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Research Paper

Allium ursinum L. in Germany – surprisingly low genetic variability

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Abstract

Allium ursinum s.l. is a widely spread species of the herb layer in beech forests throughout Europe. Little is known about its phylogenetic origin and its biogeographic history. Molecular genetic analyses of eleven populations from Germany were used to clarify the relationship between populations of *A. ursinum* s.l. and its relationship to several other species of the genus *Allium*. The study focused mainly on the Teutoburg Forest in Lower Saxony and the Franconian mountain area in Bavaria. Sequences of the nuclear internal transcribed spacer ITS, and the external transcribed spacer ETS, as well as the plastidic *trn*L-*rpl*32 and the *trn*L-*trn*F spacer regions were compared. No variation was detected within the species. Even sequences of populations from Belfast, Ireland did not differ from populations of Germany. The closest relative to *Allium ursinum* s.l. turned out to be *Allium moly* or *Allium scorzonerifolium* from the section *Molium*. Random amplified polymorphic DNA fingerprinting was performed and revealed 29% polymorphic bands. Genetic distances of the populations within the Teutoburg Forest coincided with geographical distances. Three populations (Osnabrück Westerberg, Osnabrück Honeburg and Leer, East Frisia) out of eleven analysed populations were identified as garden escapes.

Zusammenfassung

Der Bärlauch *Allium ursinum* s.l. ist eine weit verbreitet Pflanze in der Krautschicht der Buchenwälder Mitteleuropas. Trotz seines Bekanntheitsgrades ist nur sehr wenig über den Ursprung, die Besiedlungsgeschichte sowie Verbreitungsstrategien bekannt. Molekulargenetische Analysen von elf Populationen aus Deutschland wurden genutzt, um die Beziehungen zwischen den Populationen sowie die Beziehungen zu nahe verwandten Arten der Gattung *Allium* zu klären. Die Untersuchungen fokussierten sich hauptsächlich auf den Teutoburger Wald in Niedersachsen und die Fränkische Schweiz in Bayern. Die Sequenzen der nuklearen ITS und ETS Regionen sowie der plastidären *trn*L-*rpl*32 sowie des *trn*L-*trn*F Regionen wurden verglichen. Diese Bereiche sind innerhalb der Art *A. ursinum* s.l. kaum variabel. Sogar Sequenzen von Populationen aus Belfast unterschieden sich nicht von denen aus Deutschland. Als nächster Verwandter von *Allium ursinum* s.l. konnte *Allium moly* oder *Allium scorzonerifolium* aus der Sektion *Molium* identifiziert werden. Weiterhin wurde das RAPD Verfahren benutzt, um „Fingerprints“ verschiedener Populationen von *A. ursinum* subsp. *ursinum* zu erstellen. Die genetischen Distanzen der Populationen, innerhalb des Teutoburger Waldes korrelieren mit den geographischen Distanzen. Drei Populationen (Osnabrück Westerberg, Osnabrück Honeburg und Leer, Ostfriesland) von insgesamt elf analysierten stellten sich

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als Gartenflüchtlinge heraus. Die Analysen basieren auf 29 % genetischem Polymorphismus innerhalb der RAPD Merkmale.

Introduction

Allium ursinum L., also known as Ramson or Wild Garlic, is taxonomically placed in the subgenus *Amerallium* TRAUB. of the genus *Allium* L. in the monophyletic section *Arctoprasum* KIRSCHL (FRIESEN et al. 2006). The species is found widely spread in beech forests (*Fagus sylvatica*) across Europe. In fact, the distribution areas of both species (*F. sylvatica* and *A. ursinum* s.l.) are more or less identical except in Scandinavia, England and Ireland where *F. sylvatica* is rare (MEUSEL & JÄGER 1965). Ramson is commonly found from the Mediterranean to sub-atlantic regions and occurs predominantly in mountain ranges (WEBER 1995, JÄGER & WERNER 1994). It is rather rare in lowland regions where it may occur at infrequent intervals (HAEUPLER & SCHÖNFELDER 1988, MEUSEL & JÄGER 1965). As *A. ursinum* is an early spring geophyte its growing time is rather short (WEBER 1995, JÄGER & WERNER 1994). The inflorescence appears between May and June, consists of up to 30 self-compatible flowers and is capable therefore of discharging multitudes of seeds (ERNST 1979, NAULT & GAGNON 1987, JÄGER & WERNER 1994). If germination occurs, it takes up to five years until the first blossom appears (ERNST 1979, ELLENBERG 1996). The slow sexual propagation is bypassed by its ability to produce daughter bulbs vegetatively, which are able to flower the following year (EGGERT 1992). Nevertheless, sexual reproduction outranks vegetative reproduction when compared to other herb layer plants (ERNST 1979; TUTIN 1957).

There are several means of seed dispersal discussed. The seeds possess elaiosoms and, therefore, the ability to be distributed by ants. ERNST (1979) reported for the Teutoburg Forest populations that two out of one-thousand seeds were moved by ants more than ten centimetres and less than 24 cm. ERNST (1979) estimated that the spatial dispersal of the seeds is approximately two to five meters in 20 years. Zoochory via mud on animal hooves as another possibility for long distance dispersal was observed by ELLENBERG (1996). Also anthropogenic influences have been discussed by BAUCH (1937). However, it has not yet been finally established, as to how the seeds were distributed to the present area and from where. Also the phylogenetic origin of *A. ursinum* s.l. is still unknown. There are two subspecies of *A. ursinum*. The subspecies *ucrainicum*, is distributed in eastern and southern Europe and the subspecies *ursinum* is distributed in western and middle Europe.

The aim of this study was to understand the relationship between the populations of ramson (*A. ursinum*) in Germany and its taxonomical position among related species in the subgenus *Amerallium*. We focused mainly on the Teutoburg Forest in the Osnabrücker Land, as it is part of the northern border of the main distribution area in Germany. Further to the north, the occurrence of populations is rather isolated. For comparison we used three populations from the Franconian mountain area.

We sequenced the external transcribed spacer (ETS) and internal transcribed spacer (ITS) from several accessions of *A. ursinum* s.l. and several related species. Additionally, the plastidic *trn*L-*trn*F and *trn*L-*rpl*32 regions were sequenced. They are described as being effective markers to clarify interspecific relations (e.g. BALDWIN et al. 1995, BALDWIN & MARKOS 1998, DUBOUZET & SHINODA 1999, FRIESEN et al. 2006, SPALIK & DOWNIE 2006, NGUYEN et al. 2008, LI et al. 2010). Furthermore random amplified polymorphic DNA (RAPD) fingerprinting was carried out in order to determine the relationship within and between the populations of ramson. The RAPD data were used for a principal component analysis (PCA) to highlight differences between genetic and geographic distances. These methods have repeatedly proven to be reliable tools to answer phylogenetic questions in closely related groups (e.g. WILLIAMS et al. 1990, NEUFFER 1996, FRIESEN & HERRMANN 1998, FRIESEN & KLAAS 1998, NEUFFER et al. 1999a, NEUFFER et al. 1999b, FRIESEN & BLATTNER 2000, REISCH & POSCHLOD 2004, EBRAHIMI et al. 2009, JÜRGENS et al. 2010) even though they may have their limitations as e.g. restricted reproducibility and uncertainty regarding homologous fragments with equal

length. Since we investigated different accessions in one species, we assumed that all fragments of identical size are homologues (RIESEBERG 1996, ADAMS & RIESEBERG 1998).

Materials and methods

Plant material

The leaf material was collected from eleven populations of *A. ursinum* subsp. *ursinum* in Germany (Tables 1 & 2, Fig. 1 & 2). The leaves were first washed with tap water and then dried with Silica Gel. The CTAB method from DOYLE & DOYLE (1987) was used to extract the DNA of the leaves. The quality and concentration of the isolated DNA and every amplification product was checked on a 1% agarose gel stained with Ethidiumbromid. All PCRs were carried out in a Biometra Professional Thermocycler gradient. Isolated DNA was used directly for PCR amplifications.

ITS amplification

The complete ITS region (ITS-1, 5.8S and ITS-2) was amplified using primers ITS-A and ITS-B (BLATTNER 1999). The thermocycler was programmed as follows: 95 °C for 2 min [55 °C for 30 sec, 70 °C for 1 min, 95 °C for 20 sec]$_{32}$ 70 °C for 7 min. Amplification was carried out with 1 Unit Taq DNA polymerase (Gene Craft, Germany) in the supplied reaction buffer, 0.2 mM of each dNTP, 50 pmol of each primer and 10–25 ng of total DNA in 50 µl reaction volume.

ETS amplifikation

The ETS region was amplified using the primers 18S-IGS and ETS-all-f (BALDWIN & MARKOS 1998, NGUYEN et al. 2008) (manufactured by MWG-Biotech AG). The thermocycler was programmed as follows: 95 °C for 5 min [94 °C for 1 min, 54 °C for 1 min, 72 °C for 2 min]$_{40}$ 72 °C for 5 min. Amplification was carried out with 0.2 µl Taq polymerase (Gene Craft, Germany) in 3 µl 10× reaction buffer (15 mM $MgCl_2$) (Gene Craft), 3 µl 5 mM dNTP mix, 2 µl of each primer and 1µl of the sample DNA in 30 µl reaction volume.

*trn*L-*trn*F spacer amplification

The *trn*L-*trn*F spacer was amplified using the Taberlet primer "e" and "f" (TABERLET et al. 1991, SOLTIS et al. 1989). The thermocycler was programmed as follows: 95 °C for 1 min [94 °C for 20 sec; 60 °C

Table 1
Provenances of *Allium ursinum* ssp. *ursinum* populations in Germany

Code[a]	Location	Coordinates	Altitude (m)
UA	Lower Saxony Dissen, Noller Schlucht	N 52°08′20″; E 08°11′16″	211
UB	Lower Saxony Bad Laer/Bad Rothenfelde, Kleiner Berg	N 52°06′42″; E 08°07′28″	188
UC	Lower Saxony Bad Iburg, Kleiner Freeden	N 52°09′26″; E 08°04′31″	191
UD	Lower Saxony Bad Iburg/Holperdorp, Langenberg	N 52°09′41″; E 08°00′28″	203
UE	Lower Saxony Osnabrück, Westerberg	N 52°16′48″; E 08°01′28″	92
UF	North Rhine-Westphalia Brochterbeck	N 52°13′25″; E 07°45′25″	105
UG	Lower Saxony Leer (East Frisia)	N 53°13′48″; E 07°29′49″	7
UH	Lower Saxony Osnabrück, Honeburg	N 52°18′31″; E 08°02′11″	80
UK I	Bavaria SW Franconian mountain area, Hetzles	N 49°38′00″; E 11°08′00″	400–500
UK II	Bavaria N Franconian mountain area, Staffelberg	N 50°06′00″; E 10°59′00″	500
UK III	Bavaria N Franconian mountain area, Vierzehnheiligen	N 50°06′00″; E 11°02′00″	500

[a] working code for the population

Table 2
EMBL accession numbers of *Allium* species used for ITS, ETS, *trn*L-*rpl*32 and the *trn*L-*trn*F spacer. Three-letter-country code: CHN – China, DEU – Germany, FRA – France, GBR – United Kingdom, IRN – Iran, ISR – Israel, ITA – Italy, POL – Poland, THA – Thailand, TUN – Tunesia, TUR – Turkey, ESP – Spain, USA – United States of America; Code: working code for the populations

Code	Species	Country[a]	Provenence	ITS	ETS	*trn*L-*trn*F	*trn*L-*rpl*32
UA1	*A. ursinum* L.	DEU	Lower Saxony, Dissen, Noller Schlucht		FN551200		
UA32	*A. ursinum* L.	DEU	Lower Saxony, Dissen, Noller Schlucht		FN551201		
UA43	*A. ursinum* L.	DEU	Lower Saxony, Dissen, Noller Schlucht		FN551202	FN550391	
UB8	*A. ursinum* L.	DEU	Lower Saxony, Bad Laer, Kleiner Berg		FN551204		
UB17	*A. ursinum* L.	DEU	Lower Saxony, Bad Laer, Kleiner Berg		FN551203	FN550392	
UC10	*A. ursinum* L.	DEU	Lower Saxony, Bad Iburg, Kleiner Freeden		FN551206		
UC20	*A. ursinum* L.	DEU	Lower Saxony, Bad Iburg, Kleiner Freeden	FR682003	FN551205	FN550393	
UD44	*A. ursinum* L.	DEU	Lower Saxony, Bad Iburg, Langenberg		FN551208		
UD50	*A. ursinum* L.	DEU	Lower Saxony, Bad Iburg, Langenberg		FN551207	FN550394	
UE7	*A. ursinum* L.	DEU	Lower Saxony, Osnabrück, Westerberg		FN551210		
UE16	*A. ursinum* L.	DEU	Lower Saxony, Osnabrück, Westerberg		FN551209	FN550395	
UF1	*A. ursinum* L.	DEU	North Rhine-Westfalia, Brochterbeck	HE962498	FN551213		HE859948
UF32	*A. ursinum* L.	DEU	North Rhine-Westfalia, Brochterbeck		FN551211		
UF33	*A. ursinum* L.	DEU	North Rhine-Westfalia, Brochterbeck		FN551212	FN550396	
UG9	*A. ursinum* L.	DEU	Lower Saxony, Leer (East Frisia)		FN551215		
UG12	*A. ursinum* L.	DEU	Lower Saxony, Leer (East Frisia)		FN551214	FN550397	
UH5	*A. ursinum* L.	DEU	Lower Saxony, Osnabrück, Honeburg		FN551217		
UH7	*A. ursinum* L.	DEU	Lower Saxony, Osnabrück, Honeburg		FN551216	FN550398	
UKI-2	*A. ursinum* L.	DEU	Bavaria, Hetzles		FN551218		
UKI-6	*A. ursinum* L.	DEU	Bavaria, Hetzles		FN551219	FN550399	
UKII-8	*A. ursinum* L.	DEU	Bavaria, Staffelberg		FN551221		
UKII-10	*A. ursinum* L.	DEU	Bavaria, Staffelberg		FN551220	FN550400	
UKIII-16	*A. ursinum* L.	DEU	Bavaria, Vierzehnheiligen		FN551223		
UKIII-22	*A. ursinum* L.	DEU	Bavaria, Vierzehnheiligen	FR682005	FN551222	FN550401	HE859949
UL9	*A. ursinum* L.	GBR	Northern Ireland, Belfast, near Castle	FR682002			
UL14	*A. ursinum* L.	GBR	Northern Ireland, Belfast, near Castle	FR682004			
Am 32	*A. usinum* subsp. *ucrainicum* Kleop.	POL	cottage Studen Kladec S Kjustendil	FR693742	FN551224		HE859950
Am 40	*A. neapolitanum* Cirillo	TUR	n.a.	FR693743	FN551228		HE859951
Am 340	*A. neapolitanum* Cirillo	ISR	Judaean Mts	HE962510	HE859941		
Am 77	*A. moly* L.	ESP	Gatersleben, TAX 1117	AJ412743	FN551227		HE859952
Am 44	*A. moly* L.	ESP	Jaen	HE962501	FN551226		HE859953
Am 76	*A. subvillosum* Salzm. ex Schult. et Schult. f.	ESP	Tenerife, east from Masca	FR693744	FN551229		HE859956
Am 123	*A. subvillosum* Salzm. ex Schult. et Schult. f.	ESP	Tenerife, east from Masca		HE859942		HE859955
Am 82	*A. roseum* L.	ESP	Malaga, Finca Los Picachones	FR693745	FN551230		HE859957
Am 126	*A. chamaemoly* L.	ESP	Malaga	HE962504	HE859943		HE859954
Am 174	*A. scorzonerifolium* Desf. Ex DC.	ESP	n.a.	HE962500	FN551225		
Am 244	*A. triquetrum* L.	TUN	Tabarka, 45 km NW of Beja, Kroumrie, Tell Atlas	HE962507	HE859938		HE859962
Am 249	*A. paradoxum* (M.Bieb.) G.Don	IRN	Naitinga valley, east of Gorgan	HE962505	HE859939		HE859958
Am 220	*A. pendulinum* Ten.	FRA	Corsica, Dep. Haute-Corse	HE962506	HE859940		HE859961
Am 285	*A. hookeri* Thwaites	THA	Tak province Mal sot, commercial market	HE962508	HE859944		HE859963
Am 66	*A. wallichii* Kunth	CHN	Xizang	HE962502	HE859945		HE859964
Am 127	*A. insubricum* Boiss. & Reut.	ITA	Barzio, Grigna Meridionale	HE962509	HE859946		HE859959
Am 79	*A. narcissiflorum* Vill.	FRA	Alps, Villard de Lane	HE962503	HE859947		HE859960
Am 4	*A. cratericola*	USA	California	EU096146	EU162714		HE859965

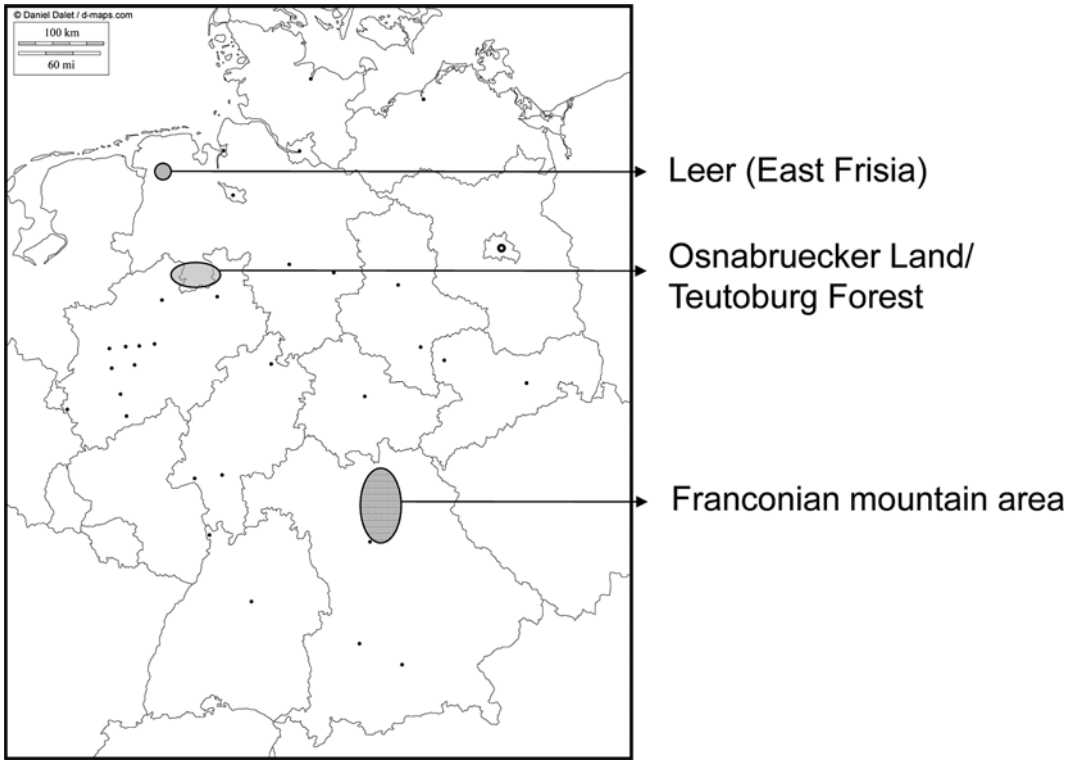

Fig. 1
Locations of the analyzed populations in Germany. Black lines: federal = and state borders; black dots = capital cities

for 20 sec; 72 °C for 1 min and 30 sec]$_{35}$ 72 °C for 4 min. Amplification was carried out with 0.2 µl Taq polymerase (Gene Craft, Germany) in 3 µl 10× reaction buffer (15 mM $MgCl_2$) (Gene Craft), 3 µl 5 mM dNTP mix, 2 µl of each primer and 2 µl of the sample DNA in 30 µl reaction volume.

*trn*L-*rpl*32 spacer amplification

The noncoding marker *trn*L-*rpl*32, which is according to SHAW et al. (2007) the most variable marker on the cpDNA, was amplified using the primer *trn*L$^{(UAG)}$ (CTG-CTT-CCT-AAG-AGC-AGC-GT) as a forward primer and *rp*L32F (CAG-TTC-CAA-AAA-AAC-GTA-CTT-C) as a reverse primer (SHAW et al. 2007). The thermocycler was programmed to: 80 °C for 5 min [95 °C for 1 min, 50 °C for 1 min, 65 °C for 4 min]$_{31}$ 65 °C for 5 min, 9 °C for 5 min. Amplification was carried out with 1 µl DMSO, 3 µl 10× reactions buffer (15 mM $MgCl_2$), 2 µl dNTP Mix, 1 µl of each primer, 0.2 µl Taq polymerase (Gene Craft) and 1 µl of the sample DNA in 30 µl reaction volume.

Sequencing

After purifying the amplicons with the NucleoSpin Gel Extraction kit (Macherey-Nagel, Düren, Germany) they were used in a sequencing reaction with the ABI BigDye Terminator kit (Applied Biosystems, Foster City, California, USA) according to the instructions of the manufacturer in a 10 µl reaction volume. Forward and reverse primers were sequenced separately. Forward and reverse sequences from each individual were edited manually with CHROMAS Lite vers. 2.0 software (Technelysium Pty. Ltd., Tweantin, Queensland, Australia) and combined into a single consensus sequence. The sequences were aligned with CLUSTAL_X (THOMPSON et al. 1997) and the alignment subsequently corrected manually in MEGA 5 (TAMURA et al. 2011).

Data analysis

A heuristic search with the tree bisection reconnection (TBR) algorithm was conducted for the sequence data in PAUP*vers.4.0b10 (SWOFFORD

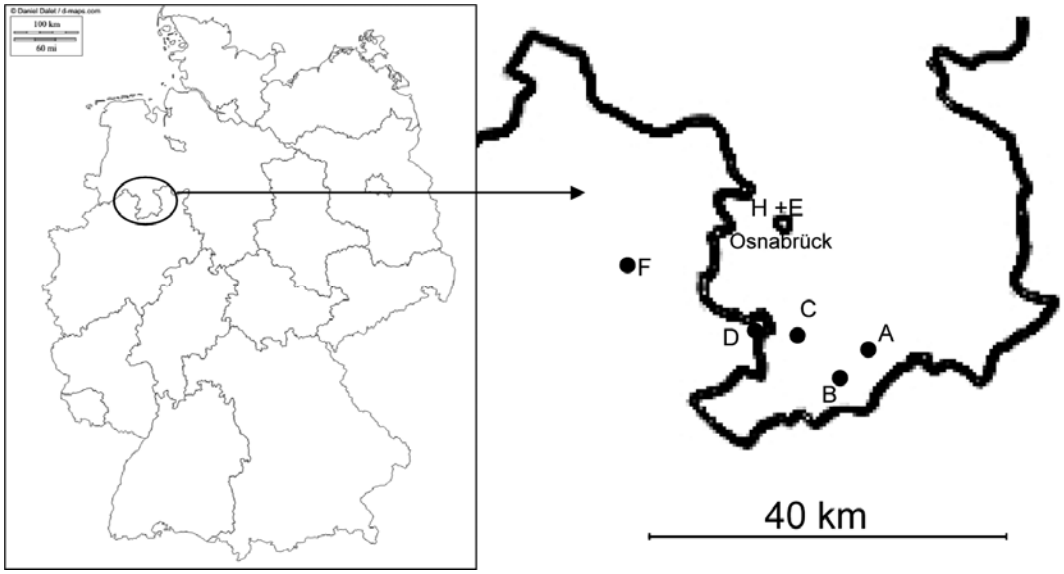

Fig. 2
Location of the populations in the Teutoburg Forest and Osnabrücker-Land; black lines: border between the federal states of Lower Saxony and North-Rhine-Westphalia in the area of Osnabrück, Germany; A: Dissen, Noller Schlucht; B: Bad Laer, Kleiner Berg; C: Bad Iburg, Kleiner Freeden; D: Bad Iburg, Langenberg; E: Osnabrück, Westerberg; F: Brochterbeck; H: Osnabrück, Honeburg

2002). Evolutionary models were tested in a hierarchical likelihood ratio test (hLRT) with Modeltest3.7 (POSADA & CRANDALL 1998). For the ITS and ETS data the sequence evolution models were HKY with a gamma rate of evolution on different sides (HASEGAWA et. al. 1985). For the *trn*L-*rpl*32 data the sequence evolution model was TIM with a gamma rate of evolution on different sides. For Bayesian inferences four Markov chains were run for two million generations in respect to the tested sequence evolution models and evolutionary rates on different sides with MrBayes 3.1.2 (RONQUIST & HUELSENBECK 2003). The calculation of the bootstrap values was conducted with 100 replicates in PAUP*. GenBank accession numbers of used sequences are shown in Table 2.

RAPD-Amplification

The RAPD-Method was carried out using 25 Operon primer (A04, A06, A07, A09, A13, B01, B04, B06, B07, B12, B14, B15, B17, B19, C08, C10, C18, D04, D05, D16, D20, H03, H07, H13, H17) (Welsh & McClelland 1990, WILLIAMS et al. 1990). Three DNA templates of each population were used for amplification (UA32, 37, 43; UB8, 17, 18; UC10, 20, 21; UD44, 49, 50; UE7, 16, 19; UF32, 33, 47; UG9, 10, 12; UH5, 7, 9; UKI 2, 5, 6; UKII 8, 9, 10; UKIII 16, 17, 22).

The two biggest populations (Noller Schlucht near Dissen (UA) and Brochterbeck near Tecklenburg (UF)) were used to detect genetic diversity within a population. Additionally, these two populations originated from quite distant locations (Fig. 2). From these two populations 28 templates were used in a PCR as mentioned above. Of the 25 screened primers nine were informative and were selected for further analysis (A7, A9, A6, C10, C18, D16, H3, H7 and H13).

The thermocycler was programmed as follows: 94 °C for 2 min [94 °C for 30 sec, 37 °C for 1 min, 72 °C for 2 min]$_2$ [94 °C for 30 sec, 35 °C for 1 min, 72 °C for 2 min]$_{35}$ 72 °C for 5 min. Amplification was carried out with 0.2 µl Taq polymerase (Gene Craft, Germany) in 3 µl 10× reaction buffer (15 mM $MgCl_2$) (Gene Craft), 3 µl 5 mM dNTP mix, 2 µl of each primer and 1 µl of the sample DNA in 30 µl reaction volume. The products were separated on a 1.8% agarose gel stained with ethidium bromide. Images were taken with BioDocAnalyze 2.0 (Biometra) and edited with Adobe Photoshop.

The RAPD patterns were evaluated manually and transferred into a binominal (0/1) matrix (1 = band; 0 = no band). The minimum evolution (ME) trees with the *p*-distance and the calculation of the bootstrap values with 100 replicates were generated with Mega 5 (TAMURA et al. 2011). A PCA was generated with the Jaccard coefficient

(JACCARD 1908) in SPSS version 18.0 (SPSS, Inc., Chicago IL) (Fig. 5). The scale factor was sqrt (Lambda).

Results

Sequence data analysis

The sequences of the ITS region from *A. ursinum* subsp. *ursinum* consisted of 642 bp and was highly conserved. Within the ITS region of the 22 accessions (five accessions of *A. ursinum* s.l. and 17 related species from the subgenus *Amerallium*), the sequences ranged from 642 bp (*A. ursinum* subsp. *ursinum*) up to 661 bp (*A. triquetrum* L.) and the alignment covered 683 bp.

Generalized parsimony analysis of the ITS data produced one tree (length = 478 steps, including parsimony uninformative characters, consistency index (CI) 0.7552, retention index (RI) 0.8289). Of the 683 detected characters, 398 characters were constant, 88 variable characters were parsimony-uninformative and 197 characters were parsimony-informative.

The sequence of the ETS region from *A. ursinum* subsp. *ursinum* consisted of 460 bp and was highly conservative. The subspecies *ucrainicum* varied only in one base pair (position 373, Guanine instead of Cytosine) within the sequence. Within the ETS region of the 42 accessions (25 accessions of *A. ursinum* s.l. and 17 related species from the subgenus *Amerallium*), the sequences ranged from 456 bp (*A. scorzonerifolium* DESF. ex DC.) to 469 bp (*A. hookeri* THWAITES) and the alignment covered 501 bp.

Generalized parsimony analysis of the ETS data produced two equally parsimonious trees (length = 578 steps, including parsimony uninformative characters, consistency index (CI) 0.7405, retention index (RI) 0.8862). Of these 501 detected characters, 202 characters were constant, 75 variable characters were parsimony-uninformative and 224 characters were parsimony-informative.

The sequence of the *trn*L-*trn*F spacer of *A. ursinum* s.l. consisted of 240 bp and was highly conservative. We detected no differences in the nucleotide sequences between different accessions of *A. ursinum* s.l. (tree not shown).

The *trn*L-*rpl*32 spacer revealed no variability between accessions of *A. ursinum* s.l. and was highly conserved with 873 bp in length. Within the spacer of the 18 accessions (three accessions of *A. ursinum* s.l. and 15 related species from the subgenus *Amerallium*), the sequences ranged from 669 bp (*A. paradoxum* (M.BIEB.) G.DON) up to 874 bp (*A. subvillosum* SALZM. ex SCHULT. et SCHULT.) and the alignment covered 1123 bp.

Generalized parsimony analysis of the sequence data produced six trees (length = 229 steps, including parsimony uninformative characters, consistency index (CI) 0.8908, retention index (RI) 0.8768). Of the 1123 detected characters, 949 characters were constant, 87 variable characters were parsimony-uninformative and 87 characters were parsimony-informative.

In all three analyses (Fig. 3 & 4) the American species *Allium cratericola* EASTW. was chosen as out-group taxon (FRIESEN et al. 2006). In the Bayesian trees of the ITS and ETS data, the Asian species were placed as a sister group to all Mediterranean and European accessions. Within the Bayesian tree of the *trn*L-*rpl*32 data (Fig. 4) the endemic species from the South-West Alps (*A. insubricum* BOISS. & REUT. and *A. narcissiflorum* VILL.-section *Narkissoprason*) were placed as a sister group to all other accessions.

The analysis of the sequence data could not clarify the relation between the populations of the subspecies *ursinum*. The analysis was not able to separate even a population from Belfast, Northern Ireland (UL) (Fig. 3). The subspecies *ucrainicum* was placed in the same clade as the subspecies *ursinum* in all trees and the topology represented a comb. Within the ETS and ITS trees the accessions from *A. ursinum* s.l. were placed as a sister group to the accessions from the section *Molium*. The section *Molium* appeared to be heterogeneous for it was divided into two groups. Of these two groups the "Moly-Group" represented by *A. moly* L. and *A. scorzonerifolium* DESF. ex DC. appeared to be the closest relative to the section *Arctoprasum.* A pairwise distance analysis of the ITS sequences revealed that the *p*-distance between *A. moly* and *A. ursinum* subsp. *ursinum* was 0.116 and the distance between *A. scorzonerifolium* and *A. ursinum* subsp. *ursinum* was

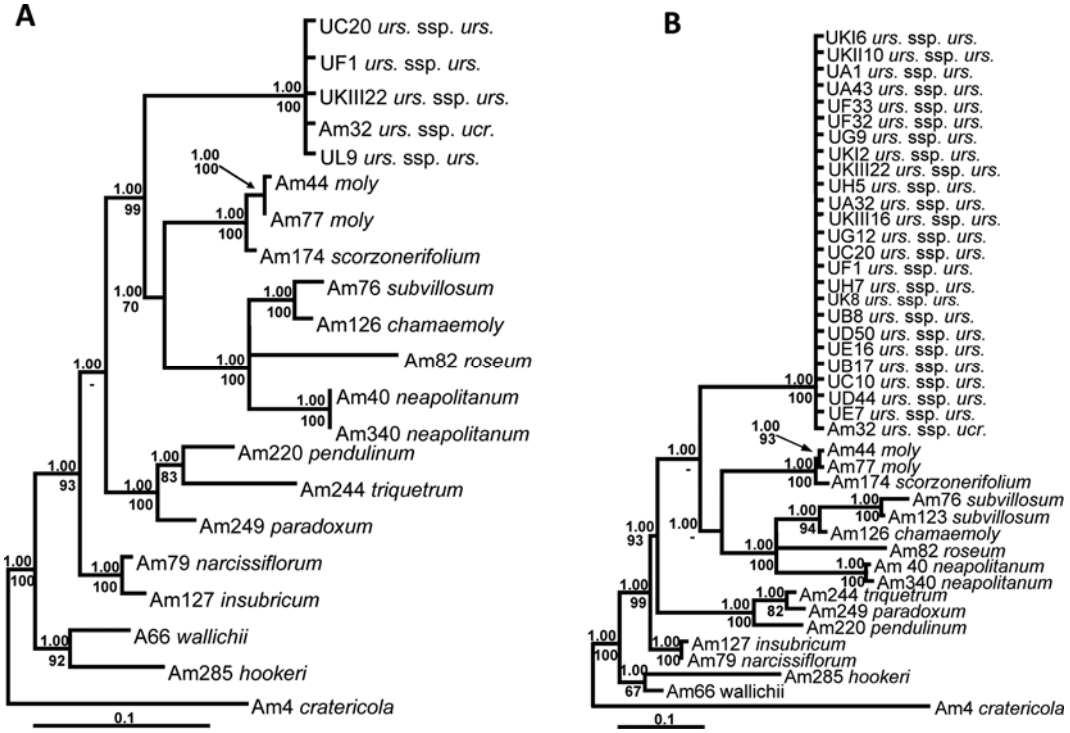

Fig. 3
Phylogenetic trees resulting from a Bayesian analysis of the (A) ITS sequences and (B) ETS sequences of *Allium ursinum* s.l. and related species, with *A. cratericola* as outgroup taxon. Bayesian posterior probabilities (BI) are given above the branches and bootstrap values with 100 replicates (>50%) are given below the branches for every notch. When given a bar, no Bootstrap values over 50% were obtained; (urs. = *ursinum*; ucr. = *ucrainicum*); (UA – Noller Schlucht, Dissen; UB – Kleiner Berg, Bad Laer; UC – Kleiner Freeden, Bad Iburg; UD – Langenberg, Holperdorp; UE – Westerberg, Osnabrück; UF – Brochterbeck; UG – Leer, East Frisia; UH – Honeburg, Osnabrück; UKI – Franconian mountain area, Hetzles; UKII – Franconian mountain area, Staffelberg; UKIII – Franconian mountain area, Vierzehnheiligen; UL – Belfast)

0.112. The *p*-distance of the ETS sequences between *A. moly* and *A. ursinum* subsp. *ursinum* was identical with the distance between *A. scorzonerifolium* and *A. ursinum* subsp. *ursinum* (0.174). The Bayesian tree obtained by the *trn*L-*rpl*32 sequence data, placed *A. ursinum* s.l. as a sister group to *A. moly* and the remaining taxa of the section *Molium* as a sister group to both (Fig. 4).

RAPD analysis

Altogether 89 fragments (33 individuals and nine different primers) with different scores per primer were amplified (Table 3). The percentage of polymorphic bands per primer was between 14.3% and 55.6%. 29.2% of the characters used for the analysis were polymorphic, 70.8% were monomorphic (Table 3). Of the 89 detected characters, 63 characters were constant, two variable characters were parsimony-uninformative and 24 characters were parsimony-informative. Despite the low polymorphism detected by RAPD fingerprinting, the analysis of the data resulted in clusters of the eleven populations, supported by the ME tree (Fig. 5) and a PCA (Fig. 6).

Geographically the eleven populations can be arranged into three main groups (Fig. 1). The first group, Teutoburg Forest, consists of the populations Brochterbeck (UF), Langenberg near Bad Iburg (UD), Kleiner Berg near

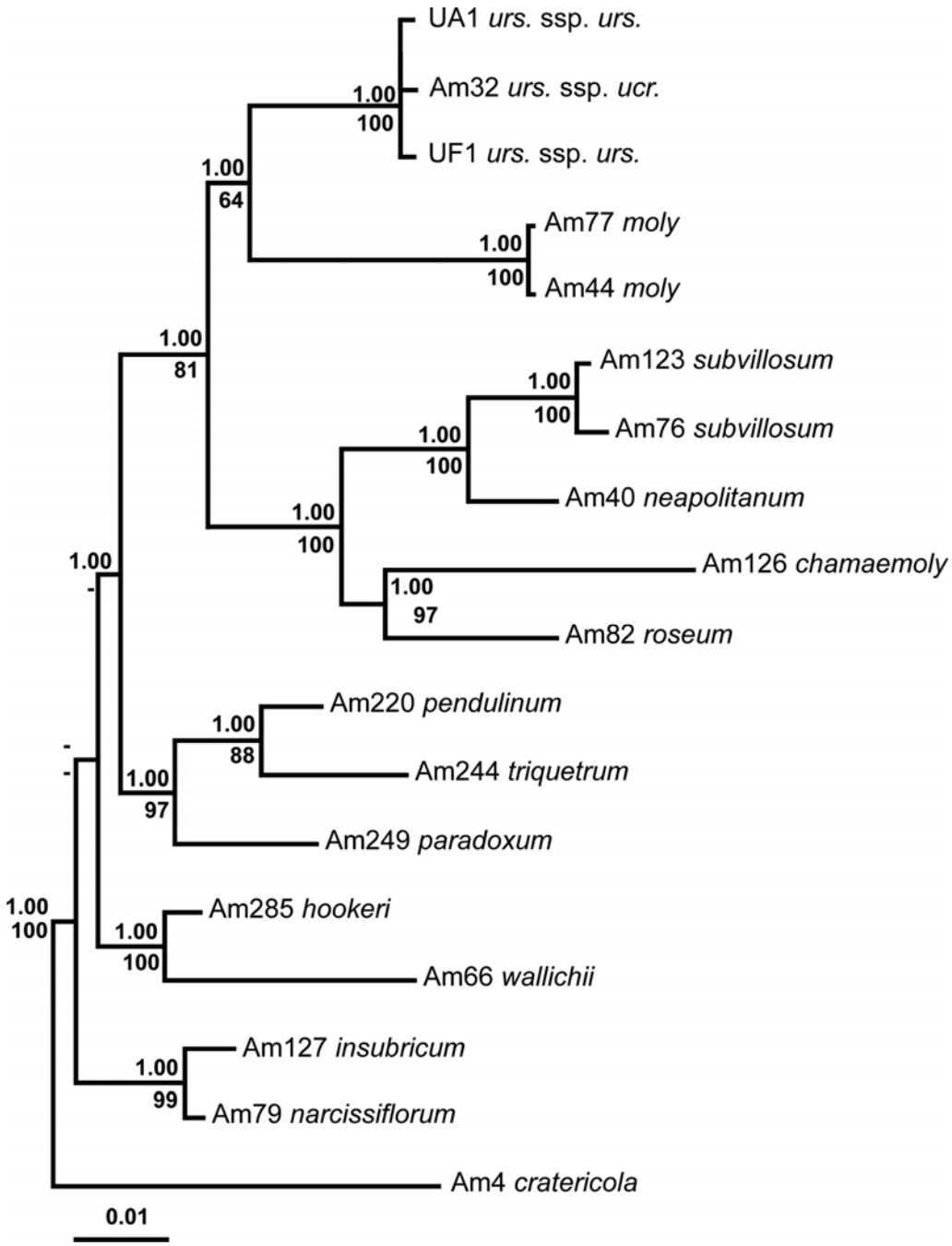

Fig. 4
Phylogenetic tree resulting from a Bayesian analysis of the *trn*L-*rpl*32 spacer located on the cpDNA of *Allium ursinum* s.l. and related species, with *A. cratericola* as outgroup taxon. Bayesian posterior probabilities (BI) are given above the branches and bootstrap values with 100 replicates (>50%) are given below the branches. When given a bar, no Bootstrap values were obtained; (urs. = *ursinum*; ucr. = *ucrainicum*)

Table 3
Absolute number of monomorphic and polymorphic markers of nine operon primers and percentage of polymorphic bands (P %)

Operon Primer	Polymorphic Bands	Monomorphic Bands	Total	*P %*
A7	3	8	11	27.3
A9	5	4	9	55.6
C10	1	6	7	14.3
A6	2	8	10	20.0
H3	4	7	11	36.4
D16	3	12	15	20.0
H13	5	4	9	55.6
H7	1	6	7	14.3
C18	2	8	10	20.0
Total	26	63	89	29.2

Bad Laer (UB), Kleiner Freeden near Bad Iburg (UC) and Noller Schlucht near Dissen (UA). The genetic distance within this group was expected to be in geographical order (Fig. 2). The second group, Franconian mountain area, consists of the populations Hetzles (UKI), Staffelberg (UKII) and Vierzehnheiligen (UKIII). The genetic distance between Vierzehnheiligen and Staffelberg (linear distance ca. 1.5 km) was expected to be lower than

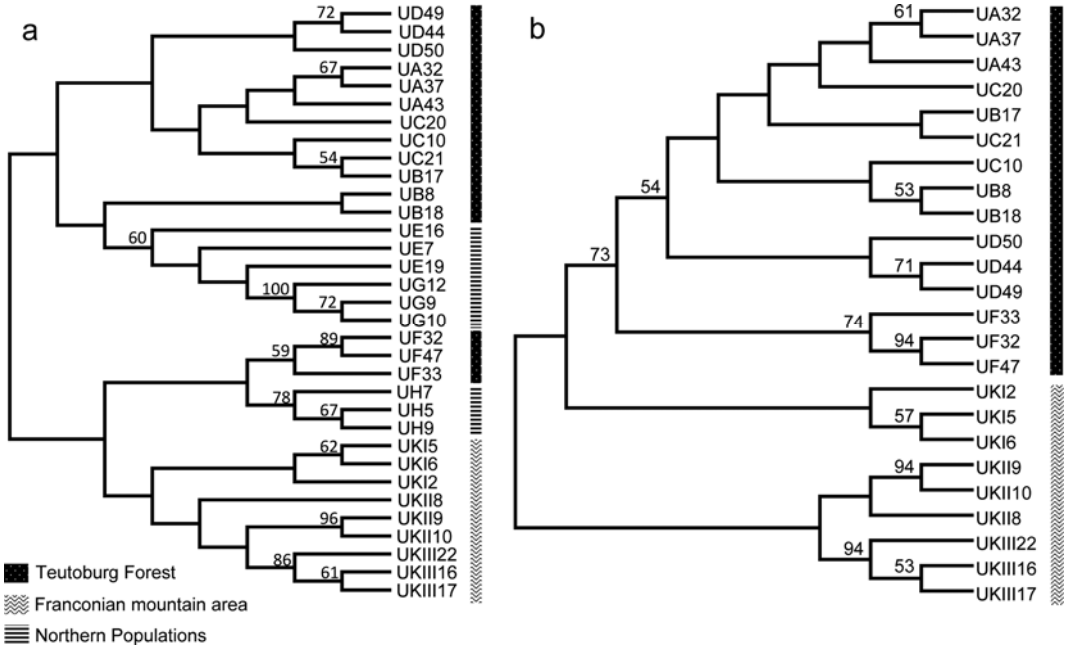

Fig. 5
Minimum evolution trees calculated with the p-distance based on 89 markers of the RAPD data. Bootstrap values (>50%) are given above the branches. (A) Analysis of all eleven populations. (B) Analysis excluding anthropogenically influenced populations; (UA – Noller Schlucht, Dissen; UB – Kleiner Berg, Bad Laer; UC – Kleiner Freeden, Bad Iburg; UD – Langenberg, Holperdorp; UE – Westerberg, Osnabrück; UF – Brochterbeck; UG – Leer, East Frisia; UH – Honeburg, Osnabrück; UKI – Franconian mountain area, Hetzles; UKII – Franconian mountain area, Staffelberg; UKIII – Franconian mountain area, Vierzehnheiligen)

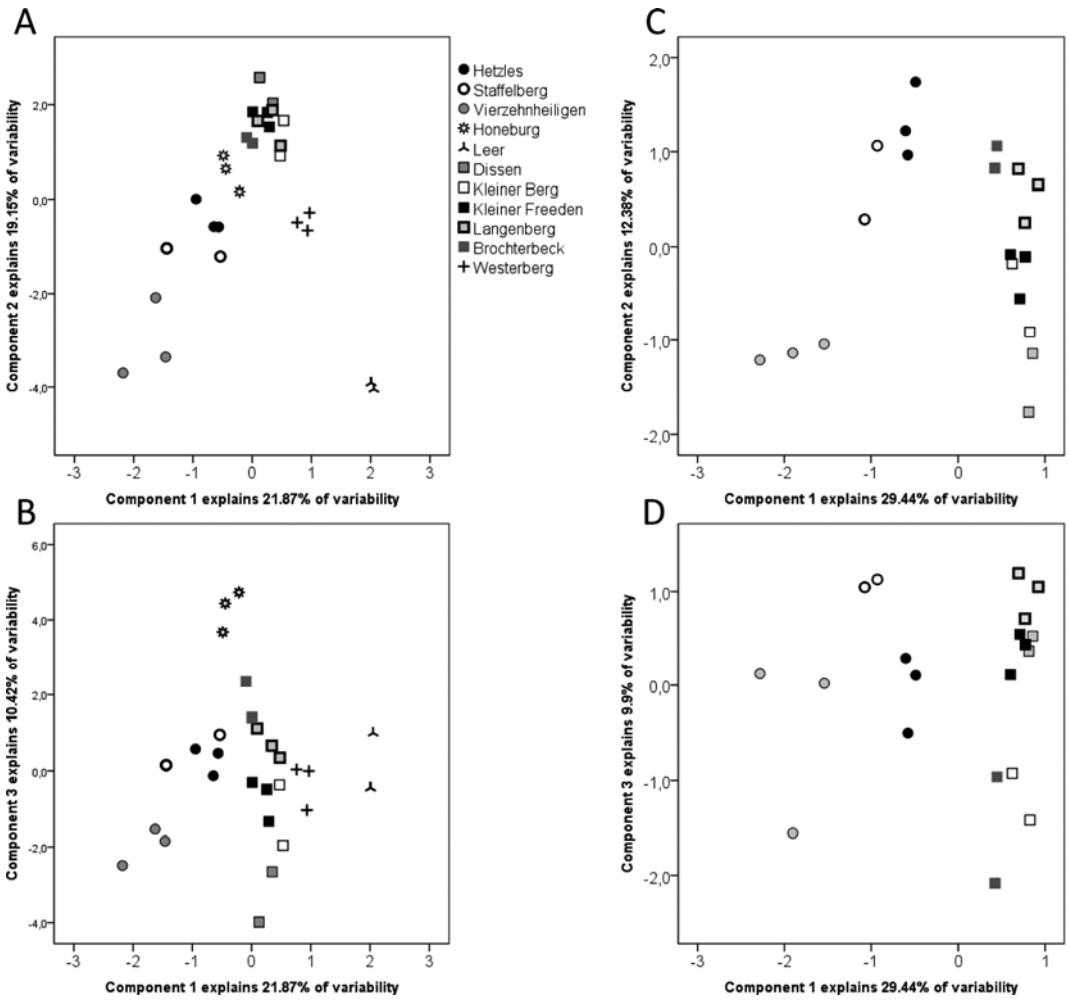

Fig. 6
(A) Principal component analysis (PCA) of 33 *A. ursinum* individuals from eleven populations with 26 informative RAPD markers plotted with factor one and factor two, (B) plotted with factor one and factor three, (C) PCA with anthropogenically influenced populations excluded (Osnabrück, Westerberg; Osnabrück, Honeburg; Leer, East Frisia) (24 Individuals, 23 informative RAPD markers) and plotted with factor one and factor two, (D) plotted with factor one and factor three

between Hetzles and Staffelberg or Hetzles and Vierzehnheiligen (linear distance ca. 50 km). The third group, here called Northern Populations consists of Leer, East Frisia (UG), Osnabrück, Westerberg (UE) and Osnabrück, Honeburg (UH). The genetic distance between Honeburg and Westerberg (linear distance ca. 3 km) was expected to be lower than between Leer and Westerberg or Leer and Honeburg (linear distance ca. 115 km).

Within the topology of the ME tree (Fig. 5a) the northern populations intermingled with the populations of the Teutoburg Forest and Franconian mountain area. The population Osnabrück, Honeburg (UH) was placed as a sister group to the population of Brochterbeck (UF) and both were placed as a sister group to all accessions from the Franconian mountain area. The components one (explained 21.87% of variability) and two (explained 19.15% of

variability) of the PCA (Fig. 6a), grouped Osnabrück, Honeburg (UH) between Brochterbeck (UF) and Hetzles (UKI). In this plot, the population Osnabrück, Westerberg (UE) seemed to be relatively distant from Honeburg. The population Leer, East Frisia (UG), which was placed as a sister group to Osnabrück, Westerberg (UE) within the ME tree, was located quite distant in the PCA plot of component one and two. However, the components one and three (explained 10.42% of variability) of the PCA placed Leer (UG) near Westerberg (UE) but Honeburg (UH) quite distant from both (Fig. 6b). All three populations (UE, UG and UH) seemed to be independent and no coincidence between the genetic distances and the geographical distances was obvious. The components one and two were not able to separate the group Leer (East Frisia) (Fig. 6a).

One plausible explanation for the populations Honeburg (UH) and Westerberg (UE) is that they are garden escapes as they are located directly beside (5–50 m away) cultured plants in private property. The population Leer in East Frisia (UG) is located in a city park and therefore in all probability also anthropogenic influenced.

After exclusion of these populations, the topology of the ME tree (Fig. 5b) obviously coincided with the geographical distance. Both remaining groups (Franconian mountain area and Teutoburg Forest) were clearly separated. The branch of the group Teutoburg Forest is supported by a bootstrap value of 73%. All populations were determined by the RAPD markers. However, the bootstrap values do not support all branches. The component one of the PCA (explained 29.44% of variability) was able to separate the groups Franconian mountain area and Teutoburg Forest (Fig. 6c). Component two (explained 12.38% of variability) was able to separate the populations within the two groups (Fig. 6c). Within the group Teutoburg Forest the geographical distance coincided with the genetic distance. However, in this plot, the populations Hetzles (UKI) and Staffelberg (UKII) which are geographically distant from one another were placed closer together. In the plot of component one and three (explained 9.9% of variability) all three populations of the group Franconian mountain area were more-or-less equally distant from one another (Fig. 6d). Within the ME tree the distance seems to coincide with the geographical distance. However, bootstrap values do not support all branches (Fig. 5b).

Within populations variation of the RAPD data is low, significant polymorphism was obvious, as shown e.g. for the populations Noller Schlucht near Dissen (UA) and Brochterbeck near Tecklenburg (UF). Only one of nine primers (C10) produced polymorphic bands and this concerns only one marker between two individuals of population Dissen, Noller Schlucht (UA).

Discussion

The nucleotide sequences of the ITS, ETS and *trn*L-*rpl*32 spacer within the species *A. ursinum* s.l. show no significant variability. No data were obtained to clarify the relation of the different populations of *A. ursinum* s.l. in Germany (Fig. 3 & 4). Even a population from Belfast, Northern Ireland had the same ITS sequences (Fig. 3). Furthermore, the *trn*L-*trn*F spacer (cpDNA) was highly conserved within the species.

It is noticeable that in both trees (ITS and ETS; Fig. 3) the accessions of *A. ursinum* s.l. were placed as a sister group to the section *Molium*. Within the section *Molium* the closest relative seems to be either *A. moly* or *A. scorzonerifolium* depending on the markers observed. Additionally, within the tree of *trn*L-*rpl*32 data (Fig. 4) *A. moly* was placed as a sister group to *A. ursinum* s.l. This seems plausible as the distribution areas of *A. moly, A. scorzonerifolium* and *A. ursinum* subsp. *ursinum* overlap in the Pyrenees and the Cantabrian Mountains in northern Spain (AEDO 2008). The result confirms the assumption of FRIESEN et al. (2006) that the section *Molium* is the closest relative to the section *Arctoprasum*.

RAPD analysis detected only a few polymorphic fragments between different populations of *A. ursinum* subsp. *ursinum* (Tables 3 & 4). Nevertheless, ME analysis was able to distinguish all populations. After excluding anthropogenically influenced populations, the topology of the ME tree coincided with the geographical distribution. Both remaining groups (Franconian mountain area and Teuto-

Table 4
Percentage and total counts of monomorphic bands between each population

		UB	**UC**	**UD**	**UE**	**UF**	**UG**	**UH**	**UKI**	**UKII**	**UKIII**
	UA	91%	94%	90%	88%	89%	83%	87%	84%	84%	81%
UB	81	**UB**	93%	90%	90%	90%	83%	88%	84%	85%	81%
UC	84	83	**UC**	82%	91%	92%	85%	90%	88%	89%	83%
UD	80	80	73	**UD**	89%	88%	85%	90%	87%	87%	80%
UE	78	80	81	79	**UE**	90%	90%	87%	83%	88%	81%
UF	79	80	82	78	80	**UF**	84%	91%	88%	87%	82%
UG	74	74	76	76	80	75	**UG**	83%	81%	82%	79%
UH	77	78	80	80	77	81	74	**UH**	84%	89%	83%
UKI	75	75	78	77	74	78	72	75	**UKI**	88%	84%
UKII	75	76	79	77	78	77	73	79	78	**UKII**	87%
UKIII	72	72	74	71	72	73	70	74	75	77	**UKIII**

100% = 89 markers of the RAPD data

burg Forest) were clearly separated within the ME tree and the PCA (Fig. 5 & 6). Even though *A. ursinum* s.l. tends to reproduce clonally by producing genets, MORSCHHAUSER et al. (2009) predicted considerable genetic diversity within a population. They observed a high recruitment of seeds even at high densities. However, within a population of *A. ursinum* subsp. *ursinum,* the RAPD primers could not detect variability. On a molecular level, every population seemed to be derived from one or very few individuals.

HEWITT (1996) and CRONBERG (2000) were able to show that the genetic variability of areas, which have been postglacially influenced, is often depleted. As *A. ursinum* is often found as an accompanying species to beech forests in Germany, it seems plausible that they may have shared a common refugia during the last glacial maximum and co-migrated northwards after the ice sheet retreated. Furthermore the variability within these refugia should be higher (HEWITT 1996). The low percentage of variability observed could be explained by a rapid spread, as HEWITT (1999) showed for several species and MAGRI (2006 and 2008) for *F. sylvatica* in central Europe, and especially in central Germany. The question whether or not the low percentage of variability is due to the manner of reproduction, human influences or postglacial influences still remains and therefore, further investigations are necessary.

Concluding remarks

Our analysis of *A. ursinum* subsp. *ursinum* populations allows us a first insight into complicated relationships and bio-geographic processes in historic times which may have been partly influenced by mankind. These findings draw our interest to a more detailed and broader study. The sampling should be widened with respect to the whole distribution area and especially to the possible glacial refugia of *F. sylvatica* discovered by MAGRI (2008). A more sophisticated marker system such as microsatellites or single nucleotide polymorphism (SNP) array should be introduced for intra- and inter-population analysis.

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