

Origin and relationships of *Astragalus vesicarius* subsp. *pastellianus* (Fabaceae) from the Vinschgau Valley (Val Venosta, Italy)

Elke Zippel & Thomas Wilhalm

Abstract

Astragalus vesicarius subsp. *pastellianus* is present in the most xerothermic parts of the Italian Alps at a few localized sites. Two populations are known from the Adige region, one at the *locus classicus* at Monte Pastello (lower Adige, Lessin Mountains) and another in the Vinschgau Valley (upper Adige), and there is a further population 250 km westwards in the Aosta Valley. The relationships of this taxon were investigated with molecular sequencing and fingerprinting methods. *Astragalus vesicarius* subsp. *pastellianus* shows a clear genetic differentiation in a Western and an Eastern lineage as it is known from several other alpine and subalpine species. The population at Monte Pastello is closely related to the populations in the Aosta Valley and to the subspecies *vesicarius* from the French Alps, whereas the populations from the Vinschgau Valley (South Tyrol) belong to the Eastern lineage, together with the subspecies *carniolicus* from the Julian Alps. Therefore, the origin of the Vinschgau populations seems to be in Eastern refugia and not in the open Southern Adige Valley which would be plausible from a geographical point of view.

Keywords: *Astragalus vesicarius* subsp. *pastellianus*, phylogeography, AFLP, nuclear and chloroplast marker, South Tyrol, Southern Alps, Italy

1. Introduction

One of the rarest plant taxa in the Alps is *Astragalus vesicarius* subsp. *pastellianus* (Pollini) Arcangeli. It was first described by POLLINI (1816) from Monte Pastello in the Lessin Mountains between Verona and Lake Garda (Northern Italy) and is currently known from a few disjunct locations in the Italian Alps such as the *locus classicus* at Monte Pastello, and two inner alpine valleys, the Vinschgau Valley (Valle Venosta) and the Aosta Valley (Valle d'Aosta), as well as from the Maurienne in the French Alps. The conspicuous, small, up to 20 cm high chamaephyte has rounded, later ovate or broad cylindric inflorescences with pale yellow or cream-coloured flowers. The inflated calyx bears short black hairs intermingled with longer white ones. The taxon belongs to *Astragalus vesicarius* L. s. lat. which occurs with several infraspecific taxa from Spain (subsp. *miltiflorus* Cuatrec) along the southern and interior parts of the Alps (subsp. *vesicarius* and subsp. *pastellianus*) to the Pannonian region (subsp. *albidus* (Waldst. & Kit.) Kožuharov & Pavlova) and the Balkan region (subsp. *carniolicus* (A. Kern.) Chater). Due to the considerable variation of *Astragalus vesicarius* s. lat. it appears inappropriate to recognize several species

such as defined by KERNER (1896), DALLA TORRE & SARNTHEIN (1909) and DEGEN (1937) which were supposed to differ in shape and length of calyx and banner, in symmetry and size of calyx teeth, and in length, shape and indument of leaves. The subspecies *vesicarius*, *miltiflorus* and partially *carniolicus* are uniformly violet flowered, subspecies *carniolicus* is sometimes, like subspecies *pastellianus*, uniformly pale yellow, whereas the pannonic subsp. *albidus* has a violet banner and pale yellow keel and wings. The chromosome number as drawn from literature varies between $2n=16$ for *Astragalus vesicarius* subsp. *albidus* (MURÍN & MÁJOVSKÝ 1976), $n=8$ for *Astragalus vesicarius* (without specifying the subspecies, PRETEL & SANUDO 1978) and $2n=32$ for *Astragalus vesicarius* subsp. *carniolicus* (as „subsp. *pastellianus* auct.“) and subsp. *vesicarius* (PAVLOVA & KOZHUHAROV 1993).

Astragalus vesicarius subsp. *pastellianus* needs open and sandy grasslands and is threatened due to decreasing pasture and the invasion of shrubs in open grasslands since the last 50 years. At the *locus classicus* at Monte Pastello, only a very small relict population has remained including some 100 plants (Salmaso, pers. comm.). The stand is situated in a protected area ("Natura 2000"). Here in the Lessin mountains *Astragalus vesicarius* subsp. *pastellianus* is a characteristic floristic element of the hot, stony and open xerothermic grasslands on limestone about 700 m a.s.l. in Festuco-Brometalia communities (BIANCHINI et al. 2004). The next known location of *Astragalus vesicarius* subsp. *pastellianus* is in the Vinschgau Valley in South Tyrol about 200 km distant upstream the river Adige (120 km bee-line). Here, *A. vesicarius* subsp. *pastellianus* is restricted to a few sites on the warm and dry slopes of the Ötztal and Ortler Alps between 700 and 1200 m a.s.l.. It grows near Glurns in open pine forests of the Ononido-Pineon (BRAUN-BLANQUET 1961), and in dry pastures near Laas (the so-called „Laaser Leiten“) which form on weakly acid soil with a pH ranging from 5.8 to 7.2 (SCHWABE & KRATOCHWIL 2004) and constitute a xerophilic steppe flora. A third Italian occurrence of *Astragalus vesicarius* subsp. *pastellianus* is documented from the Aosta Valley where it is currently known from only one location near Chambave in dry grassland and adjacent embankments. Like in the Vinschgau, this population is not under particular protection. Other populations in the Aosta Valley (OBERWINKLER 1969, FAVARGER 1970) are probably extinct.

The populations from the Vinschgau Valley have originally been named as *Astragalus vesicarius* var. *leucanthus* by SALIS-MARSCHLINS (1840), this varietal epitheton being used at specific rank by DALLA TORRE & SARNTHEIN (1909; see there and MERXMÜLLER 1960 for further synonymy). Later a valid description was given by FRITSCH (1922) as *Astragalus venostanus* (Lectotype: [Italy] Südtirol, Laas im Vintschgau, 1869, A. Kerner (WU), PODLECH 1999). Taxonomy and nomenclature of these plants remained unclear for a long time (see MERXMÜLLER 1960), until MERXMÜLLER (1960), who had no notice of the populations in the Aosta Valley, included the population from Monte Pastello and those of the Vinschgau Valley in one taxon, *A. pastellianus* Pollini emend. Merxm., because of the congruent morphology.

This study aimed at checking the genetic relationship of the populations of *Astragalus vesicarius* subsp. *pastellianus* from the Vinschgau Valley and the Monte Pastello as presumed by MERXMÜLLER (1960). In addition, the relationships between all known North Italian populations of the subspecies in question and between subsp. *vesicarius* from the Hautes Alpes (France) and subsp. *carniolicus* from the southern edge of the Julian Alps (Italy and Slovenia) were investigated by means of chromosomal and nuclear DNA sequence data as well as of the AFLP method.

2. Material and Methods

2.1 Plant material

The plant material was collected during the summers 2005 and 2007. For the genetic analysis, leaves were collected in the field into small paper bags and immediately stored in boxes with silica gel. Of each population, at least one specimen was collected and stored in the herbarium B. For sequencing, further herbarium specimens were used (Tab. 1). The DNA samples are stored in the DNA bank at the Botanical Garden and the Botanical Museum Berlin-Dahlem (DRÖGE et al. 2008).

2.2 ITS and psbA-trnH sequence analysis

ITS and psbA-trnH sequences were analyzed from each two specimens of *Astragalus vesicarius* subsp. *pastellianus* from the Vinschgau Valley, the Aosta Valley and Monte Pastello, four specimens of subsp. *vesicarius* from the French Alps and additional two herbarium specimens of subsp. *carniolicus* from the Julian Alps near Gorizia and from Mt. Nanos (Tab. 1, Fig. 1). *Astragalus alpinus* L. was chosen as outgroup taxon. Total DNA extraction was carried out using DNeasy™ Extraction Kit (Qiagen) following the manufacturer's protocol. For ITS the primer pair ITS1-P1 (5'-GGAAGTAAAGTCGTAACAAGG-3', WHITE et al. 1990) and ITS2-P4 (5'-TCCTCCGCTTATTGATATGC 3', WHITE et al. 1990) was used, for psbA-trnH the primer pair psbA3'f (5'-GTTATGCATGAACGTAATGCTC-3', SANG et al. 1997) and trnH (5'-CGCCCATGGTGGATTACAATCC-3', TATE & SIMPSON 2004). The protocol for both ITS and psbA PCR comprises the following steps: initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 20 s, 42°C for 45 s and 72°C for 1 min, final extension at 72°C for 10 min. The PCR products were purified with the Millipore Kit (Genomics) and cycle sequenced using the CEQ™ Dye Terminator Cycle Sequencing (DTCS) Quick Start Master Mix (BeckmanCoulter, USA).

Sequencing reactions were performed with a dye terminator procedure and loaded on a capillary automatic sequencer CEQTM 8000 (BeckmanCoulter) according to the manufacturer's recommendations. Primers for the sequencing reaction were those used in the amplification step; all sequences were confirmed in both directions. The data were analyzed by the BeckmanCoulter software, the sequences edited manually by eye using Chromas 1.45 (MC CARTHY 1996-98) and aligned with the multiple sequence editor ALIGN (HEPPERLE 2000). GenBank accession numbers of all sequences are provided in Tab. 1.

2.3 AFLP analysis

The AFLP studies are based on 35 individuals of *Astragalus vesicarius* subsp. *pastellianus* (Aosta Valley, Vinschgau Valley and Monte Pastello, all Italy) which were compared with 13 individuals of subsp. *vesicarius* (Durance Valley, France) and 20 of subsp. *carniolicus* (Julian Alps, Italy and Slovenia, Tab. 1, Fig. 1).

The AFLP protocol of restriction, ligation and preselective amplification followed PFEIFFER et al. (2005) with only half reaction volume and without covering the samples with liquid

wax. For the selective amplification, 3,125 µl AFLP Amplification Core Kit (Applera, Germany), each 0,208 µl of both non-radioactive fluorescent dye labelled primers (primers (*Eco*RI 5ng / µl, *Mse*I-primer 1ng / µl, proligo) and 0,61 µl of the 1:10 to 1:50 diluted pre PCR product were used. Of the 36 tested primer combinations four combinations (*Eco*RI-ACA / *Mse*I-AGC, *Eco*RI-ACA / *Mse*I-CTG, *Eco*RI-ACG / *Mse*I-AGT and *Eco*RI-ACT / *Mse*I-CGG) were chosen for PCR running with all samples. Reproducibility was tested with two randomly chosen samples per population. Only clear, reproducible and unambiguous fragments between 80 to 410 bp were included in the statistical procedures in order to limit a consideration of non homoplasy bands. To detect possible contaminations, blind samples were included in the restriction/ligation and the PCR. The selective PCR products were purified and precipitated with Na-acetate, glycogen and cold ethanol, dried, re-suspended in 25µl Sample Loading Solution (BeckmanCoulter), mixed with 0,15 µl CEQ 400 Standard Size (BeckmanCoulter) and separated in a polyacrylamide gel using a BeckmanCoulter sequencer. After fragment analysis carried out with the BeckmanCoulter software, the exported text-files were imported into Genographher 1.6 (Montana State University, <http://hordeum.msu.montana.edu/software/genographher>) which allows to check the quality of each fragment.

2.4 Data analysis

Sequences. Due to the small amount of parsimony characters, the indels were coded according to SIMMONS (2000). The alignment was exported in a nexus-file and analysed with SplitsTree 3.1 (HUSON & BRYANT 2006) with a NJ tree. In order to check reticulate evolution, the nexus file was opened in SplitsTree 3.1 with Hamming distance settings. **AFLPs.** For all populations, the Shannon index was calculated and the number of population-specific fragments (fragments which occur in one population or group only) and the fixed population-specific fragments (fragments which are restricted to all specimens of one population or group) counted. The AFLP 01 matrix was used to generate a neighbor-joining dendrogram with Nei and Lee's distance algorithm (NEI & LI 1979). For the NJ dataset, bootstrap values were calculated with 1000 replicates with PAUP*4.0b 10 (SWOFFORD 2001), and like for the sequence data, the nexus file was opened in SplitsTree 3.1 with Hamming distance settings. Fst-values, standard genetic diversity according to NEI (1987) as well as the structure of the genetic variation (molecular variance analysis, AMOVA) were analysed with Arlequin (EXCOFFIER 2005) version 3.01. Further, to visualize the variation between and within populations, a Principal Coordinate Analysis (PCO) based on the Jaccard distance matrix was performed using SPSS 12.0.1. (SPSS Inc., Chic., IL, USA).

Tab. 1: List of populations and herbarium specimens used for AFLP (A) and / or ITS and psbA trnH sequence (S) analysis.

<i>Astragalus vesicarius</i> subspecies	Country	Region	Location	altitude (a.s.l.)	Coordinates	Collection date	Collector	used for (with number of individuals used in the AFLP analysis)	GenBank accession numbers (ITS, psbA-trnH)
<i>carniolicus</i>	Italy	Julian Alps	Mt. Sabotino / Sabotin (Gorizia), top ridge	580	45°59'18"N, 13°39"E	05.2007	F. Martini	A (10)	
<i>carniolicus</i>	Italy	Julian Alps	Gorizia	600		06.1976	L. Feoli Chiapella et E. Feoli	S	GU338384, GU338393
<i>carniolicus</i>	Slovenia	Julian Alps	Lonice, Nanos-Plateau	800	45° 49'22" N, 13° 59'24" E	06.1995	R. Hand	S	GU338385, GU338394
<i>carniolicus</i>	Slovenia	Julian Alps	Nanos, Vojkova koča	785	45°47'46" N, 14°00'59" E	05.2007	F. Martini	A (10)	
<i>pastellianus</i>	Italy	South Tyrol, Vinschgau Valley	Untertrög (Laas)	1400	46°38'00" N, 10°41'33" E	07.2004	E. Zippel, Th. Wilhalm	A (12), S	GU338383, GU338392
<i>pastellianus</i>	Italy	South Tyrol, Vinschgau Valley	Glurns	1000	46°39'12" N, 10°33'31" E	07.2004	Th. Wilhalm	A (1), S	GU338382, GU338391
<i>pastellianus</i>	Italy	Lessin Mountains	Monte Pastello	1500	45°34'46" N, 10°51'57" E	06.2005	R. Salmaso	A (10), S	GU338380, GU338389
<i>pastellianus</i>	Italy	Aosta Valley	Prelaz (Pontey)	520	45°44'28" N, 7°34'07" E	06.2005	E. Zippel & Th. Wilhalm	A (13), S	GU338381, GU338390
<i>vesicarius</i>	France	Alpes de Haute Provence, Durance Valley	Gorges de Meouge	600	44°16'50" N, 5°47'15" E	06.2005	E. Zippel, E. Chas & F. le Durant	A (6), S	GU338378, GU338387
<i>vesicarius</i>	France	Haute Alpes, Durance Valley	Embrun	800	44°33'43" N, 6°30'42" E	06.2005	E. Zippel	A (8)	GU338379, GU338388
<i>Astragalus alpinus</i>	Italy	South Tyrol, Gröden	Wolkenstein, 1570 m, rocky meadows	1570		06.1991	Van Buggenhout Nr. 15184	S	GU338386

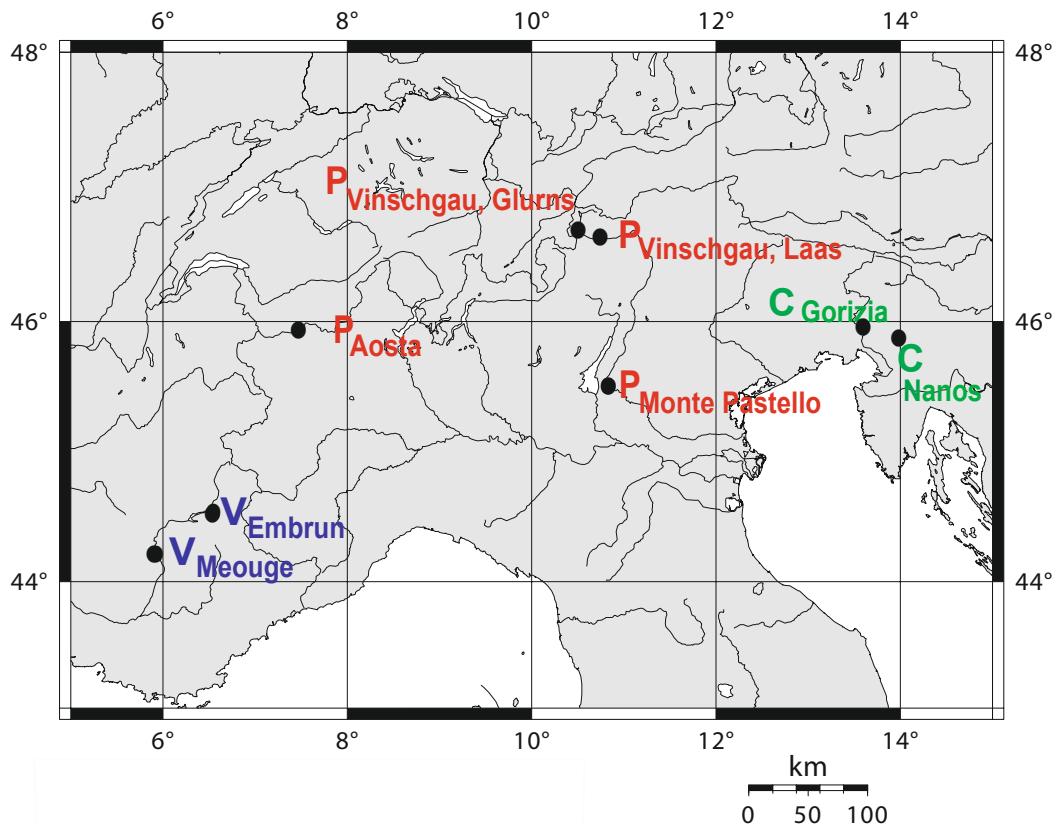


Fig. 1: Locations of the investigated *Astragalus vesicarius* populations. Map created with "create a map" (WEINELT 2006). Population abbreviations: C: subsp. *carniolicus* (Italy and Slovenia); P: subsp. *pastellianus* (Italy); V: subsp. *vesicarius* (France).

3. Results

3.1 ITS and psbA trnH sequences

The ITS sequences within *Astragalus vesicarius* s. lat. count from 633 bp to 634 bp and caused, including outgroup, an alignment of 648 bp. The ITS1 and ITS2 sequences of the investigated *Astragalus alpinus* specimen is identical with the sequence provided by WOJCIECHOWSKI et al. (1993), GenBank accession numbers L10760, L10761. The length of the *Astragalus vesicarius* psbA trnH sequences reaches from 434 bp to 441 bp, the alignment includes 463 bp. The aligned data matrix of both ITS and rbcL sequence data contains, including indels, 26 parsimony informative characters of 1 to 7 bp length (within *A. vesicarius* 18 characters) and 51 variable, but parsimony uninformative characters (within *A. vesicarius* 15 characters). The NJ phylogram summarizes the *A. v.* subsp. *pastellianus* populations from the Aosta Valley and Monte Pastello in one clade, and the subsp. *pastellianus* from the Vinschgau Valley with the subsp. *carniolicus* from the Julian Alps in another clade (tree not shown). The calculation of the NeighborNet (SplitsTree 4, character transformation

Hamming) indicates the same relationships as the NJ tree, and confirms the separation of the populations from the Vinschgau Valley from the other investigated populations of the subspecies (Fig. 2). When excluding the non parsimony characters and / or the gaps of the sequences, the network has the same topology (graphs not shown).

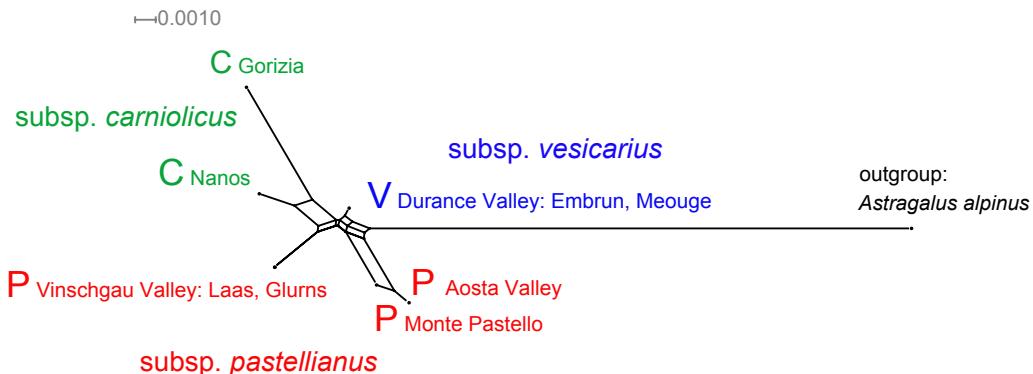


Fig. 2: Neighbor-Net network (BRYANT & MOULTON 2004) created from the combined ITS and trnH psbA nexus-file by SplitsTree (HUSON & BRYANT 2006) applying Hamming-Distance of eight *Astragalus vesicarius* subspecies from several locations and *Astragalus alpinus* as outgroup. Abbreviations see Fig. 1.

3.2 AFLP analysis

The fragments of four primer combinations amount to 151 clear and reproducible loci between 100 and 400 bp length including 6 constant and 145 variable characters. Every individual is characterized by a population-specific AFLP profile. The number of fragments per individual varies between 29 and 51, the number of variable sites within a population between 35 and 63.

The population diversity expressed in the Shannon diversity index (H_{Sh}) ranges from 8,8 in the subsp. *carniolicus* population from Nanos/Slovenia in the Julian Alps to 18,60 in the subsp. *pastellianus* from Monte Pastello/Northern Italy. The average gene diversity over loci (π_n), as another measurement for genetic diversity, ranges from 0,097 (Nanos) to 0,200 (Monte Pastello, s. Tab. 2). All groups are characterized by population-specific fragments (fragments which occur only in one population, Tab. 1): The populations from the Durance Valley (Meouge and Embrun) share 1 fixed group-specific (which occurs in all samples of the Durance Valley) and 7 group-specific fragments, the Aosta Valley/ Monte Pastello group 1 group-specific fragment and the populations from the Julian Alps 2 fixed group-specific and 6 group-specific fragments.

The Neighbor-Net graph created from the nexus-file by SplitsTree (Fig. 3) supports the groups given by the sequence data (Fig. 2). It differentiates four lineages in the two main groups: the subsp. *pastellianus* populations from the Vinschgau Valley and the subsp. *carniolicus* populations (Clade A) on the one hand, and the subsp. *pastellianus* populations from Monte Pastello and the Aosta Valley together with the subsp. *vesicarius*

populations from the Durance Valley on the other hand (Clade B). The single sample of the population from Glurns/Vinschgau (P13) falls into the population from Laas. The NJ tree (not shown) has the same topology and differentiates the populations of each region, with a close relationship of the Monte Pastello and Aosta Valley populations. Both *A. vesicarius* subsp. *vesicarius* populations from the Durance Valley are nested in one clade, sister to some individuals from subsp. *pastellianus* in the Aosta Valley. All main clades of the NJ tree are supported by bootstrap values between 76 and 90 (Fig. 3).

The Principal Coordinate Analysis plot (PCO) based on Jaccard indices among all 71 AFLP phenotypes leads to a diagram with four clouds of points (Fig. 4). It separates four groups in accordance with the NJ and network analyses: the populations from the Vinschgau Valley, the Julian Alps, the Durance Valley, and the Aosta Valley/Monte Pastello group. The first and second factors explain 44,76% and 29,11% of the total variance. The third factor (9,46% of total variance) points to a weak internal structure of the populations from the Aosta Valley and Monte Pastello (graph not shown). Within the populations from the Val de Durance there is only a weak internal structure. Like in the NJ tree, the individuals from Meouge nested between the individuals from Embrun.

The Fst of 0.52 points, according to WRIGHT (1965), to a very high genetic differentiation between the investigated populations. The Fst values for both populations from the Val de Durance (0,13), the Julian Alps (0,22), and from the Aosta Valley and Monte Pastello ($Fst=0,11$) indicate a weak variation within these populations (Tab. 3). Non-hierachical analysis of molecular variance (AMOVA) of the four main clades obtained from the MP and NJ tree indicates a total genetic variance of 48,53% among the groups, 7,67% among the populations within groups and 43,80% within populations (Tab. 4).

Tab. 2: Shannon indices and number of fixed population-specific and population-specific fragments in populations and main groups. Fixed population-specific fragments: fragments of every individual of the group; population-specific fragments: fragments restricted to the group.

	shannon index	Average gene diversity over loci (π_n)	fixed group-specific fragments	specific fragments of groups	specific fragments of populations
Durance Valley, Meouge, France	10.79	0.130667 +/- 0.078607	{ 1	{ 7	2
Durance Valley, Embrun, France	13.65	0.157429 +/- 0.088971			4
Aosta Valley, Italy	11.96	0.139556 +/- 0.077817	{ 1	{ 1	1
Lessin Mountains, Monte Pastello, Italy	18.61	0.199590 +/- 0.105447			5
Laas, Untertrög, Vinschgau Valley, Italy	14.67	0.165128 +/- 0.087747			2
Julian Alps, Gorizia, Italy	12.60	0.135644 +/- 0.074671	{ 2	{ 1	4
Julian Alps, Nanos, Slovenia	8.66	0.097422 +/- 0.054458			0

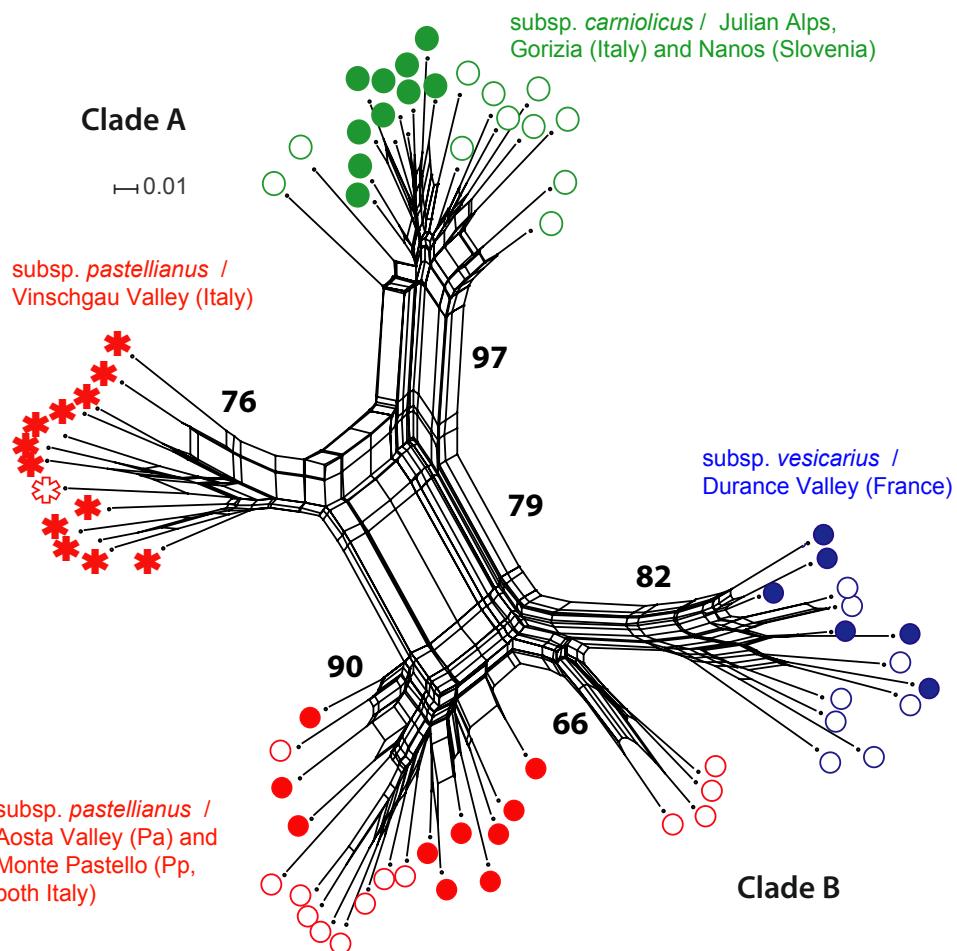


Fig.3: Neighbor-net (BRYANT & MOULTON 2004) created by SplitsTree (HUSON & BRYANT 2006) applying Hamming-Distance of selected *Astragalus vesicarius* s.lat. samples. The numbers are bootstrap values (% of 10000 replicates) of the neighbor-joining analysis based on Nei & Li's distance of AFLP phenotypes. Symbols: Green – subsp. *carniolicus*, filled dots – Nanos (Slovenia), unfilled green dots – Gorizia (Italy); red – subsp. *pastellianus*, filled dots – Monte Pastello (Italy), unfilled dots – Aosta Valley (Italy), filled stars – Laas – Vinschgau, unfilled stars – Glurns; blue – subsp. *vesicarius* (France), filled dots – Meouge (France), unfilled dots – Embrun (France).

Tab.3: Population pairwise FSTs, P value < 0,01. Grey Fields: FST < 0.25 (lower genetic variation).

	Meouge	Embrun	Aosta	Pastello	Laas	Gorizia	Nanos
Durance Valley, Meouge, France	0.00						
Durance Valley, Embrun, France	0.13	0.00					
Aosta Valley, Italy	0.53	0.50	0.00				
Lessin Mountains, Monte Pastello, Italy	0.43	0.42	0.11	0.00			
Laas, Untertrög, Vinschgau Valley, Italy	0.64	0.63	0.48	0.44	0.00		
Julian Alps, Gorizia, Italy	0.66	0.64	0.60	0.57	0.55	0.00	
Julian Alps, Nanos, Slovenia	0.73	0.69	0.66	0.60	0.60	0.22	0.00

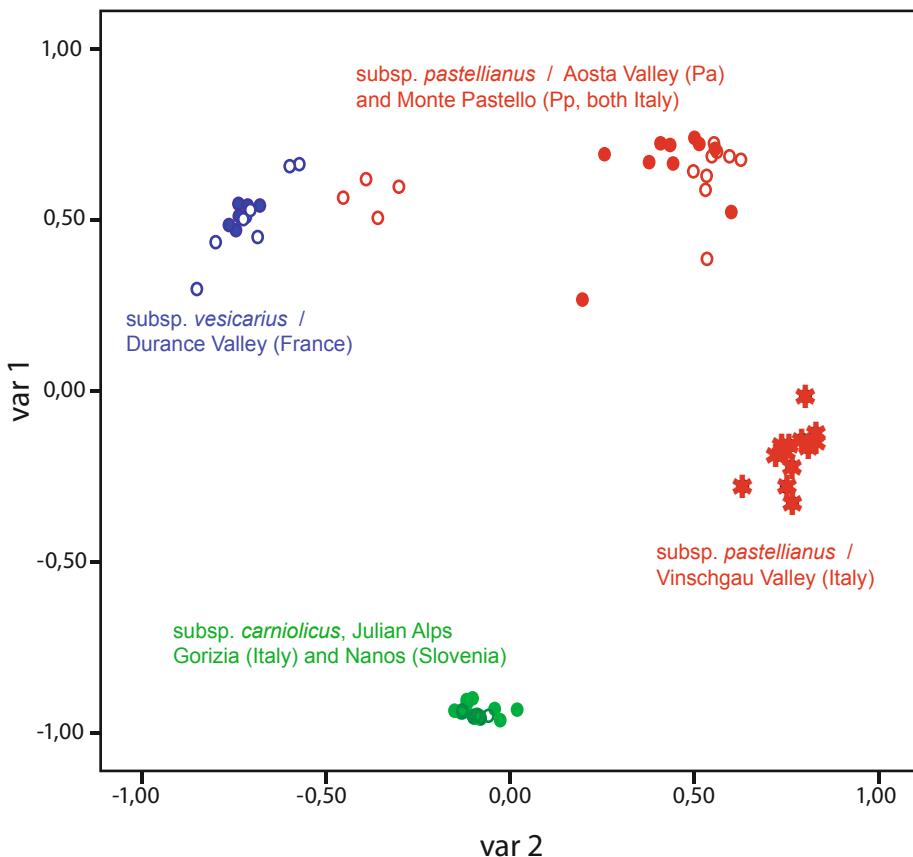


Fig. 4: PCO analysis, var1: 44,76; var2: 29,11 of total variance. Population abbreviations see Fig. 3.

4. Discussion

4.1 Genetic structure of *Astragalus vesicarius* subsp. *pastellianus*

In view of the morphologic uniformity of all *Astragalus vesicarius* subsp. *pastellianus* populations (MERXMÜLLER 1960), the conspicuous genetic variation between the populations from Monte Pastello and the Aosta Valley against the Vinschgau populations in both ITS and psbA trnH sequences and AFLP datasets is surprising. In the AFLP analysis, the amount of population-specific fragments isolates the Vinschgau populations from both other investigated *pastellianus* populations and supports the sequence data. The separated position of the Vinschgau populations is evident from both AFLP neighbor-joining and PCO analyses (Figs. 4 and 6) and is confirmed by the large amount of variation among the groups in the AMOVA (Tab. 4). The molecular data of this study exclude the assumption that the Vinschgau populations had derived from populations located at the lower Adige Valley which would be plausible from the geographical

point of view. Both, ITS and psbA phylogeny and AFLP data, point to at least two major geographic lineages within *Astragalus vesicarius*. The Western lineage includes the populations from the French Alps, from the Aosta Valley and also from Monte Pastello, and the Eastern lineage includes the populations from Vinschgau and from the Julian Alps. This split suggests that the subsp. *pastellianus* is not monophyletic and indicates different ways of immigration into the inner alpine valleys after the retreat of the Würm glaciation which had formerly covered these valleys. Such East-West-patterns in genetic variation are known from a couple of subalpine or alpine species (e.g. KROFF et al. 2002, STEHLIK et al. 2002, SCHÖNSWETTER et al. 2002, 2003).

The present occurrence of *A. vesicarius* subsp. *pastellianus* at Monte Pastello may be a hint for a survival of the Western lineage in this region which is known for its particular flora (CALZOLARI 1566, MANGANOTTI 1846, DALLA TORRE 1904) with a relatively high amount of endemic taxa (CALZOLARI 1566, TRIBSCH & SCHÖNSWETTER 2003, BIANCHINI et al. 2004) and which is known as a xerothermic refugium and nunatak at the former edge of the last glacial maximum (PROSSER & BERTOLLI 2007). The Vinschgau populations have their origin in populations of the Eastern Alps or the Balkan region. Both regions are known as important refugia (BENNETT et al. 1991, TABERLET et al. 1998, HEWITT 1999, TRIBSCH & SCHÖNSWETTER 2003, PAUN et al. 2008). The probable migration route from the East to South Tyrol leads through the Puster Valley (Val Pusteria) as it is presumed also for other species of Eastern European origin, as e.g. *Carex supina*, *Carex stenophylla* and *Seseli pallasii*. The Quaternary climatic changes lead to distribution patterns of these thermophilous plant species from lower belts similar as in those from the subalpine and alpine belts.

4.2 Genetic variation between and within populations

The genetic variation between the populations reflects the geographical distance with the exception of the populations from Aosta Valley and from Monte Pastello. The conspicuously close relationship and the weak genetic variation between these populations with a distance of 250 km beeline indicate a relative young fragmentation of a previously wider distribution range along the Southern Alps during the warmer periods of the Holocene. Due to the high genetic variation within the Monte Pastello population which does not reflect the small size of this population, long distance dispersal from a Western population seems to be very improbable. Long distance dispersal would generally lead to a reduced genetic diversity within a population due to the founder effect (ELLSTRAND & ELAM 1993, HEWITT 1996, BECKER 2003).

All investigated populations with their high to very high genetic diversity may be regarded as relict populations descended from a former wider distribution area which was reduced during the climate oscillations in the Pleistocene and / or later also due to human impact. In other *Astragalus* species a high genetic diversity of such isolated relict populations is also known. BECKER (2003) observed a high genetic diversity in populations of *Astragalus exscapus* which are supposed to be fragmented since the early Holocene. TRAVIS et al. (1996) reports a high genetic diversity among populations of *A. cremnophylax* fragmented through human impact.

Tab. 4: Analyses of molecular variance (AMOVA) based on 151 AFLP markers of together 70 individuals, grouping both populations of subsp. *vesicarius* (Durance Valley) and subsp. *carniolicus* (Julian Alps), respectively. df: degrees of freedom (WEIR & COCKERHAM 1984, EXCOFFIER et al. 2005, WEIR 1996).

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	4	673.263	10.43846 Va	48.53
Among populations within groups	2	46.670	1.65078 Vb	7.67
Within populations	62	584.125	9.42136 Vc	43.80
Total	68	1304.058	21.51061	

5. Conclusions

The investigated *Astragalus vesicarius* populations of the Southern border of the Alps are distinctly genetically separated. This study points to an extinction of the taxon in wide parts of the former range during the climatic fluctuations of the Pleistocene which caused the separation in an Eastern and a Western lineage and had a great impact to the genetic structure of the species. An in-depth study concerning the taxonomical relationships and the reconstruction of migration routes of *Astragalus vesicarius* subsp. *pastellianus* after the Quaternary glaciations is only possible if more samples of its closest relatives and more detailed morphological studies are included. Eventually, because of rareness and high genetic variability, great efforts are needed to protect the few remaining populations of *Astragalus vesicarius* subsp. *pastellianus* in Northern Italy.

Zusammenfassung

Ursprung und Verwandtschaft des *Astragalus vesicarius* subsp. *pastellianus* aus dem Vinschgau (Südtirol, Italien)

Astragalus vesicarius subsp. *pastellianus* kommt in den italienischen Alpen in den trockenwärmsten Gebieten vor und zwar an wenigen, sehr lokalisierten Fundorten. Zwei Populationen sind vom Etschtal bekannt: eine vom *locus classicus* am Monte Pastello (Lessinische Alpen nördlich Verona), die andere vom Vinschgau (Oberlauf der Etsch). Eine weitere findet sich 250 km westlich im Aosta-Tal. In der vorliegenden Studie wurde die Verwandtschaft dieses Taxons mit Hilfe von Sequenzierungen ausgewählter Marker des Kern- und Chloroplastengenoms sowie AFLP-Fingerprinting untersucht. *Astragalus vesicarius* subsp. *pastellianus* ist genetisch deutlich in eine westliche und eine östliche Linie differenziert, so wie es von einer Reihe anderer alpiden Arten der subalpinen und alpinen Stufe bekannt ist. Die Monte Pastello-Population ist eng verwandt mit jener im Aosta-Tal und mit der subsp. *vesicarius* der Französischen Alpen, während die Populationen des Vinschgaus (Südtirol) zusammen mit subsp. *carniolicus* aus den Julischen Alpen zur östlichen Linie gehören. Es liegt nahe, den Ursprung der Vinschger Populationen in östlichen Refugien zu suchen und nicht in dem nach Süden offenen Etschtal, was aus geographischen Gesichtspunkten plausibler erscheinen würde.

Acknowledgments

Thanks to Fabrizio Martini (Triest), Roberta Salmaso (Verona) for collecting samples from the Julian Alps and Monte Pastello, respectively, Maurizio Bovio (Aosta), Edouard Chas, Franck le Driant (Gap) and Roland Wieloch (Berlin) for their support in the field as well as to H. Niklfeld (Vienna) for valuable comments on the manuscript. The study was supported by a grant from the Berliner Programm/Senat von Berlin (Germany).

References

- BECKER T., 2003: Auswirkungen langzeitiger Fragmentierung auf Populationen am Beispiel der reliktischen Steppenrasenart *Astragalus exscapus* L. (Fabaceae). Diss. Bot., 380: 1-210.
- BENNETT K.D., TZEDAKIS P.C. & WILLIS K.J., 1991: Quaternary refugia of north European trees. J. Biogeogr., 18: 103-115.
- BIANCHINI F., CURTI L., DI CARLO F. & LAZZARIN G., 2004: Aspetti floristici e vegetazionali. Mem. Mus. Civico Storia Nat. Verona - 2. Serie Monografie Naturalistiche, 1: 89-120.
- BRAUN-BLANQUET J., 1961: Die inneralpine Trockenvegetation. Geobot. Selecta, 1: 1-273.
- BRYANT D. & MOULTON V., 2004: NeighborNet: an agglomerative algorithm for the construction of phylogenetic networks. Mol. Biol. Evol., 21(2): 255-265.
- CALZOLARI F., 1566: Viaggio di Monte Baldo della magnifica città di Verona. Venezia.
- DALLA TORRE K.W., 1904: Die Geschichte der floristischen Erforschung des Monte Baldo.- In: URBAN I. & GRAEBNER P. (eds.): Festschrift zur Feier des siebzigsten Geburtstages des Herrn Professor Dr. Paul Ascherson. Leipzig.
- DALLA TORRE K.W. & SARNTHEIN L., 1909: Die Farn- und Blütenpflanzen von Tirol, Vorarlberg und Liechtenstein, 2. Wagner'sche Universitäts-Buchhandlung Innsbruck.
- DEGEN Á. v., 1937: Flora Velebitica. Budapest.
- DRÖGE G., ZETZSCHE H. & GEMEINHOLZER B., (eds.) 2008 - (continuously updated): DNA samples of the DNA bank at the BGBM (Botanic Garden and Botanical Museum Berlin-Dahlem).
- ELLSTRAND N.C. & ELAM D.R., 1993: Population genetic consequences of small population size: Implications for plant conservation. Annual Rev. Ecol. Syst., 24: 217-242.
- EXCOFFIER L., LAVAL G. & SCHNEIDER S., 2005: Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evol. Bioinf. Online, 1: 47-50.
- FAVARGER C., 1970: *Astragalus vesicarius* ssp. *pastellianus* im Cognetal. Ber. Bayer. Bot. Ges., 42: 201-202.
- FRITSCH K. 1922: Exkursionsflora für Österreich und die ehemals österreichischen Nachbargebiete. 3. Aufl. Wien-Leipzig, 277 pp.
- HEPPERLE D., 2000: Multicolor Sequence Alignment Editor. Institute of Freshwater Ecology and Inland Fisheries. 16755 Neuglobsow, Germany.
- HEWITT G.M., 1996: Some genetic consequences of ice ages and their role in divergence and speciation. Biol. J. Linn. Soc., 58: 247-276.
- HEWITT G.M., 1999: Post-glacial re-colonization of European biota. J. Linn. Soc., 68: 87-112.
- HUSON D.H. & BRYANT D., 2006: Application of phylogenetic networks in evolutionary studies. Molec. Biol. Evol., 23(2): 254-267.
- KERNER A., 1896: *Astragalus carniolicus*. Schedae ad Floram Exsiccatam Austro-Hungaricam, 7: 3.
- KROPF M., KADEREIT J.W. & COMES H.-P., 2002: Late Quaternary distributional stasis in the sub-mediterranean mountain plant *Anthyllis montana* L. (Fabaceae) inferred from ITS sequences and amplified fragment length polymorphism markers. Molec. Ecol., 11: 447-463.
- MANGANOTTI A., 1846: Corrispondenza al Prof. Filippo Parlatore. Giornale Botanico Italiano, 2 (3,1): 6-17.

- MC CARTHY C., 1996-98: Chromas 1.45. Griffith University, Gold Coast Campus, Queensland, Australia. (<http://www.technelysium.com.au/chromas.html>)
- MERXMÜLLER H., 1960: Der Etschtaler Blasentragant. Jahrb. Vereins Schutze Alpenpfl. Tiere, 25: 155-160.
- MURÍN A. & MÁJOVSKÝ J.M. (eds.), 1976: [Report]. In: LÖVE Á. (ed.): IOPB Chromosome number reports LIII. Taxon, 25 (4): 487-489.
- NEI M., 1987: Molecular Evolutionary Genetics. Columbia Univ. Press, New York.
- NEI M. & LI W.-H., 1979: Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A., 76: 5269-5273.
- OBERWINKLER F., 1969: Der „Etschtaler“ Blasentragant im Aostatal. Ber. Bayer. Bot. Ges., 41: 65.
- PAUN O., SCHÖNSWETTER P., WINKLER M., TRIBSCH A. & INTRABIODIV CONSORTIUM, 2008: Historical divergence vs. contemporary gene flow: evolutionary history of the calcicole *Ranunculus alpestris* group (Ranunculaceae) in the European Alps and the Carpathians. Molec. Ecol., 17(19): 4263-4275.
- PAVLOVA D.K. & KOZHUHAROV S.I., 1993: Chromosome numbers of Bulgarian angiosperms. Fitologija, 44: 75.
- PFEIFFER T., ZIPPEL E., FRITZ S. & STECH M., 2005: Application of the non-radioactive biotin-streptavidin system to visualise AFLP fragments. Molec. Ecol. Notes, 5: 673-675.
- PODLECH D., 1999: Typification of *Astragalus* species III. (Leguminosae). Sendtnera, 6: 175-191.
- POLLINI C., 1816: Horti et Provinciae Veronensis Plantae Novae vel minus cognitae 19. Diar. Phisic. Med. Ticin, 9: 95.
- PRETEL A. & SANUDO A., 1978: Estudios cariologicos en especies españolas del genero *Astragalus* L. I. Numero y comportamiento de los cromosomas durante la meiosis. Lagascaia, 8: 25-38.
- PROSSER F. & BERTOLLI A., 2007: A new subspecies of *Guenthera repanda* (Brassicaceae) from Mt. Baldo (SE Prealps, Italy). Willdenowia, 37: 191-198.
- SALIS-MARSCHLINS 1840: *Astragalus vesicarius* var. *leucanthus*. Literaturber. Flora 12, 10:180 in nota, nom. nud.
- SANG T., CRAWFORD D. J. & STUESSY T. F., 1997: Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). Amer. J. Bot., 84: 1120-1136.
- SCHÖNSWETTER P., TRIBSCH A., BARFUSS M. & NIKLFELD H., 2002: Several Pleistocene refugia detected in the high alpine plant *Phyteuma globulariifolium* in the European Alps. Molec. Ecol., 11: 2637-2647.
- SCHÖNSWETTER P., TRIBSCH A., SCHNEEWEISS G. M. & NIKLFELD H., 2003: Disjunctions in relict alpine plants: phylogeography of *Androsace brevis* and *A. wulfeniana* (Primulaceae). Bot. J. Linn. Soc., 141: 437-446.
- SCHWABE A. & KRATOCHWIL A., 2004: Festucetalia valesiacae communities and xerothermic vegetation complexes in the Central Alps related to environmental factors. Phytocoenologia, 34: 329-446.
- SIMMONS M.P. & OCHOTERENA H., 2000: Gaps as characters in sequence-based phylogenetic analyses. Syst. Biol., 49 (2): 369-381.
- STEHLIK I., BLATTNER F.R., HOLDERECKER R. & BACHMANN K., 2002: Nunatak survival of the high Alpine plant *Eritrichium nanum* (L.) Gaudin in the central Alps during the ice ages. Molec. Ecol., 11: 2027-2036.
- SWOFFORD D.L., 2001: PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4.0b10 for 32-bit Microsoft Windows. Sunderland, MA.
- TABERLET P., FUMAGALLI L., WUST-SAUCY A. G. & COSSON J. F., 1998: Comparative phylogeography and postglacial colonization routes in Europe. Molec. Ecol., 7: 453-464.
- TATE J.A. & SIMPSON B.B., 2004: Breeding system evolution in *Tarasa* (Malvaceae) and selection for reduced pollen grain size in the polyploid species. Amer. J. Bot., 91: 207-213.
- TRAVIS S.E., MASCHINSKI J., KEIM P., 1996: An analysis of genetic variation in *Astragalus creminophylax* var. *creminophylax*, a critically endangered plant, using AFLP markers. Molec. Ecol., 5: 735-745.
- TRIBSCH A. & SCHÖNSWETTER P., 2003: Patterns of endemism and comparative phylogeography confirm palaeo-environmental evidence for Pleistocene refugia in the Eastern Alps. Taxon, 52: 477-497.

- WEINELT M., 2006: "Create a map" – online map creation. Available under http://www.aquarius.geomar.de/omc/make_map.html
- WEIR, B.S., 1996: Genetic Data Analysis II. Sinauer Associates. Sunderland, MA.
- WEIR, B.S. & COCKERHAM C.C., 1984: Estimating f-statistics for the analysis of population structure. *Evolution*, 38: 1358-1370.
- WHITE T.J., BRUNS T., LEE S. & TAYLOR J., 1990: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M., Gelfand D., Sninsky J. & White T. (eds.): PCR protocols: A guide to methods and applications. Academic Press, San Diego: 315-322.
- WOJCIECHOWSKI M., SANDERSON M.J., BALDWIN B.G. & DONOGHUE M.J., 1993: Nuclear ribosomal ITS sequence data support the monophyly of aneuploid *Astragalus*. *Amer. J. Bot.*, 80: 711-722.
- WRIGHT C.H., 1965: The interpretation of population structure by F-statistics with special regard to systems of mating systems. *Evolution*, 19: 395-420.

Addresses of authors:

Dr. Elke Zippel
Botanischer Garten und Botanisches Museum Berlin-Dahlem
Freie Universität Berlin
Königin-Luise-Straße 6-8
D-14195 Berlin, Deutschland
e.zippel@bgbm.org

Dr. Thomas Wilhalm
Naturmuseum Südtirol
Bindergasse 1
I-39100 Bozen
thomas.wilhalm@naturmuseum.it

eingereicht: 24. 06. 2009
angenommen: 19. 11. 2009

