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Phylogeny and taxonomy of *Draba pacheri*

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Erich Kucs, BSc

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To all those who helped me to come so far

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Zusammenfassung

Draba pacheri, wurde 1855 in den Hohen Tauern entdeckt. 1931 wurde von Felix Widder *Draba norica* von der Koralpe als neue endemische Art beschrieben. Er vermutete eine enge Verwandschaft zwischen beiden Arten. Nach dem Auffinden weiterer Populationen zwischen den beiden Gebieten, welche morphologische Ähnlichkeiten zu den bereits bekannten aufwiesen, und einer weiteren in der Slowakischen Tatra, wurden später alle Populationen unter dem Namen *D. pacheri* vereint. Aufgrund der unzureichend bekannten morphologischen Differenzierung zwischen den geographisch stark getrennten Teilpopulationen, besteht allerdings nach wie vor die Notwendigkeit weiterer Untersuchungen zu Taxonomie und Phylogenie von *D. pacheri*. Mit einer Kombination aus morphometrischen Untersuchungen (einschließlich Sternhaaranalysen), DNA-Sequenzierung (ITS, trnL) und AFLP-Analyse testen wir Hypothesen: (I) Es gibt, wie von Widder vor der Entdeckung der anderen Populationen vermutet, zwei Taxa, entsprechend *D. pacheri* und *D. norica*, in welche sich die dazwischen und abgelegenen Populationen (Seetaler Alpen, Gurktaler Alpen und Slowakische Tatra) zwanglos einbetten lassen. (II) Die Regionen mit *D. pacheri* Populationen sind sowohl morphologisch als auch genetisch konstant abzugrenzen und können, wie von Melzer und Prugger (1986) vorgeschlagen, als Unterarten abgegrenzt werden. (III) Wie von Buttler vermutet, stellt *D. pacheri* eine einzige, wenngleich morphologisch variable Art ohne weitere intraspezifische Taxa dar.

Da die Slowakische Population „regional ausgestorben“ ist, konnten nur österreichische Populationen aus den Alpen, im Einklang mit naturschutzfachlichen Auflagen beprobt werden. *Draba pacheri* konnte der „Core *Draba:* Group III“, entsprechend Jordon-Thaden (2010), zugeordnet werden. In dieser Gruppe befinden sich auch jene Arten (z.B.: *Draba glabella*, *D. tomentosa* oder *D. incana)*, in deren naher Verwandschaft *D. pacheri* vermutet wurde. Die genaue Position innerhalb dieser Gruppe konnte nicht aufgeklärt werden. Populationen aus den vier Regionen wiesen zwar signifikante morphologische Unterschiede in einzelnen Merkmalen und eine deutliche genetische Differenzierung in drei Gruppen (Koralpe; Seetaler Alpen; Hohe Tauern plus Gurktaler Alpen) auf, die allerdings nicht mit den Vorschlägen früherer Autoren zur taxonomischen Gliederung übereinstimmen. Entsprechend sind alle bekannten Populationen taxonomisch unter *D. pacheri* zu vereinen. Es wurde festgestellt, dass sich einige Populationen genetisch stark unterscheiden, was auf genetische Bottlenecks in Refugialräumen im Pleistozän zurückzuführen sein könnte. Durch diese genetische Eigenständigkeit der Populationen ist es unbedingt notwendig, alle Teilareale des Verbreitungsgebietes zu erhalten.

Abstract

Draba (Brassicaceae) is one of the largest genera of Brassicaceae, whose circumscription has little changed (in contrast to other groups in Brassicaceae), even after the application of molecular phylogenetic approaches. *Draba* species are important components of the flora of northern hemisphere mountain ranges as well as in the European Alps. One of those Alpine *Draba* species is *Draba pacheri*, a subendemic of the Austrian Alps.

With this paper we want to clarify the position of *Draba pacheri* within *Draba,* using nuclear and plastid sequence data (ITS and trnL). Furthermore, we combine AFLP analyses with morphometric analyses to test, whether there are differences among the four distribution areas of *D. pacheri*, and if they contradict the present-day taxonomy. We refer to the two formerly separated and acknowledged endemic species *D. pacheri* and *D. norica*, which were later merged, after finding additional populations, bridging the gap between known populations, overlapping in morphological characteristics. Further, we assess the idea by Melzer and Prugger, to distinguish subspecies, according to the distribution regions. Using a comprehensive sampling in the Alps, we further assess the phylogeographic history of *D. pacheri*.

Draba pacheri fell into the *Draba* core III clade, according to the ITS and trnL data set. Phylogenetic relationships between *D. pacheri* and its relatives could not be clarified, since the sequence data did not yield results of sufficient resolution and bootstrap support. AFLP analyses resulted in three genetically separated groups (Koralpe; Seetaler Alpen; Hohe Tauern plus Gurktaler Alpen), whereas morphometric characteristics were partially overlapping. According to the pattern in which the four regions vary morphometrically and genetically, the idea of subdividing *D. pacheri* into two constant taxa, as suggested by Widder (1931), could not be supported with our results. Furthermore, the suggestion by Melzer and Prugger (1986), to establish four subspecies, according to the regional characteristics, could not be confirmed either. Hence, as suggested by Buttler (1967), we conclude, that one species correctly embraces all known populations, even though they show regional characteristics.

Genetic differentiation between the four regions indicates a lack of gene flow between spatially fragmented populations of *Draba pacheri*. These genetic splits likely are the result of isolation in Pleistocene refugia even on regional scale, as evident from the genetic differentiation in the Hohe Tauern within a few kilometers distance. Since some of the endangered populations in Austria could not be confirmed and the single one in Slovakia is considered as “regionally extinct” since 2014, Austria remains solely responsible for the protection of *D. pacheri*.

1. Introduction

1.1 General information on the genus *Draba*

The genus *Draba* is the largest within the family of Brassicaceae, subfamily Arabideae, comprising more than 370 species worldwide (Jordan-Thaden et al., 2010). The genus is considered to be phylogenetically difficult until today (Jordon-Thaden, 2009). Mainly distributed in Arctic, subarctic, alpine and montane areas the plants are mostly perennial, only some are annual or biannual (Jordon-Thaden et al., 2010). *Draba* is a species-rich and important genus in the European Alps, comprising around 30 species of which five are endemics (*Draba hoppeana*, *D. dolomitica*, *D. sauteri*, *D. ladina*, *D. stellata*) and two are near endemics (*Draba aizoides* subsp. *beckeri* and *Draba pacheri*) (Aeschimann et al., 2004).

Due to a lack of explicit and easily accessible morphological characters for developing a coherent taxonomy, the trichome configuration early on has been considered an attribute of priority for defining species and taxa within the genus *Draba* (Widder, 1931). In the middle of the 19th century, the peak of the first period of *Draba* taxonomic history, trichomes have only been used as a quantitative character (Buttler, 1967). Due to defining different trichome types and combination patterns in the early 20th century, trichomes became soon a more powerful attribute for identification (Buttler, 1967). Beside other morphologic characters, the trichome configuration was of particular importance for the composition of the first *Draba* monography by Schulz (1923), which is still the main source for the systematics of *Draba* today (Jordon-Thaden, 2010).

1.2 Historical information about *Draba pacheri*

Draba pacheri has been found for the first time by Gussenbauer in the Austrian Alps in the Hohe Tauern on Mt. Stern (pop. 3, **Fig. 1**). He gave the plant to David Pacher, who sent it to Dionys Stur, who recognized and described it as a new species in 1855 and named it after D. Pacher (Melzer and Prugger, 1986).

Pacher already recognized the similarity of *Draba pacheri* with the Artic *Draba glabella.* Whereas Widder (1931), Buttler (1967), Melzer and Prugger (1986) agreed that *Draba pacheri* was a close relative of *Draba glabella*, they had different opinions on the origin of *Draba pacheri*. Weingerl (1923) described *Draba pacheri* as a neo-endemic, derived from *Draba tomentosa*. Widder (1934) considered it an ancient species that provides the

evolutionary link between white-flowered Alpine *Draba* species possessing basal leaf rosettes (e.g., *Draba tomentosa, D. dubia*) and the *Draba incana – Draba lanceolata* complex (characterized by a high plant stem with many cauline leaves, rich inflorescence and elongated infructescence (Buttler, 1967)). Buttler (1967) rejected both ideas and considered the species to be part of the Arctic-Central Asian *Draba glabella* complex, but with closer affinities to Central Asian taxa than the Nordic *Draba glabella*.

In 1931, F. Widder found a similar *Draba* species in eastern Austria, Lavanttaler Alpen on the Koralpe (pop. 7, **Fig. 1**). Differing in morphologic characters, such as plant height, number of cauline leaves, silique length, fruit stem length or trichome pattern, he considered it as an endemic and named it *Draba norica*, connecting it to Leucodraba D.C. (Series III Ramoso-stellatae, *D. tomentosa* group) (Widder, 1931). Regarding morphological, geographical and ecological similarities, Widder furthermore assumed that *D. norica,* together with *D. pacheri* and *D. glabella*, were evolutionary remnants of the ancestor taxon of the abovementioned Series III Ramoso-stellatae (Widder, 1934).

After discovering further populations of *D. pacheri* (respectively *D. norica),* showing extended and overlapping morphological variability and similarity, and being located in the geographical gap between the Hohen Tauern and the Koralpe, and finding one additional population in the Belianské Tatry in Slovakia, it was generally accepted to merge *D. norica* with *D. pacheri* under the latter (Fischer et al., 2008). Nonetheless, being aware of the obvious morphological differences between the regions and the possible impact on taxonomy, further research was recommended by Melzer and Prugger (1986).

In the Austrian "Red list of endangered species", *Draba pacheri* is listed as "3" (Niklfeld and Schratt-Ehrendorfer, 1999). In the federal state of Kärnten it is listed as "3 endangered" (Kniely et al., 1995), and "fully protected" in the federal state of Steiermark (Anonymus, Amt der Steiermärkischen Landesregierung, 2007). In Slovakia the plant is included in the "Red List of vascular plants of the Carpathian part of Slovakia", but is since 2014 considered as "regionally extinct".

1.3 Morphology of *Draba pacheri*

Draba pacheri is a perennial, hemicryptophyte growing in open, subalpine grassland (Buttler, 1967), on neutral soils, over calcareous mica schists with good nutrient support (Widder,

1934). The egg-shaped leaves are up to 2.5 cm long, entire or slightly toothed. The plant, including infructescence, is 4 – 20 cm high and carries 0 – 7 cauline leaves. The petals are white, rounded or emarginate and 2.8 – 4.2 mm long. The egg-shaped siliques are glabrous (Buttler, 1967).

The fully developed summer leaves, especially on the lower leaf surface, possess stellate trichomes, whose primary branches partly have secondary branchings (**Fig. 2**). All trichome branches are located on the same level, more or less parallel to the leaf surface. In 1931 and 1934, Felix Widder considered the trichome configuration as an important character to distinguish *Draba pacheri* from *Draba norica.* He referred to two types of stellate trichomes, where the (usually) four primary branches have secondary branchings of differing dimensions. Thus, according to his experiences, he assigned "Pili stellati dentati", stellate trichomes with short, tooth-like secondary branchings, to *Draba norica* and "Pili stellati ramosi", with much longer secondary branches, to *Draba pacheri* (Widder, 1931). Additional, diagnostic characteristics of the two species are listed in **Table 1**. The list also includes information about the later discovered region Seetaler Alpen taken from Buttler (1967). There is no information available about the morphometric characteristics of the plants in the Gurktaler Alpen.

Table 1. List of relevant morphological characteristics to distinguish *Draba norica* from *Draba pacheri* according to Widder (1934). The list also includes information about the characteristics of the later discovered region of *Draba pacheri* s.str. in the Seetaler Alpen, published by Buttler (1967).

	Hohe Tauern	**Gurktaler Alpen**	**Seetaler Alpen**	**Koralpe (formerly *Draba norica*)**
	(Widder, 1934)	no information	(Buttler, 1967)	(Widder, 1934)
number of cauline leaves	0 – 3 – (4)	n/a	(1) – 3 – 4	3 – 7
infructescence	dense, up to 50% of plant height	n/a	n/a	sparsely, far below 50% of plant height
fruit length	6 – 7 – (9) mm	n/a	5.9 – 8.6 mm	5 – 6 mm
fruit pedicel	strong	n/a	n/a	fine
fruit pedicel	shorter than the fruit	n/a	relatively long	as long as or longer than the fruit

2. Material and methods

2.1 Plant material collection and DNA extraction

The plant material was collected in four regions (Hohe Tauern, Gurktaler Alpen, Seetaler Alpen and Koralpe) in 2013, from June to August. In 2014, a search for the population in Slovakia, in the Belianské Tatry, was not successful. All field work was carried out according to the permissions granted by the responsible public authorities. Thus, no plant vouchers were collected, and only single leaves were taken, without harming the plants seriously. GPS coordinates were taken using a Garmin etrex 10 GPS device. The sampling locations are listed in **Table 2** and shown in **Fig. 1**.

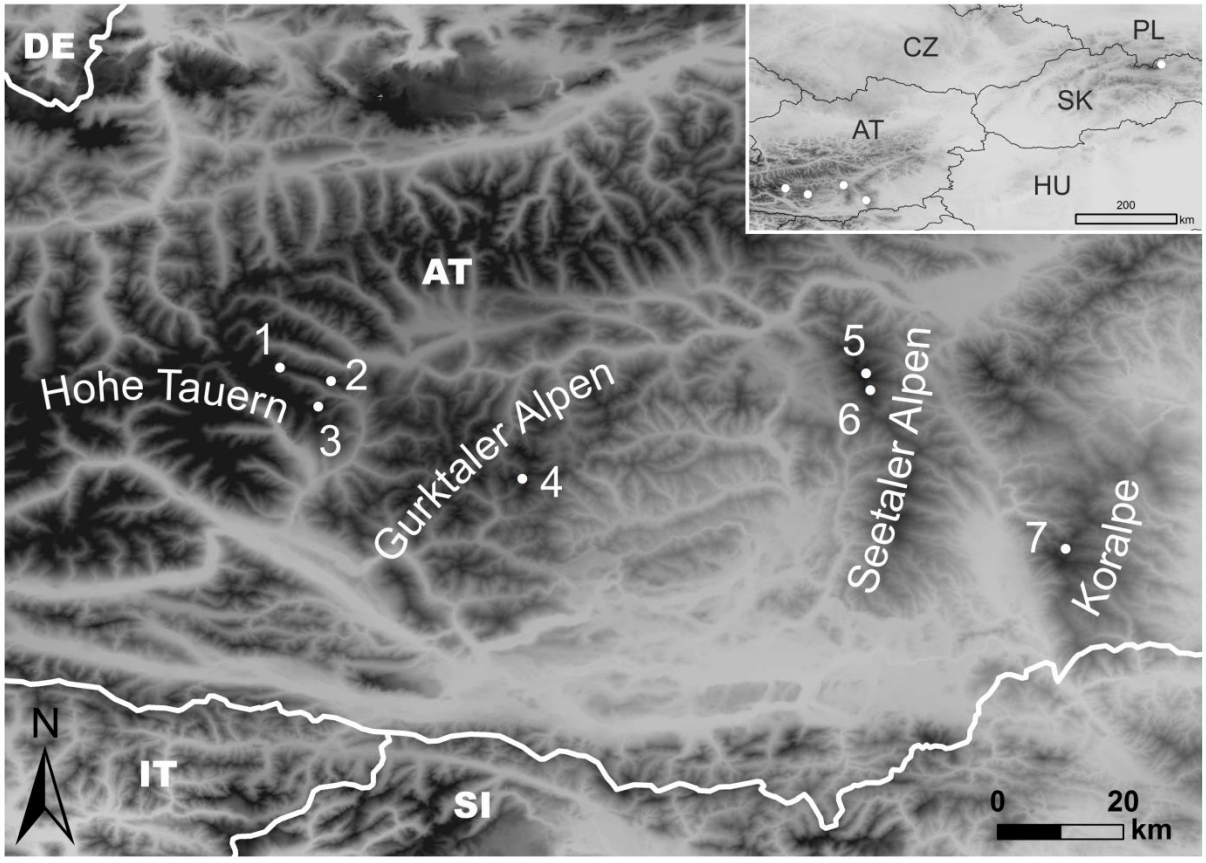

Fig. 1. Populations of *Draba pacheri (white dots)* in the Austrian Alps. Population numbers as in Table 2. The insert shows an overview of all documented European distribution areas as white dots, including the one in the Slovakian Belianské Tatry, which is considered as "regionally extinct".

Of each *Draba pacheri* population, one to five adequate plants were chosen for the collection of leaf samples for further analysis. 36 *Draba pacheri* individuals and six *Draba siliquosa* individuals, providing the outgroup, were sampled that way. If possible, rosette

leaves were collected, as they could be used for both DNA extraction and trichome analysis (Widder, 1931). In case of absence of rosette leaves or the option of only collecting dry and bald rosette leaves, also cauline leaves were sampled. In most cases three rosette leaves were collected and put into a tea bag, which was stored with silica gel in an air tight plastic bag for conservation.

The DNA was extracted using the peqGOLD Plant DNA Mini Kit (peqlab, Erlangen, Germany) according to the instruction manual. Due to the critical limitation of plant material, only 2-5 mg of each plants leaf tissue could be used for extraction, to keep some of the leaf material for a second extraction if necessary. The extracted DNA was eluted in 100 µl HPLC-water. 2 µl of the elution were mixed with the loading dye Orange G and run on an electrophoreses for 30 min at 80 V using agarose gel with GelRed (0.3 g Agarose, 30 ml TAE-Buffer, 0.3 µl GelRed) to confirm the presence of DNA under UV-light, in order to check if the extraction had worked successfully. The DNA-eluate was stored at -20°C.

2.2 DNA sequencing

Five *Draba pacheri* samples (two from Hohe Tauern, one from Gurktaler Alpen, one from Seetaler Alpen and one from Koralpe) were used for amplifying and sequencing the nuclear ITS region, using primers ITS 4 and ITS 5 (White et al., 1991), and the plastid trnL–trnF region (trnL-intron and trnL-trnF intergenic spacer) using primers c and f (Taberlet et al., 1991).

The PCRs were carried out on an Applied Biosystems GeneAmp PCR System 9700 thermocycler. The PCR mix contained 0.9 µl of DNA extract of unknown concentration and 19.1 µl Mastermix, which consisted for each sample of 9 µl 1.1X ReaddyMix PCR (Thermo Fisher Scientific, Braunschweig, Germany), 9 µl HPLC-water, 0.7 µl of each primer. For ITS, the PCR conditions were 4 min at 94°C, 35 cycles each with 1 min at 95°C, 1 min at 51°C, 1 min at 68°C and a final elongation step of 10 min at 72°C. For trnL–F , the conditions were 1 min 30 s at 94°C, then 35 cycles each: 15 s at 94°C, 15 s at 51°C, 1 min 30 s at 72°C and extension for 10 min at 72°C. Amplified DNA was cleaned with 1 µl FastAP and 0,5 µl Exo I using a Thermocycler, following the manufacturers´ instructions.

After the PCR amplification and cleaning, cycle sequencing was carried out in 10 µl, mixing 5.3 µl of the PCR product with 2 µl trehalose, 1.6 µl sequencing buffer, 0.6 µl Big Dye and 0.5 µl primers (concentration was 10 µM). After cleaning the products with Sephadex G-50 Fine

(Sigma-Aldrich, St. Louis, USA), they were loaded on the cycle sequencer (ABI 3730 DNA Analyzer capillary sequencer, Applied Biosystems).

Sequence reads were assembled using SeqMan 5.05 (DNAStar, Madison, WI, USA). Using Bioedit 7.2.5 (Hill, 1999), the ITS and trnL sequences were aligned to the datasets of Jordon-Thaden, 2010 (available as supplementary material), containing ITS and trnL sequences of 169 *Draba* species.

The three data sets (nuclear and plastid sequences separately and combined) were analyzed using maximum likelihood with the fast bootstrap option (with 1000 replicates) (Stamatakis et al., 2008) implemented in RAxML 8.0.14 (Stamatakis, 2014).

2.3 AFLPs

38 samples (36 *Draba pacheri* plants and two *Draba siliquosa* plants providing the outgroup) were used for AFLP analyses. The concentration of the samples was checked using a Nanodrop 1000 (Thermo Fisher Scientific, Waltham, USA) and amounted to a minimum of 15.2 ng per 1 µl eluate.

The AFLP protocol followed Vos et al. (1995) with the modifications described in Schönswetter et al. (2009) and Rešetnik et al. (2014). Three primer combinations were employed for the selective PCR (fluorescent dye in brackets): EcoRI (6-FAM)-ACA/MseI CAT, EcoRI (NED)-ACC/MseI-CAT and EcoRI (VIC)-AAG/MseI-CTG. Purification and visualization of PCR products were done as described in Rebernig et al. (2010). All samples were processed in a single PCR round. Eleven samples (26%) were used as replicates to test reproducibility.

The binary data matrix was further analyzed with the program SplitsTree 4.13.1 (Huson and Bryant, 2006) to create a NeighbourNet, calculating 10,000 bootstrap replicates. The program PAST 3.07 (Hammer et al., 2001) was used to calculate a PCoA, based on Jaccard´s similarity coefficient. AFLPdat (Ehrich, 2010) was used to calculate the “rarity1” (henceforth termed “rarity”) index, corresponding to the frequency down weighed marker value, proposed by Schönswetter and Tribsch (2005). Further, AFLPdat was executed to calculate “Nei’s gene diversity” (termed “genetic diversity” in the following) (Nei, 1987) for each of the four regions, as well as for the six populations.

2.4 Morphometric analysis

Herbarium material of various Austrian herbaria (GJO, GZU, KL and LI) as well as fresh plant material was examined. The plants found and chosen in the field were morphometrically analyzed on the spot in vivo, without being removed or harmed (only leaves were collected for DNA extraction).

Overall nine characters, relevant to distinguish *Draba pacheri* from *Draba norica*, could be assessed consistently on 161 herbarium vouchers (including only vouchers of wild grown individuals) and 32 living plants from the field work: (1) above ground plant height; (2) number of cauline leaves; (3) length of one rosette leaf; the mean values of the (4) fruit lengths, (5) fruit widths and (6) the ratios of fruit length : width, each measured on the 3 lowest, sufficiently developed siliques; mean values of (7) angles and (8) lengths of fruit pedicels, measured on the three lowest fruits; (9) length of the plant stem between rosette leaves and infructescence. The sample sizes for each region were: Hohe Tauern n=124, Gurktaler Alpen n=9, Seetaler Alpen n=24 and Koralpe n=36.

Many of the herbaria vouchers were dry or in bad condition, as well as some of the fresh material was in insufficient condition for trichome analysis, hence the trichome analysis was carried out only for the fresh, properly developed leaves collected in the field. 24 collected individuals (13 from Hohe Tauern, five from Gurktaler Alpen, five from Seetaler Alpen and one from Koralpe) were examined that way as well as nine additional herbarium vouchers (three from Seetaler Alpen and six from Koraple) to achieve a sufficient number of individuals for representative statistics for all populations.

Before grinding the collected leaves for DNA-extraction, trichome analysis of the lower surface of the proximal half of the rosette leaves was carried out. To this end, a binocular and a digital camera were used to take pictures of the leaf section to optimize quantification and assignment to hair types. Marking the counted trichomes with specific symbols prevented counting errors and allowed to conserve the information for future trichome studies on the rare material. In case of uncertainties concerning the classification of trichomes on the digital pictures, a binocular was used to examine the leaf surface directly.

Only stellate trichomes composed of four primary branches were counted after being classified into five types according to the structure of the secondary branching (**Fig. 2**): (A)

stellate trichomes without or with only one secondary, tooth-like branching, that had a length : width ratio < 2 : 1 and was maximally 0.15 times as long as the primary branch it was located on; (B) stellate trichomes with a minimum of two tooth-like secondary branchings; (C) stellate trichomes with none to several tooth-like branchings and one “true” secondary branch, that had a length : width ratio >= 2 : 1 and was at least 0.15 times as long as the primary branch it was located on; (D) stellate trichomes with none or more tooth-like branchings and two “true” secondary branches, both located on the same primary branch; (E) stellate trichomes with two or more true secondary branches located on at least two different branches.

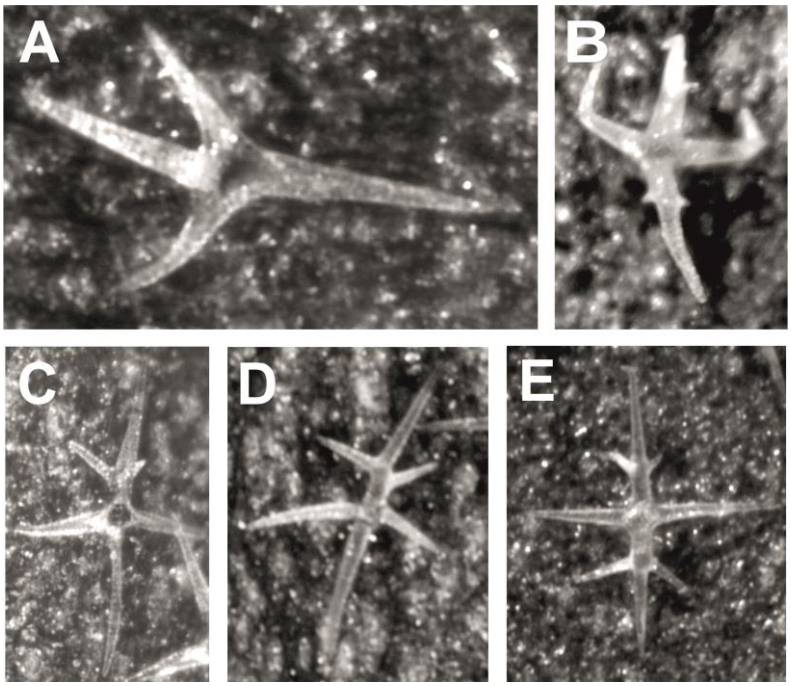

Fig. 2. Examples of the five trichome types A, B, C, D and E.

Morphometric data were analyzed using PAST 3.07 (Hammer et al., 2001). PCA was carried out on the morphometric characters. Morphometric differences between the four geographically distinct regions were tested using t-tests for normally distributed data and Mann-Whitney-tests for non-normally distributed data. For some characters, the data were log-transformed. For multiple comparisons, Bonferroni correction was applied.

3. Results

3.1 Plant material

In Austria, populations of *Draba pacheri* could be confirmed for all four regions (Koralpe, Seetaler Alpen, Gurktaler Alpen and Hohe Tauern). Since they partially embrace multiple sites, out of 16 subpopulations, eleven could be found and supplied with GPS coordinates (**Table 2**).

Table 2. All subpopulations listed with information about region and location, affiliation to population, status, GPS coordinates and altitude of the site. Population numbers refer to those illustrated on the map in Fig. 1.

mountain range location	pop# affiliation	pop code	status	n	GPS N	GPS E	altitude m.a.s.l.
Hohe Tauern, Pöllagruppe, Oblitzen	1	POB	confirmed	5	47° 04.760`	13° 27.241`	2627m
Hohe Tauern, Pöllagruppe, saddle between Oblitzen and Schurfspitze	1	PEO	confirmed	1	47° 04.719`	13° 26.807`	2547m
Hohe Tauern, Pöllagruppe, West of the saddle PEO	1	PWE	confirmed	1	47° 04.693`	13° 26.615`	2572m
Hohe Tauern, Pöllagruppe, East of Schurfspitze	1	POS	confirmed	1	47° 04.676`	13° 26.527´	2586m
Hohe Tauern, Pöllagruppe, Schurfspitze	1	PSC	not found	0			
Hohe Tauern, Pöllagruppe, Kareck	2	PKE	confirmed	5	47° 03.534`	13° 33.237`	2479m
Hohe Tauern, Pöllagruppe, Steinwandeck	2	PSW	not visited	0			
Hohe Tauern, Ankogelgruppe, Stern	3	AST	confirmed	1	47° 01.187`	13° 32.020`	2486m
Hohe Tauern, Ankogelgruppe, Wandspitze	3	AWS	not found	0			
Hohe Tauern, Ankogelgruppe, Wandkessel	3	AWK	not found	0			
Hohe Tauern, Ankogelgruppe, Poisnig	3	APG	confirmed	5	47° 00.771`	13° 31.784`	2520m
Gurktaler Alpen, Nockberge, Bretthöhe	4	GBH	confirmed	5	46° 54.604`	13° 55.988`	2149m
Lavanttaler Alpen, Seetaler Alpen, Linderseekar	5	SLK	confirmed	6	47°04.240`	14° 34.290`	2022m
Lavanttaler Alpen, Seetaler Alpen, Wildseekar	6	SWK	confirmed	1	47° 02.692`	14° 34.923`	2137m
Lavanttaler Alpen, Koralpe, Großes Kar	7	KGK	confirmed	5	46° 48.190`	14° 58.602`	1797m
Lavanttaler Alpen, Koralpe, Seekar	7	KSN	not found	0			
Belianské Tatry, Košiare	8	BTK	regional extinct	0			

3.2 ITS and trnL sequencing

Log-likelihood scores of the three phylogenetic trees were: for the ITS sequences -6418.974544, for the trnL sequences -6333.986215 and for the combination of ITS and trnL -14489.788324. *Draba pacheri* consistently fell into the clade of Core *Draba* Group III, described by Jordon-Thaden, in 2010. Specific relationships with other *Draba* species within the clade could not be revealed, since the resolution of the trees was low and clades were insufficiently supported by bootstrap values. The calculated trees did not show any differences among the *Draba pacheri* samples (**Fig. 3**).

Fig. 3. Phylogenetic relationships of *Draba pacheri* inferred from maximum likelihood analysis of combined ITS and trnL–F data (-ln likelihood of -14489.788324). Only core clade III from Jordon-Thaden (2010) is shown, whose position in the overall *Draba* phylogeny is indicated in grey in the insert phylogeny.

3.3 AFLPs

194 polymorphic fragments were detected and visualized in a binary presence/absence matrix. The average error rate was 0.0007.

Based on the AFLP presence/absence matrix, excluding outgroup individuals but including monomorphic markers and those present/absent in all but one individuals, a two-dimensional PCoA was calculated with PAST 3.07 (Hammer et al., 2001), using the Jaccard similarity index. Three separated main groups were revealed, in which one was Koralpe, one the Seetaler Alpen and one group comprised the Hohe Tauern and the Gurktaler Alpen populations (**Fig. 4**).

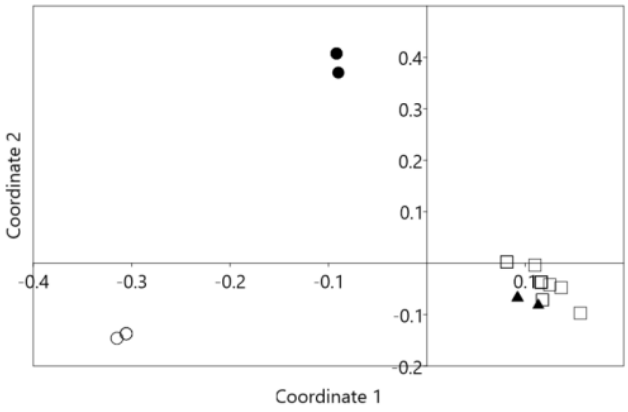

Fig. 4. PCoA of AFLP binary data matrix separated three main groups. □ =Hohe Tauern, ▲=Gurktaler Alpen, ○= Seetaler Alpen, ●= Koralpe

For AFLPdat calculations, the outgroup individuals were removed from the original binary matrix. Further, of 157 remaining fragments all monomorphic (88 fragments) and those present/absent in all but one individual (ten fragments) were removed. The final matrix consisted of 59 polymorphic fragments among 36 individuals.

Calculated for the four regions (Hohe Tauern, Gurktaler Alpen, Seetaler Alpen and Koralpe) the gene diversity (Nei, 1987) varied approximately 13-fold, ranging from 0.0068 (Gurktaler Alpen) to 0.0866 (Hohe Tauern) (**Table 3**).

The rarity values varied approximately 2-fold from 1.2575 (Hohe Tauern) to 2.5122 (Koralpe) among the four regions (**Table 3**).

Table 3. Rarity and genetic diversity values of the geographical regions containing *Draba pacheri* populations.

Mountain range	n	rarity	genetic diversity (NEI)
Hohe Tauern	19	1.2575	0.08663
Gurktaler Alpen	5	2.0658	0.00678
Seetaler Alpen	7	1.6026	0.00807
Koralpe	5	2.5122	0.01356

Furthermore, the regions were divided into six spatially separated groups, consisting each of a minimum of five individuals. Hence, only Hohe Tauern was divided into three populations (**Fig. 1**). The gene diversity (Nei, 1987) varied approximately 2.7-fold among the six populations, ranging from 0.0068 (pop. 4 and pop. 2) to 0.01808 (pop. 3) (**Table 4**). The rarity values varied approximately 2-fold from 1.2119 (pop. 1) to 2.5122 (pop. 7) among the six populations (**Table 4**).

Table 4. Rarity, and genetic diversity values of *Draba pacheri* populations are listed, each including a minimum of 5 samples. Hence, the Seetaler Alpen population number 6, containing only one sample, was not listed.

pop. #	Location	n	rarity	genetic diversity
1	Hohe Tauern, Poellagruppe West	8	1.2119	0.00726
2	Hohe Tauern, Kareck	5	1.4077	0.00678
3	Hohe Tauern, Ankogelgruppe	6	1.2202	0.01808
4	Gurktaler Alpen	5	2.0862	0.00678
5	Seetaler Alpen, Linderseekar	6	1.7953	0.00904
7	Koralpe	5	2.5486	0.01356

The NeighbourNet revealed three major groups, supported by high bootstrap values (**Fig. 5**): One group for Koralpe (pop. 7), one for Seetaler Alpen (pops. 5, 6) and one group comprising Gurktaler Alpen (pop. 4) and Hohe Tauern (pops. 1, 2 and 3). In this latter group, a further separation into three subgroups was evident. One well separated subgroup included only samples from the Gurktaler Alpen, another one only samples from Kareck (PKE) in the eastern Pöllagruppe of the Hohe Tauern (pop. 2). The third subgroup showed a trend for further separation between Hohe Tauern Ankogelgruppe (pop. 3) and Hohe Tauern western Pöllagruppe (pop. 1).

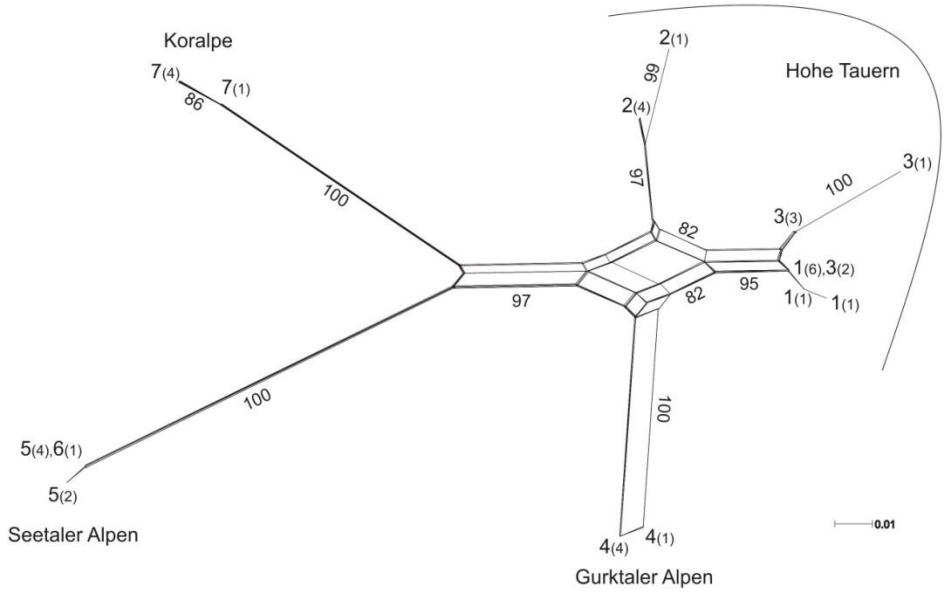

Fig. 5. NeighbourNet of AFLP data (using 10,000 bootstraps).

3.4 Morphometric analysis

Comparisons of six morphometric characters (plant height, fruit pedicel length, number of cauline leaves, angle between fruit pedicel and stem, ratio of fruit pedicel length to fruit length and fruit length) are shown in **Fig. 6**.

The plant height of specimens from Seetaler Alpen differed significantly from those of the other three regions (Koralpe: t=4.4495, p=3.9602E-05; Gurktaler Alpen: t=-2.8534, p=0.00764 and Hohe Tauern: t=-5.9867, p=1.5874E-08).

Concerning fruit pedicel length, plants in the Hohe Tauern (U=178, p=0.0006563), as well as the Koralpe (U=64.5, p=0.005713) were significantly shorter than those of the Gurktaler Alpen. Further, the fruit pedicels of the plants in the Hohe Tauern (U=615, p=5.438E-06), as well as the Koralpe (U=187.5, p=0.0002206) were significantly shorter than those of Seetaler Alpen.

Plants in the Hohen Tauern had significantly less cauline leaves than those in Koralpe (U=1334, p=9.308E-05) and significantly less than those in Seetaler Alpen (U=800.5, p=0.0001272).

Comparison of the angle between fruit pedicel and plant stem revealed that individuals in Gurktaler Alpen have a significantly smaller angle than those in Hohe Tauern (U=255.5, p=0.006811) and Seetaler Alpen (U=37.5, p=0.004613).

Regarding the ratio of fruit pedicel length and fruit length, values were significantly lower in Hohe Tauern than in Gurktaler Alpen (U=80.5, p=1.916E-05), in Seetaler Alpen (U=575, p=2.052E-06) and in Koralpe (U=813, p=6.722E-09).

Fruit length was significantly shorter in Koralpe than in Hohe Tauern (t=4.3926, p=2.0443E-5) and Seetaler Alpen (t=4.8473, p=9.739E-06).

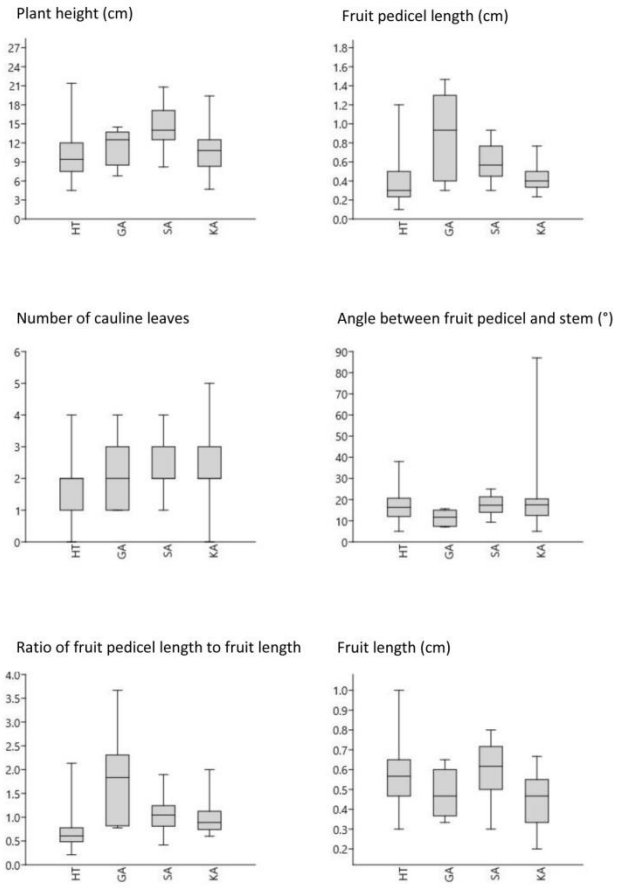

Fig. 6. Boxplot diagrams of six morphometric characters: plant height, fruit pedicel length, number of cauline leaves, angle between fruit pedicel and stem, ratio of fruit pedicel length to fruit length and fruit length. Illustrated data is untransformed. Hohe Tauern = HT, Gurktaler Alpen = GA, Seetaler Alpen = SA, Koralpe = KA

After exclusion of the single discrete character in the data set (number of cauline leaves), PCA for those 33 individuals, for which information about the proportion of trichomes of type D and E was available, did not show any clear separation according to the different geographic regions (**Fig. 7**). Only Gurktaler Alpen population was separated from the others, mainly due to three characters (**Table 5**): plant height, angle of the fruit pedicel and length of the fruit pedicel (**Fig. 6**).

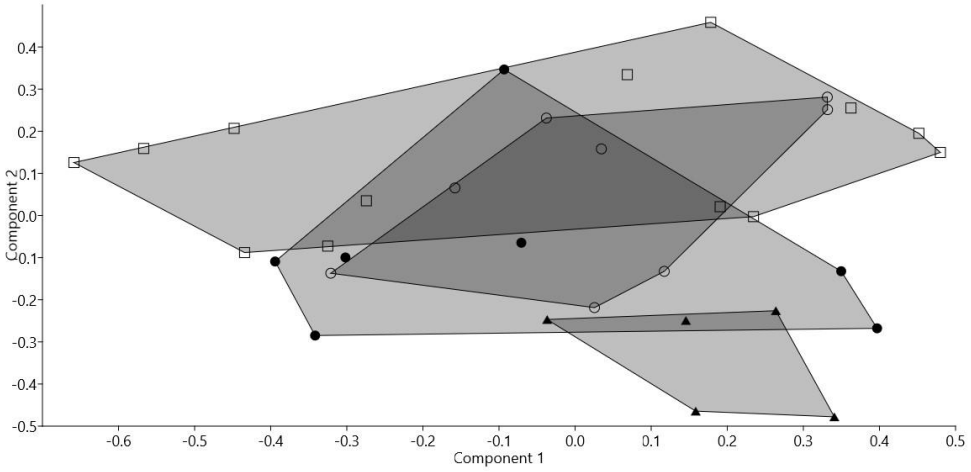

Fig. 7. PCA of morphometric data including trichome configuration information, excluding number of cauline leaves. □ =Hohe Tauern, ▲=Gurktaler Alpen, ○= Seetaler Alpen, ●= Koralpe. Eigenvalues, variances and loadings of the components 1 and 2 are listed in Table 5.

Table 5. Eigenvalues, variances and loadings of components 1 and 2 of the PCA of morphometric data (Fig. 7)

PCA	**PC 1**	**PC 2**
Eigenvalues	0.1023	0.0559
Variance	45.60%	24.93%
morphometric characters (loading >= 0,2)	**PC 1**	**PC 2**
plant height	0.4	3.67E-05
length of the fruit pedicel	0.573	-0.2953
angle of the fruit pedicel	-0.4	0.528
fruit length	0.361	0.401
fruit width	0.268	0.29
fruit form (l/w ratio)	0.0345	0.0281
leaf length	0.297	0.242
length of stem between rosette and infructescence	0.194	-0.006
% of trichomes with 2 or more sec. branchings	-0.15	-0.574

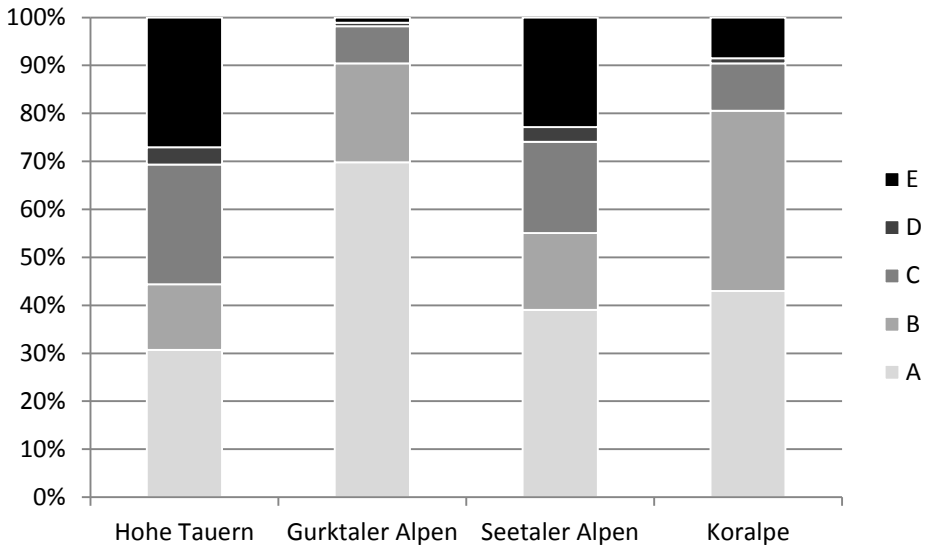

Fig. 8. Proportions of stellate trichome types A, B, C, D and E for each geographical region.

Region Hohe Tauern showed the highest proportion of trichomes with two or more secondary branches (types D and E) with over 30% of all trichomes considered. With more than 25% of all trichomes counted belonging to type D or E, the region Seetaler Alpen resembled Hohe Tauern, regarding the trichome configuration. In plants from Koralpe, only around 10% of leaf trichomes belonged to types D and E, but had the greatest proportion of trichomes with tooth-like branching (more than 35%). Plants from the Gurktaler Alpen had the greatest proportion of trichomes of types A, B and C with more than 95% and hence the lowest proportion of trichomes with two or more “true” secondary branchings. Around 70% of all trichomes belonged to type A, which was the highest proportion of this type among all four regions.

4. Discussion

4.1 Phylogeny and taxonomy

ITS and trnL marker based sequencing revealed clearly the affiliation of *Draba pacheri* to the *Draba* core III, as circumscribed by Jordon-Thaden in 2010 (**Fig. 3**), but due to the low resolution of the phylogenetic tree, as well as low bootstrap support values, clear relationships between *Draba pacheri* and other species could not be detected. Thus, neither hypothesis about the derivation of *Draba pacheri* could be tested. Taxonomic connections to putative closest relatives, such as the Nordic *Draba glabella*, suggested by Widder (1934), could also not be confirmed within this thesis. Further studies, using better resolving phylogenetic markers will be necessary to address this issue.

Two hypotheses have been suggested with respect to taxonomic treatment of *Draba pacheri* s.l.. Widder (1931, 1934) suggested to distinguish two taxa, *Draba norica* in Koralpe and *Draba pacheri* in Hohe Tauern, according to morphological differences. But at the time, the geographically intermediate populations from Seetaler Alpen and Gurktaler Alpen were not known yet. Melzer and Prugger (1986) suggested to distinguish four subspecies. Neither of these hypotheses is supported by our data. Instead, genetic data indicate the presence of three groups (**Fig. 4** and **Fig. 5**): Koralpe, Seetaler Alpen and one comprising Hohe Tauern and Gurktaler Alpen. These groups, however, cannot be defined morphologically, as morphological characters either only define single groups (e.g., individuals in Seetaler Alpen were significantly higher than in all other regions (**Fig. 6**)) or are incongruent with genetic groups (trichome types join Seetaler Alpen with Hohe Tauern to the exclusion of Gurktaler Alpen (**Fig. 8**)).

Since the sites of *Draba pacheri* differ in features like altitude (Koralpe around 1800 m.a.s.l. and Hohe Tauern around 2400 to 2600 m.a.s.l.) or geomorphology, morphologic characteristics could be influenced by ecological differences (such as variation of temperature, wind velocity or UV radiation). The plants are also randomly fertilized by sheep and chamois feces, which has a strong influence on the plant growth (Melzer et al., 1986).

The deep genetic split separating Koralpe and Seetaler Alpen from each other and from the other populations (**Fig. 5**), likely is due to persistent isolation in Pleistocene refugia (both areas are well known refugia: Tribsch and Schönswetter, 2003). This is also in line with the

high rarity values (**Table 3** and **Table 4**). The unexpected clear differentiation in the Hohe Tauern (**Fig. 5**) probably is also due to Pleistocene patterns (Tribsch et al., 2002; Escobar et al., 2012).

4.2 *Draba pacheri*, an endangered species

Draba pacheri in Slovakia is considered as "regionally extinct" (Turis et al., 2014). Furthermore, we could not confirm its presence in some subpopulations in Austria either. Even though we searched for the plants in Koralpe, Seekar, the type locality of *Draba norica* several times, we were not successful and cannot exclude the possibility, that *Draba pacheri* may be locally extinct. Since we could confirm most of the subpopulations in Austria, the species is not threatened with total extinction today. Nonetheless, low genetic diversity within some populations (**Table 4**) suggests that they underwent a genetic bottleneck and long-term isolation. Due to the resulting high level of genetic divergence among the *Draba pacheri* populations, it is necessary to protect all geographically separated populations. We further note, that Austria has sole responsibility for the conservation of this species.

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7. Curriculum vitae

Erich Kucs, BSc

Citizenship: Austria

Email: erich.kucs@univie.ac.at

Education

Since 2013 Curriculum Master "Ökologie"
University Vienna

June 12th, 2013 Bachelor´s degree in "Biologie"
University Vienna

March 19th, 2007 Final degree Mag. Art. in Instrumental-(Gesangs)pädagogik - Gitarre (klassik)
University of Music and Performing Arts

Since 2003 Curriculum Diplomstudium Biologie
University Vienna

June 16th, 1999 School leaving examination
Bundesrealgymnasium Pichelmayergasse
Vienna

Occupational career

2014–2015 Employment at Nationalpark Donau-Auen, Habitat mapping

2014 Employment at University Vienna
Project DISEQU-ALP

2010–2014 Tutor at the University Vienna for:
-Kenntnis mitteleuropäischer Lebensgemeinschaften
-Experimente aus Zoologie
-Diversität und Systematik der Höherer Pflanzen
-Feldkurs Ötschergräben

Since 2009 Guitar teacher in the Vienna International School

2002 Guitar teacher in the music school Wolkersdorf

1999–2013 Guitar teacher in Volkshochschule Favoriten, Vienna