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RESEARCH ARTICLE

Arnica montana subsp. *atlantica*: Really a subspecies?

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Abstract In *Arnica montana* L. (Asteraceae) two subspecies are described, *A. montana* subsp. *atlantica* (AMA), present only on the Iberian Peninsula and *A. montana* subsp. *montana* (AMM) with a very wide distribution area. The morphological differences between the two subspecies are small and variable. Therefore, this concept is sometimes questioned. To establish the genetic background of the two subspecies, populations of AMA and AMM together with herbarium samples and DNA Bank material of AMM were tested with 12 microsatellite markers. *A. montana* propagates by seeds or by clonal propagation of its rhizome. In AMA, clonality was frequent while in AMM only one case of clonality could be identified. Therefore, further results were clone-corrected. Genetically, AMA separated very well from AMM with a G_{ST} between the subspecies of 0.81, genetically justifying the subspecies concept of *A. montana*. Genetic variability in AMA (H_{exp}=0.28) was lower than in the AMM populations (H_{exp}=0.70). A somewhat higher fixation index of AMA (F_{ST}=0.17, compared to an F_{ST}=0.08 for AMM) may indicate that geneflow in AMA is a bit more restricted than in alpine AMM. However, the fixation index of AMA is not deviating from Hardy–Weinberg equilibrium. No inbreeding was observed for AMA (F_{IS}=0.10) and AMM (F_{IS}=0.08).

Keywords *Arnica montana* · *Arnica montana* subsp. *atlantica* · *Arnica montana* subsp. *montana* · Genetic analysis · Microsatellites

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Introduction

Arnica L. (Asteraceae, Heliantheae s.l.) is a circumboreal genus of about 30 species mostly of montane habitats. *Arnica montana* L. (Asteraceae, mountain arnica, wolfs' bane) is a perennial, facultative apomictic species (Yankova-Tsvetkova et al. 2016), predominantly self-incompatible, insect pollinated which reproduces sexually with seeds and vegetatively with short rhizomes (Luijten et al. 1996, 2000). *A. montana* grows on acidic grass- and shrublands and is distributed from the Iberian Peninsula to the

Ukraine (Maurice et al. 2012). Bolos y Vayreda (1945) distinguished two subspecies, *A. montana* subsp. *atlantica* A. Bolos (AMA) and *A. montana* subsp. *montana* (AMM), where AMA is present only in SW-France, N-Spain and Portugal. AMA is morphologically different in its smaller height, thinner floral stems, lanceolate leaves and smaller flower heads with fewer bracts. This subspecies differs from AMM also in its habitat preferences, it occurs between 0 and 440 m (max. 1000 m) in areas of oceanic climate while AMM occurs from 0 to 3000 m. This subspecies concept was recently questioned by the analysis of biometrical data from different populations in Galicia where the only significant difference between low- and highland plants was found in plant height (Romero et al. 2011).

The two major proveniences of arnica flowers for the pharmaceutical/cosmetics industry in Europe are the Romanian Carpathians and NW Spain (Galicia) (Vera et al. 2016). Mountain arnica is an old folk medicine still popular for the treatment of pain, swelling and bruises. Due to its topical application of flower extracts in gel or cream form it is regarded as 'cosmeceutical' (Baumann 2007). Sesquiterpene lactones (SL) are responsible for the anti-inflammatory activity of arnica (Wagner et al. 2004) with helenalin esters (H) showing higher anti-inflammatory activity than dihydrohelenalin esters (DH) (Klaas et al. 2002) while DH are less allergenic than H (Lass et al. 2008). Lowland arnica (AMA) is a DH-chemotype, while AMM possesses predominantly helenalin esters (cf. for example chromatograms in the European Pharmacopoeia where the DH-chemotype is described as the 'Spanish type', while the Helenalin-chemotype is called 'East-European type' (EDQM 2014)).

In general, *A. montana* is so abundant in many countries that The IUCN Red List of Threatened Species classifies *A. montana* as "Least Concern", although monitoring population trends is suggested due to the decline in some countries (Falniowski et al. 2011). Following this suggestion, Luijten et al. (1996, 2000) studied the effect of habitat fragmentation on population structure of AMM in the Netherlands and found reduced levels of genetic variation and limited gene flow between the populations. A strong genetic differentiation and a suggested restricted gene flow with signs of genetic erosion in lower altitudes were also recently found in the large-scale genetic study of Duwe et al. (2017) on AMM. Furthermore, in recent decades a significant decline in the populations of arnica were also observed in Galicia (Lange 1998; Romero et al. 2011).

Cultivation of *A. montana* subsp. *montana* is possible, but not without problems. The species need a loose, well-aerated soil with an ample supply of water and a lime content of less than 1%. Otherwise, the plant reacts immediately with chlorosis. Seed germination is another difficulty in cultivation (von Raison et al. 2000).

Since lowland proveniences of AMA are significantly different in their chemical composition from AMM, it would be interesting to know if the genetic distance justifies the proposed division into two subspecies. To address this question, individuals from NW Spain and Central Europe were classified as subspecies AMA or AMM according to the criteria defined by Bolos y Vayreda (1945) and compared with a set of microsatellite markers recently published by Duwe et al. (2015).

Materials and methods

Sample material

In total a sample set of 89 individuals was analysed and classified according to Bolos y Vayreda (1945) as *A. montana* subsp. *montana* (33 samples, AMM) or *A. montana* ssp. *atlantica* (55 samples, AMA). One sample of *A. chamissonis* Less. was used as outgroup (Table 1). The samples were obtained from the herbarium of the University of Vienna (WU), collected from the wild in 2016 (aerial plant parts only) and were obtained from the DNA Bank of the Botanic Garden and Botanical Museum Berlin-Dahlem (BGBM) (see Table 1). All tissue samples from the BGBM and the underlying voucher specimens are deposited at the Botanic Garden and Botanical Museum Berlin and are available via the Global Genome Biodiversity Network (GGBN) (Droege et al. 2016) and the Global Biodiversity Information Facility (GBIF). Specimens collected in Spain were deposited in the herbarium of Kräuter-Mix, specimens collected in Austria in WU.

Table 1 Locality and specimen information of reference samples used in this study

Pop.	n	ng	Species	Origin (location; elevation in m.s.m., GPS coordinates; collection date)
ES01	10	8	AMA	Spain, Galicia, 0.4 km NNE of Vilouris; 480 m, 43°12.30 N, 8°2.33′W; 2016-06-28
ES02	10	6	AMA	Spain, Galicia, 2.6 km NW of Vilouris; 482 m, 43°12.70′N, 8°04.12′W; 2016-06-28
ES03	10	3	AMA	Spain, Galicia, 1.4 km SW of Vilouris; 513 m, 43°11.56′N, 8°03.10′W; 2016-06-28
ES04	5	1	AMA	Spain, Galicia, 3 km NNE of Xermade; 451 m, 43°22.97′N, 7°48.45′W; 2016-06-29
ES05	10	4	AMA	Spain, Galicia, 3.3 km WNW of Susana; 705 m, 43°28.52′N, 7°40.40′W; 2016-06-29
ES06	10	6	AMA	Spain, Galicia, 1.5 km NNE of Susana; 607 m, 43°28.86′N, 7°37.48′W; 2016-06-29
OG01	1	1	ACH	WU: Austria, Vienna, cultivated at HBV; 1995-09-04
CE01	10	9	AMM	Austria, Styria, Steinplan; 1640 m, 47°9.73′N, 14°54.09′E; 2016-07-21
CE02	10	10	AMM	Austria, Carinthia, 6 km N of Millstatt, Hansbauerhütte; 1720 m, 46°51.47′N, 14°53.10′E; 2016-07-24
CE03	1	1	AMM	WU: Switzerland, Grisons, NW Ravaisch; 2004-08-22
CE04	1	1	AMM	WU: Italy, S-Tyrol, Central Alps, Passeier, Platt; 1995-07-10
CE05	1	1	AMM	WU: Austria, E-Tyrol, Defereggen, Oberberg, N St. Jakob; 1987-08-04
CE06	1	1	AMM	WU: Austria, Carinthia, Hohe Tauern, Hafnergruppe; 2003-07-07
CE07	2	2	AMM	WU: Austria, Lower Austria, SW Waldviertel; 2009-06-05
			AMM	WU: Austria, Lower Austria, Waldviertel, Langsehschlag; 1913-06-05
CE08	1	1	AMM	WU: Austria, Vienna, NW-Plateau of Sophienalpe; 1950
CE09	1	1	AMM	BGBM (BGT 0008920): Germany, Saxony, Oelsen, Osterzgebirge; 632 m, 50°47′N, 13°56′E; 2013-06-03
CE10	1	1	AMM	BGBM (BGT 0012009): Germany, Mecklenburg-Western Pomerania, Zarrendorf, Stralsund; 0 m, 54°14′N, 13°05′E; 2013-06-17
CE11	1	1	AMM	BGBM (BGT 0013108): Germany, Brandenburg, Naturpark Niederlausitzer Heidelandschaft; 97 m, 51°30′N, 13°46′E; 2013-06-20
CE12	1	1	AMM	BGBM (BGT 0011921): Italy, S-Tyrol, 3 km ENE of Badia; 2062 m, 46°37′N, 11°56′E; 2013-06-29
CE13	1	1	AMM	BGBM (BGT 0013144): Germany, Baden-Württemberg, Black Forest; 1424 m, 47°52′N, 8°01′E
CE14	1	1	AMM	WU: France, E-Pyrenees, Superbolquere; 1750 m; 1944-07-17.-20

Pop. … population, n … number of sampled individuals per population, ng … number of individuals with different multilocus genotypes (genets), *AMA* ... *Arnica montana* subsp. *atlantica*, *AMM* ... *A. montana* subsp. *montana*, *ACH* ... *A. chamissonis, BGBM* … Botanical Garden and Botanical Museum Berlin-Dahlem, Germany, HBV … Botanical Garden of the University of Vienna, Austria, WU … Herbarium of the University of Vienna, Austria

Extract for HPLC analysis of sesquiterpene lactones

Extraction for the analysis of sesquiterpene lactones was performed using a modification of the European Pharmacopoeia Monograph Arnica flower (EDQM 2014). In detail, dried flowers (approx. 5 g) were milled. 1.0 g of the powdered drug was weighed exactly into a 250 mL flat bottom flask. After addition of 2.0 mL internal standard solution containing 1 mg/mL santonin in MeOH, immediately prepared prior to use, and 50 mL MeOH, the mixture was extracted for 1 h using a reflux condenser. The cooled solution was centrifuged for 15 min at 4500 rpm. To the supernatant, 7 g of neutral aluminium oxide was added. After shaking for 120 s the mixture was filtered through a folded filter into a 100 mL flask. The filtrate was brought to dryness in vacuo and re-suspended in 3.0 mL of a mixture of equal volume parts of MeOH and H_2O. After filtration, the solution was used for HPLC analysis.

HPLC analysis

HPLC analysis of sesquiterpene lactones were performed using an Alliance 2695 high pressure gradient system (Waters GmbH, Eschborn, Germany) equipped with a DAD detector. The following parameters were applied: column, Merck Superspher 100 RP 18e 125 × 4 mm (4 µm particle size); guard

column, Merck LiChrospher 100 RP 18e, 4 × 4 mm (5 µm particle size); mobile Phase A, H_2O; mobile Phase B, MeOH; flow rate, 1.2 ml/min; injection volume, 20 µL; detection wavelength 225 nm; oven temperature, 20 °C; isocratic 0–3 min 38% B; linear gradient 3–20 min 45% B; isocratic 20–30 min 45% B; linear gradient 30–55 min 55% B; linear gradient 55–57 min 100% B, 70 min stop. The assignment of chemotypes was deduced by comparing the chromatograms with the chromatograms in the European Pharmacopoeia representing the two chemotypes ('Spanish Type' and 'East European Type') (EDQM 2014).

DNA extraction

Genomic DNA was extracted from air dried specimens using a modified CTAB-protocol (Schmiderer et al. 2013) based on Doyle and Doyle (1990). DNA concentrations of the extracts were determined using a NanoDrop ND-2000c (Peqlab Biotechnologie GmbH, Erlangen, Germany). DNA extracts were diluted with Milli-Q water to 5 ng/µL.

Microsatellite analysis

Microsatellite markers and part of the primer sequences were adopted from Duwe et al. (2015). Remade primers with an optimum melting temperature ranging from 51 to 53 °C were designed using Primer Express 2 (Applied Biosystems, Foster City, California, USA). Primer dimers were evaluated using NetPrimer software (http://www.premierbiosoft.com/netprimer). Multiplexing of different loci was performed using Multiplex Manager (www.multiplexmanager.com).

PCR amplification was performed using tailed locus-specific forward primers, fluorescence labelled nested forward primers (5′ modified with 6-FAM, ATTO532, ATTO550 or ATTO565) binding to the forward primer extensions and "PIG-tailed" reverse primers to reduce stutter bands (with 3–4 bp extensions to achieve a GTTT consensus sequence at the 5′-end, according to Brownstein et al. 1996). Unlabelled and 6-FAM labelled primers were obtained from Sigma-Aldrich (Vienna, Austria), all ATTO labelled primers were obtained from Microsynth (Vienna, Austria).

For 15 µL PCR reactions, 10 ng of genomic DNA was added to a master mix containing 1 × PCR buffer B, 2 mM $MgCl_2$, 200 µM dNTPs (each), 0.6 U Taq HOTFIREPol DNA Polymerase (all reagents from Solis BioDyne, Tartu, Estonia), 200 nM fluorescent labelled forward primers, 50 nM locus specific forward primers and 250 nM locus specific reverse primers. Samples with no or insufficient amplification were repeated with different DNA amounts (0.25–10 ng) and 0.9 U polymerase. The PCR conditions included a denaturation step at 95 °C for 15 min, followed by 30 cycles at 95/58/72 °C for 30/45/45 s, 15 cycles at 95/53/72 °C for 30/45/45 s, and a final elongation step at 72 °C for 10 min. PCR products were checked on 2% agarose gels stained with PeqGreen (VWR International, Vienna, Austria; 4 µL/100 mL agarose solution; products including 6-FAM) or without staining (PCR products including ATTO dyes). Six amplified loci per sample (1 µL PCR product each; Table 2) were mixed and diluted with 24 µL ddH_2O. The determination of the sequence lengths was performed by Microsynth (Balgach, Switzerland) using GeneScan™ 500 LIZ™ dye size standard (Thermo Fisher Scientific, Waltham, MA, USA). The obtained chromatogram files were edited using Peak Scanner 2.0 software (Applied Biosystems, Waltham, Massachusetts, USA).

Statistical analysis

The number of multilocus genotypes (MLG) standardized by sample numbers, Stoddard and Taylors' index of MLG diversity (Stoddart and Taylor 1988), Simpsons' index (Simpson 1949) corrected by N/(N-1), Evenness, E.5 (Grünwald et al. 2003), Nei's expected heterozygosity (H_{Exp}), Nei's genetic distances and a Neighbor-joining tree were calculated using R 3.3.0 (R Core Team 2016) with poppr 2.2.0 (Kamvar et al. 2014, 2015). For more detailed population analysis, accessions with just one sample (i.e., herbarium specimen) were excluded. Hence, two Austrian AMM populations were compared to the six Spanish AMA populations. Putative clonality and AMOVA were calculated with Genalex 6.5 (Peakall and Smouse 2006, 2012). Putative clonality was determined by multilocus genotypes (MLG). Individuals with the same MLG may either be parts of a clone (ramets of a genet) or—after sexual

Table 2 Primers used for analysis

Primer name	T_m^+	Primer sequence	Repeat motif* allele size range	Analysis reaction
Arm02(565) F	58*	gaatcaccatcgtcgcatAACACACATCCACGTTTGGC	TACA	2
Arm02 R	58*	gtttAACCGTGCATCATTCTGTGG	190-274	
Arm03(532) F2	52	tgtaaaacgtcggcgactCAAAAACCCTAATTCTCCATC	TACA	2
Arm03 R2	52	gtttCTGCGCAATGGGTTTACT	109-145	
Arm05(532) F	58*	tgtaaaacgtcggcgactACTGTCACCTAGGGGTGTTC	AACA	2
Arm05 R	58*	gttTAAGCGGGGAGTCTTTCTGG	186-206	
Arm06(550) F	58*	ccaagtagggcggtatctTGTCGCCTCAATCCTTGGTG	ACAT	1
Arm06 R	58*	gtttGCTGAAGTCCTTCCTTGGAC	119-271	
Arm07(M13) F	58*	tgtaaaacgacggccagtACATGACGCAAAAAGCGTAG	TATG	2
Arm07 R	58*	gtttCCATGTTACCACCATGTCGC	211-251	
Arm08(M13) F	58*	tgtaaaacgacggccagtAGATGAGGTTCTTGCAGCATC	TGTA	2
Arm08 R	58*	gttTGCTTGCAGTTGAAGTAAAGGG	134-180	
Arm09(565) F	58*	gaatcaccatcgtcgcatTAGGCGTGAGTTTGTACTCG	TATG	1
Arm09 R	58*	gtttAAGCGTGTTAACTTCGTGAG	236-264	
Arm10(565) F	58*	gaatcaccatcgtcgcatACCAGCTGACTCTCTTTCCG	CATA	1
Arm10 R	58*	gtttCAAGGATGAACATCGGCCTC	147-207	
Arm11(532) F2	52	tgtaaaacgtcggcgactGCACAAGGTATGTGTTGCA	GT	1
Arm11 R	58*	gttTCTTCGACCGAATGTTTTCACC	167-183	
Arm12(550) F2	52	ccaagtagggcggtatctCTTGCTTCTTCTCTTTATAGATGTC	AG	2
Arm12 R2	52	GGTTACCATTTTGGGTTCA	96-126	
Arm13(532) F (= Armo02 F*)	58*	tgtaaaacgtcggcgactGGTTTGAACACGAGATAGCG	AT	1
Arm13 R (=Armo02 R*)	58*	gtttACAAACTTCCTGTTGTCCCG	224-254	
Arm14(M13) F (= Armo03 F*)	58*	tgtaaaacgacggccagtTCAAACAGTCACCAGCAACC	ACCTGG	1
Arm14 R (=Armo03 R*)	58*	gtttCAGAGGCTGCAACCCTAATG	213-241	
M13-FAM	53	[6FAM]-TGTAAAACGACGGCCAGT		
ATTO532	53	[ATTO532]-TGTAAAACGTCGGCGACT		
ATTO565	53	[ATTO565]-GAATCACCATCGTCGCAT		
ATTO550	51	[ATTO550]-CCAAGTAGGGCGGtATCT		

Capital letters of the primer sequences indicate the locus specific sequences; small form letters indicate artificial primer tails. The microsatellite repeat motif was published by Duwe et al. (2015). The allele size range was obtained with the used sample set including *Arnica chamissonis*

*Allele specific primer sequence and T_m according to Duwe et al. (2015)

$^+$Primer melting temperatures without asterisks were calculated with Primer Express 2, excluding primer tails

reproduction—equal by chance. Briefly, the program estimates the probability (P_{sex}) of the occurrence of an MLG in a randomly mating population and gives statistical significance levels based on observed allele frequencies. Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992) was calculated separately for AMA and AMM with the Codom-Allelic distance with 999 permutations. The division allowed us a closer insight into the structure of the two subspecies that would have been covered by the high genetic distance between the two subspecies.

Results

In total 6 populations of lowland *Arnica montana* from Spain with 5–10 individuals each were classified as *A. montana* ssp. *atlantica* (AMA) according the proposed criteria by Bolos y Vayreda (1945) and were compared to two populations of *A. montana* ssp. *montana* (AMM) from Central Europe (Austria) with 10 individuals each. This sample set was complemented by a geographically wide range of individual samples from Germany to Northern Italy and the East-Pyrenees (France). All AMA plants belonged in their sesquiterpene lactone profile to the 'Spanish type', while AMM plants were of the 'East-European type' (EDQM 2014). The genetic study with 12 microsatellite loci showed between 6 and 15 different alleles, the expected heterozygosity H_{exp} ranged from 0.46 to 0.82 (mean=0.61) and the mean evenness ranged from 0.37 to 0.86 (mean=0.58) (Table 3).

Clonality

Arnica montana has two propagation strategies, a sexual strategy (a facultative apomictic species with predominant sexual reproduction (Yankova-Tsvetkova et al. 2016)) and an asexual strategy by rhizomes (Sugier et al. 2013). To avoid clonal influence on the estimation of variability, probabilities of equal multilocus genotypes (MLG) were estimated that an individual of a MLG was either a ramet of a genet or sexually reproduced and equal by chance. Of the 75 samples in the population sample subset only 45 MLG could be detected. 10 MLG were present in multiple copies, all of them with a P_{sex}-value lower than 0.05 indicating that the probability of equal MLG by sexual reproduction is rather low and clonality is more likely. Apart from one genet in AMM with only two ramets all other 9 genets were found in AMA. Subsequently, only one ramet of a genet was left in the sample set for further analysis (Table 4).

Genetic difference between AMA and AMM

The results show a clear genetic distinction of Spanish AMA from Central European AMM individuals (G_{ST}=0.81, Table 3, Fig. 1). Although the genetic variability was much smaller in AMA, the separation of populations within this group is far better supported than amongst AMM. Especially the population from Xermade (ES04) is distinctively different, but also the other two population groups from Susana (ES05) and Vilouris (ES03) are well separated from each other, indicating limited gene flow between AMA populations.

AMM samples were only in some cases grouped by their geographic distance. Samples from Styria (CE01) and Carinthia (CE02) are geographically close, as well as from Vienna (CE08) and Lower Austria (CE07) and from E-Tyrol (CE05) and

Table 3 Characteristics of the microsatellites used in this study (Alleles … number of different alleles detected; H_{Exp} … expected heterozygosity; evenness … distribution of the different alleles; G_{ST}… proportion of genetic diversity that resides among the two subspecies)

Locus	Alleles	H_{Exp}	Evenness	G_{ST} between AMA and AMM
Arm14	8	0.53	0.67	0.993
Arm11	6	0.54	0.74	0.152
Arm13	9	0.64	0.53	0.829
Arm06	15	0.57	0.41	0.690
Arm10	15	0.58	0.37	0.939
Arm09	8	0.67	0.63	0.998
Arm08	10	0.60	0.62	0.343
Arm07	12	0.57	0.43	0.976
Arm03	7	0.46	0.60	0.898
Arm05	6	0.68	0.86	0.898
Arm12	14	0.82	0.68	0.983
Arm02	13	0.64	0.48	0.991
mean	10.25	0.61	0.58	0.808

Table 4 Characteristics of AMA and AMM (n … number of samples, eMLG … number of multilocus genotypes standardized by sample numbers, G … Stoddard and Taylors' index of MLG diversity, lambda … Simpsons' index corrected by N/(N-1), E.5 … Evenness, H_{Exp} … Nei's expected heterozygosity)

Subspecies	n	eMLG	G	lambda	E.5	H_{exp}
Before clone correction						
AMA	55	13.2	13.7	0.736	0.726	0.249
AMM	20	19	18.2	0.986	0.973	0.657
Total	75	15.9	23.1	0.970	0.69	0.518
After clone correction						
AMA	28	9.76	24.5	0.959	0.965	0.278
AMM	32	10	32	0.969	1	0.702
Total	60	9.95	57.2	0.983	0.982	0.704

S-Tyrol (CE04). The German populations (Saxony, Brandenburg, Mecklenburg, Baden-Württemberg), however, grouped separately with completely different geographical locations, but bootstrap support is generally very weak in the AMM group (Table 1; Fig. 1).

Population structure

To compare population structures between AMA and AMM populations, populations with just one individual (herbarium samples) were excluded. So, two AMM populations from Austria were compared to the six Spanish AMA populations. The linear distance of the two Austrian populations was 107 km while the linear distance between the most distant AMA populations was 47 km. The number of expected multilocus-genotypes (eMLG), the standardized MLG for unequal sample numbers, as well as Simpsons' index and evenness were almost identical between AMA and AMM (Table 4). Expected heterozygosity (gene diversity) was low in AMA (H_{exp}=0.28) and high in AMM (H_{exp}=0.70). These results were also reflected in AMOVA analysis. Both subspecies, analysed separately, showed here significant variation between populations (Table 5). However, AMA populations differed to a higher degree from each other than the two AMM

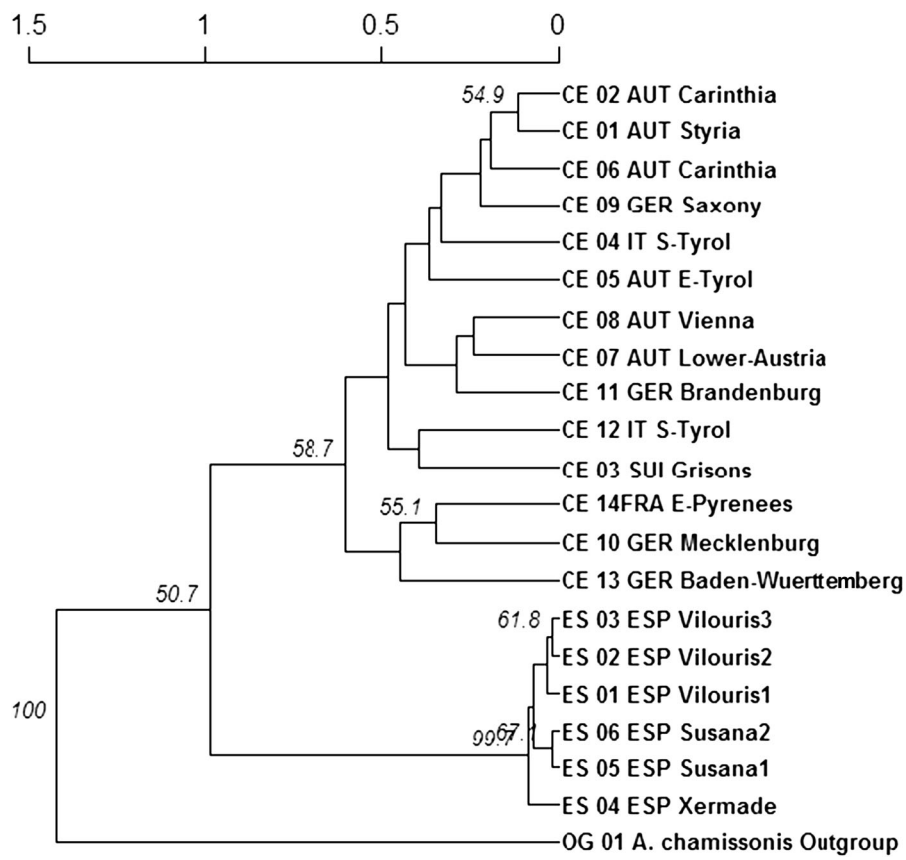

Fig. 1 Neighbor joining tree based on Nei's genetic distances of *Arnica montana* of Spanish (ES) AMA, and Central European (CE) AMM samples. *Arnica chamissonis* was used as outgroup (OG)

Table 5 Results of analysis of molecular variance (AMOVA), calculated separately for AMA and AMM

Source	df	SS	MS	Est. Var.	%		*F* value	*P* value
AMA								
Among populations	4	18.721	4.680	0.294	17	F_{ST}	0.171	0.001
Among individuals	22	34.742	1.579	0.151	9	F_{IS}	0.105	0.074
Within individuals	27	34.500	1.278	1.278	74	F_{IT}	0.258	0.002
AMM								
Among populations	1	10.050	10.050	0.312	8	F_{ST}	0.075	0.001
Among individuals	17	70.450	4.144	0.322	8	F_{IS}	0.084	0.020
Within individuals	19	66.500	3.500	3.500	85	F_{IT}	0.153	0.001

populations did (17% of variation among populations in AMA compared to 8% in AMM, indicating a higher degree of panmixis in AMM). The degree of inbreeding (variation among individuals) was almost equal for AMA and AMM (F_{IS}=0.105 (*P*=0.07) and F_{IS}=0.084 (*P*=0.02*) for AMA and AMM, respectively).

Discussion

The subspecies concept

The subspecies concept in *A. montana* was recently questioned by morphological analysis of an extensive sample set (Romero et al. 2011) where the authors found that the defined criteria to distinguish AMA from AMM were highly variable not allowing a clear distinction. However, AMA was genetically highly distinguishable from AMM in our microsatellite study. Vera et al. (2015) found also a phylogenetic grouping of the two sesquiterpene lactone chemotypes by sequencing two polymorphic chloroplast markers (*rps16* intron and *ycf4-cemA*). From the chloroplast data they could even deduce that the Spanish chemotype is ancestral to the Central-European Chemotype and Galicia may be the source for the post-glacial colonization of *A. montana* in Europe (Vera et al. 2015).

Genetic diversity and population structure

Although AMA showed a much lower expected heterozygosity compared to AMM (0.28 and 0.70, respectively) the lower genetic variability of AMA was also found by Vera et al. (2015) in sequencing two chloroplast markers. Genetic variability in Dutch AMM populations (H_{exp}=0.09) (Luijten et al. 2000) were even much lower than in AMA from this study (H_{exp}=0.28). As in AMA (F_{ST}=0.17), the Dutch populations showed moderately significant population differentiation (F_{ST}=0.14).

Clonality

The elevated level of clonality in AMA is either an indication of negative influences on sexual reproduction or more favourable conditions for vegetative growth. Many reasons can negatively influence seed propagation. Decreased pollination and seed development, low seed longevity and poor possibilities for seeds to germinate in densely covered vegetation (competition) may be reasons linked to flower and seed biology. Attacks on and diseases of floral tissues caused e.g., by herbivore slugs and fruit flies specialized on *A. montana* (*Tephritis arnica*e L., Diptera, Tephritidae) (Sugier et al. 2013) which parasites in flower heads may lead to low seed yields. Nutrient-rich (especially nitrogen-rich) soils are promoting vegetative growth over flower and seed development. Grassland management (early cutting or grazing, intensity of use) has also influence on successful propagation by seeds. Finally, flower collection intensity may also promote clonality.

Conservation

Applying a decision-making framework based on genotyping developed for threatened species (Ottewell et al. 2016), management for AMM should focus on habitat quality and maintaining large populations rather than managing genetic diversity

(Duwe et al. 2017). For AMA, which shows higher genetic differentiation than AMM, lower genetic variability and no inbreeding, this framework proposes to increase artificially gene flow to increase genetic diversity. Introduction of AMA cultivation in the region collection could support gene flow by bridging natural populations. In future, cultivating AMA could supplement wild collection.

Conclusion

The recognition within *Arnica montana* of two infraspecific taxa at subspecific rank, *A. montana* subsp. *montana* and *A. montana* subsp. *atlantica*, is supported by the data presented in this paper.

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