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Indigenous and synanthropic populations of *Galanthus nivalis* L. in Baden-Württemberg

CHRISTINA SCHÜSSLER, MIKE THIV & ARNO WÖRZ

Abstract

Galanthus nivalis L. is a widely distributed species. The boundaries of its natural area of distribution, however, are fuzzy due to cultivation and naturalisation. For Germany, indigenous populations have been reported from Baden-Württemberg and Bavaria. To establish the genetic identity of the populations from Baden-Württemberg, we performed molecular phylogenetic analysis based on nuclear marker ITS and on chloroplast marker *trnL-trnF* spacer. In addition to that, morphometric data of indigenous and synanthropic plants were analysed. Our results indicate that the indigenes are genetically distinct from the synanthropes. Furthermore, a close relationship of the indigenes to specimens from the supposed centre of origin for the species has been suggested. Morphometrically, the identity of synanthropes and indigenes is hard to establish and contradicts findings from previous studies.

Keywords: Amaryllidaceae, Baden-Württemberg, *Galanthus nivalis* L., indigenous and naturalised populations, molecular phylogenetic analysis, morphometry.

Zusammenfassung

Galanthus nivalis L. ist eine weitverbreitete Art, deren Verbreitungsgebiet aufgrund von Kultivierung und Verwilderung jedoch nicht eindeutig abgegrenzt ist. Innerhalb Deutschlands gibt es in Baden-Württemberg und Bayern mutmaßlich indigene Populationen. Um die genetische Identität der Populationen in Baden-Württemberg zu ermitteln, wurden molekulare Phylogenien auf Basis des Markers ITS aus dem Kerngenom und des Chloroplastenmarkers *trnL-trnF* spacer berechnet. Außerdem wurde indigene und synanthrope Individuen morphometrisch untersucht. Unsere molekulargenetischen Analysen ergaben, dass indigene und synanthrope Populationen genetisch verschieden sind. Die indigenen Akzessionen sind eng verwandt mit Akzessionen aus dem vermuteten Ursprungsgebiet der Art. In unseren morphometrischen Untersuchungen lassen sich indigene und synanthrope Populationen allerdings nur schwer abgrenzen. Die sich abzeichnenden morphometrischen Tendenzen stehen des Weiteren im Widerspruch zu Ergebnissen früherer Studien.

Contents

1 Introduction

Galanthus L., the Snowdrop genus, comprises ca. 20 species (RØNSTED et al. 2013). Nearly half of them are on The IUCN Red List of Threatened Species (IUCN 2015). Their distribution ranges from the Pyrenees to Iran and from Italy to Central Europe (STERN 1956). The common Snowdrop, *Galanthus nivalis* L., is the most widely distributed *Galanthus* species. Its geographical range covers the Pyrenees and France in the west and extends over Central Europe, Italy and the Balkan Peninsula towards the Ukraine in the east (STERN 1956, HEGI 1939, SEYBOLD 1998). The Northern boundary of its distribution is hard to establish, as it is a popular horticultural plant and widely naturalized (WEBB 1980). The IUCN (2015) classifies *G. nivalis* as near threatened, as indigenous subpopulations often occur in small relictual forests or woodlands and are likely to suffer from habitat loss, for example due to climate change, plucking and forestry (CROOK & DAVIS 2013, SEYBOLD 1998). For Germany, indigenous populations have been reported from the Swabian Jura and the Southern Black Forest in Baden-Württemberg and from South Eastern Bavaria (SEYBOLD 1998, HEGI 1939, FLORAWEB 2013). According to SEYBOLD (1998), the Swabian Jura indigenes differ from the synanthropes by not growing in clusters, by possessing longer tepals and broader leaves and by bearing fruits more frequently. He hypothesised that the plants from the Swabian Jura might be identical with *Galanthus nivalis* var. *major* Tenore or *G. nivalis* subsp. *imperati* (Bertol.) Baker. However, the affinities of the indigenous populations from the Swabian Jura, the Southern Black Forest and the adjacent Swiss Jura, have not been confirmed yet. Thus, a relationship to broad-leafed *Galanthus elwesii* Hook. f. which occurs as a horticultural plant and naturalised in Baden-Württemberg (Seybold 1998, Florabw 2015), may also be proposed.

The aim of our study is to assess the genetic identity of the presumed indigenous populations from the Swabian Jura and the Southern Black Forest. By performing a molecular analysis of nuclear and chloroplast DNA markers, we test a) for the genetic affinity of the presumably indigenous populations and b) whether indigenous and synanthropic plants are genetically distinct. Additionally, we gather morphometric data in order to morphologically delimit indigenes and synanthropes.

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We would like to thank the Regierungspräsidium Tübingen (department 55/56) for providing the collection permit (file number 55-2/8841.06 N 4,112).

2 Material and methods

2.1 Sampling

Specimens and silica dried leaf samples of *Galanthus nivalis* were collected from eight indigenous and two synanthropic populations in Baden-Württemberg (Table 1). The criteria for indigenous populations were their near-natural habitats in beech forests and their caespitose habit of growth covering large areas in forest floors due to sexual reproduction. Synanthropic populations were garden escapes near settlements growing in small non-caespitose, tussock-like groups due to mostly asexual reproduction. From each population, 10 plants of different age were gathered. The vouchers have been deposited at the State Museum of Natural History Stuttgart (STU). To increase the sample size of synanthropic *G. nivalis*, further specimens from STU were included in the analyses. Additionally, one specimen of *G. nivalis* indigenous to Bavaria was included.

In total, 59 indigenous samples from nine different locations were analysed morphologically and nine samples from nine different locations genetically. Of the synanthropic samples, 41 from 21 different locations were analysed morphologically and four samples from four different locations genetically.

Our newly created sequences were combined with previously published sequences of *Galanthus* and of the outgroup taxa *Acis* Salisb. and *Leucojum* L., mainly derived from Larsen et al. (2010) and Rønsted et al. (2013). Sequence data from these authors was selected in a way that the major clades recovered in their studies were represented. Of *Galanthus nivalis*, eight of the nine accessions analysed by Rønsted et al. (2013) were included. One was excluded as its identity seemed to be ambiguous (listed as *Galanthus reginae-olgae* at GenBank but as *Galanthus nivalis* in Rønsted et al. 2013).

2.2 DNA isolation, PCR and Sequencing

Total genomic DNA was isolated applying the CTAB extraction protocol by Ivanova (2008). In case of herbarium material, we used the NucleoSpin Plant II kit (Macherey-Nagel) which according to our experience provides higher yields of DNA for degraded samples.

To test for sequence variation between samples from different localities, we amplified nrITS for one sample from each of the newly collected populations and for three plants obtained from STU (Tab. 1). The reaction mix contained 16.55 µl ddH$_2$O, 2.5 µl PCR buffer (10x), 2.5 µl dNTPs (2 mM), 1 µl of each primer (10 µM), 0.2 µl *Taq* polymerase (5 U/µl), 0.25 µl BSA (10 mg/ml) and 1 µl DNA template. As primers we used ITSA (5›-GGA AGG AGA AGT CGT AAC AAG G-3›) and ITSB (5›-CTT TTC CTC CGC TTA TTG ATA TG-3›) (Blattner 1999). The PCR conditions were 95 °C for 3 min, 9 cycles of (95 °C for 30 s, 60 °C (-0.5 °C per cycle) for 30 s, 72 °C for 50 s), 29 cycles of (95 °C for 30 s, 55 °C for 30 s, 72 °C for 50 s), 72 °C for 10 min. As almost no sequence variation was detected among the indigenes from different localities and also no sequence variation was present among the synanthropes from different localities, no intra-population sequences of ITS were created.

As markers from different genetic compartments can sometimes provide different phylogenetic hypothesis, the cp-marker *trnL-trnF* spacer was amplified in addition to nrITS. Although Rønsted et al. (2013) showed that cp data does not suffice to phylogenetically resolve relationships in *Galanthus*, we aimed at detecting if sequence variation between indigenes and synanthropes, whether phylogenetically informative or not, can also be observed in the chloroplast data. As indigenes and synanthropes were clearly separated by ITS data, one indigenous and one synanthropic sample were chosen to represent each clade. The reaction mix contained 16.33 µl ddH$_2$O, 2.5 µl PCR buffer (10x), 2.5 µl dNTPs (2 mM), 0.5 µl MgCl$_2$ (25 mM), 0.94 µl of each primer (10 µM), 0.2 µl *Taq* polymerase (5 U/µl), 0.1 µl BSA (10 mg/ml) and 1 µl DNA template. As primers we used c (5›-CGA AAT CGG TAG ACG CTA CG-3›) and f (5›-ATT TGA ACT GGT GAC ACG AG-3›) by Taberlet (1991). PCR conditions for *trnL-trnF* spacer were set to 94 °C for 1 min, 30 cycles of (94 °C for 50 s, 52 °C for 60 s, 72 °C for 80 s), 72 °C for 10 min according to Wu et al. (2013). PCR products were cleaned up and sequenced by the external service of LGC Genomics.

2.3 Phylogenetic analysis

Raw sequences were checked and assembled in Geneious 7.1.7 (Biomatters). Alignments were performed using the muscle algorithm implemented in PhyDE 0.9971 (Edgar 2004, Müller et al. 2010) and in case of obvious misalignment adjusted manually. We conducted Maximum Likelihood Analysis with 100 bootstrap replicates using the RAxML webserver at http://embnet.vital-it.ch/raxml-bb/index.php (Stamatakis et al. 2008). The best-fitting substitution model was determined under the AIC criterion in jmodeltest 2.1.5 (Darriba et al., 2012). For ITS, GTR+G was selected. For *trnL-trnF,* GTR+I+G was indicated. Additionally, we conducted Bayesian analysis using MrBayes v3.2.6. on CIPRES (Miller et al. 2010, Ronquist et al. 2012) with two independent runs. Each run consisted of one cold chain and three heated chains with a heating parameter of 0.2, sampling every 1000th generation. The total number of generations was chosen so that convergence was indicated by split frequencies and Potential Scale Reduction Factor (PSRF) values. After removing 25% of the trees as burn-in, a 50% majority rule consensus tree was generated. The nrITS and the *trnL-trnF* datasets were analysed separately as topological incongruences between nuclear and chloroplast phylogenies have been recovered for *Galanthus* by Rønsted et al. (2013).

2.4 Morphometric analysis

We measured the length of the outer tepals and the width of the leaves of suitable herbarium specimens using scale paper. Where more than one tepal or leaf of a plant was analysable, the mean value from all measurements for this plant was recorded. The arithmetic means of tepal length and leaf width were calculated a) for all indigenous populations and b) for all synanthropic samples and compared using two sample t-tests for equal/unequal variances. We checked for equality of variance using two sample F-tests.

3 Results

3.1 Phylogenetic analysis and DNA sequence variation

In total, the nrITS dataset comprised 48 accessions from 17 species and 764 characters. 49.6% of these characters were variable, 39.1% parsimony informative. The topology of our nrITS phylogeny (Fig. 1) is in accordance with the previously published molecular phylogenies by Larsen et al. (2010) and Rønsted et al. (2013). Indigenous as well as synanthropic populations were recovered within *G. nivalis* and clearly distinct from *G. elwesii*. *Galanthus nivalis* is well-supported as monophyletic (bootstrap value 100%, posterior probability 1.0). Within *G. nivalis* 1.2% of the characters were variable, 0.7% parsimony informative. Two different clades, clade A (bootstrap

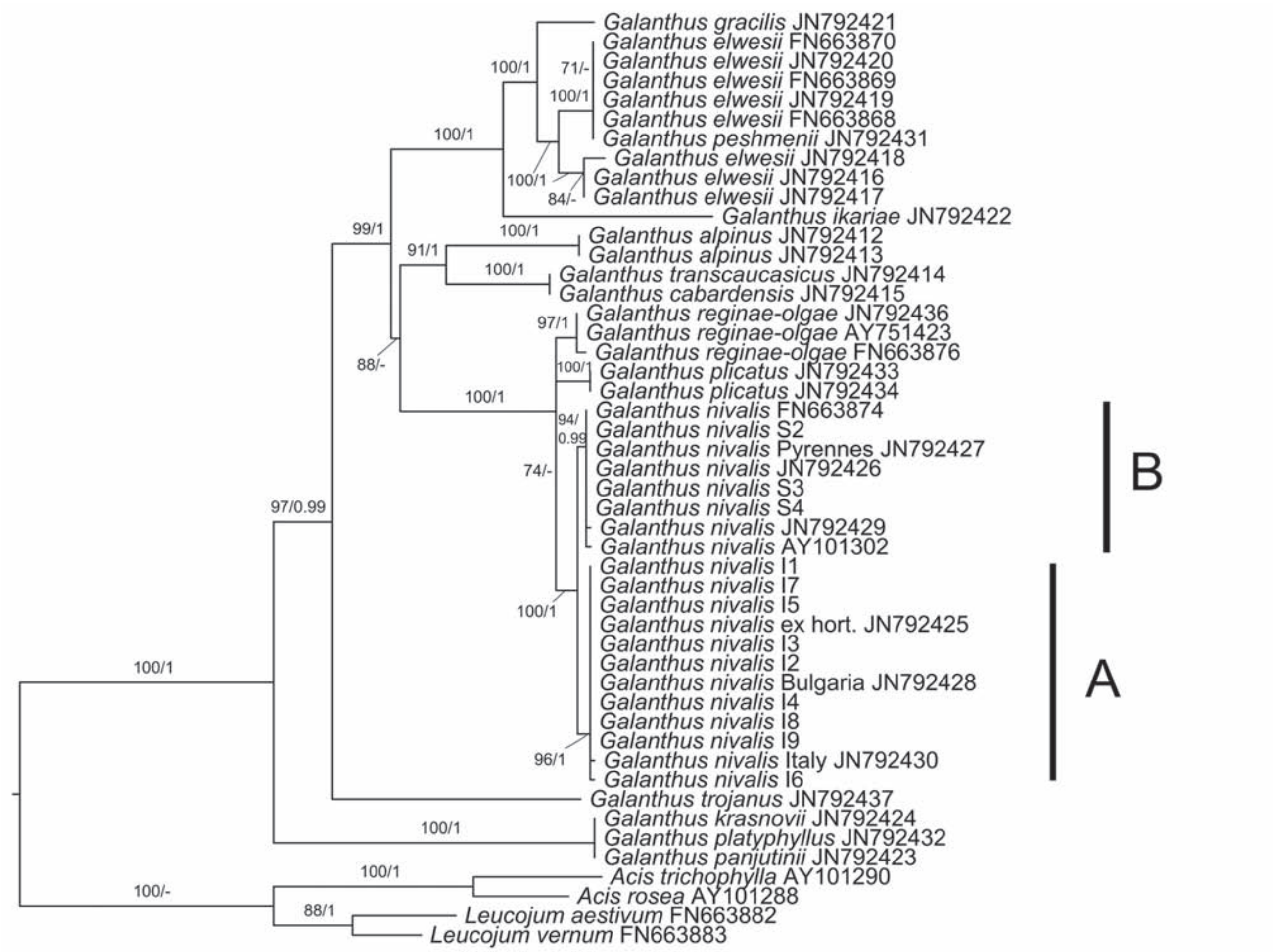

Fig. 1. Maximum likelihood phylogeny of *Galanthus* based on nrITS. *Acis* and *Leucojum* serve as outgroup. Bootstrap values >70 from Maximum likelihood analysis and posterior probabilities ≥0.90 from Bayesian analysis are shown along the branches (Bootstrap/posterior probability).

value 96%, posterior probability 1.0) and clade B (bootstrap value 94%, posterior probability 0.99), were recovered. The indigenous populations from the Swabian Jura as well as from the Southern Black Forest and a sample from South Eastern Bavaria are members of clade A, the synanthropic populations belong to clade B. The sequence obtained from synanthropic *G. nivalis* from Schafhausen (S1) has been excluded from analysis as it contained too many ambiguous base positions, which lowered the support values for clades A and B. In each of the two clades recovered within *G. nivalis*, less than 0.1%, that is 2 or less of 764 characters from 8 or 12 sequences respectively, were variable and none of them parsimony informative. Phylogenetic relationships within clade A and clade B are not resolved.

For the *trnL-trnF* spacer, the dataset comprised 20 accessions from 16 species and 926 characters. 8.1% of the characters were variable and 3.1% parsimony informative. As regards the *trnL-trnF* phylogeny (Fig. 2), the nivalis clade indicated in the cp-phylogeny of Rønsted et al. (2013) was recovered (bootstrap value 86%, posterior probability 1.0). It comprised *Galanthus nivalis*, *G. reginae-olgae*, *G. plicatus* and *G. gracilis*. Within this clade, relationships were largely unresolved. Of *Galanthus nivalis*, only clade B was statistically supported (bootstrap value 93%, posterior probability 1.0). Among the sequences obtained from one indigenous (I8) and one synanthropic sample (S2), less than 0.1%, that is 3 of 929, variable and parsimony informative characters were found.

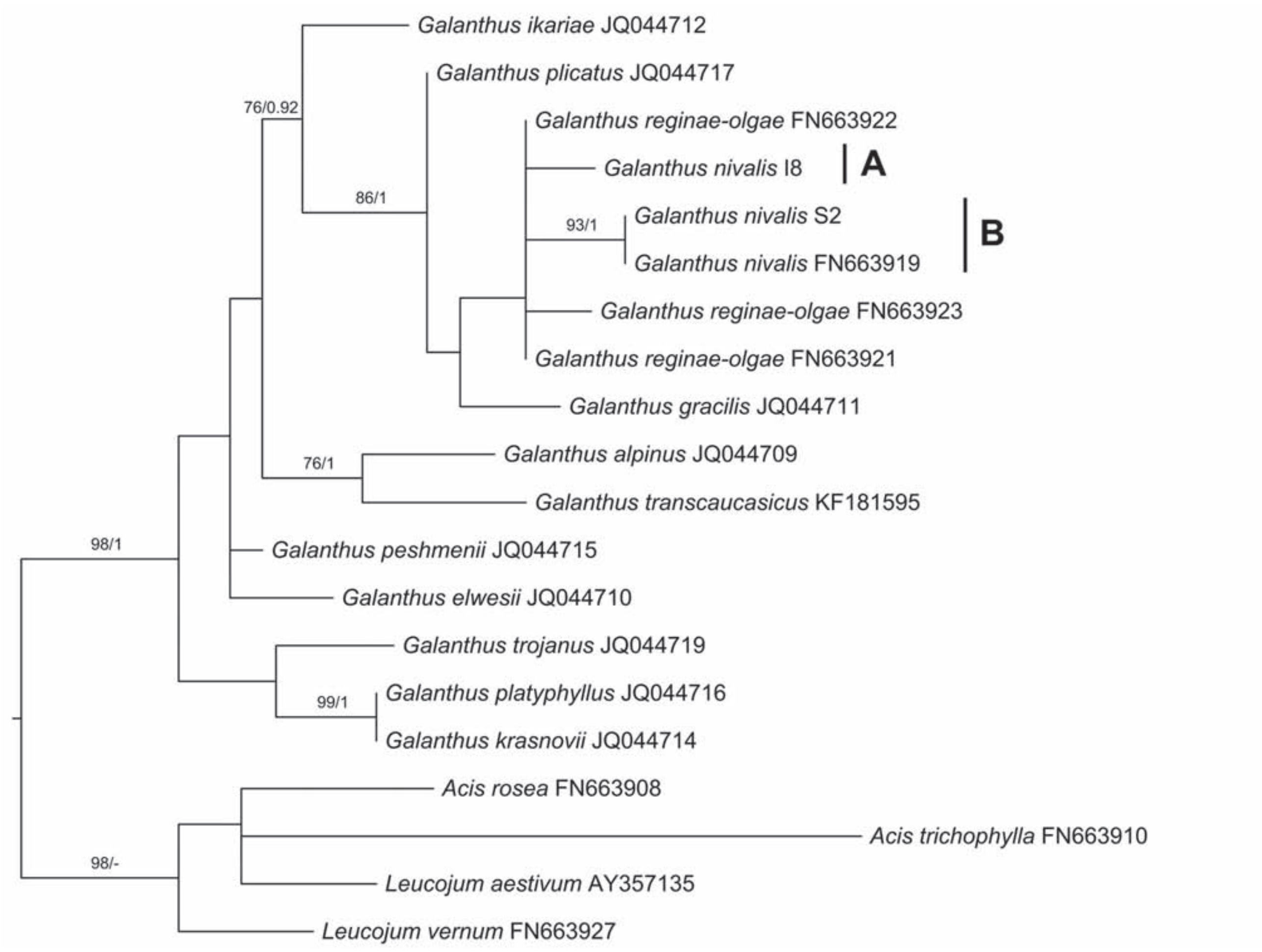

Fig. 2. Maximum likelihood phylogeny of *Galanthus* based on *trnL-trnF* spacer. *Acis* and *Leucojum* serve as outgroup. Bootstrap values >70 from Maximum likelihood analysis and posterior probabilities ≥0.90 from Bayesian analysis are shown along the branches (Bootstrap/posterior probability).

3.2 Tepal length and leaf width

Synanthropic *Galanthus nivalis* was found to have significantly longer tepals (19.6±3.0mm) than indigenous *G. nivalis* (18.2±2.2mm; t-test, df=62, p=0.00; Tab. 2). Leaf width of synanthropic (5.7±1.5mm) and indigenous (5.5±1.3mm) *Galanthus nivalis* did not differ significantly (t-test, df=77, p=0.64; Tab. 2).

4 Discussion

Our nrITS phylogeny shows that the analysed plants from Baden-Württemberg can be genetically confirmed as *Galanthus nivalis*. Like Rønsted et al. (2013), we recovered a clade containing South Eastern and Central European accessions and a second clade containing South Western European accessions within *G. nivalis*. The indigenous plants from the Swabian Jura and the Southern Black Forest as well as the sample from South Eastern Bavaria are part of the South Eastern and Central European clade. The synanthropic species are found to be genetically distinct from them, they group with the South Western European species. This genetic distinctness adds further evidence for treating the Swabian Jura and the Black forest populations as indigenes which should be of interest for conservation measures, for example with respect to forestry (cf. Seybold 1998), in these areas.

Chloroplast marker *trnL-trnF* spacer hints towards a distinctness of indigenes and synanthropes but neither supports nor rejects the phylogenetic hypothesis provided by nrITS due to a lack of phylogenetic resolution.

As regards morphology, synanthropes and indigenes cannot be distinguished easily. *Galanthus nivalis* is very variable in size (Webb 1980). Larger variants like G. *nivalis* subsp. *imperati* have been reported from Southern and Southern Central Europe and are connected to typical *G. nivalis* by various intermediates (Webb 1980, Trotter 1915). According to Seybold (1998), the Swabian Jura indigenes might be identical with these larger variants. Our morphometrical analyses, however, are in contrast to the observations by Seybold (1998). We found larger tepals in synanthropes. However, no significant differences in leaf size between synanthropes and indigenes were seen. This probably also contrasts Rønsted et al. (2013), who stated that they could not find any morphological and systematic differences between plants from clade A and clade B. The reasons for these discrepancies remain speculative. Possible causes might be a large amount of phenotypic plasticity, variation in nutrients and water in the soil over time and space, sampling bias/small sample size or drying artefacts like bent tepals. To get more conclusive results, fresh material should be measured.

Our results suggest that there are at least two different genetic variants of *Galanthus nivalis* in Baden-Württemberg. The first has already been classified as indigenous based on morphology and occurrence as it can be found in unconnected tributary valleys of a valley system (Seybold 1998). Our molecular data show that it is part of a clade containing accessions from the Balkans, the region where *G. nivalis* possibly originated (Meerow et al. 2006). Its area of distribution might have been shrunken during glaciations. Afterwards, the Swabian Jura could have been recolonised from a relict population within the Jura by ant dispersal (Seybold 1998). The second variant is represented by the synanthropes, which are part of a clade containing plants from Spain and France. Thus, *G. nivalis* used in horticulture in Baden-Württemberg probably descended from South Western European plants. Despite being probably morphologically distinct from the Swabian Jura indigenes, the Black Forest indigenes could not be distinguished genetically from the Swabian Jura indigenes. To further resolve the relationship within the Baden-Württemberg indigenes, a more variable marker system than ITS or a combination of more markers will be needed.

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Table 1. Taxa used for molecular analysis and GenBank accession numbers. Sequences created newly in this study are marked in bold, the rest of the sequences were downloaded from Genbank. I1 to I8 mark the newly collected indigenous populations, S1 and S2 the newly collected synanthropic populations. I9, S3 and S4 were obtained from herbarium material.

	Species	Collector	Coll. number	Coll. date	Herbarium	Locality	Status	ITS	*trnL-trnF*
	Acis rosea (F.Martin bis) Sweet	Chase	1524		K	Corsica		AY101288	FN663908
	Acis trichophylla G. Don.	Chase	1526		K	Morocco		AY101290	FN663910
	Galanthus alpinus Sosn.	Davis	s.n.		K	Caucasus		JN792412	JQ044709
	Galanthus alpinus Sosn.	Davis	s.n.		K	Caucasus		JN792413	-
	Galanthus cabardensis Koss	Zubov & Didenko	s.n.		K	Caucasus		JN792415	-
	Galanthus elwesii Hook. f.	Zubov & Didenko	s.n.		K	Ukraine		JN792416	-
	Galanthus elwesii Hook. f.	Zubov & Didenko	s.n.		K	Ukraine		JN792417	JQ044710
	Galanthus elwesii Hook. f.	Davis	353		K	Greece		JN792418	-
	Galanthus elwesii Hook. f.	Davis	s.n.		K	Turkey		JN792420	-
	Galanthus elwesii Hook. f.	Allison & Ball	s.n.		K	Turkey		JN792419	-
	Galanthus elwesii Hook. f.	Rønsted	328		C	Ex hort.		FN663868	-
	Galanthus elwesii Hook. f.	Rønsted	335		C	Ex hort.		FN663869	-
	Galanthus elwesii Hook. f.	Rønsted	341		C	Ex hort.		FN663870	-
	Galanthus gracilis Celak.	Davis	s.n.		K	Turkey		JN792421	JQ044711
	Galanthus ikariae Baker	Davis	s.n.		K			JN792422	JQ044712
	Galanthus krasnovii A.P. Khokhr.				BONN	Turkey		JN792424	JQ044714
I1	***Galanthus nivalis*** **L.**	**Wörz**	**15.03.12.01.**	**12.III.2015**	**STU**	**Baden-Württemberg, Swabian Jura, Tiefental north of Trochtelfingen**	**I**	**KU739398**	**-**
I2	***Galanthus nivalis*** **L.**	**Wörz**	**15.03.12.02.**	**12.III.2015**	**STU**	**Baden-Württemberg, Swabian Jura, Burgstall close to Oberstetten**	**I**	**KU739397**	**-**
I3	***Galanthus nivalis*** **L.**	**Wörz**	**15.03.12.03.**	**12.III.2015**	**STU**	**Baden-Württemberg, Swabian Jura, Tiefental east-northeast of Aichstetten**	**I**	**KU739396**	**-**
I4	***Galanthus nivalis*** **L.**	**Wörz**	**15.03.12.04.**	**12.III.2015**	**STU**	**Baden-Württemberg, Swabian Jura, Bärental south of Anhausen**	**I**	**KU739395**	**-**

	Species	Collector	Coll. number	Coll. date	Herbarium	Locality	Status	ITS	*trnL-trnF*
I5	***Galanthus nivalis* L.**	**Wörz**	**15.03.12.05.**	**12.III.2015**	**STU**	**Baden-Württemberg, Swabian Jura, Valley south of Lindehof close to Buttenhausen**	**I**	**KU739394**	**-**
I6	***Galanthus nivalis* L.**	**Wörz**	**15.03.12.06.**	**12.III.2015**	**STU**	**Baden-Württemberg, Swabian Jura, Hochberg west of Buttenhausen**	**I**	**KU739393**	**-**
I7	***Galanthus nivalis* L.**	**Wörz**	**15.03.12.07.**	**12.III.2015**	**STU**	**Baden-Württemberg, Swabian Jura, Tiefental northwest of Buttenhausen**	**I**	**KU739391**	**-**
S1	***Galanthus nivalis* L.**	**Wörz**	**15.03.18.01.**	**18.III.2015**	**STU**	**Baden-Württemberg, Großes Härtle east of Schafhausen**	**S**	**KU739401**	**-**
I8	***Galanthus nivalis* L.**	**Wörz**	**14.03.12.01.**	**12.III.2014**	**STU**	**Baden-Württemberg, Southern Black Forest, Alb Gorge close to Buch**	**I**	**KU739400**	**KU739388**
I9	***Galanthus nivalis* L.**	**Schmid**	**MS-99-2**	**13.III.1999**	**STU**	**Bavaria, hardwood floodplains of the Röthenbach close to Bad Aibling**	**I**	**KU739392**	**-**
S2	***Galanthus nivalis* L.**	**Wörz**	**15.02.27.01**	**15.II.2015**	**STU**	**Baden-Württemberg, Höfingen, Eselspfad**	**S**	**KU739399**	**KU739387**
S3	***Galanthus nivalis* L.**	**Frey**	**s.n.**	**03.III.2010**	**STU**	**Baden-Württemberg, Nürtingen, Old Cemetery**	**S**	**KU739390**	**-**
S4	***Galanthus nivalis* L.**	**Thiv**	**s.n.**	**15.VI.2015**	**STU**	**Baden-Württemberg, Stuttgart, courtyard of the State Museum of Natural History**	**S**	**KU739389**	**-**
	Galanthus nivalis L.	Rønsted	343		K			FN663874	FN663919
	Galanthus nivalis L.	MCLW	9302			Bulgaria		JN792428	-
	Galanthus nivalis L.	HEPF	4326		K	Italy		JN792430	-
	Galanthus nivalis L.	Davis	s.n.		K	France, Pyrennes		JN792427	-
	Galanthus nivalis L.	Nutt	189		K	Spain		JN792429	-
	Galanthus nivalis L.	Chase	11194		K	Spain		AY101302	-
	Galanthus nivalis L.	Davis	s.n.		K	Ex hort.		JN792425	-
	Galanthus nivalis L.	Davis	s.n.		K	Greece		JN792426	-

	Species	Collector	Coll. number	Coll. date	Herbarium	Locality	Status	ITS	*trnL-trnF*
	Galanthus panjutinii Zubov & A.P. Davis	Zubov & Didenko	s.n.		K	Russia		JN792423	-
	Galanthus peshmenii A.P. Davis & C.D. Brickell	Rønsted	458		C	UnitedK ingdom		JN792431	JQ044715
	Galanthus platyphyllus Traub & Moldenke	Rix	s.n.		K	High Caucasus		JN792432	JQ044716
	Galanthus plicatus M. Bieb.	Davis	s.n.		K	Crimea		JN792434	-
	Galanthus plicatus M. Bieb.	Zubov & Didenko	s.n.		K	Ukraine		JN792433	JQ044717
	Galanthus reginae-olgae Orph.	Meerow	2611		FTG			AY751423	-
	Galanthus reginae-olgae Orph.	Rønsted	327		C	Greece		FN663876	FN663921
	Galanthus reginae-olgae Orph.	Davis	s.n.		K	Sicily		JN792436	-
	Galanthus reginae-olgae Orph.	Rønsted	342		C	Greece		-	FN663923
	Galanthus reginae-olgae Orph.	Rønsted	334		C	Greece		-	FN663922
	Galanthus transcaucasicus Fomin	Zubov & Didenko	s.n.		K	Armenia		JN792414	-
	Galanthus transcaucasicus Fomin	Whir	089		KWHA	Iran		-	KF181595
	Galanthus trojanus A.P. Davis & Ozhatay	Davis	s.n.		K	Turkey		JN792437	JQ044719
	Leucojum aestivum L.	Graham & Barrett	00-4-2		ALTA			-	AY357135
	Leucojum aestivum L.	Rønsted	345		C	Ex hort.		FN663882	-
	Leucojum vernum L.	Rønsted	339		C			FN663883	FN663927

Table 2. Leaf width and tepal length of Baden-Württemberg indigenes and synanthropes. Values shown are the arithmetic mean (m), standard deviation (s) and sample size (n).

Sample	**Region**	**Tepal length (mm)**			**Leaf width (mm)**		
		m	**s**	**n**	**m**	**s**	**n**
Synanthropes	Baden-Württemberg	19.6	3.0	40	5.7	1.5	35
Indigenes	Baden-Württemberg	18.2	2.2	56	5.5	1.3	44