct arbuscular mycorrhizal fungi (Schechter & Bruns 2008), whereas for Estonian mine tailing hills no different mycorrhizal interactions were found when compared to natural habitats (Shefferson et al. 2008). Stark et al. (2009) found a high taxonomic and ecological diversity of the fungal community associated with *G. conopsea* in Germany, with a clear spatial structure suggesting a non-random distribution. The fact that five out of six populations in the post-mining area are of G. *densiflora*, although in the surrounding area predominantly *G. conopsea* occurs, might be indicative for a complex interaction of intrinsic and extrinsic factors determining the fungal communities associated with *G. conopsea* and needs further investigation.

Reproductive success and plant performance

Overall reproductive success in the natural habitats was generally high (82% fruit set). The populations in the post-mining area in East Germany showed a significant reduction in fruit set, whereas no such effect was found for the quarry populations. While for *G. densiflora* no correlation between fruit set and population size or genetic diversity was found, fruit set of *G. conopsea* was positively affected by both genetic diversity and population size. However, plant performance was similar for all investigated populations.

Reasons that may lead to a reduced reproductive success are manifold. Plants are immobile and depend on abiotic and/or biotic vectors to transport pollen for sexual reproduction (Ashman et al. 2004). Hence, an inadequate quantity or bad quality of pollen can reduce plant reproductive success (Bierzychudek 1981). *G. conopsea* and *G. densiflora* depend on pollination by insects for fruit set (Gustafsson 2000) and are pollinated by a variety of Lepidopteran taxa (Meyer et al. 2007; Proctor & Harder 1993). In animal-pollinated plants pollen quantity may be reduced as a result of fewer pollinator visits or less pollen delivered per visit (Ashman et al. 2004). Pollinator abundance has been shown to be strongly influenced by plant population size, because small and isolated populations are faced the problem to sustain pollinator interest (Ackerman et al. 1996; Brys et al. 2008). Jacquemyn et al. (2007) showed that a minimum of 50 flowering individuals is needed to produce a sizeable number of fruits and seedlings for *Orchis purpurea*. Therefore, our result of a correlation of fruit set to population size and the generally small populations in the post-mining area in combination with its landscape character and the surrounding hostile agricultural landscape, indicate that the pollinator community is depauperate and plant-pollinator interactions are disrupted (Cole & Firmage 1984; Dafni & Ivri 1981). Furthermore, the reproductive success of *G. conopsea s.l.* has been shown to be influenced by pollinator group as pollination efficiency varied between nocturnal (86%) and diurnal (67%) pollinators (Meyer et al. 2007). Previous investigations showed that with increasing isolation of a habitat, besides abundance also species richness of pollinators

may decrease (Steffan-Dewenter & Tscharntke 1999). A changing guild composition, due to e.g. unsuitable/changing habitat conditions as caused by proceeding succession of open habitats, is likely to influence the reproductive effect. Hence, changes in plant-pollinator interactions, either due to a decreased abundance or changed guild composition, is likely to impact the reproductive success of *G. conopsea* and *G. densiflora*. However, the degree may be different for the two taxa as their floral scent bouquet differs (Jersáková et al. 2010). Although Jersáková (2010) showed that pollinators (two nocturnal taxa) did not use floral scent to distinguish between the *Gymnadenia* taxa, this may attract different suites of taxa with differing ecological preferences.

Pollen quality, however, can be reduced if self or otherwise incompatible pollen is delivered, for example as a result of inbreeding (Ashman et al. 2004). Inbreeding is a factor that has long been known to have deleterious consequences for reproduction and survival in naturally outbreeding species (Charlesworth & Willis 2009). With decreasing population size, populations are more prone to genetic drift and the probability of selfing or biparental inbreeding increases. As a consequence, small populations are more vulnerable to inbreeding depression (Barrett & Kohn 1991; Falconer & Mackay 1996) as an increasing number of deleterious recessive alleles may become homozygous and will be expressed (Ellstrand & Elam 1993). This has been shown to lead to e.g. an increasing number of abortions and reduced fecundity (Barrett & Kohn 1991; Ellstrand & Elam 1993). We found only limited evidence for an effect of genetic pollen quality, because although relatively high inbreeding coefficients were found for the *G. densiflora* populations in the post-mining area, they were not correlated with fruit set. However, in *G. conopsea* fruit set was positively correlated with genetic diversity, and its effect was much stronger than that of population size.

In addition fruit set may be resource limited when resources are not sufficient to mature all flowers. Hence, unsuitable or suboptimal site conditions like lack of water or nutrients can contribute to a decreased fruit set (Bierzychudek 1981; Horvitz & Schemske 1988; Stephenson 1981). Assuming suboptimal habitat conditions one would expect a reduced plant performance, but a similar plant performance for all investigated *Gymnadenia* populations indicates that resource limitation is negligible. However, for field observations a strict distinction between pollination and resource limitation is difficult to achieve as other factors like the patch size and density of flowering plants, occurrences of competing alternative flowers, abundance of herbivores may additionally influence the actual fruit set in the field (see Steffan-Dewenter & Tscharntke 1999 and references therein).

Conclusions

Our study shows that anthropogenic habitats like post-mining areas and quarries provide valuable habitat for endangered species like e.g. orchids. Anthropogenic habitats contribute to the conservation of biological diversity not only on the level of species diversity, but also on the level genetic diversity. Hence, they can play a key role for the maintenance of biodiversity, for example in intensively used landscapes. If so, they are worth legal protection and should be an integral part of national conservation programs.

On the other hand our study emphasizes the important role the intensity, size and frequency of disturbance events may play for the colonization process (Turner et al. 1998). The quarries were relatively small, and of considerable age and are spatially intermingled with natural habitats. This favours high gene flow via seed dispersal and pollinator activity by which populations become functionally connected. In contrast, in the post-mining area in East Germany natural populations are distant and spatially separated in another region. Our results indicate that both seed arrival and pollinator activity might be influenced negatively, leading to genetic depauperation and the establishment of only small and isolated populations. The combination of small population size, low genetic diversity and reduced fruit set may threaten the long-term persistence of these populations. Nevertheless, in this intensively used agricultural landscape which is bare of structural elements, the post-mining area plays an important role for the maintenance of biodiversity. However, in order to prevent succession and to ensure that populations are functionally connected, appropriate management strategies like e.g. mowing or grazing are essential.

Synthesis

Today human impact is the main driver of global environmental change (Rockström et al. 2009). One of the most severe anthropogenic changes is land transformation, which has altered 40- 50% of Earth's surface into urban and agricultural systems (Chapin et al. 2000) and 1% is represented by mining areas (Walker 1992). Direct consequences of land transformation are habitat loss, degradation and fragmentation, the most important causes of species extinctions. Indirect consequences are e.g. the loss of genetic diversity and locally adapted populations, inbreeding due to decreasing population sizes and breakdown of ecological connections (e.g. mycorrhizal associations, pollinator services) (Vitousek et al. 1997). These aspects are diminishing the chances of population persistence and influence the future survival of a species in the wild.

Orchids are particularly vulnerable to environmental changes, because with germination and pollination two important life cycle stages directly depend on symbiotic interactions (Rasmussen 1995; Swarts & Dixon 2009b). As a consequence, the persistence as well as the establishment of new populations are determined by a highly interlinked combination of abiotic and biotic factors (Swarts & Dixon 2009b). Therefore a thorough understanding of orchid biology and the factors that determine habitat suitability and orchid distribution is essential for an effective conservation of orchid diversity. Using *Gymnadenia conopsea s.l.* as study system, a common orchid that occurs over a wide ecological amplitude, this thesis addresses aspects of orchid biology that might be critical for orchid recruitment and the long-term persistence of populations in the wild, namely the specificity of the mycorrhizal symbiosis, population genetic diversity and genetic differentiation.

Fungi from the roots of the common terrestrial orchid Gymnadenia conopsea

A key feature for the persistence and establishment of orchid populations in the wild is the recruitment of young individuals. In the orchid family, this is inevitably connected with the availability of mycorrhizal fungi providing nutrients for germination and initial growth (Leake 1994; Rasmussen 1995). As a consequence of this obligatory symbiotic relationship, the degree of specificity between orchid and fungus influences the chances of successful seedling establishment (Bidartondo & Read 2008) and is likely to be a determinant factor also for the colonization ability of a species. In a first study (chapter 1) the fungal communities of six *G. conopsea s.str.* populations in two study regions in East and North Germany were analysed. The 28 identified taxa revealed a high diversity of fungi associated with *G. conopsea.* A wide variety of fungi associating with an orchid indicates a low specificity of the orchid-mycorrhizal symbiosis (Timms & Read 1999). Species with a broad taxonomic spectrum of potential fungal partners are expected to have relatively wide distributions and good colonisation abilities as the probability to find a compatible fungus after dispersal should be high (Batty et al. 2002; Currah et al. 1997). Previous investigations on Australian orchids showed that an invasive species that is rapidly spreading over the continent

and a native, widespread species that occupies an unusually diverse range of habitats have broad webs of compatible fungi (Bonnardeaux et al. 2007). The high diversity of fungi found to associate with *G. conopsea* indicates that this orchid shows only little specificity to certain fungal clades, which is likely to contribute to its ability to grow in very different habitat types with the respective fungal communities.

Most so far identified orchid mycorrhizas (OM) are Basidiomycetes of the *Rhizoctonia* group (Rasmussen 1995; Warcup & Talbot 1967; Warcup & Talbot 1971). In all populations taxa of the known OM genera *Tulasnella*, *Ceratobasidium*, *Thanatephorus* and *Sebacina* (Moore 1987; Warcup & Talbot 1967) were detected, suggesting that *G. conopsea* utilizes typical OM fungi as mycorrhizas. However, also ascomycetous taxa from the Pezizales and Helotiales were identified that are known to form ectomycorrhizas on other plants. So far these morphologically delicate fungi have been less studied than their basidiomycetous counterparts and comparatively little is known about their taxonomy and ecology (Tedersoo et al. 2006). Hence, their importance as mycorrhizas is probably seriously underestimated (Egger 2006). The continuous detection in the roots of *G. conopsea* indicates that this orchid is also able to utilize ascomycetous ectomycorrhizal taxa. The replacement of the usual *Rhizoctonias* by ectomycorrhizas may be a strategy to secure access to fungal carbohydrates where *Rhizoctonias* are either not available or where photosynthesis rate is limited due to insufficient light availability like in forest habitats (Selosse et al. 2004). *G. conopsea* is typically occurring in open grassland sites, but it is also found in shaded forest habitats (e.g. Gustafsson 2000; Scacchi & de Angelis 1989). Hence, the adoption of ectomycorrhizal taxa as orchid mycorrhizas would mean a more stable carbon resource and could be another aspect contributing to its ability to grow in a variety of different habitats.

Strong genetic differentiation between Gymnadenia conopsea and G. densiflora despite morphological similarity

Gymnadenia conopsea (L.) R.BR. *s.l.* is a controversial taxon. The two most commonly distinguished taxa are *G. conopsea* (L.) R.BR. ssp. *conopsea* and *G. conopsea* ssp. *densiflora* (WAHLENB.) K. RICHT., for which also a species status has been suggested (Bateman et al. 1997; Campbell et al. 2007; Marhold et al. 2005). The taxa are described to differ in morphology, phenology, scent emission and habitat preferences (e.g. Jersáková et al. 2010; Marhold et al. 2005). Genetic differentiation has been found between flowering time variants (Gustafsson & Lönn 2003) and ecotypes (Scacchi & de Angelis 1989). Hence, for taxon assignment populations were morphologically and genetically analysed and in order to investigate the relationships on a larger geographic scale, additional samples from other European regions were included (chapter 2). The analysis of the ITS region revealed a 2% nucleotide divergence, similar to the divergence between

other *Gymnadenia* species. This, together with largely non-overlapping sets of microsatellite alleles supports the view that *Gymnadenia conopsea* (L.) R.BR. *s.str*. and *Gymnadenia densiflora* (WAHLENB.) DIETRICH deserve species status. *G. conopsea* s.str. and *G. densiflora* are not even sister species as the sequences of *G. densiflora* form a well supported monophyletic group, sharing a most recent common ancestor with *G. nigra* and *G. austriaca*. The evolutionary history of *G. conopsea s.str*. remains unclear, because based on ITS data it cannot be separated from the morphologically well distinguished *G. odoratissima. G. conopsea s.str*. was either diploid or tetraploid, while *G. densiflora* was diploid throughout. As the microsatellite patterns of the two ploidy levels of *G. conopsea s.str.* were hardly differentiated and the most frequent ITS haplotypes occurred in both of them, an autopolyploid origin of tetraploid from diploid *G. conopsea s.str*. is likely. This suggests that *G. conopsea s.str.* and *G. densiflora* have separated prior to the split of other groups and that this occurred before polyploidy has arisen in *G. conopsea s.str*.

However, morphological differentiation is less clear. Although some traits (e.g. flower number and density) allow a fairly good distinction, considerable variation makes an unequivocal identification difficult. Orchids are prime examples for selection on flower morphology by insect pollinators (Thompson 1994). Both *G. densiflora* and *G. conopsea* s.str. have fairly specialized, rewarding flowers and are pollinated by the same taxa of moth. Thus, it may be hypothesized whether convergent selection of pollinators has led to similar flower morphology. Furthermore, as *G. densiflora* is described to be strongly scented and *G. conopsea* s.str. appears to be less scented (Schmeil 1996) the latter may be under selection to morphologically resemble *G. densiflora* to attract the same pollinators.

Another aspect that is considered to contribute to orchid diversification is their symbiotic relationship with mycorrhizal fungi (Waterman & Bidartondo 2008). For example, the phylogenetic divergence of floral variants within the *Hexalectris spicata* complex is partly attributed to differences in the fungal associates (Taylor et al. 2003). Fine-scale distribution patterns are thought to promote diversification. Taxon richness of the fungal community associated with *G. conopsea* ranged from 5 to 14 taxa per population and the regions shared only 43% of the taxa (chapter 1). Therefore, both the overall similarity between, and the morphological variation within the two species may be related to its association with mycorrhizal fungi. So far nothing is known about fungi associating with *G. densiflora* and further studies are needed to investigate the role of mycorrhizal fungi for phylogenetic and ecological differentiation between of *G. conopsea s.str*. and *G. densiflora*.

The value of anthropogenic habitats for conservation: A case study on G. conopsea and G. densiflora

Mineral extraction represents one of the most severe anthropogenic changes as it destroys the original vegetation and soil structure. On the other hand mining activity creates new habitats, because at these sites a diverse array of substrates with special physico-chemical properties provides habitat for specialized and therefore endangered species (Brändle et al. 2003; Ratcliffe 1974; Varela et al. 1993). However, in order to contribute to the conservation of endangered species, the genetic diversity of the respective populations has to be comparable to this one of natural habitats. Hence, in a third study (chapter 3) the genetic diversity and general plant performance of *G. conopsea* and *G. densiflora* populations of anthropogenic habitats (lignite post-mining area in East Germany and quarries in North Germany) were compared with populations of surrounding natural sites. The results revealed a reduced genetic diversity and lowered fruit set for the populations in the lignite post-mining area, whereas no such effects were found for the quarry populations. However, the general plant performance was similar for all investigated populations.

The results indicate that the size and intensity of a disturbance event may play an important role for the colonization of new habitat. Two important factors that influence the colonization process are seed dispersal and seedling recruitment (Vekemans & Hardy 2004). Seed dispersal patterns are determined by the distribution of reproductive adults and their seed outputs (Nathan & Muller-Landau 2000). Mining sites are often fairly large and distances to potential seed sources might be great, like in the post-mining area in East Germany, where natural *Gymnadenia* populations were distant in another region. Here, limited seed arrival may be one reason for the reduced genetic diversity, leading to the establishment of only a few and/or small populations. The reduced fruit set may be a consequence of altered pollinator interactions, either due to reduced abundance or changed species composition; and there was limited evidence for inbreeding depression due to increased, but not with fruit set correlated inbreeding coefficients for *G. densiflora* in the post-mining area, and a correlation between fruit set and genetic diversity for *G. conopsea*. Hence, the combination of small population sizes, low genetic diversity and reduced fruit set may threaten the long-term persistence of these populations.

Human impact on the genetic diversity of plants has also been found for e.g. *Epipactis helleborine,* for which the average level of genetic variation was lower for urban than for rural populations (Hollingsworth & Dickson 1997). However, no difference between the genetic diversity of populations from anthropogenic and natural sites was found for *Epipactis palustris,* investigated in the same post-mining area in East Germany (Esfeld et al. 2008). These results are in line with those for the quarry populations of *G. conopsea* in North Germany. Here, smaller excavation sites are spatially intermingled with natural sites, favouring repeated and independent colonisations from

different source populations. Hence, high levels of gene flow, either by seed dispersal or pollinator activity, prevent founder effects and contribute to the high genetic diversity and weak genetic differentiation of these populations.

However, the small population sizes in the post-mining area may also be indicative of low recruitment rates, because germination depends on the availability of suitable microsites (Nathan & Muller-Landau 2000). In the orchid family recruitment success depends on the presence of compatible mycorrhizal fungi (Jersáková & Malinová 2007). Spatial distribution patterns of soil fungi have been found to be determined by e.g. large scale soil carbon gradients, land use or small-scale soil textures produced by plant growth (Ettema & Wardle 2002; Kasel et al. 2008). As the environmental conditions of anthropogenic habitats are often characterized by extreme conditions (low ph-values, lack of nutrients), ecological and nutritional differences of mycorrhizal fungi may lead to differentiation between fungal communities of anthropogenic and natural habitats. This has been found for e.g. *Collinsia sparsilfora*, for which serpentine and non-serpentine ecotypes associated with distinct arbuscular mycorrhizal fungi (Schechter & Bruns 2008). The regional differentiation of the fungal community associated with *G. conopsea* together with the high variability on the population level (chapter 1) suggest that factors at the local scale may strongly affect local species composition and hence diversity at the regional level. The fact that five out of six populations in the post-mining area are of G. *densiflora*, although in the surrounding area predominantly *G. conopsea* occurs, might indicate a complex interaction of intrinsic and extrinsic factors determining the fungal community associated with *G. conopsea* and *G. densiflora*.

The results of this thesis show that anthropogenic habitats contribute to the conservation of biological diversity not only on the level of species diversity, but also on the level genetic diversity. Mining sites typically contain periodically disturbed, early successional and heterogeneous surfaces, with extreme abiotic conditions and minimum productivity (Novák & Prach 2003; Schulz & Wiegleb 2000). Similar conditions have become rare in modern landscapes, because humans are increasing the productivity of land, which favours middle phases of succession (Hoekstra et al. 2005). Hence, in many regions species depending on early successional and sparsely vegetated habitats are highly threatened and post-mining sites provide valuable habitat in otherwise industrialized landscapes (Lundholm & Richardson 2010; Thomas et al. 1994; Wenzel et al. 2006).

On the other hand, however, the results emphasize how important it is that populations are connected via gene flow in order to prevent founder effects. Hence, in order to prevent succession and to ensure that populations are functionally connected, appropriate management strategies like e.g. mowing or grazing are essential.

G. conopsea s.l. is still relatively common and the low specificity of the orchid-fungus-relationship is likely to contribute to its ability to grow in very different habitats. However, *G. conopsea s.l.*

comprises the two genetically divergent species *Gymnadenia densiflora* (WAHLENB.) DIETRICH and *Gymnadenia conopsea* (L.) R.BR. *s.str*. So far little is known of *G. densiflora* as a separate species. For example, differences in the diversity and composition of the fungal community associated with *G. densiflora* and *G. conopsea* may lead to different habitat preferences and colonization abilities. Future investigations should include questions like e.g. which role genetic divergence has for the fungal community associated with the two taxa and how this might influence their distribution and colonization ability, of e.g. anthropogenic habitats. Little is known about the factors that determine the diversity and composition of fungal communities associated with orchids and its consequences for habitat suitability, population dynamics and genetics. Hence, *G. conopsea*/ *G. densiflora* is a good study system to gain a better understanding of orchid biology and which role ecological and genetic factors play for orchid diversification and distribution.

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Eigenständigkeitserklärung

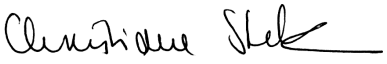
Hiermit versichere ich, dass ich meine Dissertation mit dem Titel

„Population genetics on anthropogenic and natural sites, subspecies differentiation and fungal community of *Gymnadenia conopsea s.l.* (Orchidaceae)"

selbständig und ohne unerlaubte Hilfe angefertigt habe. Ich habe mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient. Die den benutzen Werken wörtlich oder inhaltlich entnommenen Stellen wurden als solche von mir kenntlich gemacht.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Ich erkläre weiterhin, dass ich mich noch nie um einen Doktorgrad beworben habe.



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Work Experience and Education

Since 07/ 2008	Scientific officer at the Federal Environment Agency (UBA) FG IV1.1 International Chemicals Management, Dessau Germany
06/ 2008 - 01/ 2005	PhD student at the Helmholtz Centre for Environmental Research (UFZ), Department of Community Ecology, Halle/S. Germany
12/ 2004 - 07/ 2004	Research Assistant at the Max-Planck-Institute for Limnology (since 2007 MPI for Evolutionary Biology), Plön Germany
06/ 2004 – 10/ 1997	Studies of Biology at the Philipps-University Marburg, Germany Diploma thesis at the Leibnitz Centre for Agricultural Landscape and Land Use Research (ZALF), Müncheberg, Germany: "Comparative genetic studies on bolters and varieties of *Beta vulgaris* (L.) on selected fields in Brandenburg, Germany."
06/ 1997	A-Levels at the Sophie-Hedwig-Gymnasium in Diez, Germany

International Experience

09- 08/ 2002	Work placement at Sturt National Park, New South Wales, Australia
06/ 2001- 10/ 2000	Erasmus student at Imperial College of Science, Technology & Medicine, London Great Britain
06- 04/ 2001	Trainee and Final Year Project at the "Web of Science" at London Zoo: "Distribution mapping of endemic and introduced snail species on Tahiti in French Polynesia"

Publications

Hauck, M. & C. Stark (2010): The Importance of Large-Scale Cattle Pastures as a Flower Resource. In "Large-scale Livestock Grazing. A Management Tool for Nature Conservation". Harald Plachter & Ulrich Hampicke (Eds.) 478 pp, Springer-Verlag Berlin Heidelberg.

Stark, C., Babik, W. & W. Durka, (2009): Fungi from the roots of the common terrestrial orchid *Gymnadenia conops*ea. Mycological Research 113, 952-959.

Stark C., Liepelt S., Dieckvoss M., Bartsch D., Ziegenhagen B. & A. Ulrich (2006): Fast and Simple Monitoring of Introgressive Gene Flow from Wild Beet into Sugarbeet. Journal of Sugar Beet Research (43), 145- 154.

Stark, C., Babik, W., Michalski, S.G., Winterfeld, G. & W. Durka. Strong genetic differentiation between *Gymnadenia conopsea* and *G. densiflora* despite morphological similarity. (submitted to Plant Systematics and Evolution).

Stark, C. & W. Durka. The value of anthropogenic habitats for conservation: A case study on *Gymnadenia conopsea* and *G. densiflora*. (to be submitted).

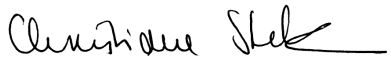
Talks (selected)

Stark, C. (2009): Von der Legaleinstufung zur Harmonisierten Einstufung. REACH in der Praxis- Fachworkshop „Die Umsetzung des GHS in die Praxis - Orientierung & Unterstützung für kleine und mittlere Unternehmen". 06. Oktober 2009, Bundespresseamt Berlin/ Germany.

Stark C. & W. Durka (2007): The importance of anthropogenic habitats for the maintenance of the genetic diversity of an orchid species. International Workshop on Molecular Methods in Conservation, 10. - 14. October 2007, Bialowieza/ Poland.

Stark C. & W. Durka (2007): The role of anthropogenic habitats as substitutes for natural habitats: a case study on *Gymnadenia conopsea*. Annual Conference of the Ecological Society of Germany, Austria and Switzerland, 10. - 14. September 2007, Marburg/ Germany.

Stark C. & W. Durka (2006): Genetische Untersuchungen an *Gymnadenia conopsea*. 13. Wuppertaler Orchideentagung/ Naturwissenschaftlicher Verein Wuppertal, 11.- 12. November 2006, Wuppertal/ Germany.



Halle/ Saale, den 18.12.2010