Relicts Within the Genus Complex *Astragalus/Oxytropis* (Fabaceae), and the Comparison of Diversity by Objective Means

Matthias Schlee, Markus Göker, GuidoW. Grimm, and Vera Hemleben

Abstract *Astragalus* and *Oxytropis* represent one of the largest angiosperm genus complexes. It is widely discussed why – and if at all – it contains so many species. Here, we discuss how the genetic divergence in a postglacial relict species, *Oxytropis pilosa*, can provide insights into the relative genetic variance of taxa. Methods are introduced to obtain sequence clusters of similar genetic diversity and to detect statistically significant differences. These approaches provide objective means to identify taxa that deserve the same protection efforts, independent of a fixed species concept. *O. pilosa* ITS sequences display an inter- and intra individual variance, comparable to clusters comprising numerous accepted species. Regarding genetic diversity, some *O. pilosa* populations represent relicts that deserve the same or a higher protection status than some species of *Astragalus*. We discuss the need of objectivity in identifying the evolutionary hot spots as well as the measures to define and protect them.

1 Introduction

Astragalus (~2.500 species; Podlech 1986) and *Oxytropis* (~300 species) are closely related genera, representing one of the largest angiosperm genus complexes. Taxonomic instability within the genus complex comprising both *Astragalus* and

M. Schlee (✉) and V. Hemleben
Center for Plant Molecular Biology (ZMBP) – General Genetics, University of Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany
e-mail: matthias.schlee@gmx.de

M. Göker
DSMZ – German Collection of Microorganisms and Cell Cultures, Inhoffenstraße 7B, D-38124 Braunschweig, Germany

G.W. Grimm
Department of Palaeobotany, Swedish Museum of Natural History, Box 50007, S-10405 Stockholm, Sweden

J.C. Habel and T. Assmann (eds.), *Relict Species: Phylogeography and Conservation Biology*, 105
DOI 10.1007/978-3-540-92160-8_5, © Springer-Verlag Berlin Heidelberg 2010

Oxytropis results in a high number of similarities, which may differ in their assignment to the genus. In fact, the separation of both genera is based on a single morphological criterion only: *Astragalus* exhibits a mucronate keel along the adaxial side of the legume, whereas *Oxytropis* is characterized by an abaxial carinate legume (Candolle 1802; Barneby 1964; Chater 1968). Molecular studies encountered difficulties in providing sufficient resolution at the species level, independent of the sequence marker used (nuclear and/or chloroplast DNA spacer: Sanderson 1991; Sanderson and Doyle 1993; Wojciechowski et al. 1993, 1999; Kazempour Osaloo et al. 2003). Efforts in chemotaxonomy were promising (Williams and Barneby 1977), but have been only sporadically applied (Tin et al. 2007).

It has been widely discussed why so many species are recognized for *Astragalus* (Sanderson and Wojciechowski 1996), but it has seldom been addressed whether this large number of species, which is continuing to increase in local floras, optimally represent the natural relationships. *Astragalus* underwent a considerable radiation in all its main centers of biodiversity: Eurasia (mainly Southwest Asia), Africa, North America, and South America. The high number of morphologically defined species – and varieties – is caused by numerous endemics, often referred to as "relict" species because of their isolated mountainous stands and small populations. It is rare to find widespread *Astragalus* species. *Astragalus* covers various climates within its biogeographical areas, such as continental to arctic steppes, stony alpine slopes, and Mediterranean mountainous rocky places or even tropical African highland (Barneby 1964; Gillett 1964; Chater 1968). The preference for mountainous habitats, in conjunction with (post)glacial rapid environmental changes, may have facilitated differentiation processes leading to a split up into numerous species and varieties.

Oxytropis shows a quite similar distribution area in Eurasia and North America compared to *Astragalus*, but is absent from South America. This distribution is comparable to that of another large genus – *Pedicularis* (Sauer et al. 1990). *Oxytropis* exceeds more to the (arctic) North of the Northern Hemisphere than *Astragalus*, and some remarkable species such as *O. campestris* with its distinct sub species can be found on both continents. *Oxytropis pilosa* (Fig. 1) is a rare relict species exhibiting a highly fragmented distribution area. It is mainly found in the Pannonian Basin (Hungary to Austria) and in the Alps with prompt occurrences in Scandinavia (including the seashore of Gotland), the Baltic as well as in the Abruzzo (Hegi 1924; Meusel et al. 1965; M. Schlee, pers. observation). In a number of other accounts including *O. pilosa* (M. Schlee, G.W. Grimm, M. Göker, V. Hemleben, in prep.), the internal transcribed spacers (ITS1 and ITS2) of the nuclear ribosomal DNA (nrDNA) allowed reconstruction of detailed evolutionary pathways (Schlee et al. 2003; Denk and Grimm 2005; Grimm et al. 2007) as they are biparentally inherited, part of a multicopy gene region and undergo (to some degree incomplete) concerted evolution as well as frequent intragenomic recombination (Arnheim et al. 1980; Hemleben et al. 1988; Baldwin et al. 1995; Volkov et al. 2007). We detected significant intraspecific and intraindividual genetic variability in *O. pilosa* by cloning and sequencing a large number of nrDNA internal transcribed spacer sequences (ITS1 and ITS2; M. Schlee, G.W. Grimm, M. Göker,

Fig. 1 *Oxytropis pilosa*, xerothermic habitat on gypsum, Ascoli Piceno, Marche, Italy

V. Hemleben, in prep.). The variability found allows discussion of migratory and evolutionary patterns for this species, which will form the subject of an upcoming study.

Here, we make use of the large amount of sequence data obtained for *O. pilosa* to conduct a comparison of the genetic diversity of clades by objective means. This is done to find an alternative to classical methods, such as morphotaxonomy-based approaches for the identification of units that represent hot spots of biodiversity (here, genetic resources). We apply non-hierarchical single-linkage clustering to the combined *Astragalus* and *Oxytropis* ITS dataset after determining the distance threshold that optimally delimits *O. pilosa*. Thus, the resulting clusters represent estimates for species boundaries obtained by the use of an operational and fully-automated method treating *O. pilosa* as the standard. The clusters are expected to be uniform, given the distance threshold and the clustering algorithm (i.e., non-hierarchical single-linkage, NHSL). The obtained clusters are discussed with respect to the taxonomic affiliation (assigned species) of the comprised sequences. A character-based approach is used to determine whether the genetic diversity of selected clusters is significantly distinct. If one agrees that in conservation management major emphasis needs to be laid on equal protection efforts for taxa that are equally diverse (Watt 1947; Stock et al. 1999; Potthast 2000; Schlee 2004), it is crucial to compare diversity by appropriate means. Taking into account that taxonomic affiliations and taxonomic ranks often only poorly reflect relative genetic variance, we wonder whether they are sufficient to determine valuable genetic resources. Here, we thus address the question if biodiversity assessment based on substantial molecular data is superior to estimating diversity from data linked to the recognition of morphospecies and if the proposed techniques are valuable to estimate the relative need for conservation efforts as a means to discriminate protection units. Finally, we

briefly discuss the conservation measures that are appropriate in the case of *O. pilosa*, once the most valuable genetic resources have been identified.

2 Material and Methods

In addition to our own dataset of *Oxytropis* ITS clones comprising the ITS1, 5.8S rDNA, and ITS2 regions, and flanking 18S and 25S rDNA (also including some European *Astragalus*; EMBL accession numbers AM401376 to AM401574; AM943374 to AM943384; FM205750 to FM205773), we added all ITS sequences stored in DDBJ, EMBL, and NCBI gene banks (sequences usually obtained by direct sequencing, not by cloning; downloaded on January 2008) for the genera *Astragalus* and *Oxytropis*. Solitary ITS1 and ITS2 sequences were combined if they belonged to the same source according to the original literature/description. The sequences were aligned using the POA program (Lee et al. 2002). The 5.8S rDNA was excluded from the analyses. Phylogenetic inference was performed under the maximum likelihood (ML) optimality criterion using RA x ML 7.0 (Stamatakis 2006; Stamatakis et al. 2008). The program implements a new fast ML bootstrapping and subsequent search for the best topology. Duplicate sequences were eliminated prior to ML analyses. Tree inference and 100 bootstrap replicates were conducted under the CAT approximation (Stamatakis 2006), but final parameter optimization was done under a GTR+Γ model. A plain text (NEXUS) file containing the complete alignment, GenBank accession numbers, and information on the sets of identical sequences reduced by RAxML to a single one, respectively, is available at http://www.goeker.org/mg/clustering/.

To obtain a non-nested sequence classification based on the given distance (or similarity) threshold, we use NHSL clustering, as in the case of the popular tool blastclust (ftp://ftp.ncbi.nih.gov/blast/documents/blastclust.html; regarding hierarchical single-linkage clustering, see, e.g., Legendre and Legendre 1998, pp. 308–312). NHSL is based on the notion of a "link". A link is defined as any distance between two objects (here, sequences) that is smaller than or equal to the predefined threshold. NHSL starts by assigning the first object to the first cluster. For each of following objects in turn, it assigns that object to the same cluster than a previously clustered object if the distance between them is a link. If no such previously classified object is found, the current object is assigned to a new cluster. If several such previously classified objects are found that belong to distinct clusters, these clusters are joined.

Obviously, larger threshold values will result in larger but less numerous clusters, whereas small thresholds will lead to numerous small clusters. A biologically sensible threshold is usually not known a priori. However, a particularly well studied (and monophyletic) taxon (here, *O. pilosa*), can be used as a standard by determining the lowest possible threshold that results in all sequences obtained from that taxon being assigned to a single cluster. It is easy to implement an algorithm that calculates this value for a predefined group. Wirth et al. (1966, p. 61) who used similarities

instead of distances define "…a similarity value, *c*, which is the largest fixed linking similarity value for which the cluster is still an interlinked aggregate of specimens". For each of the *n* objects within the group, the distance to the least distant object that belongs to the same group is determined; the largest of these *n* values represents the result, analogous to *c*. This is due to the fact that in NHSL, a *single* link is sufficient for an object to be assigned to a cluster. This algorithm and NHSL have been implemented in the program OPTSIL (Göker et al. 2009) which is available upon request. The determination of the standard threshold and NHSL relied on uncorrected distances (also called "Hamming" or "*p*" distances; e.g., Swofford et al. 1996, p. 455), which represent the relative number of deviations between two sequences. For downloaded sequences, we relied on the NCBI taxonomy provided in the same files to assign them to genera and species. The optimal threshold calculated for *O. pilosa* was then applied in NHSL.

A disadvantage of the NHSL approach is that clustering methods such as single-linkage clustering cannot be considered as valid methods of phylogenetic inference, mainly because lower pair wise distances (or higher similarities) do not necessarily indicate a closer phylogenetic relationship (e.g., Felsenstein 2004, pp. 165–167). This issue has led to the widespread avoidance of UPGMA clustering (Sokal and Michener 1958) in phylogenetic studies. However, the difficulties may be tempered or even disappear if non-nested clustering is applied and if species are to be distinguished, because within-species sequence dissimilarities are expected to be rather low. The same rationale applies to the usual arguments against uncorrected distances because unobserved, superimposed nucleotide substitutions only play a role if distances are large (e.g., Felsenstein 2004, p. 158).

Göker and Grimm (2008) used the well-known Shannon entropy formula (Shannon 1948) to calculate the character data of *hosts* (plant individuals) from the character data of their *associates* (cloned sequences obtained from the respective plant individuals). For all sequences belonging to the same individual, the entropy of each alignment column was calculated to represent the amount of genetic divergence within each individual using the program G2CEF designed and implemented by M. Göker (downloadable from http://www.goeker.org/mg/distance/). An alignment of length *n* will thus result in *n* corrected entropy values per group. Because the variance in nucleotide characters may depend on the number of sequences, each entropy value was corrected by division through the maximum possible entropy for the given number of associates, which is 0 in the case of a single associate. Accordingly, the corrected entropy values for individuals present with a single sequence only are undefined.

For each pair of groups, *n* differences between the *n* corresponding corrected entropy values can be determined. Subsequently, a non-parametric Wilcoxon signed-rank test or a parametric t-test (or any other appropriate statistical test) can be applied to assess whether the distribution of these differences significantly deviates from 0 and, thus, the two original entropy distributions are significantly different from each other. This procedure is similar to paired-site tests used to assess whether the scores of two phylogenetic trees are significantly different, given a sequence alignment of the same taxa (Felsenstein 2004, p. 364 ff.). Pair wise tests

were conducted with R (R Development Core Team 2005) and restricted to those clusters obtained by NHSL that comprised more than five sequences.

3 Results

Our final data matrix comprised 437 ITS sequences of *Astragalus* and *Oxytropis* species comprising 464 characters from ITS1 and ITS2. Of these 437 sequences, 184 were exact sequential duplicates of others; 291 of the 464 characters were variable and 195 were parsimony-informative. After elimination of duplicates, the alignment analyzed under ML comprised 358 distinct alignment patterns, including a proportion of gaps and completely undetermined characters of 7.48%. The best ML tree obtained is shown in Fig. 2. The tree was rooted along an unresolved backbone polytomy giving respect to a bipartition that separated all *Astragalus* s.str. from the remaining *Astragalus* lineages and *Oxytropis* (in 98% of ML bootstrap replicates). The remaining backbone could be considered to be rather well-resolved, indicating several lineages with bootstrap support (BS) between 70 and 100. Towards the tip of the tree, a general decrease in BS was observed (details not shown). Particularly, the well-known *Neo-Astragalus* clade received little BS. Multiple accessions of the same *Astragalus* species (also listed in Table 1) were found within the same clade (with varying support), as were all ITS sequences of *O. pilosa* (BS = 74, new cloned data plus one directly sequenced NCBI GenBank accession). In contrast, the two other extensively sampled species of *Oxytropis*, *O. arctica* and *O. campestris*, were intermixed with each other and with several other *Oxytropis* species.

Optimal distance thresholds calculated for the present species are shown in Table 1. We obtained an optimal value for *O. pilosa* of about 0.0069. Results of NHSL obtained with the optimal threshold are shown together with the ML tree in Fig. 2.

The largest clusters and their properties are shown in Table 2. *Oxytropis campestris* appeared inseparable from *O. arctica* as well as from a number of less intensively sampled *Oxytropis* species. The clusters 39, 42, 44 and 45 each contained a number of distinct *Astragalus* species, indicating that genetic divergence within many accepted *Astragalus* species is much lower than within either *O. pilosa* or the *O. arctica–O campestris* complex.

The results of the Wilcoxon signed-rank and t-tests, which are shown in Table 3, confirmed this conclusion. According to the Wilcoxon test, genetic divergence of *O. pilosa* was not significantly different from the *O. arctica–O. campestris* complex, but significantly different (lower) from *Astragalus* clusters 44 and 45 (species numbers given in Table 2), and significantly different (higher) from *Astragalus* cluster 42 and *Astragalus* cluster 39, respectively. According to the t-test, genetic divergence of *O. pilosa* was not significantly different from any of the other clusters, whereas the *O. arctica–O. campestris* complex was significantly more divergent than *Astragalus* clusters 42 and 39, respectively.

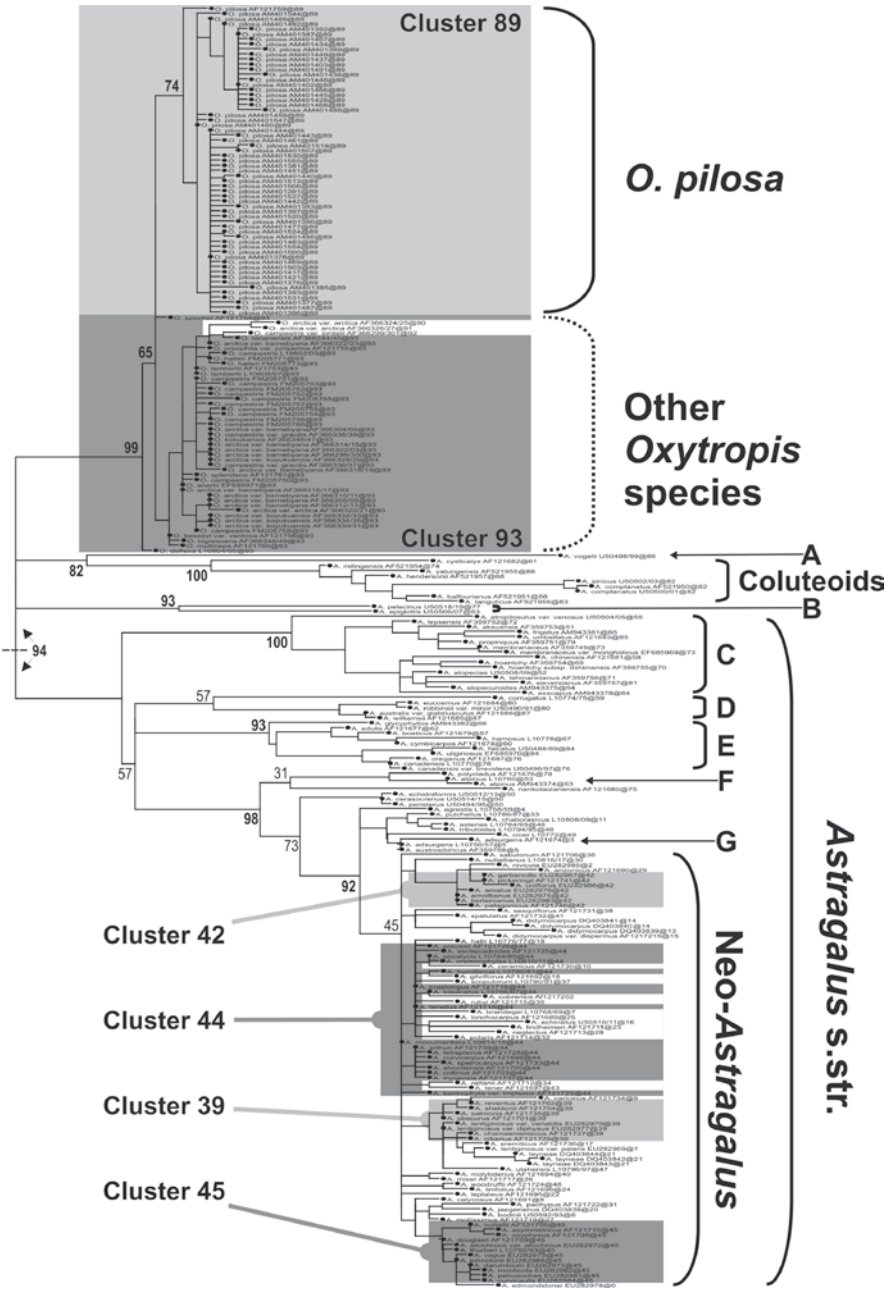

Fig. 2 ML phylogram based on ITS data of *Astragalus* and *Oxytropis*, with major clusters (see text) and clades annotated. Numbers at nodes indicate non-parametric bootstrap support based on 100 ML bootstrap replicates. Clusters (see text) are highlighted by distinct background shading, clade names are given at right. A, *A. vogelii;* B, *A. epiglottis* and *A. pelecinus;* C, clade of Eurasian *Astragalus;* D, *A. corrugatus* and allies*;* E, clade of Eurasian and North American *Astragalus*; F, *A. alpinus*; G, *A. adsurgens* (cf. Table 1)

Table 1 Optimal distance thresholds (i.e., smallest thresholds for which the group is retained in a non-hierarchical single-linkage clustering; NHSL) for the *Astragalus* and *Oxytropis* taxa present in the molecular dataset

Taxon	Optimal threshold	Number of sequences
A. hoantchy, clade C	0.01609195	3
A. didymocarpus, *Neo-Astragalus* clade	0.01395349	4
A. adsurgens, G	0.01173709	2
O. campestris	0.01162791	23
A. lentiginosus, *Neo-Astragalus* clade	0.00697674	6
O. arctica	0.00692841	18
O. pilosa	0.00688073	200
A. membranaceus, clade C	0.00458716	3
A. alpinus, F	0.00440529	2
A. layneae, *Neo-Astragalus* clade	0.00232558	3
O. halleri	0.00229358	7
A. complanatus, Coluteoid clade	0.00229358	2
Others	0	1

An optimal threshold of 0 is due to the presence of no more than a single sequence for the respective taxon or due to all sequences being identical. Single uppercase letters (C, F, G) after the taxon name refer to Fig. 2

Table 2 Clusters containing more than five sequences obtained by using non-hierarchical single-linkage clustering and the optimal distance threshold determined for *O. pilosa*

Cluster no.	Number of species	Number of sequences contained	Taxonomy	Average corrected entropy
89	1	200	*O. pilosa*	0.005865
93	16	59	*O. arctica*, *O. campestris*, *Oxytropis* spp.	0.009686
44	19	19	*A.* spp. (*Neo-Astragalus*)	0.008344
45	14	16	*A.* spp. (*Neo-Astragalus*)	0.008122
42	13	16	*A.* spp. (*Neo-Astragalus*)	0.002533
39	12	16	*A.* spp. (*Neo-Astragalus*)	0.002326

The reference numbers of each cluster, the numbers, and the taxonomic affiliations of the contained sequences, and the average corrected entropy values are shown

Table 3 Results of the Wilcoxon signed-rank tests (lower left half) and t-tests (upper right half) applied to the five largest clusters found by NHSL

Cluster no.	89	93	44	45	42	39
89	–	0.1466	0.4906	0.4288	0.08392	0.2013
93	0.4882	–	0.7527	0.6992	0.0005*	0.0279*
44	0.0099*	0.7671	–	0.9497	0.0291*	0.0694
45	0.0025*	0.4939	0.7987	–	0.0326*	0.0853
42	<0.0001*	<0.0001*	0.1052	0.0393*	–	0.9373
39	<0.0001*	<0.0001*	0.004*	0.0160*	0.3964	–

Differences significantly ($p \leq 0.05$) deviating from 0 are marked with an asterisk

4 Discussion and Conclusion

Genetic variability of populations within the *Astragalus-Oxytropis* complex has most often been addressed by techniques, such as allozyme polymorphisms (Karron et al. 1988; two endemic and two widespread species), RFLP (Kulshreshtha et al. 2004; one *Astragalus* species comprising six varieties, two *Oxytropis* species, pea as outgroup), AFLP, or RAPD (Travis et al. 1996; one variety). Only a few studies used cloned ITS sequences to compare populations (Dong et al. 2003, eight widespread species, one comprising two varieties, another comprising two sub species; Yip and Kwan 2006, one widespread species from 23 localities divided into at least two undefined varieties). Both studies found the marker highly conserved, and proposed the use of 5S rDNA spacers for the future. In contrast, our results indicate that if a sufficiently large number of cloned sequences are retrieved and if populations are sampled from a broad biogeographic range, valuable insights into the genetic divergence of populations can be obtained. Such substantially sampled taxa (here, *O. pilosa*) can be used as standards to optimize distance thresholds for non-hierarchical clustering algorithms. The adapted clustering techniques will result in a division of sequences into subsets of comparable genetic divergence as a basis to define conservation units. Statistical tests based on multiple sequence alignments allow us to compare the molecular diversity of different groups by objective means and to identify the significant differences regarding genetic variance. In contrast, traditional approaches such as AFLP, RAPD, RFLP, and microsatellite studies are often methodologically restricted to the species level and are statistically difficult to interpret (Felsenstein 2004, pp. 240f, 246f).

Results of our molecular comparison approach are in agreement with previous studies. For instance, the fact that *O. campestris* and *O. arctica* are hardly separable has been addressed by Jorgensen et al. (2003). Likewise, the considerably high genetic variance observed in our *O. pilosa* data confirms the interpretation of that species as a relict species. Particularly, the comparison of *O. pilosa* and the *Neo-Astragalus* species underlines how much divergence is preserved within the former. In fact, single populations of *O. pilosa* are as divergent as or even considerably more divergent than some *Astragalus* species. As a consequence, they deserve the same conservation efforts as the latter. This result is striking regarding the tendency in literature to suggest the protection of even single alleles within a variety of *A. cremnophylax* (Travis et al. 1996; for further examples, see the discussion in Soltis and Gitzendanner 1999), one of the numerous species included in our cluster 44 (Fig. 2 and Tables 1–3). Already the limited data available on *Astragalus* indicates that some species within the *Neo-Astragalus* clade (Table 1) are genetically more diverse than others, which should be taken into account in course of conservation.

In our view, not each unique individual and/or morphotype should per se be considered a relict or endemic. Biodiversity hotspots and genetic resources can be detected by objective means, similar to the approach presented here. Suitable nature conservation efforts should, therefore, also rely on molecular markers and algorithms that identify those relict stocks with the highest potential for ongoing speciation

processes, and those stocks that are endangered to become extinct. A highly adaptive species complex will frequently evolve (and lose) new, unique, and locally restricted morphotypes, but will not necessarily significantly increase its genetic diversity (Table 3). As a consequence, such a species complex will show a high biodiversity as reflected by the number of accepted (endemic) "species", with only little genetic divergence between them. On the other hand, true relict species such as *O. pilosa* are morphologically and ecologically restricted or conservative (Hegi 1924), but can harbor a high genetic diversity (cf. Tables 1–3).

Focusing on the genetic diversity within the ITS regions may only be part of the story. Most likely, amplifying additional genes will result in additional insight into ecology and morphology. However, because of a lack of both time and funding, it is hardly realistic to do such multi gene sequencing for a broad sample size, and to detect and protect each individual that is a potential founder organism. Rather, one should focus on the identification and protection of at least some of the remaining true relicts in our rapidly changing world. Even though the cost of sequencing is continuously decreasing (because of recent advances in high-throughput techniques), there will always remain a trade-off between the number of loci amplified per individual and the number of individuals examined. As demonstrated above, it is easy to quantitatively determine units with similar genetic diversity, as long as a sufficient number of sequences is amplified for an appropriate reference taxon (here, *O. pilosa*). Accordingly, focusing on a single molecular locus, preferably one for which a large number of sequences are already deposited in public databases, is likely to remain the method of choice in many future studies. Once the most valuable genetic resources have been identified, using the methods described above (or any other appropriate means), protection measures for these resources can be laid out. Phytosociological studies can provide valuable insights into a species' habitat needs. Such studies are necessary, particularly if the genetic divergence within single individuals is so high that they deserve considerable conservation efforts, as confirmed by the present study for *O. pilosa*. In such a case, conservation should aim at preserving as much of the estimated genetic richness as is possible. The monitoring of relicts should be intensified, in general, using the combination of molecular and phytosociological methods to detect the evolutionary hotspots and endangered genetic resources as well as the means to protect them. Phytosociological surveys were conducted for *O. pilosa* using the Braun-Blanquet approach to quickly obtain detail-rich relevées (Schlee et al. 2003; M. Schlee, G.W. Grimm, M. Göker, V. Hemleben, in prep.). As a conclusion from these surveys, conservation efforts need to include more suitable land-use of the habitats. A management system should prefer a grazing over a cutting regime. The toxicity of *Astragalus* and *Oxytropis* species, in general, is here of lesser importance because food aversions can be conditioned if they do not already exist in grazing animals (Ralphs and Provenza 1999). Hairiness of the plants (e.g., *O. pilosa*) or spines (many *Astragalus* species) then remain the species' natural means of protection. If disturbances are too weak, networking concepts for habitats (e.g., NATURA 2000 for Europe) fail. However, if the natural linkage between habitats cannot be restored, networking is not necessarily desirable when speciation is in progress because it might be

thwarted by artificial hybridization or homogenization among populations. The protection of ecological processes is, therefore, highly recommended instead of pursuing a networking concept at all cost. As a consequence, conservation efforts must hastily be improved to protect the disjunct populations of *O. pilosa* and to retain their genetic diversity as a valuable natural resource.

Acknowledgments Special thanks go to Angela Dressel and Karin Stögerer for technical assistance, and to Wilhelm Sauer for discussion. Reiner Schmahl, Eberhard Koch, Natalie Schmalz and Matthias Kropf, Ludmilla Barjaewa, Ludmilla Mantschenkowa, and Erich Kalmbach, Friedrich Schlee, Evgenia Zeidler-Schewzowa, and the University of Barnaul (Altai, Siberia) are kindly acknowledged for contributing plant material, GZU, LY, M, and MA for sending specimens for revision. Hospitality of the visited herbaria BC, BOLO, C, DR, E, FB, FI, FR, G, HBG, K, LE, LI, LJU, M, MPU (incl. SIGMA), P, RO, S, STU, TUB, UPS, W, WU, and Z was enjoyed with great pleasure. The project was supported by a grant of the "Landesgraduiertenförderung Baden-Württemberg"; grants for travelling were obtained from "Reinhold-und-Maria-Teufel-Stiftung" and the German Academic Exchange Service (DAAD).

References

Arnheim N, Krystal M, Schmickel R, Wilson G, Ryder O, Zimmer E (1980) Molecular evidence for genetic exchanges among ribosomal genes on nonhomologous chromosomes in man and apes. Proc Natl Acad Sci USA 77(12):7323–7327

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ (1995) The ITS region of nuclear ribosomal DNA: a valuable source of evidence of angiosperm phylogeny. Ann Mo Bot Gard 82(2):247–277

Barneby RC (1964) Atlas of North American *Astragalus*. Mem NY Bot Gard 13(1–2):1–1188

Candolle AP de (1802) [Astragalogia] Augustini-Pyrami Decandolle, Astragalogia nempe Astragali, Biserrulae et Oxytropidis, nec non Phacae, Colutae et Lessertiae, Historia Iconibus Illustrata. Joann. Bapt. Garnery, Parisiis

Chater AO (1968) *Astragalus*. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA (eds) Flora europaea, vol 2, 1st edn, Rosaceae to Umbelliferae. Cambridge University Press, Cambridge, pp 108–124

Denk T, Grimm GW (2005) Phylogeny and biogeography of *Zelkova* (Ulmaceae s.str.) as inferred from leaf morphology, ITS sequence data and the fossil record. Bot J Linn Soc 147(2):129–157

Dong TTX, Ma XQ, Clarke C, Song ZH, Ji ZN, Lo CK, Tsim KWK (2003) Phylogeny of *Astragalus* in China: molecular evidence from the DNA sequences of 5S rRNA spacer, ITS, and 18S rRNA. J Agric Food Chem 51(23):6709–6714

Felsenstein J (2004) Inferring phylogenies. Sinauer Associates, Sunderland, MA

Gillett JB (1964) *Astragalus* L. (Leguminosae) in the highlands of Tropical Africa. Kew Bull 17(3):413–423

Göker M, Grimm GW (2008) General functions to transform associate data to host data, and their use in phylogenetic inference from sequences with intra-individual variability. BMC Evol Biol 8:86

Grimm GW, Denk T, Hemleben V (2007) Evolutionary history and systematics of *Acer* section *Acer* – a case study of low-level phylogenetics. Plant Syst Evol 267(1–4):215–253

Hegi G (1924) Flora von Mitteleuropa. IV. Band, 3. Teil: Dicotyledones 2. Teil Leguminosae – Tropaeolaceae, 1st edn. Carl Hanser, München

Hemleben V, Ganal M, Gerstner J, Schiebel K, Torres RA (1988) Organization and length heterogeneity of plant ribosomal RNA genes. In: Kahl G (ed) Architecture of eukaryotic genes. VCH, Weinheim, pp 371–383

Jorgensen JL, Stehlik I, Brochmann C, Conti E (2003) Implications of ITS sequences and RAPD markers for the taxonomy and biogeography of the *Oxytropis campestris* and *O. arctica* (Fabaceae) complexes in Alaska. Am J Bot 90(10):1470–1480

Karron JD, Linhart YB, Chaulk CA, Robertson CA (1988) Genetic structure of populations of geographically restricted and widespread species of *Astragalus* (Fabaceae). Am J Bot 75(8):1114–1119

Kazempour Osaloo S, Maassoumi AA, Murakami N (2003) Molecular systematics of the genus *Astragalus* L. (Fabaceae): Phylogenetic analyses of nuclear ribosomal DNA internal transcribed spacers and chloroplast gene *ndhF* sequences. Plant Syst Evol 242(1–4):1–32

Kulshreshtha S, Creamer R, Sterling TM (2004) Phylogenetic relationships among New Mexico *Astragalus mollissimus* varieties and *Oxytropis* species by restriction fragment analysis. Weed Sci 52(6):984–988

Lee C, Grasso C, Sharlow M (2002) Multiple sequence alignment using partial order graphs. Bioinformatics 18(3):452–464

Legendre P, Legendre L (1998) Numerical ecology, 2nd English edn. Elsevier, Amsterdam

Meusel H, Jäger E, Weinert E (1965) Vergleichende Chorologie der zentraleuropäischen Flora. Band 1, Teil 1: Text; Band 1, Teil 2: Karten. Gustav Fischer, Jena

Podlech D (1986) Taxonomic and phytogeographical problems in *Astragalus* of the Old World and South-West Asia. Proc R Soc Edinb B 89:37–43

Potthast T (2000) Funktionssicherung und/oder Aufbruch ins Ungewisse? Anmerkungen zum Prozeßschutz. In: Jax K (ed) Funktionsbegriff und Unsicherheit in der Ökologie. Beiträge zu einer Tagung des Arbeitskreises "Theorie" in der Gesellschaft für Ökologie vom 10. bis 12. März 1999 im Heinrich-Fabri-Institut der Universität Tübingen in Blaubeuren (= Theorie in der Ökologie; 2). Peter Lang, Frankfurta a.M, pp 65–81

Ralphs MH, Provenza FD (1999) Conditioned food aversions: principles and practices, with special reference to social facilitation. Proc Nutr Soc 58(4):813–820

R Development Core Team (2005) R: A language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria

Sanderson MJ (1991) Phylogenetic relationships within North American *Astragalus* L. (Fabaceae). Syst Bot 16(3):414–430

Sanderson MJ, Doyle JJ (1993) Phylogenetic relationships in North American *Astragalus* (Fabaceae) based on chloroplast DNA restriction site variation. Syst Bot 18(3):395–408

Sanderson MJ, Wojciechowski MF (1996) Diversification rates in a temperate legume clade: are there "so many species" of *Astragalus* (Fabaceae)? Am J Bot 83(11):1488–1502

Sauer W, Gebert S, Shu-Kun C, Li H (1990) Beitrag zur Kenntnis chinesischer *Pedicularis*-Arten (Scrophulariaceae). Razprave IV. razreda SAZU [Razprave / Slovenska Akademija Znanosti in Umetnosti, Razred za Naravoslovne Vede = Dissertationes / Academia Scientiarum et Artium Slovenica, Classis 4]. Ljubljana 31(19):291–316

Schlee M (2004) Probleme der Erhaltung biologischer Vielfalt in der Kulturlandschaft – Ökologische Schäden durch verfehlte Pflegekonzepte. In: Potthast T (ed) Ökologische Schäden – begriffliche, methodologische und ethische Aspekte (= Theorie in der Ökologie; 10). Peter Lang, Frankfurt a.M, pp 95–120

Schlee M, Sauer W, Hemleben V (2003) Molekulare und pflanzensoziologische Analyse von pontisch-pannonischen Reliktarten aus wärmebegünstigten Saum-Gesellschaften Süddeutschlands und benachbarter Gebiete. Nova Acta Leopold NF 87(328):379–387

Shannon C (1948) A mathematical theory of information. Bell System Tech J 27:379–423

Sokal RR, Michener CD (1958) A statistical method for evaluating systematic relationships. Univ Kansas Sci Bull 38(Pt 2, No 22):1409–1438

Soltis PS, Gitzendanner MA (1999) Molecular systematics and the conservation of rare species [Review]. Conserv Biol 13(3):471–483

Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22(21):2688

Stamatakis A, Hoover P, Rougemont J (2008) A rapid bootstrap algorithm for the RAxML webservers. Syst Biol 57(5):758–771

Stock M, Eskildsen K, Gätje C, Kellermann A (1999) Evaluation procedure for nature conservation in a national park – a proposal for the protection of ecological processes. Z Ökologie Naturschutz 8(1):81–95

Swofford DL, Olsen GJ, Waddell PJ, Hillis DL (1996) Phylogenetic inference. In: Hillis DM, Moritz C, Mable BK (eds) Molecular systematics. Sinauer Associates, Sunderland, MA, pp 407–514

Tin MMY, Cho C-H, Chan K, James AE, Ko JKS (2007) *Astragalus* saponins induce growth inhibition and apoptosis in human colon cancer cells and tumor xenograft. Carcinogenesis 28(6):1347–1355

Travis SE, Maschinski J, Keim P (1996) An analysis of genetic variation in *Astragalus cremnophylax* var. *cremnophylax*, a critically endangered plant, using AFLP markers. Mol Ecol 5(6):735–745

Volkov R, Komarova N, Hemleben V (2007) Ribosomal DNA in plant hybrids: inheritance, rearrangement, expression. Syst Biodiver 5(3):261–276

Watt AS (1947) Pattern and process in the plant community. J Ecol 35(1/2):1–22

Williams MC, Barneby RC (1977) The occurrence of nitro-toxins in North American *Astragalus* (Fabaceae). Brittonia 29(3):310–326

Wirth M, Estabrook GF, Rogers DJ (1966) A graph theory model for systematic biology, with an example for the Oncidiinae (Orchidaceae). Syst Zool 15(1):59–69

Wojciechowski MF, Sanderson MJ, Baldwin BG, Donoghue MJ (1993) Monophyly of aneuploid *Astragalus* (Fabaceae): Evidence from nuclear ribosomal DNA internal transcribed spacer sequences. Am J Bot 80(6):711–722

Wojciechowski MF, Sanderson MJ, Hu JM (1999) Evidence on the monophyly of *Astragalus* (Fabaceae) and its major subgroups based on nuclear ribosomal DNA ITS and chloroplast DNA *trn*L intron data. Syst Bot 24(3):409–437

Yip PY, Kwan HS (2006) Molecular identification of *Astragalus membranaceus* at the species and locality levels. J Ethnopharmacol 106(2):222–229