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REGULAR PAPER

Evaluation of genetic differentiation of autochthonous sloe (*Prunus spinosa*, Rosaceae) populations across Germany using molecular markers

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Background and aims – Sloe is a woody plant often used for plantings in the open landscape in Germany. As the use of autochthonous plant material is now required by the new German Nature Conservation Act six regions of origin have been designated according to eco-geographical parameters. As little is known about the actual genetic situation of most species affected by the new law we investigate the genetic diversity/differentiation of autochthonous sloe populations across Germany and discuss our findings with respect to conservation law and its practical implication.
Methods – Fifteen autochthonous populations of sloe from all officially designated regions of origin were analysed using a highly reproducible high-annealing-temperature (HAT-) RAPD protocol. Genetic differentiation was assessed using distance based and Bayesian approaches.
Key results – General heterozygosity detected within the populations was in the same range as described for other woody species (he 0.171–0.213). While the observed values of genetic differentiation between populations varied considerably (F_{ST} 0.025–0.226) the majority was found in the moderate range. Only two moderately differentiated genetic clusters were identified for sloe in Germany.
Conclusions – Moderate genetic differentiation was observed between the two main clusters of sloe populations in Germany. Here, no strong evidence was found for isolation by distance (IBD) or by adaption (IBA). The genetic constitution of sloe populations across Germany rather seems to support isolation by colonialization (IBC) as the main driver of the moderate genetic differentiation in this species. The observed genetic differentiation and the geographic location of the identified genetic clusters only partially coincide with the designated regions of origin defined by German authorities for the implementation of the Nature Conservation Act. In our opinion, those regions can only be considered a first step in the preservation of genetic diversity. Upon availability of data on genetic structure and differentiation in a given species, the regions of origin should gradually be adapted to reflect those structures for each analysed species.

Key words – Autochthonous, differentiation, genetic diversity, *Prunus spinosa*, regions of origin, residential.

INTRODUCTION

Genetic diversity has widely been accepted as an important factor for the success of a species in its given environment and a prerequisite for colonialization of new niches or survival of changing environmental conditions (Mitton 1994, Sambatti et al. 2001, Crawford & Whitney 2010). Thus, not only the conservation of species themselves has been the focus of nature conservation, but also the maintenance of their genetic diversity (Moritz 2002, Iriondo et al. 2012) as stipulated by the Convention on Biological Diversity (1992).

In Germany, those demands were also implemented in the 2010 amendment of the Federal Nature Conservation Act (BNatSchG 2009). Accordingly, from 2020 on, the exclusive use of autochthonous plant material for all plantings in the open landscape is compulsory. The implementation of the law raised some interesting questions, though. There is an ongoing discussion on what the term "autochthonous" actually implies, how to identify such resources and whether there are differences/overlaps to terms like residential or indigenous (Kowarik & Seitz 2003, Mátyás et al. 2002, Rumpf 2003). In spite of this, in 2012 the Federal German

Ministry for Environment, Nature Conservation and Nuclear Safety recommended six regions of origin (called "Vorkommensgebiete" in the German nature conservation legislation) for woody plants which are not covered by forestry or agricultural legislation (BMU 2012). This was done to fulfil the demands of § 40 of the Federal Nature Conservation Act and to facilitate the practical organization, regulation and control of usage of autochthonous plant material. The designated regions of origin (called RO in this paper) follow in principal the proposal of Schmidt & Krause (1997). They are combinations of neighbouring geographic main units as given in the classification of Meynen & Schmithüsen (1953–1962) or parts of them and it is assumed that the fused units have similar ecological conditions. By this approach, the delineation of the RO follows main landscape features rather than clearly defined and verified ecological borders.

Sloe (*Prunus spinosa* L.), also known as blackthorn, is a wild tetraploid (2n = 4x = 32) shrub native to Europe, North Africa and West Asia (Schütt et al. 1992). The origin of tetraploidy has not been unambiguously resolved, yet. There is evidence for allotetraploidy (Reynders-Aloisi & Grellet 1994, Mohanty et al. 2000) but also some support for autotetraploidy (Leinemann 2000). This allogamous, insect-pollinated, chiefly mammalian and bird dispersed plant (Guitián et al. 1993, Fitter & Peat 1994a, 1994b) is very wide-spread over Germany (and most of Europe in general). Sloe is heliophilous but also grows in moderately shaded habitats (till 30% illuminance). It prefers moderately moist conditions and ranges from lower to montane elevations. Concerning soil conditions this species indicates weakly acidic to weakly alkaline dry to fresh soils (Ellenberg et al. 1992). Due to its relatively wide ecological range sloe is one of the species most often used in plantings in the open landscape such as renaturation and compensation measures.

In the last few years, progress has been made in the investigation of sloe population genetics and differentiation. Using chloroplast markers Mohanty et al. (2000, 2002) found a rather high diversity but low differentiation between the tested European populations – no clear correlation could be established between genetic and geographic distances on a European scale. Allozyme studies revealed moderate (Leinemann et al. 2002) to low (Fronia 2009) diversity within and differentiation between regional German sloe populations. Eimert et al. (2012) used HAT-RAPD markers to assess nuclear diversity in a limited number of autochthonous seedstock populations reporting no genetic differentiation between the populations and commercial seedstocks. Vander Mijnsbrugge et al. (2013) used AFLP markers and morphometric data to investigate the diversity of regional (Flemish) Belgian sloe populations. They reported a relatively low diversity within populations and appreciable differentiation between them. However, no correlation of the observed genetic clusters with their geographic situation was found. Leinemann et al. (2014) combined chloroplast and AFLP markers to examine the genetic structure of sloe in a larger region of Germany (covering three of the six designated regions of origin and including Italian and Hungarian plant material as outgroups). Despite generally high haplotype variability the authors found little evidence for geographic/genetic structure at the chloroplast level. When restricted to the German populations only, nuclear markers revealed a certain genetic structure, seemingly unrelated to geographic distances.

The aim of this research was to evaluate the genetic diversity and differentiation of sloe populations from all designated RO across Germany. Observed genetic structures within those populations were then correlated to the mentioned RO and the results discussed in view of possible improvements to the legislation on the use of autochthonous plant material in Germany.

MATERIALS AND METHODS

Plant material

Fully expanded leaves from at least forty individuals of fifteen populations of sloe from across Germany were sampled (fig. 1, for details see electronic appendix 1A). With the exception of RO 3 (population F), at least two populations were sampled from within each RO. The distance between sampled populations ranged from 30 km (populations I and K within RO 4) to over 700 km (populations A and L from RO 1 and 5, respectively).

The evaluation of autochthony in the field is critical but challenging. We followed the procedures summarized by Vander Mijnsbrugge et al. (2013). In short, autochthonous populations are characterized by the following criteria: old shrubs; no obvious plantation (ordered growth patterns); sample site is within the natural geographic range and ecological requirements; well sized population in areas of undisturbed woodlands and old growth forests. Not all conditions could be met at all times. As mentioned before, sloe often occurs in hedgerows along forest borders or farm tracks (Leinemann 2000, Vander Mijnsbrugge et al. 2013). Even the latter can be considered autochthonous populations if they are old enough to pre-date industrial area import and planting of non-resident plant material (Leinemann et al. 2014, Vander Mijnsbrugge et al. 2013). Since extended clonal stands of sloe have been reported (Fronia 2009, Leinemann 2000, Leinemann et al. 2014), we strove for a minimal distance of at least 50 m between sampled individuals whenever possible (but never less than 10 m). Pairwise geographic distances of the populations are shown in the electronic appendix 2A.

RAPD-fingerprinting

DNA extraction followed the procedure of Eimert et al. (2012). In short, plant material was homogenized in uria extraction buffer (0.05 M Tris–HCl pH 8.0, 0.02 M ethylenediaminetetraacetic acid [EDTA] pH 8.0, 0.3 M NaCl, 7 M urea, 1% sarcosine) containing 1% (vol.) insoluble polyvinylpolypyrrolidone (PVPP, SIGMA) and RNase A (c. 1 Kunitz-unit/mg fresh weight leaf material). After centrifugation the supernatant was extracted twice in phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated in high salt concentration (3.3 M NaCl) resuspended in TE buffer and stored at -70°C until use. On average, 100 mg leaf tissue yielded 2–8 µg clean DNA (as judged by the OD260/280 ratio).

A subset of the RAPD primers used in earlier works (Eimert et al. 2012) was utilized in this study (see electronic appendix 1B). The primers were chosen from the University of British Columbia (UBC, Vancouver, Canada) RAPD primer sets no. 1, 4, and 8 for their characteristics suitable for HAT-RAPD polymerase chain reaction (PCR). The higher GC content (70–90%) and/or microsatellite-like di- or trinucleotide composition give them inter-simple sequence repeat (ISSR)-anchored primer (Zietkiewicz et al. 1994) or SPAR (Gupta et al. 1994) like properties.

The PCR protocol was described earlier (Eimert et al. 2012). In essence, the reaction was performed in volumes of 20 µl containing 1 ng/µl DNA, 1 µM primer, 1.5 mM MgCl2, 0.2 mM each of four deoxynucleotide triphosphates, and 0.1 unit/µl Taq DNA polymerase (Fermentas, Fisher Scientific – Germany GmbH) in a Primus Advanced thermocycler (PeqLab, VWR International GmbH). Amplification was initiated with a denaturation step at 95°C for 5 min, followed by 38 cycles of 1 min at 95°C, 1 min at 45°C, and 2 min at 72°C. The resulting DNA fragments were separated by horizontal gel electrophoresis (1.3% agarose, Tris-borate-EDTA (TBE) buffer), stained with ethidium bromide, and documented (sample gel see in appendix 1C).

PCR and electrophoresis were performed at least in two repeats for each sample to visually assess the reproducibility of band patterns.

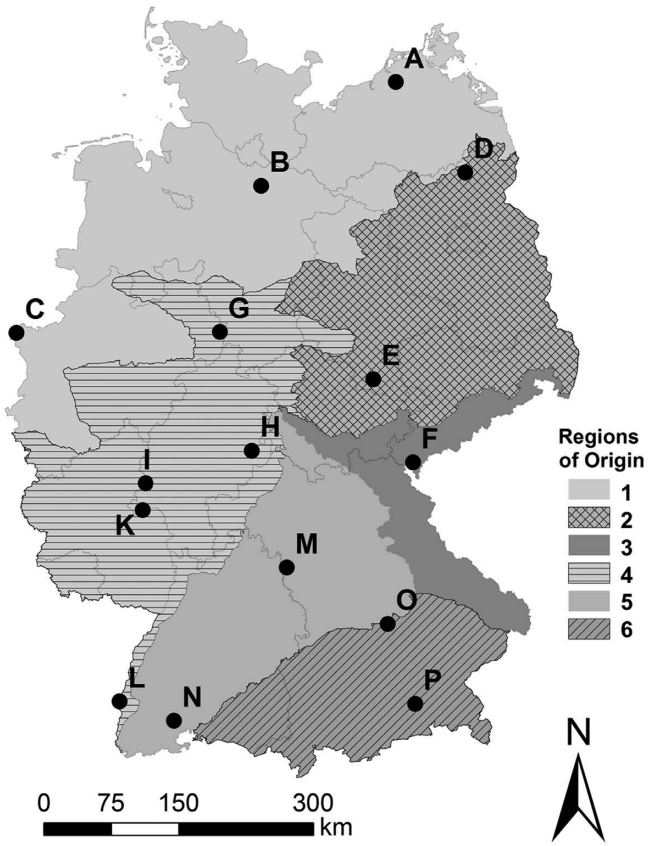

Figure 1 – Overview of the fifteen sampling locations (A–P) and the six delineated regions of origin for autochthonous shrubs. Borders of federal countries (thin gray lines) are marked for better orientation.

Data analysis

Band calling and matching for the dominant markers were conducted using the BioNumerics software package (version 6.6; Applied Maths BVBA, Sint-Martens-Latem, Belgium). The resulting binary character table was exported and used in the following analyses.

Allele frequencies over all loci were calculated using FAMD (Schlüter & Harris 2006) using Lynch & Milligan's (1994) estimation recommended for RAPD markers. Following that, markers were tested for selective neutrality using Tajima's D test (with 1,000 simulations; Tajima 1989) implemented in Arlequin version 3.5 (Excoffier et al. 2005). Pairwise F_{ST} (Fixation index, Wright 1969, Holsinger & Weir 2009) values were calculated in FAMD based on standard similarities (Jaccard 1912, average coefficient calculated from 100 random draws, d=1-s).

F_{ST} values of all markers were tested for outliers using Mcheza software (DFDIST based and adapted for use with dominant markers, Antao & Beaumont 2011) applying 99% confidence intervals and a 0.05 false discovery rate (FDR) over 100,000 simulations. We also applied a Bayesian approach to test for outliers using BayeScan 2.0 (Fischer et al. 2011) with a FDR of 0.05 over 100,000 simulations. Two datasets were prepared for further calculations – one consisting of all loci and a second one consisting only of putatively neutral loci (i.e. without outlier loci).

The software Structure 2.3 (Falush et al. 2007, Hubisz et al. 2009) was used in a model-based approach to detect genetic structure in our samples (using for all models 100,000 steps as burnin, 50,000 steps in the Markov chain and 20 runs). The most likely number of clusters (K) was calculated according to Evanno et al. (2005) using the Structure Harvester webservice (Earl & vonHoldt 2012).

Using the above F_{ST} data sets, distance based cluster analysis (NJ – neighbour-joining, Saitou & Nei 1987) for populations and RO were calculated in FAMD and a major consensus tree (50% majority) was constructed from 1,000 repeats (bootstrap support values for the nodes are displayed). A principal coordinate analysis (PCoA, Gower 1966) for the populations was also conducted in FAMD with 1,000,000 iterations.

Main genetic boundaries were calculated using Monmonier's maximum difference algorithm in the Barrier software package vers. 2.2 (Manni et al. 2004).

The GenAlEx plug-in version 6.5 (Peakall & Smouse 2006, 2012) was used for AMOVA (set to 999 permutations; Excoffier et al. 1992) to assess hierarchical partitioning of genetic variation within and among populations and/or RO.

Spatial patterns in genetic distances were evaluated using Mantel tests (Mantel 1967, Sokal 1979) at the Isolation by Distance Web Service (Jensen et al. 2005, http://ibdws.sdsu.edu) with 10,000 simulations on three datasets: all loci, neutral loci and non-neutral loci. These results were also used for inference of the main driving forces for genetic differentiation in sloe.

The correlation of all markers to climate data was assessed using the Vegan package (Oksanen et al. 2016) in R (R Development Core Team 2008) with the detailed climatic

Table 1 – **Descriptive population statistics.**
[a] monomorphic band presence in a given population; [b] bands found only in one given population; [c] private bands monomorphic in a given population; [d] expected heterozygosity = 2 × p × q.

Population (RO)	Polymorphic bands	Polymorphic loci (%)	Fixed bands[a]	Private bands[b]	Fixed private bands[c]	h[d] Mean (SE)
A (1)	217	76.41	14	1	0	0.207 (0.011)
B (1)	222	78.17	11	0	0	0.204 (0.011)
C (1)	204	71.83	13	0	0	0.191 (0.011)
D (2)	217	76.41	15	1	0	0.197 (0.011)
E (2)	208	73.24	8	0	0	0.178 (0.010)
F (3)	196	69.01	18	0	0	0.187 (0.011)
G (4)	228	80.28	9	4	0	0.190 (0.011)
H (4)	214	75.35	8	1	0	0.199 (0.011)
I (4)	225	79.23	11	0	0	0.213 (0.011)
K (4)	191	67.25	9	0	0	0.175 (0.011)
L (4)	216	76.06	11	0	0	0.182 (0.011)
M (5)	209	73.59	11	0	0	0.171 (0.010)
N (5)	219	77.11	7	0	0	0.199 (0.011)
O (6)	235	82.75	8	1	0	0.221 (0.011)
P (6)	234	82.39	10	2	0	0.212 (0.011)
Total	280 (out of 284)					0.195 (0.003)

data (1 × 1 km grid) from the Climate Data Center of the federal German weather service "Deutscher Wetterdienst" (DWD, http://www.dwd.de). Continentality according to Ivanov (1959), continentality by maximum summer and minimum winter temperatures (DWD), mean annual temperature and mean annual precipitations were used for PCoA (with 10,000 permutations). Redundancy analysis (RDA) for the climatic data was also conducted in Vegan using stepwise forward selection with 1,000 permutation steps and the actual contribution of the variables was judged by an adjusted R square (Borcard et al. 2011).

Geographic (linear) distances between the centroids of the sampled populations were determined using the Google EarthTM (Google 2015) "ruler" tool. Basic geographic maps were provided by Bundesamt für Kartographie und Geodäsie (Federal Agency for Cartography and Geodesy, http://www.bkg.bund.de) and used with ArcGIS (ESRI, Singapore) to visualise geographic data.

RESULTS

HAT-RAPD-analysis of all 607 individuals from fifteen populations with nine primers resulted in 284 scorable band classes. Fixed bands were observed in all populations and a few private bands in some populations (table 1). However, no fixed private bands could be identified.

Tajima's D test for selective neutrality revealed only slight (non-significant at the $P < 0.05$ level) deviations from 0. These results suggested no substantial deviation from neutrality in any of the populations, although, slight differences in total values were observed among the different populations (see electronic appendix 2B).

On the other hand, outliers could be detected using both a distance based and a Bayesian approach, suggesting selective pressure on some loci. With both methods, 28 (out of 284) loci appear to be under selective pressure (see electronic appendix 3). While both approaches identified almost identical loci putatively under positive selection, the loci flagged for negative selection varied between the approaches.

All following calculations were conducted first using all markers and then repeated using only the neutral markers. The pairwise F_{ST} values calculated for all populations (using all loci) revealed a broad range of genetic differentiation among populations (from 0.025 to 0.226, see electronic appendix 2A). When compared to the data calculated from the neutral loci no significant changes could be observed between the results from both datasets (electronic appendix 2C).

Bayesian estimations of genetic structure within our samples were calculated assuming different ancestry models (admixture vs. no admixture), different allele frequency models (correlated vs. independent) and different sampling information (with or without prior information). As the assumption of no admixture seems very unlikely, given the wide distribution of sloe (see electronic appendix 1D), only the results for the admixture models are shown below. When all loci were included into the calculations, the most likely K-value was established at $K = 2$ for all tested models (fig. 2, table 2). Support for this weak genetic structure was generally low, but strongest ($\Delta K = 51.93$) for the admixture/ correlated alleles model without local priors. In this case the populations A-F, I, M, O, P belong to one cluster and the populations G, H, K to a smaller second cluster. The populations L and N consist of individuals of both clusters to approximately the same extend and are considered to be admixed (fig. 2A). In

Table 2 – **The Evanno table output for most likely K (ΔK) for all populations including outliers.**
[a] order of models according to their "sensitivity" to detect structures (lower first); only K-values with the two highest ΔK-support are shown; [b] models included: admix = admixture; corr = correlated alleles; indep = independent alleles; (no) priors = local priors (not) included; K-values with higher support are printed bold.

Model[a]	Most likely K (ΔK)					
	all loci		neutral loci only		outlier loci only	
admix/ corr / no priors[b]	**2 (51.93)**	3 (29.61)	**4 (03.27)**	14 (02.63)	**2 (31.21)**	3 (4.24)
admix / indep / no priors[b]	**2 (07.09)**	5 (06.64)	**6 (26.72)**	2 (03.13)	**2 (56.16)**	3 (6.30)
admix / corr / priors[b]	**2 (15.31)**	3 (06.37)	**2 (02.78)**	10 (01.32)	**2 (09.79)**	4 (5.46)
admix / indep / priors[b]	**2 (16.94)**	3 (12.53)	**3 (14.67)**	2 (06.67)	**2 (12.58)**	3 (7.09)

most cases, including local priors into the calculations did not increase the according ΔK-values. Using only the selectively neutral loci, no clear structure could be assigned with certainty. There, the highest ΔK-support was actually observed for K = 6. When using only loci under apparent selection a relatively strong support could be shown for K = 2, demonstrating the differentiating influence of those loci on population structure.

NJ cluster analysis of the populations was conducted based on all loci (fig. 3). Generally, only two main clusters could be observed, with the smaller one (cluster II) consisting mostly of populations from RO4 (G, H, K, L). The larger cluster I consisted of populations from a rather wide geographic area occupying, apparently, quite different ecological habitats. Within the clusters, geographically neighbouring populations tended to form smaller common subclusters (i.e. A, B, D or L, N). But, that was not always the case and only once (except for F as a single representative) all populations of the same RO clustered tightly together in one subcluster (populations O and P of RO 5, within the cluster I). No significant difference in the basic clustering could be observed using the neutral markers only (see electronic appendix 4A).

A PCoA with the complete dataset supported the relative genetic distance of the populations to each other with the first two coordinates explaining 49.65% and 18.66% of the differentiation, respectively (fig. 4). Using neutral markers only, the differentiation explained by the first two coordinates sank to 39,8% while the overall clustering remained almost unchanged (see electronic appendix 4B).

The established pairwise F_{ST} values were used to identify main genetic barriers employing Monmonier's maximum difference algorithm and plot them onto a geographic grid. Since differences were relatively low, the number of calculated barriers was restricted to K = 5 (fig. 5A). The strongest genetic boundary (i) was calculated between populations C and I (belonging to RO 1 and 4, respectively) and all remaining populations. Populations G, H and K (all of RO 4) are enclosed between barrier i and the second strongest barrier (ii). Population pairs L/N and O/P were also separated from each other and from the remaining populations (barriers iii, iv and v).

An AMOVA with the complete dataset of all populations found the majority of variation within the populations (85%) and the smaller part among populations (10%) and among RO (5%). Using the dataset consisting of neutral loci, only a slight shift was observed (87% within populations, 9% among populations and 4% among RO) (see electronic appendix 4C) demonstrating a slightly lower hierarchical partitioning of genetic variation between the populations and the RO. Separate AMOVAs for populations in each region indicated that variation among populations within regions varied between 3% (RO6) and 13% (RO5) (table 3). Also, exemplary AMOVAs were calculated for population pairs from the same and from differing RO to compare their relative values (see electronic appendix 4D). The relative diversity between different populations varied significantly and seemingly independently of geographic locations and proximities. It is noteworthy that geographically close populations may be strongly differentiated at the genetic level (i.e. K and I)

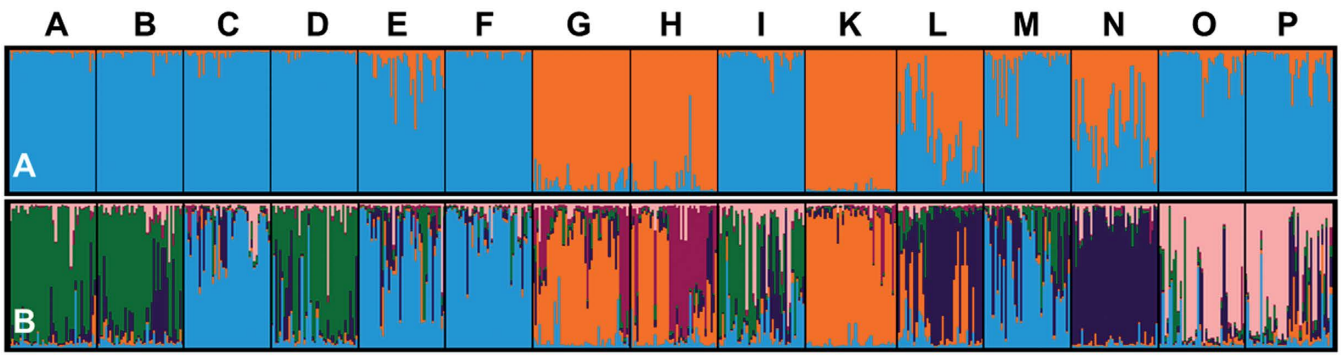

Figure 2 – Bayesian estimation of genetic structure for all fifteen populations (A–P top label, designation as in table 1): A for K = 2 (assuming admixture and correlated alleles, no local priors given, using all loci); B for K = 6 (assuming admixture and correlated alleles, no local priors given, using neutral loci).

Table 3 – **Summary AMOVA for RO.**
[a] distribution of total variation among and within populations within the RO in %; P = 0,001.

	Estimated Variation in % [a]				
RO	1	2	4	5	6
Among Pop	11	11	11	13	3
Within Pop	89	89	89	87	97

while geographically distant populations may be genetically similar (i.e. A and I).

Mantel tests were then conducted to assess the correlation between genetic and geographic distances (e.g. test for isolation by distance, IBD). Neither a test using all markers nor a test using only neutral markers yielded a significant correlation between genetic and geographic distances. Also, the Mantel test did not support a significant correlation for the markers apparently under selective pressure. When Mantel tests were conducted for the individual subclusters (defined by the above cluster analysis) no correlation to geographic distances was observed within the subclusters, either.

Correlation of the genetic structure and climatic factors (continentality, mean annual temperature, mean annual precipitation) was assessed using Mantel tests and PCoA. The Mantel tests showed no correlation of the mentioned climatic factors with the general genetic structure using either the complete or the neutral marker datasets (see electronic appendix 5). Also, no significant correlation could be observed using only the loci under apparent selection. Thus, no evidence was found for IBA, while the data indicate IBC as a driver of genetic differentiation. That scenario is also supported by the PCoA of genetic and climatic distances. Here too, no significant influence of the climatic distances could be observed. The stepwise RDA revealed the maximum influence for any of the tested climatic factors on the differentiation at well below 4% (for mean temperature, data not shown). However, when we conducted Mantel tests for the subclusters using only the loci under apparent selection, we observed a clear correlation between the genetic structure and geographic and climatic distances in subcluster II (see electronic appendix 5). That indicates that, in subcluster II, a combined effect of the pure scenarios (isolation by dispersal limitation [IBDL] and isolation by serial colonisation) is probable (Orsini et al. 2013).

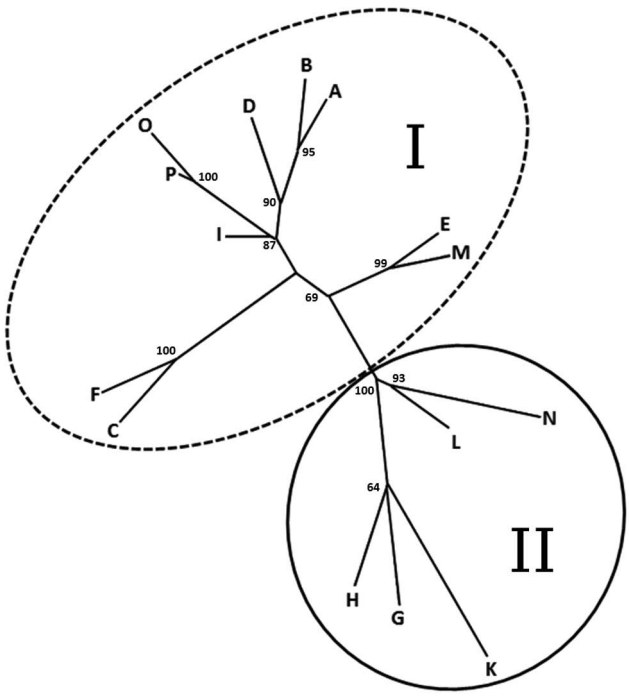

Figure 3 – Dendrogramm of cluster analysis of *Prunus spinosa* populations using all loci (NJ with distance transformation d = 1-s); A–P: populations analysed; areas bordered by dashed and solid lines: main clusters I and II, respectively; arabic numerals: bootstrap values supporting the nodes (1000 repeats).

DISCUSSION

Observed Nei's gene diversity for sloe in the analysed populations is in the upper range reported for other outbreeding, self-incompatible, long-lived, perennial woody plants (Hamrick et al. 1992). It is also higher than the diversity seen in seedstock populations of sloe collected in Germany (Eimert et al. 2012). That is not unusual as seed collections tend to be locally restricted and, thus, by necessity can only represent a part of the total diversity of the total population in question. Also, the distribution shown in electronic appendices (see Supplementary Data section), of the total genetic variation observed in the fifteen analysed populations (85% within populations vs. 10% and 5% between populations and regions of origin, respectively) is in good agreement with data observed in autochthonous populations of sloe in parts of Germany (Leinemann et al. 2014) and also in Flanders, Belgium (Vander Mijnsbrugge et al. 2013). That differs from data reported for seedstock populations of sloe collected in

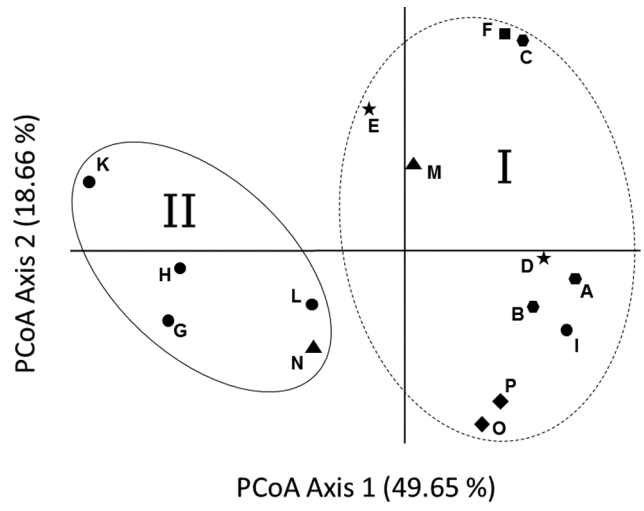

Figure 4 – PCoA of genetic distances of *Prunus spinosa* populations (1,000,000 iterations) using all loci; areas bordered by dashed and solid lines: main clusters I and II, respectively; symbols denote affiliation of the populations to the delineated RO: triangles – RO 1, stars – RO 2, squares – RO 3, dots – RO 4, crosses – RO 5, diamonds – RO 6; first two axes explain about 68% of the differentiation.

Germany (92.22% within vs. 7.78% between populations, Eimert et al. 2012). Again, this is most likely due to the limited genetic range represented in the seedstocks. While this is not the subject of this work, these differences underline the importance of defining appropriate source stands of plants for seed collection in sufficient numbers. Diverse sources from designated regions should be harvested to ensure maximum genetic diversity within the seedstocks.

Similar to Leinemann et al. (2014) we could identify genetic structures within the analysed sloe populations. For the populations as a whole, Tajima's D test revealed no significant deviations from neutral evolution patterns. The absence of any fixed private bands in any of the populations (table 1) also seemed to support the notion of lack of significant selection in correspondence to geographic location and its ecological conditions, i.e. adaption. Nevertheless, several outliers were detected using different approaches.

The two genetically differentiated clusters of sloe populations in Germany revealed by distance based approaches (NJ and PCoA calculations; figs 2 & 3) coincide well with the two genetic clusters identified in a Bayesian approach where populations A–F, I, M, O, P also belong to a larger cluster and populations G, H, K belong to a smaller cluster. Populations L and N consist of individuals of both groups to approximately the same extend and are considered to be admixed (fig. 2A).

Plotting the genetic borders onto a geographic map (fig. 5A) only partial conformity with geographic features was observed. As the barriers are mathematically calculated and the results abstractly drawn by computer software an exact mapping of the barriers onto the landscape will not always depict reality well. Therefore, some eco-geographical interpretation seems needed. As the populations G and H are located in the northern and eastern section of RO 4 it appears that the barriers i and ii approximately follow in the real landscape the designated borders between RO 4 and the neighbouring RO (RO 1, 2, 3 and 5) also concurring in this part with the boundary between the observed clusters I and II. Within RO 4, the strongest barrier (i) separates population I from the closely neighboured population K (approx. 30 km linear distance) and also from populations G and H with the consequence that population I is not part of the cluster II (figs 2, 5A & B). Barrier i in this section corresponds geographically to the southern and eastern rim of the Rhenish Mountain Massif (Rheinisches Schiefergebirge), effectively removing the Massif from RO4. As sloe is a heliophilous species that needs open landscapes over a longer period of

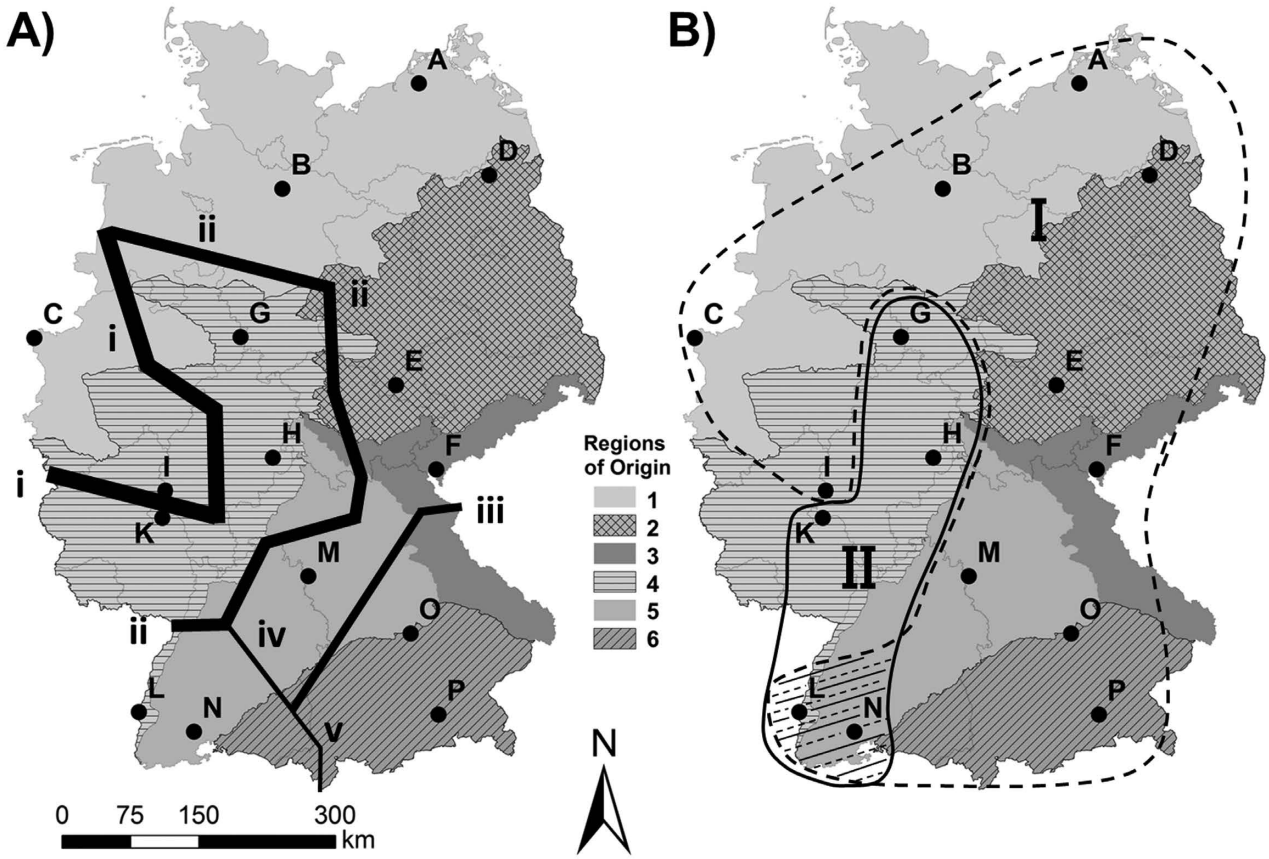

Figure 5 – Genetic barriers and clusters: A, genetic barriers between populations with K = 5 (straight lines, numbered from i to v, decreasing thickness of lines indicates declining order of strength) as identified with Monmonier's maximum difference algorithm (overlayed on the geographic map of figure 1 and using the same labelling); B, geographic situation of the calculated genetic clusters (NJ) in sloe populations in Germany, dashed and closed lines show the borders of the main clusters I and II, respectively; admixture is assumed in the south-western overlapping areas (hatched area, populations L and N). Labels are according to figure 1.

years to establish new populations (Bleicher & Herbig 2010), large connected forests and less favourable climatic conditions in the summit region of the Rhenish mountains may restrict the expansion of sloe and hinder the genetic exchange between populations from each side of the mountain range. The south-eastern part of the genetic barrier ii concords with the Upper Rhine rift, whereas the southernmost stretch crosses the rift valley separating L and N from all other populations. This genetic division does not relate to any obvious geographical features but confirms the special position of L and N within cluster II as identified by NJ cluster analysis. Likewise, we could not assign any landscape ecological features to genetic barriers IV or V separating populations L and N from NJ cluster I and from the population M of RO 5. Barrier iii follows the rim of the Schwäbisch-Fränkische Alp mountains and separates populations O and P from all other populations in cluster I. It is the only genetic barrier we could identify within cluster I.

The geographic situation of the two genetic clusters only partially reflects the designated RO. These RO are solely based on main geographic units. Thus, they would only be biologically meaningful if there was actual genetic differentiation of plant populations due to evolutionary adaption to an according ecological differentiation. Assuming that adaption to certain landscapes involves the adaption to the corresponding ecological features we evaluated the correlation of genetic distances and a set of climatic factors. No significant influence of the tested climatic factors on the genetic structures was observed using Mantel tests and PCoA. While not the subject of study, we note that the absence of correlation between genetic and geographic distances in conjunction with the absence of correlation between genetic and ecological distances for neutral loci and loci under selective pressure indicate IBC as the main driver for differentiation (Orsini et al. 2013). Within the subclusters we observed weaker genetic structures corresponding to certain geographic areas (populations A/B/D, O/P, G/H/K and L/N). While this could be due to isolation by adaptation (IBA) there is no evidence for it. On the contrary, detailed Mantel tests for each subcluster revealed no correlation of genetic and geographic distances or climatic conditions when neutral markers or the complete marker set (including outliers) were used. However, for subcluster II, a correlation could be established between genetic structure and geographic/climatic conditions when using the outlier-only data set. This partial combination indicates a collective effect of the pure scenarios (isolation by dispersal limitation and isolation by serial colonisation, (Orsini et al. 2013).

Thus, it seems most likely that the observed genetic structures of sloe in Germany are due to colonisation events and the associated founder effects. This is also supported by the fact that the calculated barriers do not always follow landscape ecological borders and in some cases even cross-sect RO. How these proposed colonisation events could come to pass is yet unknown and not subject of this study. However, while it could be that we see the effects of different post-glacial colonisation routes/sources, studies of chloroplast DNA of sloe from Germany and supposed southern refugia could not establish associations between geographic and genetic distances, which would be expected in such case (Leinemann et al. 2014, Puhlmann 2014). As sloe has long been exploited and presumably distributed by humans (Karg & Märkle 2002, Arobba et al. 2003, Martin et al. 2008, Bleicher & Herbig 2010) we suspect that colonisation events for sloe in Germany were random, possibly (even likely) anthropogenic and occurred at different historic periods. On the other hand, we cannot exclude the possibility of a common ancestor population immigrating from the south with subsequently diverging migration routes along both sites of the southern Black Forest or several such founder populations migrating from diverse sources and following different expansion routes. Such re-colonialization routes have been demonstrated for deciduous forest trees. *Fagus sylvatica* L. appears to have recolonised Europe from eastern European refugia, including routes along the slopes of the Alps (Demesure et al. 1996, Magri et al. 2006). But even in this case, we cannot not be sure whether for sloe such immigration was a purely natural process or at least partially anthropogenically influenced.

CONCLUSION

We determined the genetic structure of sloe populations in Germany. Genetic distances between the analysed populations revealed two main clusters – a larger cluster covering northern and eastern Germany (cluster I) and a smaller southwestern/central one (cluster II) with a small area of admixture in the southern locations (fig. 5B). While it is not a general rule, we would like to mention that geographically neighbouring populations tend to cluster together, seemingly independent of the differing existing geo-ecological conditions (e.g. populations A/B/D, G/H/K or L/N). This situation is also visible in the structure of the second Bayesian model with the weaker statistical support (K = 6, fig. 2B). While we did not investigate the reason for the genetic differentiation in depth, our data seem to support isolation by colonisation as a major driver of this differentiation.

Our results show that the six RO designated for woody plants in Germany (for use outside forestry) only partially reflect the genetic structure of sloe populations in Germany. This is mainly due to the fact that the RO to date are solely defined by main geographic units assuming that species will invariably show signs of adaptation to the given conditions along those lines. For sloe (and probably also for other synanthropic woody species predominantly occurring in agricultural landscapes) this concept completely neglects the different species-specific biological characteristics and only partially considers other driving forces for the spatial establishment of populations (e.g. agricultural expansion or incidental seed distribution by man).

Thus, the delineated RO can be seen as a first step to conserve the existing genetic structures of species as intended by the Nature Conservation Act. But, even in this case the use of a seed source from within the one RO could alter the existing genetic structure in another part of the same RO. In fact, the strongest genetic barrier (highest genetic differentiation) for sloe in Germany was found between two geographically close (30 km) populations of the same proposed RO (fig. 5A, RO 4, populations I and K). Using seedstock from any of both populations for use in the vicinity of the other, as possi-

ble under the recent regulations, would breach those barriers and falsify the existing genetic structure of the other.

Therefore, we propose to improve the present preservation strategy relying on six delineated RO solely based on geographic main units by considering species-specific genetic diversity and structures of existing autochthonous populations of the species in question (whether caused by postglacial colonisation, biological characteristics of the species, evolutionary adaption or other driving forces of differentiation).

Thus, existing genetic structures should be investigated for each woody species used in plantings in the open landscape so that RO for each investigated species could then be delineated according to the actual biological situation rather than by theoretically derived parameters, only. Such an approach of detailed population genetic studies would allow for more accurate determination of species specific regions of origin and improve on current regulations which, at present, might lead to inappropriate conservation actions and management measures.

SUPPLEMENTARY DATA

Supplementary data are available in pdf at *Plant Ecology and Evolution*, Supplementary Data Site (http://www.ingentaconnect.com/content/botbel/plecevo/supp-data), and consist of the following: (1) methods and materials: A, sampled populations; B, primers; C, example of an agarose gel after RAPD used for analysis; D, map: occurrence of *Prunus spinosa* in Germany; (2) pairwise F_{ST} values and Tajima's D: A, pairwise F_{ST} values using all loci; B, Tajima's selective neutrality test; C, pairwise F_{ST} values using neutral loci; (3) F_{ST} outlier detection; (4) distance-based analyses: A, NJ analysis using neutral loci; B, PCoA using neutral loci; C, summary AMOVA for all populations; D, AMOVA for selected population pairs from the same or different RO; and (5) Mantel test results.

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