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Bioactive constituents in *Prunus africana*: Geographical variation throughout Africa and associations with environmental and genetic parameters

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ABSTRACT

Prunus africana – an evergreen tree found in Afromontane forests – is used in traditional medicine to cure benign prostate hyperplasia. Different bioactive constituents derived from bark extracts from 20 tree populations sampled throughout the species' natural range in Africa were studied by means of GC–MSD. The average concentration [mg/kg w/w] in increasing order was: lauric acid (18), myristic acid (22), *n*-docosanol (25), ferulic acid (49), β-sitostenone (198), β-sitosterol (490), and ursolic acid (743). The concentrations of many bark constituents were significantly correlated and concentration of *n*-docosanol was highly significantly correlated with all other analytes. Estimates of variance components revealed the highest variation among populations for ursolic acid (66%) and the lowest for β-sitosterol (20%). In general, environmental parameters recorded (temperature, precipitation, altitude) for the samples sites were not correlated with the concentration of most constituents; however, concentration of ferulic acid was significantly correlated with annual precipitation. Because the concentration of compounds in bark extracts may be affected by tree size, the diameter of sampled plants at 1.3 m tree height (as proxy of age) was recorded. The only relationship with tree diameter was a negative correlation with ursolic acid. Under the assumption that genetically less variable populations have less variable concentrations of bark compounds, correlations between variation parameters of the concentration and the respective genetic composition based on chloroplast and nuclear DNA markers were assessed. Only variation of β-sitosterol concentration was significantly correlated with haplotypic diversity. The fixation index (F_{IS}) was positively correlated with the variation in concentration of ferulic acid. Principal Components Analysis (PCA) indicated a weak geographic pattern. Mantel tests, however, revealed associations between the geographic patterns of bioactive constituents and the phylogenetic relationship among the populations sampled. This suggests an independent evolution of bark metabolism within different phylogeographical lineages, and the molecular phylogeographic pattern is partly reflected in the variation in concentration of bark constituents. The results have important implications for the design of strategies for the sustainable use and conservation of this important African tree species.

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1. Introduction

African cherry [*Prunus africana* (Hook.f.) Kalkman] is a long-lived monoecious evergreen tree found in Afromontane forests from Ethiopia in the north to South Africa in the south, as far west as Nigeria and as far east as Madagascar (Hall et al., 2000). Besides its use for timber it is employed as a medicinal plant, whose leaves, roots and bark are used as traditional medicines in Africa. This is not surprising since various bioactive substances with anti-inflammatory, anti-cancer or antiviral effects have been found in different members of the genus *Prunus* (e.g., Donovan et al., 1998; Takeoka and Dao, 2003; Ferretti et al., 2010). African cherry is predominantly exploited for its bark, extracts of which are used to treat benign prostatic hyperplasia, a disorder of the prostate that is common in older men. These extracts significantly improve urologic symptoms (for review see: Ishani et al., 2000; Edgar et al., 2007), having anti-proliferate and apoptoptic effects on the prostate (Quiles et al., 2010). The retail value of this pharmacological use of *P. africana* exceeds several hundred million US $ each year (Hall et al., 2000). The pharmacological efficacy is believed to be due to various compounds known and unknown. Among the known compounds, three groups are of great importance: (1) phytosterols, especially β-sitosterol, have anti-inflammatory properties that inhibit the swelling of the prostate gland (Carbin et al., 1990), (2) pentacyclic triterpenoids that provide anti-edematous activity (Bombardelli and Morazzani, 1997) and (3) ferulic acid esters, or their chemical derivatives, which have a powerful hypocholesterolemic activity in the prostate (Seetharamaiah and Chandrasekhara, 1993) as well as anti-tumor activity (Kampa et al., 2004).

Intraspecific variation of chemical compounds is common in many plant species, and often shows defined geographical patterns, that may reflect environmental differences within the range of a species. Hence, chemical races are often described and provide taxonomists with a powerful tool (Bohm, 2009). One of the most-cited examples of spatio-chemical diversity in secondary metabolites, including its genetic background, is the variation of cyanogenesis in *Trifolium repens* throughout Europe (e.g., Hayden and Parker, 2002 and references therein). However, in plants from which useful natural products are extracted, despite the vast body of literature, spatio-chemical differences among populations have been only occasionally described and studies linking a spatial pattern in chemical concentrations with genetic differences among natural populations are even more limited (but see Prida et al., 2007). Correlations between the metabolic contents and DNA fingerprints among geographically distinct plant populations have been reported for phenolic acids in *Fructus xanthii* (Han et al., 2008) and for volatile oil constituents in *Ocimum gratissimum* (Vieira et al., 2001) and in *Tanacetum vulgare* (Keskitalo et al., 2001). However, Trindade et al. (2008) reported that in *Thymus caespititius*, the variation of volatile oils was not associated with the genetic substructure of the populations, but their results were based on a very small sample of only 31 individuals.

Regardless of whether the concentrations of certain bark constituents in African cherry are linked to genetic parameters, knowledge about their regional variation is essential in order to optimize the sustainable conservation and use of this vulnerable tree species (Heywood, 2002). A previous pilot study looking at three countries of origin, Cameroon, Zaire and Madagascar, has already shown that the composition of *P. africana* extracts depends on the origin of the bark (Martinelli et al., 1986). From a genetic point of view, recent studies employing both chloroplast and nuclear DNA markers in *P. africana* throughout Africa have led to the identification of five distinct regions (Kadu et al., 2011; unpublished data), which reflect divergent population histories across the continent, associated with region-specific climatic conditions during the Pleistocene. In particular, genetic divergence between populations on the African mainland and on Madagascar was high, as was the genetic divergence between populations west and east of the Rift Valley. This pronounced spatio-genetic pattern spurred the hypothesis that *P. africana* would also exhibit intraspecific diversity, i.e. variation within and among populations, in the concentrations of chemical constituents in the bark.

Thus bark and DNA samples were collected in 20 populations of African cherry throughout its natural range in order to:

(i) quantify any intraspecific chemical diversity in eight bioactive compounds extracted from bark,
(ii) estimate within- and among-population variation of concentration of these constituents,
(iii) assess whether concentration of bark constituents is correlated with tree size and environmental parameters and whether variation in the bark constituent is correlated with genetic diversity measures; and eventually
(iv) identify associations between the spatio-chemical pattern of *P. africana* and its molecular phylogeography.

2. Results and discussion

2.1. Concentration of bark constituents and associations with environmental factors

The average concentration [mg/kg w/w] based on 20 bulked population samples (population mix; see Section 4.2 below) in increasing order was: lauric acid (18), myristic acid (22), *n*-docosanol (25), ferulic acid (49), β-sitostenone (198), β-sitosterol (490), and ursolic acid (743). Friedelin was not detected with a concentration above the limit of detection (7 mg/kg) in any of the samples. This finding was quite surprising because this triterpenoid, which is known for its anti-inflammatory effects (Antonisamy et al., 2011), had previously been identified in *P. africana* bark extracts (Catalano et al., 1984) as well as in *Prunus lusitanica* (Biessels et al., 1974) and *Prunus turfosa* (Sainsbury, 1970). However, friedelin was not detected in *Prunus serotina* (Biessels et al., 1974). The concentrations of the other seven bark constituents varied considerably among and within populations. However, for lauric and myristic acid the content in individual samples within certain populations varied erratically. Both of these saturated fatty acids were previously reported in *P. africana*, but each with very low concentrations compared to other free fatty acids (Ganzera et al., 1999). In *Prunus amygdalus* (Munshi and Sukhija, 1984) and in *Artocarpus heterophyllus* (Chowdhury et al., 1997) low concentrations of lauric and myristic acid were also reported. However, Munshi and Sukhija (1984) found that the concentrations of both constituents decreased rapidly with time and eventually only traces were found in developing kernels of almonds (*P. amygdalus*). An open question is whether genetic, environmental or even methodological factors are causing the observed pattern, but in any case due to the high variation of individual concentrations in lauric and myristic acid they were not considered for further analysis.

The variation in concentration among different populations of the other key compounds studied is shown in Fig. 1. *n*-Docosanol was found to be highest in Ngashie Mt. Oku (CA-Cameroon) and lowest in Kidabaga (TE-Tanzania) with concentrations of 10–28 mg/kg. Data on natural variation of this long-chain alcohol are virtually non-existent. In crops, highly significant genotypic differences were observed in association with variable concentration of *n*-docosanol (Irmak et al., 2008) and concentration is also significantly affected by the environment (Dunford and Edwards, 2010). In the present study the concentrations varied strongly and significantly among populations; however, the variation de-

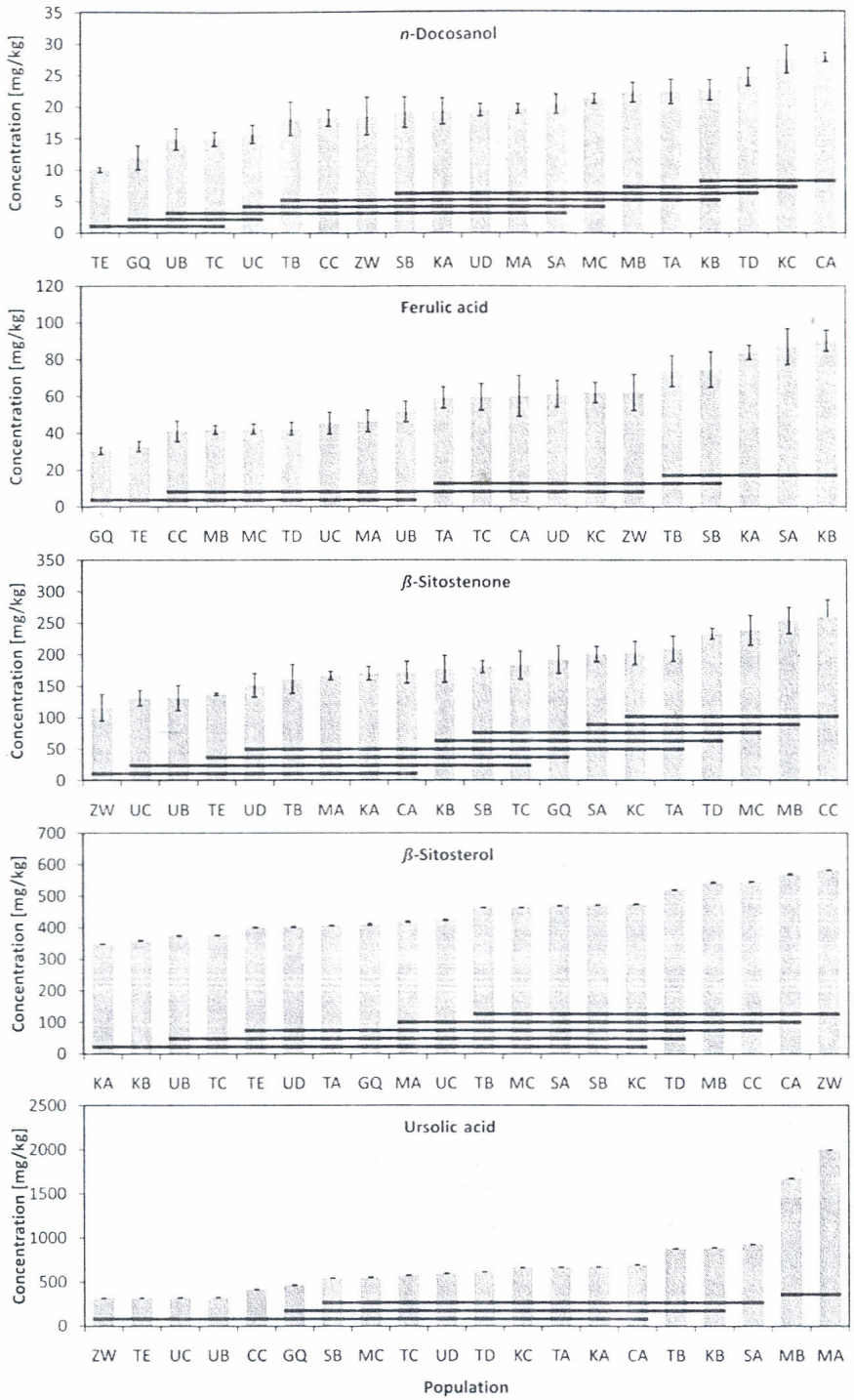


Fig. 1. Mean concentration (mg/kg) in bioactive bark constituents of *Prunus africana* individuals originating from 20 populations within the natural range of the species ranked in ascending order. Populations that are connected with a horizontal line are statistically not different. Respective vertical lines indicate standard errors. For population acronyms see Table 2.

tected did not reflect the patterns of variation in the environmental parameters studied.

Ferulic acid was found to be highest in Kinale (KB-Kenya) and lowest in Moka (GQ-Equatorial Guinea) with concentrations of 30–90 mg/kg. This hydroxycinnamic acid is found in many plants and had already been detected in *P. africana* bark (Fourneau et al., 1996). It is also present in leaves of different *Prunus* species (Liakopoulos et al., 2001). It may effectively scavenge deleterious radicals and suppress radiation-induced oxidative reactions (Kikuzaki et al., 2002). Given that ferulic acid is found in many plants as a component of lignocelluloses, it is unexpected that its variation in natural plant populations has not yet been studied. However, in cultivars of wheat (*Triticum aestivum*) significant differences in concentration have been reported (Mpofu et al., 2006; Irmak et al., 2008). In our samples concentration of ferulic acid was significantly correlated with tree diameter ($r = 0.23$, $p \leq 0.05$) and with annual precipitation ($r = -0.50$, $p \leq 0.025$). Also an earlier study in wheat (Mpofu et al., 2006) has shown, that ferulic acid concentration in different wheat cultivars varied at sampling sites differing in environmental conditions.

Concentration of β-sitostenone was found to be the highest at Mt. Danoua (CC-Cameroon) and was the lowest in Chimanimani (ZW-Zimbabwe) with concentrations of 116–260 mg/kg. These concentrations are much lower than those of β-sitosterol (see below). Catalano et al. (1984) also found several-fold lower concentration of β-sitostenone compared to β-sitosterol in African cherry bark extracts. β-Sitosterol was found in concentrations of 349 to 583 mg/kg with the highest value in Chimanimani (ZW-Zimbabwe) and lowest value in Chuka (KA-Kenya). This compound, which is mostly known for its cholesterol-lowering activity (Satou, 2003) is believed also to have anti-cancer effects (Award and Fink, 2000). It is found in the bark of African cherry in higher concentrations than in many fruits and the bark values are even similar to those found in avocado, a very rich source of β-sitosterol (Duester, 2001). *Prunus spinosa* (Wolbiš et al., 2001) and *Moringa oleifera* (Anwar et al., 2007) both have high concentrations of β-sitosterol are used in traditional medicine for their diuretic properties in traditional medicine to increase urine flow. In *Glycine max* (Yamaya et al., 2007) and *Brassica napus* (Amar et al., 2009) genotypic variation in β-sitosterol concentration was high, but to the best of the authors' knowledge, data on genetic variation in natural plant populations are not available. The concentration of β-sitosterol was independent of environmental conditions in the present study. This is in contrast to findings in soybean (*G. max*); when plants were grown in warmer areas they produced seeds with higher β-sitosterol content than when the same varieties were grown at colder sites (Yamaya et al., 2007).

Ursolic acid, was present in the highest concentration of all the compounds studied, varying from 317 to 2000 mg/kg. Its concentration was highest in Marovoay (MA-Madagascar) and lowest in Chimanimani (ZW-Zimbabwe). Ursolic acid is a natural pentacyclic triterpenoid carboxylic acid that has been identified as a major component in many plants used in traditional medicine (for a recent review see Ikeda et al., 2008) and that serves as a basic material for the synthesis of more stronger bioactive derivatives, such as anti-tumor agents (Ma et al., 2005). For instance, it has been detected in the peel of loquat (*Eriobotrya japonica*) fruits (Zhou et al., 2011) at concentrations comparable to those found in the present study.

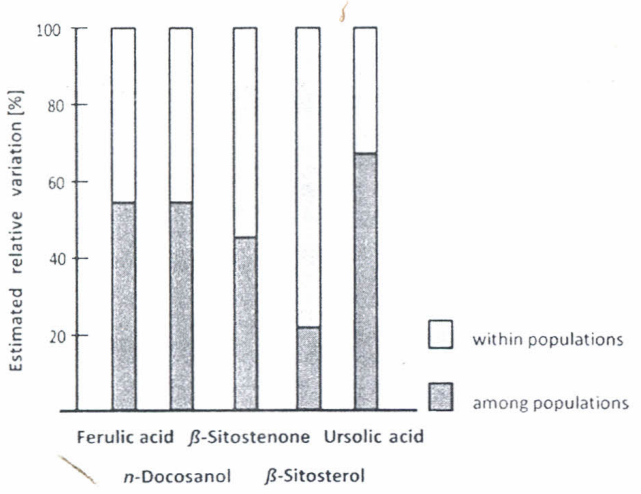


Fig. 2. Estimated relative variation due to the among and within population component of different bioactive bark constituents in *Prunus africana*.

Ursolic acid concentration was negatively correlated with tree diameter ($r = -0.33$, $p \leqslant 0.01$). This finding is to be expected because tree diameter is a good proxy for age and many triterpenoids, including ursolic acid, act as plant defenses (Arnason and Bernards, 2010) whose concentrations are likely to decrease with age, as a result of reduced pressure from herbivores. In *Betula pendula*, the concentration of triterpenoids declined roughly one hundred-fold from 1-year to 20-year old trees, demonstrating the anti-browsing effect of triterpenoids (Laitinen, 2003).

In order to facilitate efficient provenance selection or breeding, it is essential to know how much of the variation in the concentration of constituents is found among populations and how much within populations. For three constituents, i.e. ferulic acid, *n*-docosanol and β-sitostenone, the variation was roughly equal within and between populations (Fig. 2). The lowest among-population variation was for β-sitosterol, approximately 20%, and the highest for ursolic acid, approximately 67%. The high among-population variation and the lack of environmental effects on the concentration of ursolic acid bespeak population genetic effects. The findings that the content of ursolic acid was found to vary considerably among cultivars of *E. japonica* (Zhou et al., 2011) are in line with the results of the present study.

Concentration of many of the measured bark constituents were significantly correlated with one another (Table 1). For certain constituent pairs, a close correlation was to be expected. For example, β-sitostenone is an oxidized product of β-sitosterol and both saturated fatty acids share a very similar chemical structure. More importantly, the concentration of *n*-docosanol was significantly correlated with all the other constituents and can thus be used as a good predictor of the concentration of the other compounds. Note, however, that *n*-docosanol concentration was negatively correlated with lauric acid and myristic acid concentration.

The relative mean concentration of the five compounds studied more in detail was estimated in order to enable comparisons of the compound mix between populations (Fig. 3). The concentration of each of these compounds was expressed as a percentage of the maximum concentration for that compound found over all the sampled populations. Ursolic acid was found in very high concentrations in two (MA and MB) of the three Madagascan populations, while in most other populations the concentration amounted to only approximately 30% of the maximum found in Marovoay (MA-Madagascar). This population is exceptional as the only one where concentrations of all constituents exceeded 50% of the respective maxima. By contrast, relatively low concentrations of all constituents were found in the Tanzanian sample Kidabaga (TE).

2.2. Associations of bark constituents with genetic patterns

The molecular markers used in this study do not encode any of the bark constituents studied. However, both haplotypic and nuclear genetic diversity are good indicators of genetic drift, which

Table 1
Correlations between the concentrations of different bark constituents of *Prunus africana*. Asterisks indicate significance level (***$p \leqslant 0.001$, **$p \leqslant 0.01$, *$p \leqslant 0.05$).

	n-Docosanol	Lauric acid	Myristic acid	β-Sitostenone	β-Sitosterol	Urolic acid
Ferulic acid	0.266**	0.118	0.175	−0.153	−0.041	0.114
n-Docosanol		−0.420***	−0.315***	0.508***	0.557***	0.339***
Lauric acid			0.365***	−0.294**	−0.177	−0.038
Myristic acid				−0.281***	−0.198*	−0.053
β-Sitostenone					0.391***	0.263**
β-Sitosterol						0.153

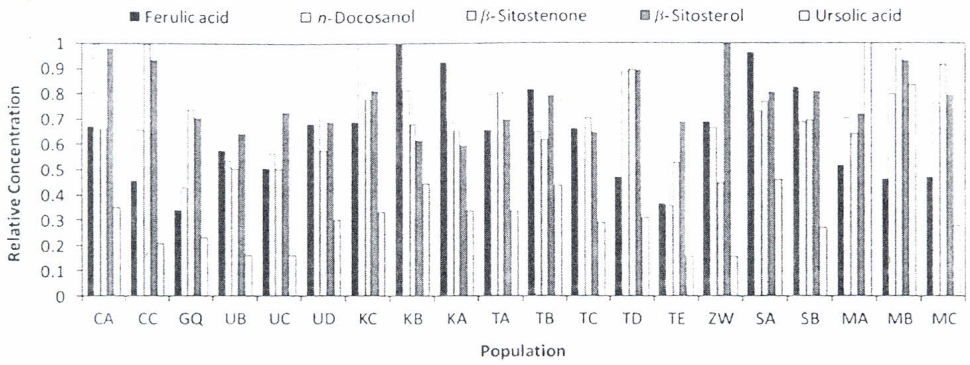
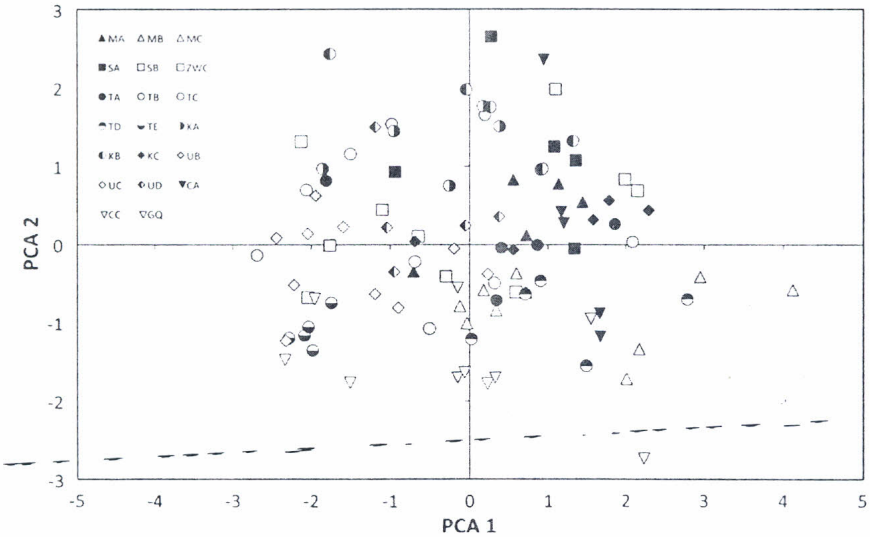


Fig. 3. Relative mean concentration of the various chemical constituents expressed as a percentage of the maximum concentration found over all sampled populations. For population acronyms see Table 2.

affects the whole genome equally and which depends only on gene frequencies and population sizes. Genes associated with direct or indirect syntheses of the bark constituents and the molecular markers used to characterize the populations will be equally affected by genetic drift. Thus, one may assume that the variation in concentration of these compounds is correlated with genetic diversity (in the chloroplast and nuclear genome) and/or with deviation from Hardy–Weinberg expectations (HWE) of the population structure, quantified as fixation index (F_{IS}). Variation of β-sitosterol concentration of the populations was significantly correlated to the effective number of haplotypes ($r = 0.63$, $p \leqslant 0.05$) and haplotypic diversity ($r = 0.65$, $p \leqslant 0.05$) (see also Supplementary data, Table S1 [available online]). Populations with high haplotypic diversity might be the result of the merging of different phylogeographical lineages with genetic variation at those genes that encode β-sitosterol synthesis. If this is the case, the populations after merging would not necessarily also have a higher genetic diversity at the nuclear SSRs studied, if these populations did not differ in SSR population structure. This could explain why nuclear SSR diversity was not significantly correlated with the concentration of β-sitosterol. Variation in ferulic acid concentration was positively correlated with F_{IS} ($r = 0.48$, $p \leqslant 0.05$). This indicates a higher variation in ferulic acid concentration in populations with an increasing excess of homozygotes compared to HWE. This could be the case if several inbred lines, which differ in ferulic acid concentration merged in the populations or the findings were due to a Wahlund-effect. Regardless of the true biological reasons, the association between measures of genetic diversity and the variation in bark concentrations must be interpreted as weak.

Previous nuclear and chloroplast DNA analyses have shown a strong population divergence among the Afromontane regions and led to the identification of five main geographical groups: (1) West Africa, (2) East Africa west of the Eastern Rift Valley, (3) East Africa east of the Eastern Rift Valley, (4) Southern Africa and (5) Madagascar (Kadu et al., 2011, unpublished data). In particular, populations from mainland Africa and Madagascar as well as from east and west of the Rift Valley system exhibited a distinct population history. In contrast, the spatio-chemical pattern of the present study was relatively weak, as shown by the first two PCA components; nevertheless, certain phylogeographic regions and populations therein were identified (Fig. 4). Even though the clustering of populations based on differences of concentrations in the bark constituents was very weak (Fig. 5), some significant correlations were found between matrices of chemical distances (based on the concentration of certain bark compounds) and genetic distances (see Supplementary data, Table S2 [available online]). Significant correlations were detected for both chloroplast ($r = 0.24$, $p \leqslant 0.05$) and nuclear data ($r = 0.24$, $p \leqslant 0.05$) and concentrations of ursolic acid. Moreover, matrices of chloroplast and nuclear genetic distances also resulted in significant correlation when all bark constituents were considered for a single Mantel test. The Mantel correlation between the overall chemical distances and



. 4. Grouping of 20 populations based on the first two PCA components. For population acronyms see Table 2. Individual populations are represented through specific nbols and their respective shapes indicate the associations with the phylogeographic groups (see Fig. 6).

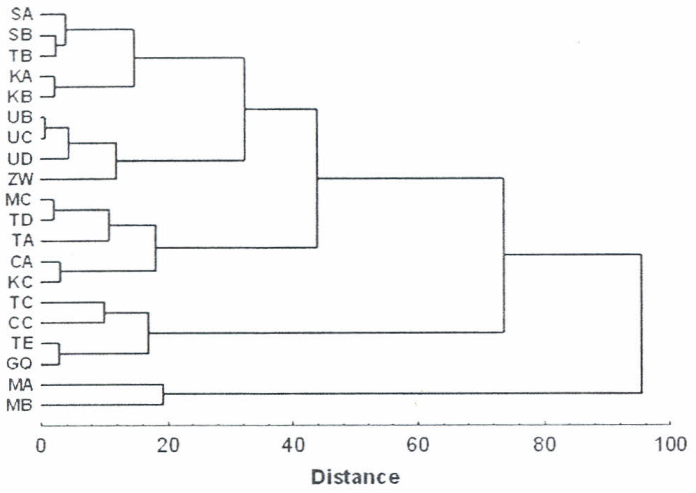


Fig. 5. Clustering of populations based on chemical distance. For population acronyms see Table 2.

the chloroplast data distances was $r = 0.23$ ($p \leqslant 0.05$) while for nuclear genetic data distances it was $r = 0.25$ ($p \leqslant 0.01$). An association between chemical differentiation and genetic differentiation is to be expected, especially in cases of plant species characterized by obligatory selfing, by limited natural seed dispersal, and by an extensive distribution range where various selective forces are at play. *Prunus africana* does not completely fit this model, being characterized by pronounced gene flow and a mixed-mating system with selfing limited by self-incompatible genes (unpublished data). However, its range is wide and geographic selection is very likely to be operating. Thus, certain associations between chemical and genetic diversity are to be expected. In general, studies that compare genetic and chemical differences, based on an intensive sampling within and among plant populations, are very rare (but see Eisenman, 2010 for the medicinal plant *Artemesia dracunculus*). Most commonly, only few accessions (Vieira et al., 2001) or genotypes (Keskitalo et al., 2001) have been studied, or information on numbers of individuals sampled per population is not reported (Nan et al., 2003). Despite these limitations, the concentration of volatile oil constituents can differentiate very well among accessions or genotypes (e.g., Luro et al., 2012).

3. Conclusions

A pronounced variation in the concentration of selected bark constituents was found among populations. This suggests that spatial (= population) genetic effects are very likely to be present, especially for ursolic acid. The concentration of *n*-docosanol was correlated with the concentration of the other constituents and thus can be used as a good predictor of the concentration of the other constituents studied. With exception of ferulic acid, environmental parameters did not significantly correlate with constituent concentrations. Concentration of ursolic acid was negatively correlated with tree size. Informal reports from pharmaceutical companies and bark traders indicate that trees older than 15 years produce a type of bark that is more highly appreciated, which suggests that ursolic acid may not be therapeutically so important. However, it is also possible that traders may simply prefer a thicker bark for handling reasons. Further investigations are needed to cast more light on the environmental factors affecting bark quality. To fully understand opportunities for genetic gains through selection of populations and individuals, genetic field tests are necessary. The concentration of bark constituents originating from different African cherry populations did not present a very distinct geographical pattern. Nevertheless, it is concluded that the molecular phylogeographic pattern is reflected in the spatial patterns of certain bark constituents, notably in ursolic acid. This suggests an independent evolution of bark metabolism within different phylogeographic lineages. The data indicate that populations with very high concentrations of the studied constituents are located in Madagascar, a region that is also genetically distinct from the African mainland. The colonization of this island was probably the result of a single dispersal event that dates back to the oldest splitting events within the species (Kadu et al., 2011). The present study also shows that data on spatio-chemical diversity are expedient for a conservation strategy for African cherry. If a conservation strategy focuses exclusively on molecular diversity, populations in Kenya, Uganda and Tanzania will be prime regions since Madagascan populations are not quite as diverse as the mainland populations from East Africa. However, populations from Madagascar feature a relatively high concentration of all bark constituents (cf. Fig. 3) and, although it is unknown which role bark constituents may play for adaptation, it is highly recommended to include Madagascan populations into a pan-African conservation strategy. These island populations appear also to be highly vulnerable due to bark overexploitation.

4. Experimental

4.1. Plant material and sites

For each tree sampled, equal-sized bark disks were collected at 1.3 m above ground in 4 orientations (N, E, S, W). Diameters at 1.3 m height of sampled trees were recorded. Samples were air-dried, stored in calico bags and shipped to the Austrian lab. In total, 20 populations (20 trees each) were sampled (Table 2, Fig. 6). Altitude of the sampling sites was recorded by hand-held GPS. Climatic data were extracted from WorldClim (http://www.worldclim.org/). Genetic data were also available for all individually sampled trees (see below). More details on the sampling sites can be found in Kadu et al. (2011).

4.2. Bark processing

All four samples per tree were crushed through a coarse mill and further processed by fine-milling using a 1 mm grid. Five tree samples per population were randomly selected and an aliquot of 50 g of fine-milled bark was removed. A sixth sample from each population was an aliquot mix of bark powder (5 g each) originating from all 20 trees. This sample was called "population mix".

4.3. Chemical analysis of the bark samples

In total, eight antioxidants probably having the most significant pharmaceutical properties, i.e. ferulic acid, friedelin, *n*-docosanol, lauric acid, myristic acid, β-sitostenone, β-sitosterol and ursolic acid were analysed. One g of the bark powder was extracted with 20 ml of chloroform/methanol (80/20 v/v) in a 100 ml Erlenmeyer flask using an orbital shaker (180 min^{-1} for 70 min). The extracts were filtered through S&S 595½ folded filters (110 mm diameter, Schleicher & Schüll, Dassel, Germany) into 24 ml glass vials and stored at 4 °C.

A 1 ml aliquot of the raw extract was transferred into a 2 ml glass vial, put on a heating plate (45 °C) and evaporated to dryness under a gentle stream of nitrogen. Residues were first dissolved in 50 µl dimethylformamide (DMF) and shaken for 10 s. Subsequently, 50 µl of the derivatisation reagent N-methyl-N-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) was added, samples were shaken again for 10 s and were left in an oven at 60 °C for 60 min.

Table 2
Description and location of 20 populations of *Prunus africana* sampled.

Name of population	Code	Country	Longitude (°)	Latitude (°)	Elevation asl (m)	Annual mean temperature (°C) Worldclim	Annual mean precipitation (mm) Worldclim	Diameter at breast height (cm)
1. Ngashie Mt. Oku	CA	Cameroon	10.5092	6.2048	2729	13.2	2088	58.4
2. Mt. Danoua	CC	Cameroon	11.9413	7.1871	1731	19.1	1687	35.2
3. Moka	GQ	Equatorial Guinea	8.6559	3.3625	1510	20.6	2364	Not available
4. Chuka	KA	Kenya	37.3645	−0.1798	1175	20.8	1021	69.5
5. Kinale	KB	Kenya	36.4151	−0.5278	2165	15.5	1177	50.1
6. Kapcherop	KC	Kenya	35.2161	1.0315	2721	13.5	1111	91.3
7. Marovoay	MA	Madagascar	48.3069	−18.8023	1062	18.8	1487	18.4
8. Lakato Forest	MB	Madagascar	48.2779	−19.1971	937	19.3	1705	14.4
9. Antsahabiraoka	MC	Madagascar	49.2164	−14.4015	1272	19.0	1430	28.6
10. Mpumalanga	SA	South Africa	30.7306	−25.0225	1474	16.3	926	72.8
11. KwaZulu Natal	SB	South Africa	30.2725	−29.2872	1566	13.3	889	Not available
12. Meru	TA	Tanzania	36.8074	−3.2925	1884	16.5	971	75.8
13. Kilimanjaro	TB	Tanzania	37.5237	−3.0036	1976	16.3	999	77.7
14. Kindoroko	TC	Tanzania	37.6267	−3.7389	1875	17.6	1005	66.8
15.Shume/Magamba	TD	Tanzania	38.2521	−4.7544	1738	15.4	985	84.7
16. Kidabaga	TE	Tanzania	35.9296	−8.1101	1989	17.0	1052	64.2
17. Kalinzu	UB	Uganda	30.1101	−0.3742	1100	22.1	1015	59.9
18. Bwindi	UC	Uganda	29.7754	−1.0476	2264	15.3	1268	54.0
19. Mabira	UD	Uganda	33.015	0.3806	1266	21.2	1319	53.6
20. Chimanimani	ZW	Zimbabwe	32.8004	−19.5781	1343	17.0	1159	63.2

Fig. 6. *Prunus africana* bark sampled throughout the natural range. Different symbols of the sampling sites indicate phylogeographic groups [triangle (peak down) = West Africa, rhombus = East Africa west of the Eastern Rift Valley, circle = East Africa east of the Eastern Rift Valley, triangle (peak upside) = Madagascar, quadrat = Southern Africa). For population acronyms see Table 2.

After derivatisation 0.45 ml isooctane and 1 ml KH_2PO_4-Buffer (pH = 7.00; 50 ml 0.1 M KH_2PO_4 mixed with 29 ml 0.1 M NaOH) were added and vials were vortexed for at least 30 s until the upper organic layer was clear. Approximately 300 µl of the organic layer containing the analytes or their derivates were transferred into a HPLC-microvial which was closed tightly and analysed by a GC–MS-system (GC 6890N and MSD 5975b inert XI/CI, Agilent, Santa Clara, USA).

Injection volume was 1 µl using the splitless injection mode. Chromatographic separation was carried out by means of an Agilent HP-5-MS column (30 m × 0.25 mm × 0.25 µm), using Helium as carrier gas (1 ml/min) and a temperature gradient (starting with 80 °C for 0.5 min, heating up to 340 °C with 25 °C/min and holding for 17 min). Analytes were detected using SCAN- and SIM-mode simultaneously. For each compound one target ion was chosen as quantifier ion namely m/z 257, 285, 365, 383, 412, 426, 471 and 627 used for lauric acid, myristic acid, ferulic acid, *n*-docosanol, *β*-sitostenone, friedelin, *β*-sitosterol and ursolic acid, respectively. The retention times and the area of the extracted ion chromatograms (EIC) of the target ions (SIM-mode) were used for quantification of the analytes. Data of the SCAN-mode were additionally used for verification by means of the spectra. For calibration of the GC–MS instrument, analyte mixtures containing isooctane between 2.5 µg/ml and 75 µg/ml per analyte were measured. Quantification of the target compounds in the bark extracts was carried out by evaluating EIC-peak areas.

Chloroform, methanol (Merck, Darmstadt, Germany) and isooctane (J.T. Baker, Deventer, Netherlands) were of p.A. quality. Myristic acid, lauric acid, ursolic acid and *n*-docosanol were purchased from Acros Organics (Geel, Belgium), *β*-sitosterol, *β*-sitostenone, friedelin from Chromadex (Santa Ana, USA) and ferulic acid from ABCR (Karlsruhe, Germany), respectively. From each standard a 50 µg ml^{-1} stock solution was prepared in chloroform and stored at 4 °C. For the derivatisation dimethylformamid (DMF) absolute (Fluka Chemie, Seelze, Germany) and N-methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) containing 1% *tert*-butyldimethylchlorosilane (TBDMCS) (Pierce, Rockford, USA) were used. The washing buffer was prepared with KH_2PO_4 and sodium hydroxide, both p.A. grade from Merck (Darmstadt, Germany).

4.4. Genetic data

Genetic data were based on seven chloroplast (*cp*) microsatellites and six nuclear (*n*) microsatellites as described in detail by Kadu et al. (2011; unpublished data). Genetic parameters, effective number of alleles in the chloroplast (N_{e_c}) and nuclear genome (N_{e_n}), unbiased expected haplotypic diversity (h_c), unbiased expected heterozygosity (H_e) as well as fixation index (F_{IS}) were calculated using GenAlEx v. 6 (Peakall and Smouse, 2006). For correlations of chemical distances to genetic distances, pairwise differentiation matrices were calculated for both chloroplast and nuclear microsatellites. For the chloroplast data, Φ_{PT}, the population differentiation for haploid loci was used, whereas for nuclear microsatellites R_{st}, the population differentiation for codominant markers assuming stepwise mutations was applied. Both, Φ_{PT} and R_{ST} estimate the proportion of pairwise differentiation among populations, relative to the total differentiation using the analysis of molecular variance approach developed by Excoffier et al. (1992) and extended by Peakall et al. (1995) and Michalakis and Excoffier (1996) to codominant data.

4.5. Statistical analysis

The measurements of ferulic acid, friedelin, *n*-docosanol, lauric acid, myristic acid, *β*-sitostenone, *β*-sitosterol and ursolic acid were scored for 20 populations along with those for the population mixes and the control samples. The method detection limits (LOD) and method quantification limits (LOQ), respectively, were calculated based on 20 total procedure blanks with LOD three times the standard deviation and LOQ 10 times the standard deviation of mean blank concentrations (see Supplementary data, Table S3). The means for the quantified chemical compounds of the five single trees per populations were calculated to establish an overview of relationships, and then coefficients of variation within populations were calculated to have a normalized comparison of variation. Intercorrelations of contents of different constituents and correlations with environmental conditions and tree size were calculated. If needed, significance was Bonferoni corrected. ANOVA and a subsequent Duncan-test were used to test for differences among populations.The proportion of variance within and among populations was estimated by variance component analysis using a restricted maximum likelihood technique.

In order to evaluate whether populations that show high variation also show higher genetic diversity, correlations of various measures of genetic diversity for chloroplast and nuclear markers with the coefficient of variation of constituents within populations were calculated.

To test for a geographical structure in the bark chemical data, a principal component analysis (PCA) was carried out. In addition, to test similarity of populations, matrices of pairwise chemical distances were calculated for each single compound (Euclidean distance) and for the complete spectra of substances, using Ward's (1963) technique for joining substances. In addition, a discriminant analysis was carried out to reveal a matrix of Mahabalonis-distances between populations as a basis for subsequent cluster analysis. The various chemical distance matrices (for single compounds and all compounds) were used to test for associations between geographical and genetic distances (Φ_{PT} and R_{ST}, respectively) by Mantel tests. Significance was tested at 999 permutations using the Mantel procedure of GenAlEx, v.6 (Peakall and Smouse, 2006). Other statistical calculations were done by means of STATISTICA (HTTP://WWW.STATSOFT.DE/).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2012.06.001.

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