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Diversity of pyrrolizidine alkaloids in native and invasive *Senecio pterophorus* (Asteraceae): implications for toxicity

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ABSTRACT

Changes in plant chemical defenses after invasion could have consequences on the invaded ecosystems by modifying the interactions between plants and herbivores and facilitating invasion success. However, no comprehensive biogeographical studies have yet determined the phenotypic levels of plant chemical defenses, as consumed by local herbivores, covering large distributional areas of a species. *Senecio pterophorus* is a perennial shrub native to Eastern South Africa, expanded into Western South Africa and introduced into Australia and Europe. As other Asteraceae, *S. pterophorus* contains pyrrolizidine alkaloids (PAs) toxic to vertebrate and invertebrate herbivores. Here we analyzed *S. pterophorus* PAs by LC-MS/MS on foliage sampled across its entire distributional range, including the native and all non-native areas. PA concentrations and diversity was very high: we found 57 compounds belonging to 6 distinct necine base-types, including the highly toxic 1,2-unsaturated PAs (retronecine and otonecines) and the less toxic 1,2-saturated PAs (platynecine and rosmarinecines). Plants from different origins diverged in their PA absolute and relative concentrations. Rosmarinine was the most abundant compound in Australia and South Africa, but it was nearly absent in Europe. We characterized three plant chemotypes: retrorsine-senkirkine chemotype in Eastern South Africa, rosmarinine chemotype in Australia and Western South Africa, and acetylseneciphylline chemotype in Europe. PA absolute concentrations were highest in Australia. The increased absolute and relative concentrations of retronecine PAs from Australia and Europe, respectively, indicate that *S. pterophorus* is potentially more toxic in the invasive range than in the native range.

Keywords: Senecio pterophorus; Asteraceae; geographical diversity; pyrrolizidine alkaloids; chemotypes; invasive species, toxicity, LC-MS/MS

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1. Introduction

Pyrrolizidine alkaloids (PAs) are plant secondary metabolites characteristic of several genera within the *Asteraceae* and *Boraginaceae,* and sporadically present in the *Fabaceae* and *Orchidaceae* (Hartmann, 1999). PAs are formed by the esterification of a necic acid and a necine base, and comprise more than 400 chemical structures identified from ca. 6000 species (Langel et al., 2011; Witte et al., 1993). In *Senecio* (*Asteraceae*), one of the largest genera of flowering plants distributed worldwide, PAs are mostly in a macrocyclic diester form (Hartmann and Ober, 2000; Witte et al., 1993).

PAs are acutely toxic, genotoxic and teratogenic to vertebrates and invertebrates (Chen et al., 2010; Cheng et al., 2011a; Frei et al., 1992; Moyano et al., 2006; Stegelmeier et al., 1999). Chronic ingestion of PA-containing plants and contaminated hay, straw or silage, causes livestock poisoning (Cheeke, 1988; Wiedenfeld and Edgar, 2011). In humans, exposure to PAs contained in herbal teas, medicines, contaminated cereals, pollen and honey has been related to acute and chronic liver toxicity, such as the veno-occlusive disease, especially in children (Edgar et al., 2002; Kempf et al., 2010; Molyneux et al., 2011; Wiedenfeld and Edgar, 2011). The role of PAs as plant chemical defenses against phytophagous insects has been widely documented (Hartmann, 1999). Indeed, PAs may have evolved in response to the consumption pressure from invertebrate herbivores (Doorduin and Vrieling, 2011; Hartmann, 1999).

PAs are largely stored in the plant foliage in the non toxic N-oxide form (Joosten et al., 2009; but see Joosten et al., 2011). However, after ingested by herbivores they are reduced into tertiary amines in the gut and metabolized by cytochrome P_{450}s, originating reactive metabolites that interact with proteins and nucleic acids (Hartmann, 1999; Fu et al., 2004). The toxicity of PAs strongly depends on some molecular features (Hartmann,

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1999; Moyano et al., 2006; Stegelmeier et al., 1999). For example, the presence of a double bond at C1-C2 in the necine base, as occurring in retronecine and otonecine bases (often referred to as 1,2-unsaturated PAs or dehydroPAs), is necessary for their bioactivation and it has been associated to a greater toxicity in comparison with saturated necine bases (Hartmann, 1991; Li et al., 2013; Wiedenfeld and Edgar, 2011). Accordingly, the toxic potential of PA-containing plants will depend on the PA absolute concentrations but also on the PA profile, that is the relative abundance of individual PAs.

One important aspect of plant phytochemistry scarcely explored is the changes in chemical defenses and plant toxicity as a result of biological invasions. Intraspecific divergence in plant chemistry could have ecological and evolutionary consequences on the invaded ecosystems by modifying the interactions between plants and herbivores and facilitating invasion success (Keane and Crawley, 2002). Genetically determined concentrations of plant toxins, including PAs, were higher in native populations compared with introduced populations of several plant species (Doorduin and Vrieling, 2011). However, whether plant toxicity is increased in the invaded areas is yet unclear. To date no comprehensive biogeograhical studies have determined phenotypic concentrations and profiles of chemical defenses, i.e. as consumed by local herbivores, covering the entire distributional area of a plant species in its native and invasive range.

Here we aimed to determine whether PAs changed after invasion in *Senecio pterophorus* DC (Asteraceae), a perennial shrub native to South East South Africa which was expanded into Western South Africa (~100 years ago), and introduced into Australia (> 70-100 years ago) and continental Europe (> 30 years ago) (Barberis et al., 1998; Casasayas, 1989; Castells et al., 2013; Hilliard, 1977; Levyns, 1950; The Council of Heads of Australasian Herbaria, 2014). *S. pterophorus* in Australia is classified as a

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Declared Noxious Weed subject to eradication by the Department of Primary Industries (State of Victoria, Australia) because it causes heavy productivity losses in agricultural areas and outcompetes the native vegetation in natural communities (Parsons and Cuthbertson, 1992). Regardless the negative effects that *S. pterophorus* may cause on the invaded ecosystems and the potential toxicity for vertebrates and invertebrates, its chemical composition has been poorly explored. Early work on *S. pterophorus* in (Eastern) South Africa by de Waal (1941) resulted in the isolation of retrorsine, senecionine and seneciphylline. Liddell and Logie (1993) investigated the PA content of *S. pterophorus* specimen from Grahamstown (Eastern South Africa). Six compounds were detected, the major one being seneciphylline (64.1%), followed by rosmarinine (18.1%) and acetylseneciphylline (10.8%). Trace amounts (3% or less) of spartioidine, senecionine and *iso*-rosmarinine were also found and a total PA content of 0.192 mg/g dry plant material was calculated. The presence of pterophorine, a PA with a dihydropyrrolizinone necine structure, and reported as a constituent of South African *S. pterophorus* by Bohlmann et al. (1977), could not be confirmed by Liddell and Logie (1993). The only published study on the PA content of *S. pterophorus* outside South Africa is that of Edgar et al. (1976). It was reported that Australian *S. pterophorus* contained seneciphylline, senecionine, rosmarinine and acetylseneciphylline, but no further information on concentration or relative composition was given. Although to date several studies on European *S. pterophorus* invasive populations have been conducted, not more is known that the total PA content of the investigated populations varied from 0.6 to 1.5 mg/g dry weight (Caño et al., 2009).

We compared the PA concentration and profile of 54 individual plants of *S. pterophorus* sampled across the species distributional area including the native range (Eastern South Africa), the expanded range (Western South Africa), and two introduced

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ranges (Australia and Europe). The relatively restricted worldwide distribution of *S. pterophorus* enabled us to conduct a broad biogeographical survey that covered the entire species distributional area (Castells et al., 2013). We aimed 1) to characterize the PA composition of *S. pterophorus*, and 2) to determine the geographical patterns of PAs across the plant distributional area including the native range (Eastern South Africa), the expanded range (Western South Africa), and two introduced ranges (Australia and Europe). We finally discuss whether toxicity of *S. pterophorus* has changed after plant invasion.

2. Results and discussion

2.1. Identification of PAs

We found 57 macrocyclic diester PAs in *S. pterophorus*, 18 of them as *N*-oxides and 39 as tertiary amines, belonging to 6 necine bases: retronecine, otonecine, platynecine, rosmarinecine, dihydrootonecine and hydroxy-dihydrootonecine (Table 1, Fig. 1). These necine bases were classified into three groups according to their structural features: 1,2-unsaturated bases (retronecine and otonecines), 1,2-saturated retronecine bases (platynecine and rosmarinecine) and 1,2-saturated otonecine bases (dihydrootonecine and hydroxy-dihydrootonecines) (Fig. 1).

PAs were identified based on their chromatographic and mass spectrometric properties, and by comparison with available standards (Table 1). Additional evidence was obtained by treating samples with sodium meta bisulfite ($Na_2S_2O_5$), which efficiently reduces N-oxides to the corresponding tertiary amines (Joosten et al., 2010). Reduction of an N-oxide should result in an increased concentration of the

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corresponding PA tertiary amine. PAs only present in the non-reduced samples were considered N-oxides. Several compounds that remained unaffected by the reduction procedure were suggestive of having an otonecine structure, as this type of bases only occur as tertiary amines and do not form the corresponding N-oxide forms (Hartmann, 1999).

Compounds for which no analytical standard was available, but which showed typical PA mass fragmentations, were classified by their necine groups according to their molecular mass and fragmentation behavior according to literature (Li et al., 2008; These et al., 2013; Xiong et al., 2009; Zhou et al., 2010). Thus, retronecine-type PAs typically produce major fragments with mass 138, 120 and 94 that can be linked to the necine core structure, and the corresponding N-oxides produce additional fragments of mass 136, 119 and 118 (Table 1, Fig. S2). Otonecine-type PAs typically produce fragments with mass 168, 150 and 122 (Table 1, Fig. S2). Platynecine-type PAs contain a saturated necine structure and these can be identified based on their main fragments with mass 140, 122 and 82 plus fragments 138 and 120 for the N-oxides (Table 1, Fig. S2). Rosmarinecine-type compounds are typified by the same fragments as the retronecine-type PAs, but they produce an additional fragment of 156, which is indicative of the presence of the 2-hydroxy group. Similarly, rosmarinecine N-oxides produce the same fragments as the retronecine N-oxides plus a 156 fragment (Table 1, Fig. S2). Dihydrootonecine compounds containing a 2-hydroxy group were identified based on the typical otonecine-type fragments (168, 150, 122) together with a fragment with mass 186, indicative for the presence of an additional hydroxyl group (most likely on position 2) in the necine base. The dihydrootonecine esters with protonated m/z 466 were characterized by the typical otonecine fragments (168, 150 and 122) together with two mass fragments (m/z 366 and 83) that were linked to the loss of the ester group

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(Table 1, Fig. S2). The absence of a mass fragment with m/z 186 indicates that the necine hydroxy group is esterified. However, the specific configuration of the ester group (e.g. angeloyl, tigloyl or senecioyl) is unknown. These PAs were tentatively identified as 1,2-dihydrosenkirkine (dihydrootonecine base) and hydroxy derivatives and esters of 1,2-dihydrosenkirkine (hydroxy-dihydrootonecine base) (Table 1).

Finally, the identity of rosmarinine was elucidated by ^{1}H and ^{13}C NMR spectroscopy through the performance and the concerted analyses of 1D and 2D NMR experiments, and the stereochemistry was determined based on 1D selective NOE (Nuclear Overhauser Effect) experiments (Fig. S1, Table S1). These results were consistent with previous reports of rosmarinine in *S. rosmarinifolius* (Richardson and Warren, 1943), *S. triangularis* (Roitman, 1983) and *S. hadiensis* (Were et al., 1991).

2.2. High concentrations and diversity of PAs

Mean total PA concentrations in *S. pterophorus* foliage including plants from the four regions ranged from 2.0 to 42.7 mg/g dry wt., with an average of 11.8 mg/g dry wt. These concentrations are high compared with most other *Senecio* species and much higher than the reports on *S. pterophorus* from Liddell and Logie (1993) and Caño et al. (2009). For example, PA concentrations in *Senecio jacobaea*, *S. alpinus*, *S. aquaticus*, *S. rupestris*, *S. erucifolius*, *S. viscosus*, *S. sylvaticus*, *S. inaequidens* and *S. adonidifolius* ranged from 0.30 mg/g dry wt. to 2.70 mg/g dry wt. (Macel et al., 2002). The concentration is similar to that reported for *S. longilobus* (5.3-54.1 mg/g dry wt.), but lower than that of *S. riddellii* (16.2-130.9 mg/g dry wt.) (Johnson et al., 1985). Comparatively to other congeners, *S. pterophorus* also displayed a high PA diversity based on the number of compounds, their necine bases and necine groups (Table 1). *S.*

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pterophorus contained 37 PAs with 1,2-unsaturated necine bases (29 retronecines and 8 otonecines), 12 PAs with 1,2-saturated bases (4 platynecines and 8 rosmarinecines), and 8 PAs with a 1,2-saturated otonecine base (1 dihydrootonecine and 7 hydroxydihydrootonecines) (Table 1). Previous studies had identified only 7 different PAs in *S pterophorus*, with either a retronecine or a rosmarinecine base. In a recent review of the PA diversity in the Senecioneae nearly all *Senecio* contained PAs from one or two necine bases, one of them being retronecine, but the presence of three or more necine bases was very uncommon (Langel et al., 2011). Indeed, 1,2-saturated otonecine base PAs have been poorly reported in the literature, as far as we know only in *Senecio integrifolius* and the *S. speciosus* / *S. macrocephalus* complex (Langel et al., 2011; Roeder and Liu, 1991; Ruth, 1991).

Retronecine and rosmarinecine PAs were the most abundant necine bases in *S. pterophorus*. Averaged for all plants, retronecine and rosmarinecine PAs represented a 45.5% and a 29.6% of the total PA concentrations, respectively. These two necine bases have a common biosynthetic precursor, homospermidine, but the subsequent steps leading to 1,2-unsaturated (retronecine and otonecine) and 1,2-saturated bases (platynecine and rosmarinecine) follow two differentiated pathways (Hartmann and Ober, 2000). Retronecine PAs, by far the most common PAs in *Senecio*, originate from trachelanthamidine, which is transformed to senecionine N-oxide in the roots, translocated to the shoots via the phloem, and transformed into a bouquet of different retronecine and otonecine PAs by specific enzymatic reactions (Hartmann, 1999; Hartmann and Dierich, 1998; Hartmann and Ober, 2000; Kelly and Robins, 1987; Kunec and Robins, 1986; Robins, 1989). In contrast, platyphylline and rosmarinecine bases are derived from isoretronecanol, an isomer of trachelanthamidine (Hartmann and

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Ober, 2000, Robins, 1989), although the biosynthetic routes of these base moieties have not been completely characterized.

The structural features characteristic of retronecine (1,2-unsaturated base) and rosmarinecine PAs (1,2-saturated base) have been related to their potential toxicity (Li et al., 2013). PAs are hepatotoxic, carcinogenic, genotoxic and teratogenic after a bioactivation by cytochromes P_{450} which occurs under the presence of a double bond at C1-C2 (Chen et al., 2010; Fu et al., 2004; Hartmann, 1999; Stegelmeier et al., 1999; Wiedenfeld and Edgar, 2011). Cytotoxic assays have demonstrated a higher toxicity of retronecine and otonecine PAs compared with platynecine PAs (Li et al., 2013). While comparative toxicity of rosmarinecine PA remains untested, rosmarinine did not form the hepatotoxic reactive pyrrole intermediates (Culvenor et al., 1971; Styles et al., 1980). All these results strongly suggest that retronecine PAs are potentially more toxic than rosmarinecine PAs.

2.3. Biogeographical divergence in PAs

We compared PA concentrations and profiles in *S. pterophorus* across its entire distributional area at the native range (Eastern South Africa), the expanded range (Western South Africa) and two cross-continental introductions (Australia and Europe). Plants from the native region in South Africa contained the highest number of compounds (55 PAs) but this diversity was partially lost in the non-native areas (Table 2). Thus, jacobine-type PAs were completely missing in the expanded populations in South Africa and Australia, and the number of otonecine-type PAs was also reduced in all non-native regions, especially in Western South Africa and Europe (Table 2). Total PA concentrations in *S. pterophorus* varied significantly across regions (P = 0.0018,

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ANOVA). Plants from Australia had the highest total PA concentrations compared with plants from Eastern South Africa and Europe, while no significant differences were found between Australia and Western South Africa, and between Europe and Eastern South Africa (Fig. 2).

Spatial differences in individual PA concentrations were found for most retronecine and otonecines and all platynecine and rosmarinecines, but not for saturated otonecines (Table 2). Rosmarinine N-oxide was the most abundant PA in Australia, Western South Africa and Eastern South Africa. In Europe, however, it was comparatively scarce, and the predominant compounds were acetylseneciphylline N-oxide, seneciphylline N-oxide and senecionine N-oxide (Table 2). When PA absolute concentrations were averaged by necine bases, geographical differences were only found for rosmarinecine and platynecines (Fig. 2). Rosmarinecines absolute and relative concentrations were extremely higher in plants from South Africa and Australia compared with plants from Europe (Fig. 2). Indeed, the abundance of rosmarinine and isomers were strong indicators of plant origin to differentiate European from non-European populations.

2.3. Chemotypes

In order to determine the differences in the PA profile across regions we clustered tertiary amines and N-oxides. Plants from the native populations in Eastern South Africa had higher levels of senecivernine, retrorsine and senkirkine, as well as lower levels of seneciphylline compared with the non-native regions (Fig. 3, 4). Plants from Western South Africa and Australia had similar PA profiles, with higher levels of platyphylline and rosmarinines (Fig. 3, 4). Finally, individuals from Europe had higher

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relative concentrations of senecionine, acetylseneciphylline and acetylspartioidine and lower concentrations of platyphylline and rosmarinines (Fig. 3, 4).

Based on the presence of individual PAs in *S. pterophorus*, and their absolute and relative concentrations across the four distributional regions, we differentiate three plant chemotypes: 1) "Retrorsine-senkirkine chemotype", found in plants from the native region in Eastern South Africa characterized by a high abundance of retrorsine, senecivernine and otonecine PAs and low abundance of seneciphylline and derivatives (spartioidine and hydroxyseneciphylline); 2) "Rosmarinine chemotype", found in plants from Western South Africa and Australia characterized by high abundance of platynecine and rosmarinecine PAs and absence of most jacobine-type and otonecine PAs; and 3) "Acetylseneciphylline chemotype" present in plants from Europe characterized by high abundance of senecionine, acetylseneciphylline and their isomers (integerimine and acetylspartioidine) and low abundance of otonecine, platynecine and rosmarinecine PAs (Table 2, Fig. 3, 4).

Geographical differences in PA composition could be originated by the plant dispersal routes during invasion or neutral events associated to the dynamics of invasion, such as founder effects or genetic drift (Lee, 2002). Because the synthesis of secondary metabolites has a genetic basis, PA profiles in the invaded areas could be related to the chemical composition of the source populations. Neutral markers (AFLPs) showed that populations in Australia and Europe had probably independent origins (Vilatersana et al., unpublished), and this could explain the divergence in PA chemotypes between these regions. The chemical similarity between Western South African and Australian plants is consistent with the closer phylogenetic relationship between these populations. An alternative explanation of the biogeographical divergence of *S. pterophorus* PAs is the evolution of PAs in response to new abiotic and

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biotic conditions (Orians and Ward, 2010). In a previous study we found that climate was driving evolutionary changes in plant traits, e.g. biomass and total leaf area (Colomer-Ventura et al., unpublished), and thus it could also have effects on PAs. Current hypotheses on biological invasions also predict changes in plant toxins due to the herbivore selective pressures in the invaded areas (Bloosey and Notzold, 1995; Doorduin and Vrieling, 2011; Orians and Ward, 2010). The higher concentrations of PAs and the predominance of a specific chemotype in the invasive populations of *Jacobaea vulgaris* (formerly *Senecio jacobaea*) compared with the native populations was related to the lost of specialist herbivores in the novel areas (Joshi and Vrieling, 2005). The divergence in *S. pterophorus* PAs between the two cross-continental introductions, Australia and Europe, suggests that different factors, including both neutral events and natural selection, could affect post-invasive changes in plant chemistry.

2.4. Consequences on plant toxicity

The biogeographical divergence of PAs in *S. pterophorus*, especially the absolute and relative abundance of retronecine (1,2-unsaturated bases) and rosmarinecines PAs (1,2-saturated base), suggests that plant toxicity could be increased after invasion. Plants from Australia had the highest absolute concentrations of total PAs than in any other region, and the highest concentrations of the highly toxic retronecine PAs. Therefore *S. pterophorus* growing in Australia were more toxic than individuals from any other region. Plants from Europe had similar absolute PAs concentrations compared with the native region. However, the absence rosmarinecine PAs in Europe resulted in a higher relative abundance of retronecine PAs compared

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with the native plants in Eastern South Africa, which is also suggestive of an increased toxicity after invasion. We acknowledge that other structural features related to PA toxicity (Hartmann 1999) have not covered here and could also contribute to *S. pterophorus* toxicity. In view of the recent large scale human poisoning on Afghanistan and Ethiopia caused by PAs ingestion (Molyneux et al., 2011) the potential of *S. pterophorus* on contaminating grain crops should be taken into consideration, especially in South Australia where large populations of *S. pterophorus* occur close to agricultural areas (Castells et al., 2013).

3. Conclusions

Plant chemical defenses often differ between native and invasive populations (Doorduin and Vrieling, 2011; Müller and Martens, 2005; Wolf et al., 2011). Here we found strong biogeographical differences in pyrrolizidine alkaloids (PAs) across the entire distributional area of *S. pterophorus* in its native (Eastern South Africa) and three non-native ranges (Western South Africa, Australia and Europe). This is the first comprehensive biogeographical study analyzing plant toxins in the original growing area, as fed by local herbivores, thus accounting for the genetic and environmental effects on chemical defense production. Our results suggest that plant toxicity was increased in the invaded areas. Future studies determining the preference and performance of herbivores consuming *S. pterophorus* of the three reported chemotypes in a dose-response fashion are necessary to elucidate whether geographical variation of PAs affect plant-herbivore interactions in a new habitat. An increase in plant toxicity after invasion could add to the negative impacts that exotic plants cause on invaded ecosystems.

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4. Experimental

4.1. General experimental procedures

Analysis of plant extracts was performed on a Waters Acquity UPLC system coupled to a Waters Quattro Premier XE tandem mass spectrometer (Waters, Milford, MA, USA) and on a Waters Acquity UPLC system coupled to a Waters Xevo TQ-S tandem mass spectrometer (Waters, Milford, MA, USA). Both systems were operated in positive electrospray mode. Pyrrolizidine alkaloids were obtained from commercial sources (Phytolab, Vestenbergsgreuth, Germany; Phytoplan, Heidelberg, Germany), were isolated from plant material (PRISNA, Leiden, the Netherlands) or were synthesized in house as described before (Cheng et al., 2011b).

4.2. Species description and sampling

Senecio pterophorus DC (Asteraceae) is a perennial shrub native to the Eastern Cape and southern KwaZulu-Natal provinces in South Africa (Hilliard, 1977). Its range expanded to the Western Cape during the early 20th century (Levyns, 1950). Introduced into Australia in 1908, *S. pterophorus* currently forms persistent populations along the southeastern coast from Port Lincoln to Melbourne and around Sydney and Newcastle in New South Wales (The Council of Heads of Australasian Herbaria, 2014). In continental Europe *S. pterophorus* was first found in 1982 near Barcelona in the northeastern Iberian Peninsula (Casasayas, 1989) and additional populations in the Mediterranean basin were found in 1990 on the Ligurian coast in northwestern Italy (Barberis et al., 1998).

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Leaf sampling was conducted during the plant flowering period, in December 2009 and January 2010 in the southern hemisphere (South Africa and Australia) and in July 2010 and July 2011 in the northern hemisphere (Catalonia and Liguria) (Castells et al., 2013). This survey covered the entire known distribution of *S. pterophorus*, including its native, expanded and introduced ranges. A total of 52 populations were surveyed, including 16 native (Eastern Cape and KwaZulu-Natal, South Africa), 5 expanded (Western Cape, South Africa) and 31 introduced (14 in Australia and 17 in Europe). We attempted to choose populations that were spread across the territory, including populations from the distributional limits. Plant foliage from one individual per population was dried and stored in silica gel. Voucher specimens used in this study were deposited at the herbarium of the *Centre de Documentació de Biodiversitat Vegetal*, University of Barcelona (BCN 78083, 78085, 78086, 78089-94, 78096, 78098-99 and 78101).

4.2 Alkaloid analysis

Dried, ground leaf material was extracted according to the procedure described by Cheng et al. (2011a). Powdered material was extracted with 2% HCOOH solution in a 1 to 100 ratio (w/v). The extract was then filtered and 25 µL were diluted 40 times with 10 mM NH_4OH. PA composition and content was determined using a Waters Acquity chromatographic system coupled to a Waters Quattro Premier XE tandem mass spectrometer, run in multiple reaction monitoring mode (MRM). Separation of components was accomplished on a Waters UPLC BEH C_{18} (150 x 2.1 mm, 1.7 µm) analytical column, kept at 50^{o}C and run at 0.4 ml/min using a CH_3CN/H_2O gradient containing 6.5 mM NH_4OH. The gradient started at 100% H_2O and was changed

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linearly to 50% CH_3CN in 12 min. Mass spectrometric data were processed using Masslynx 4.1 software (Waters, Milford, MA, USA).

Based on their fragmentation spectra for each compound two ions (typically the most abundant ones) were selected to be included in the MRM. See Table 1 for the mass spectrometric settings selected for each compound. PAs were quantified against a calibrant of available PA standards. For a number of PAs no reference standard was available. For those PAs for which no reference standard, a semi-quantificative result was obtained by comparison of the peak area with that of a structurally related compound, indicated in Table 1. Some representative chromatograms of plant extracts are provided as Supplementary Material.

Some representative samples were screened on the Xevo TQ-S mass spectrometer for the presence of novel pyrrolizidine alkaloids using parent ion scanning. Typical product fragments were selected such as m/z 94, 120, 122, 138 and 168. Mass scanning range was from m/z 200 to 500 with a mass resolution of 0.1 D and a scan time of 250 ms. Spectra were recorded using a fixed cone voltage of 40 V and a fixed collision energy of 30 eV. Compounds producing a protonated molecular ion with an even mass and displaying fragmentation behavior typical for (specific sub-types of) PAs were assumed pyrrolizidine alkaloids. Putative PAs were further identified by collecting individual fragmentation spectra at a collision energy range (20-40 eV). These compounds have been included in the MRM method and in Table 1 and 2. Some representative mass fragmentation spectra and putative fragmentation pathways are provided as Supplementary Material.

4.3 NMR analysis

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NMR analysis was performed on a Bruker Avance II 600 spectrometer (Bruker Biospin, Rheinstetten, Germany) fitted with a 5 mm TBI probe with Z-gradients and operating at 600.13 and 150.90 MHz ^{1}H and ^{13}C NMR frequencies respectively. The probe temperature was set to 298.0 K. Foliage of one individual of *S. pterophorus* collected in South Africa that contained high concentrations of the unknown PA was extracted with CH_3OH, acidified with HCl and partitioned with CH_2Cl_2 to obtain a bulk PA sample. The sample (2.3 mg of dried alkaloid extract) was dissolved in 600μL of $CDCl_3$ (99.96 % D, Cortecnet, Voisins-le-Bretonneux, France).

1D ^{1}H NMR spectra were acquired using a standard 90° pulse sequence, with an acquisition time of 1.71 s and a relaxation delay of 2 s. The data were collected into 32 K data points, with a spectral width of 9590 Hz and as the sum of 128 transients. The resulting free inductions decay (FID) was Fourier transformed, manually phased, and baseline corrected. 2D NMR experiments, ^{1}H-^{1}H COSY (Correlation Spectroscopy), ^{1}H-^{1}H TOCSY (Total Correlation Spectroscopy), ^{1}H-^{13}C HSQC (Heteronuclear Single Quantum Correlation) and ^{1}H-^{13}C HMBC (Heteronuclear Multiple Bond Correlation) were performed using standard pulse sequences (Bruker Biospin) and acquired under routine conditions. 1D ^{1}H selective NOE (Nuclear Overhauser Effect) experiments were performed in order to elucidate the stereochemistry of the alkaloid. All spectra were calibrated using the residual solvent signal ($CHCl_3$, 7.27 and 77.0 ppm for ^{1}H and ^{13}C respectively). Chemical shift data are expressed in ppm and coupling constant values in Hz.

4.4 Statistical analyses

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Total PA concentrations were squared-root transformed and differences in PA concentration among regions were tested with a one-way ANOVA. A Tukey posthoc test was performed to determine differences between regions. Individual PA could not be transformed to meet normality and thus differences between individual PAs were tested with a Kruskal–Wallis test. Significance for all statistical analyses was accepted at $p < 0.05$. All statistical analyses were performed using the R software (R Development Core Team 2008).

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Table 1

Chromatographic and mass spectrometric properties of pyrrolizidine alkaloids (PAs) of *Senecio pterophorus* identified by LC-MS/MS. PAs are classified by their necine base and necine group according to the molecular structural features: I (1,2-unsaturated necine bases), II (1,2-saturated retronecine bases) and III (1,2-saturated otonecine bases).

Group	Necine Base	PAs[1]	RT (min)	Mass $[M+H]^+$	Fragment mass[2]	Ref.[3]
I	Retronecine	senecionine	9.45	336	308, 138, 120, 94	sen
		senecionine *N*-oxide	6.61	352	138, 136, 120, 119, 118, 94	senox
		integerrimine	9.26	336	308, 138, 120, 94	int
		integerrimine *N*-oxide	6.49	352	138, 136, 120, 119, 118, 94	intox
		senecivernine	9.62	336	308, 138, 120, 94	int
		senecivernine *N*-oxide	6.67	352	138, 136, 120, 119, 118, 94	intox
		retrorsine	8.09	352	324, 138, 120, 94	ret
		retrorsine *N*-oxide	5.68	368	138, 136, 120, 119, 118, 94	retox
		usaramine	7.90	352	324, 138, 120, 94	ret
		eruciflorine*	7.33	352	324, 138, 120, 94	ret
		eruciflorine *N*-oxide*	4.78	368	138, 136, 120, 119, 118, 94	retox
		seneciphylline	8.71	334	306, 138, 120, 94	sph
		seneciphylline *N*-oxide	6.01	350	138, 136, 120, 119, 118, 94	sphox
		spartioidine	8.51	334	306, 138, 120, 94	sph
		spartioidine *N*-oxide	5.93	350	138, 136, 120, 119, 118, 94	sphox
		riddelliine	7.50	350	322, 138, 120, 94	rid
		riddelliine *N*-oxide	5.14	366	138, 136, 120, 119, 118, 94	ridox
		hydroxy-seneciphylline*	7.25	350	322, 138, 120, 118, 94	rid
		hydroxy-seneciphylline *N*-oxide*	4.75	366	136, 120, 119, 118, 94	ridox
		hydroxy-spartioidine*	7.11	350	322, 138, 120, 118, 94	rid
		acetylseneciphylline	11.31	376	316, 138, 120, 91	acsph
		acetylseneciphylline *N*-oxide	8.47	392	332, 136, 120, 119, 118, 94	acsphox
		acetylspartioidine	11.14	376	316, 138, 120, 91	acsph
		acetylspartioidine *N*-oxide	8.37	392	332, 136, 120, 119, 118, 94	acsphox
		jacobine	7.46	352	155, 123, 122, 120, 94	jac
		jacobine *N*-oxide	5.13	368	296, 138, 120, 119, 118, 94	jacox
		jacoline	5.75	370	156, 138, 120, 94	jac
		jaconine	8.31	388	352, 156, 138, 120, 94	jac
		jaconine *N*-oxide	5.43	404	368, 138, 120, 119, 118, 94	jacox
	Otonecine	senkirkine	6.92	366	168, 150, 125, 122, 107	snk
		otosenine	5.25	382	168, 150, 122, 107	oto
		onetine	4.04	400	168, 150, 122, 107, 97	oto
		desacetyldoronine	5.90	418	168, 150, 122, 107	oto
		florosenine	7.93	424	168, 150, 122, 107	flo
		floridanine	6.44	442	382, 168, 150, 122, 107	flo
		doronine	8.60	460	400, 168, 150, 122, 107	flo
		senkirkine-like otonecine*	7.67	366	168, 150, 125, 122, 107	snk
II	Platynecine	platyphylline (I)*	10.23	338	140, 122, 82	sen
		platyphylline (II)*	9.94	338	140, 122, 82	sen
		platyphylline (III)*	9.38	338	140, 122, 82	sen
		platyphylline (III) *N*-oxide*	6.40	354	140, 138, 122, 120, 108	senox
	Rosmarinecine	rosmarinine	7.96	354	156, 138, 120, 94	sen
		rosmarinine *N*-oxide	6.21	370	156, 138, 136, 120, 118, 94	senox
		rosmarinine isomer (I)*	8.43	354	156, 138, 120, 94	sen
		rosmarinine isomer (I) *N*-oxide*	6.11	370	156, 138, 136, 120, 118, 94	senox
		rosmarinine isomer (II)*	8.16	354	156, 138, 120, 94	sen
		rosmarinine isomer (III)*	7.87	354	156, 138, 120, 94	sen

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		rosmarinine isomer *N*-oxide*	6.15	370	138, 136, 120, 118, 94	sen
		hydroxy-rosmarinine *N*-oxide*	5.34	386	156, 154, 138, 120, 94	senox
III	Dihydrootonecine	1,2-dihydrosenkirkine	8.05	368	268, 168, 150, 125, 122	snk
	Hydroxydihydrootonecine	2-hydroxy-1,2-dihydrosenkirkine (I)*	6.59	384	186, 168, 150, 125, 122	snk
		2-hydroxy-1,2-dihydrosenkirkine (II)*	6.71	384	186, 168, 150, 125, 122	snk
		2-hydroxy-1,2-dihydrosenkirkine (III)*	7.12	384	186, 168, 150, 125, 122	snk
		2-hydroxy-1,2-dihydrosenkirkine ester (I)*	10.06	466	366, 250, 168, 150, 122, 83	snk
		2-hydroxy-1,2-dihydrosenkirkine ester (II)*	10.21	466	366, 250, 168, 150, 122, 83	snk
		2-hydroxy-1,2-dihydrosenkirkine ester (III)*	10.41	466	366, 250, 168, 150, 122, 83	snk
		2-hydroxy-1,2-dihydrosenkirkine ester (IV)*	10.78	466	366, 250, 168, 150, 122, 83	snk

[1]Tentative identifications, based on fragmentation spectra, MW, RT and presence or absence of N-oxides, are indicated by *

[2]Major mass fragments detected at collision energy 30 eV. Underlined fragments were used for quantification

[3]Reference standards used for identification and quantification: sen = senecionine, senox = senecionine *N*-oxide, int = interregimine, intox = interregimine *N*-oxide, ret = retrorsine, retox = retrosine *N*-oxide, sph = seneciphylline, sphox = seneciphylline *N*-oxide, rid = riddelliine, ridox = riddelliine *N*-oxide, acsph = sectylseneciphylline, acsphox = acetylseneciphylline *N*-oxide, jac = jacobine, jacox = jacobine *N*-oxide, snk = senkirkine, oto = otosenine, flo = florosenine

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Table 2

Concentrations of pyrrolizidine alkaloids (PAs) (µg/g dry wt.) of *Senecio pterophorus* foliage collected at the native and expanded ranges (South Africa) and two introduced ranges (Australia and Europe). Mean (s.e.) are shown, and dominant compounds within region are highlighted in bold. Significant differences among regions on a Kruskal-Wallis test are indicated as *p <0.05, **p <0.001 and ***p <0.001. PAs are classified by their necine base and necine group according to the molecular structural features: I (1,2-unsaturated necine bases), II (1,2-saturated retronecine bases) and III (1,2-saturated otonecine bases).

Group	Necine Base	PAs	South Africa		Australia	Europe	
			Native N = 16	Expanded N = 5	Introduced N = 14	Introduced N = 17	
I	Retronecine	senecionine	114 (63)	73 (31)	163 (34)	112 (29)	
		senecionine *N*-oxide	**817** (259)	**452** (93)	**1987** (393)	**1151** (243)	*
		integerrimine	15 (8)	6 (2)	12 (3)	11 (3)	
		integerrimine *N*-oxide	122 (40)	35 (8)	223 (53)	147 (33)	
		senecivernine	39 (14)	6 (3)	4 (2)	4 (2)	*
		senecivernine *N*-oxide	517 (213)	nd	nd	25 (17)	**
		retrorsine	154 (52)	7 (2)	30 (14)	9 (3)	*
		retrorsine *N*-oxide	**949** (52)	56 (13)	184 (42)	76 (19)	*
		usaramine	22 (10)	nd	nd	nd	**
		erucifoline	16 (5)	40 (14)	23 (6)	15 (3)	
		erucifoline *N*-oxide	8 (1)	9 (4)	14 (2)	4 (1)	**
		seneciphylline	133 (51)	**437** (144)	**521** (122)	192 (45)	**
		seneciphylline *N*-oxide	**668** (237)	**1636** (136)	**2777** (485)	**1167** (230)	***
		spartioidine	16 (6)	34 (11)	36 (11)	18 (4)	*
		spartioidine *N*-oxide	88 (28)	172 (22)	313 (58)	137 (24)	**
		riddelliine	12 (5)	15 (6)	42 (18)	8 (3)	
		riddelliine *N*-oxide	39 (17)	50 (19)	206 (52)	62 (27)	**
		hydroxy-seneciphylline	12 (8)	28 (16)	21 (9)	9 (4)	*
		hydroxy-seneciphylline *N*-oxide	60 (19)	317 (120)	275 (52)	107 (23)	***
		hydroxy-spartioidine	2 (1)	5 (3)	3 (1)	1 (1)	
		acetylseneciphylline	66 (26)	69 (26)	195 (42)	241 (58)	**
		acetylseneciphylline *N*-oxide	**517** (207)	**513** (171)	**1830** (411)	**1467** (438)	*
		acetylspartioidine	7 (3)	12 (4)	18 (4)	14 (2)	*
		acetylspartioidine *N*-oxide	37 (12)	26 (7)	113 (41)	78 (21)	*
		jacobine	nd	nd	nd	5 (3)	*
		jacobine *N*-oxide	4 (2)	nd	4 (3)	27 (8)	***
		jacoline	3 (1)	nd	nd	7 (1)	***
		jaconine	1 (0)	nd	nd	4 (2)	***
		jaconine *N*-oxide	1 (0)	nd	nd	2 (1)	*
	Otonecine	senkirkine	115 (104)	2 (1)	48 (43)	nd	*
		otosenine	28 (14)	nd	6 (2)	nd	**
		onetine	42 (18)	nd	6 (2)	nd	***
		desacetyldoronine	15 (7)	nd	2 (1)	nd	***
		florosenine	2 (2)	nd	nd	nd	
		floridanine	3 (2)	nd	nd	nd	

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		doronine	3 (2)	nd	nd	nd	
		senkirkine-like otonecine	27 (9)	51 (27)	14 (6)	45 (17)	
II	Platynecine	platyphylline (I)	67 (33)	151 (89)	146 (46)	23 (8)	***
		platyphylline (II)	5 (3)	14 (9)	26 (7)	4 (2)	***
		platyphylline (III)	nd	3 (1)	5 (2)	1 (0)	***
		platyphylline (III) *N*-oxide	9 (8)	12 (4)	51 (12)	11 (8)	***
	Rosmarinecine	rosmarinine	243 (99)	**797** (249)	**796** (235)	27 (19)	***
		rosmarinine *N*-oxide	**1652** (606)	**4159** (1058)	**5165** (1206)	160 (86)	***
		rosmarinine isomer (I)	64 (23)	176 (57)	164 (16)	15 (8)	***
		rosmarinine isomer (I) *N*-oxide	234 (107)	341 (83)	**481** (148)	15 (9)	***
		rosmarinine isomer (II)	6 (2)	7 (3)	16 (2)	1 (0)	***
		rosmarinine isomer (III)	15 (5)	24 (2)	25 (4)	16 (3)	*
		rosmarinine isomer *N*-oxide	17 (6)	nd	nd	34 (6)	***
		hydroxy-rosmarinine *N*-oxide	18 (12)	1 (1)	3 (1)	nd	**
III	Dihydrootonecine	1,2-dihydrosenkirkine	60 (15)	110 (47)	151 (38)	108 (28)	
	Hydroxydihydrootonecine	2-hydroxy-1,2-dihydrosenkirkine (I)	6 (1)	9 (4)	8 (2)	7 (1)	
		2-hydroxy-1,2-dihydrosenkirkine (II)	28 (5)	41 (14)	26 (4)	34 (8)	
		2-hydroxy-1,2-dihydrosenkirkine (III)	46 (6)	53 (18)	69 (8)	49 (13)	
		2-hydroxy-1,2-dihydrosenkirkine ester (I)	32 (8)	35 (9)	21 (4)	30 (8)	
		2-hydroxy-1,2-dihydrosenkirkine ester (II)	173 (34)	232 (74)	225 (60)	116 (20)	
		2-hydroxy-1,2-dihydrosenkirkine ester (III)	**1062** (164)	**1536** (353)	**832** (155)	**1009** (192)	
		2-hydroxy-1,2-dihydrosenkirkine ester (IV)	**1213** (230)	**1702** (586)	**1405** (325)	**962** (155)	

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Figure Legends

Fig. 1 Structures of the six types of PAs classified by their necine base: retronecine (pictured senecionine), otonecine (pictured senkirkine), platynecine (pictures platyphylline), rosmarinecine (pictured rosmarinine), dihydrootonecine (pictured 1,2-dihydrosenkirkine) and hydroxydihydrootonecine (pictured 2-hydroxy-1,2-dihydrosenkirkine). Necines bases are classified according to a structural feature related to toxicity: I) PAs with a 1,2-unsaturated necine bases, II) PAs containing a 1,2-saturated retronecine bases and III) PAs containing a 1,2-saturated otonecine base.

Fig. 2 A) Concentrations and B) relative concentrations of PAs grouped by necine bases from plants collected at the native region in South Africa, the expanded region in South Africa, and two introduced regions in Australia and Europe. Bars represent the mean (± s.e.) by region and necine base (RET = retronecine, OTO = otonecine, PLA = platynecine, ROS = rosmarinecine, DIHYD = dihydrootonecine and OH-DIHY =hydroxydihydrootonecine). Statistical differences across regions were tested on a Kruskal-Wallis test and significant differences are indicated by **$p < 0.01$ and ***$p < 0.001$. Inserted graph shows total PA concentrations by region (mean ± s.e). Different letters indicate significant differences between regions at $p < 0.05$ based on a Tukey *post-hoc* test.

Fig. 3 PA relative concentrations averaged by region (mean ± s.e.). Only PAs with a relative concentration higher than 1% are included. PAs from the retronecine and platynecine groups include both amines and N-oxides. Numbers correspond to: 1 = senecionine, 2 = integerrimine, 3 = senecivernine, 4 = retrorsine, 5 = seneciphylline, 6 =

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spartioidine, 7 = riddelliine, 8 = hydroxyseneciphylline, 9 = acetylseneciphylline, 10 = acetylspartioidine, 11 = senkirkine, 12 = platyphylline (I), 13 = rosmarinine, 14 = rosmarinine isomer (I), 15 = 1,2-dihydrosenkirkine, 16 = 2-hydroxy-1,2-dihydrosenkirkine ester (II), 17 = 2-hydroxy-1,2-dihydrosenkirkine ester (III) and 18 = 2-hydroxy-1,2-dihydrosenkirkine ester (IV). Significant differences among regions obtained by a Kruskal-Wallis test are indicated by *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Fig. 4 Percent deviation from the mean of PA relative concentrations averaged by region. Only PAs with a relative concentration higher than 1% are included. PAs from the retronecine and platynecine groups include both amines and N-oxides. Numbers correspond to the following PAs: 1 = senecionine, 2 = integerrimine, 3 = senecivernine, 4 = retrorsine, 5 = seneciphylline, 6 = spartioidine, 7 = riddelliine, 8 = hydroxyseneciphylline, 9 = acetylseneciphylline, 10 = acetylspartioidine, 11 = senkirkine, 12 = platyphylline (I), 13 = rosmarinine, 14 = rosmarinine isomer (I), 15 = 1,2-dihydrosenkirkine, 16 = 2-hydroxy-1,2-dihydrosenkirkine ester (II), 17 = 2-hydroxy-1,2-dihydrosenkirkine ester (III) and 18 = 2-hydroxy-1,2-dihydrosenkirkine ester (IV).

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Figure 1

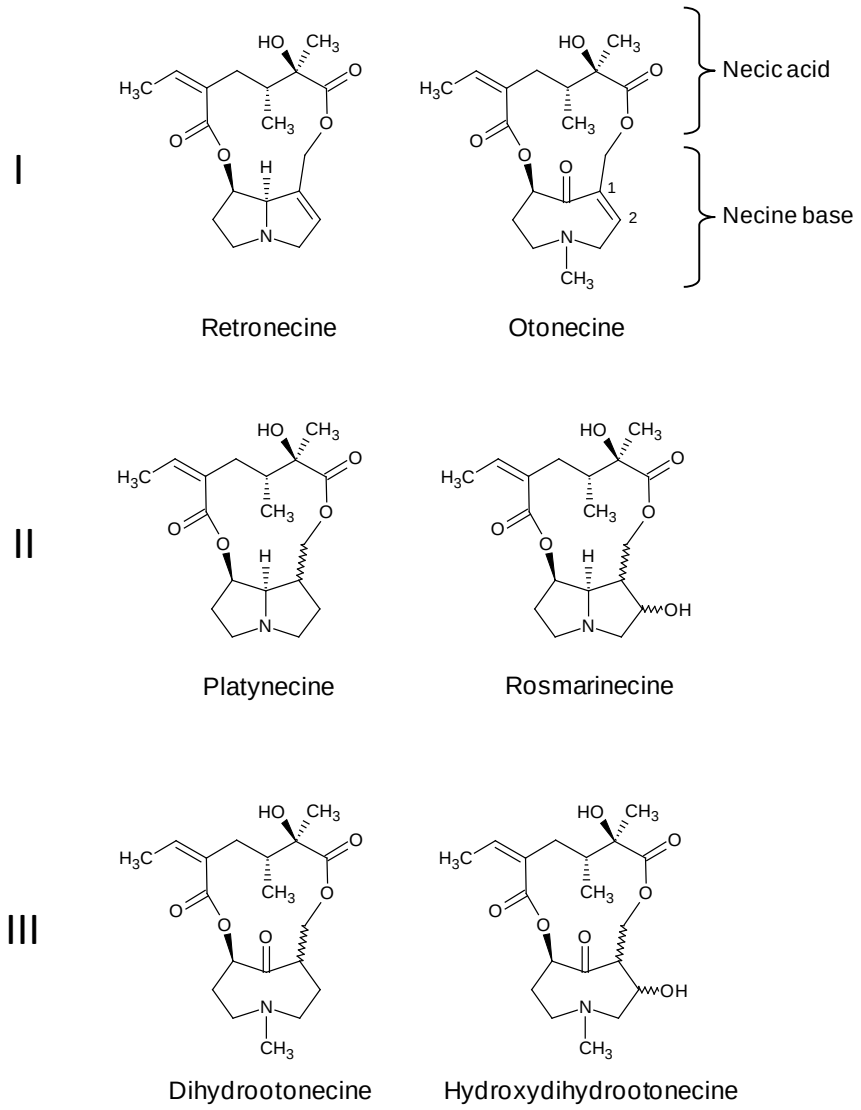

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Figure 2

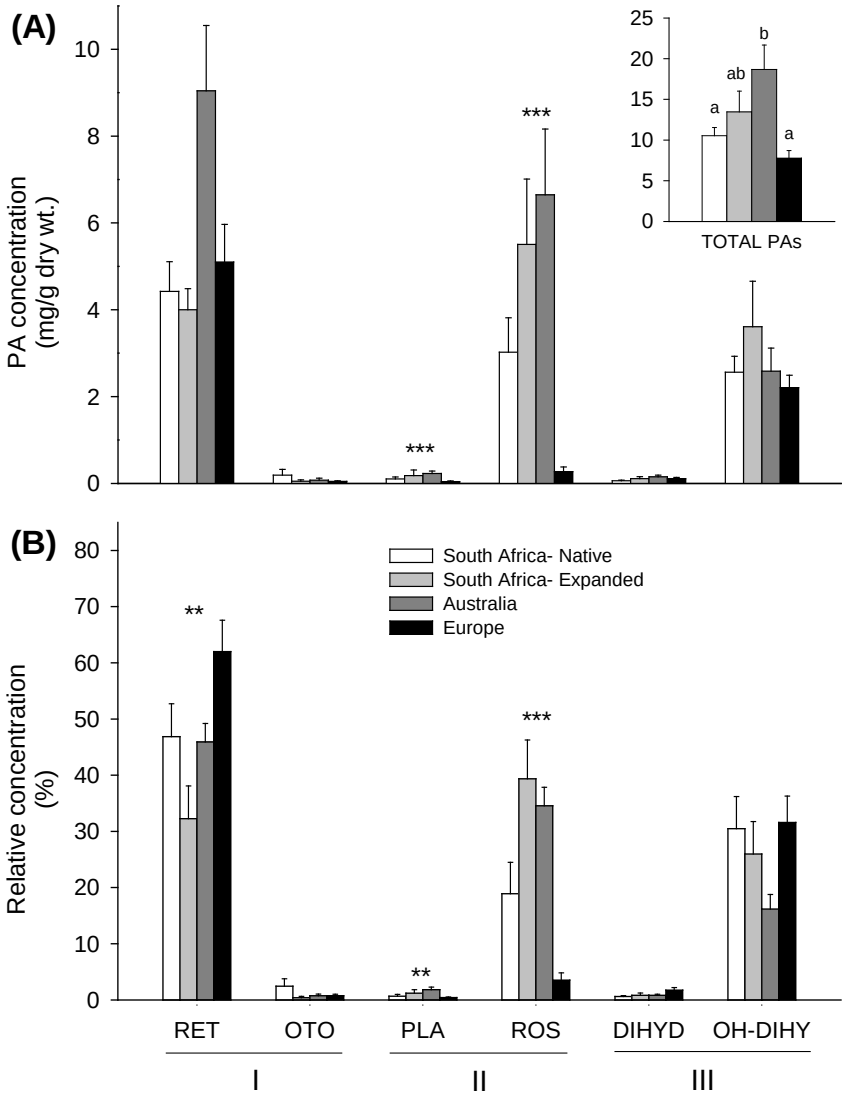

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Figure 3

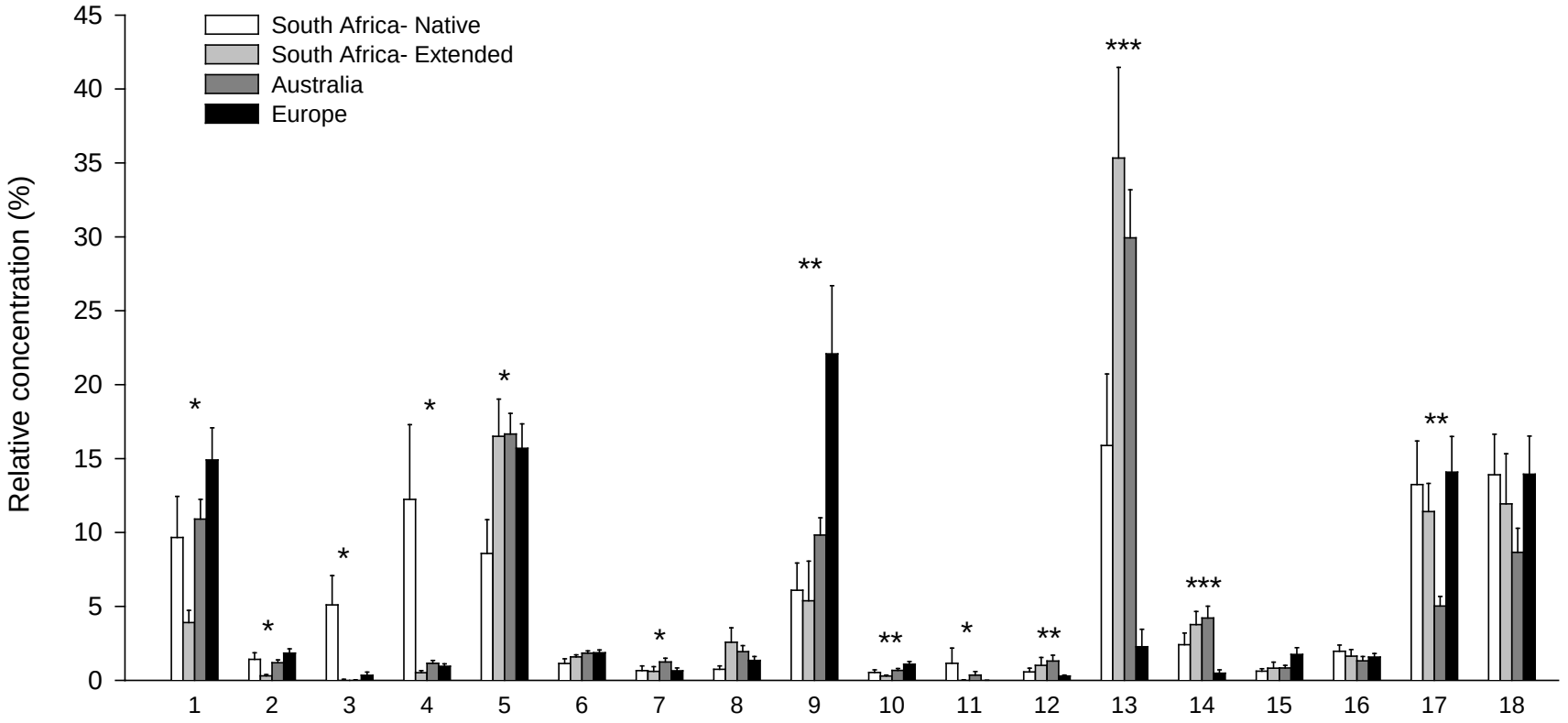
South Africa- Native
South Africa- Extended
Australia
Europe
Relative concentration (%)
0
5
10
15
20
25
30
35
40
45
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
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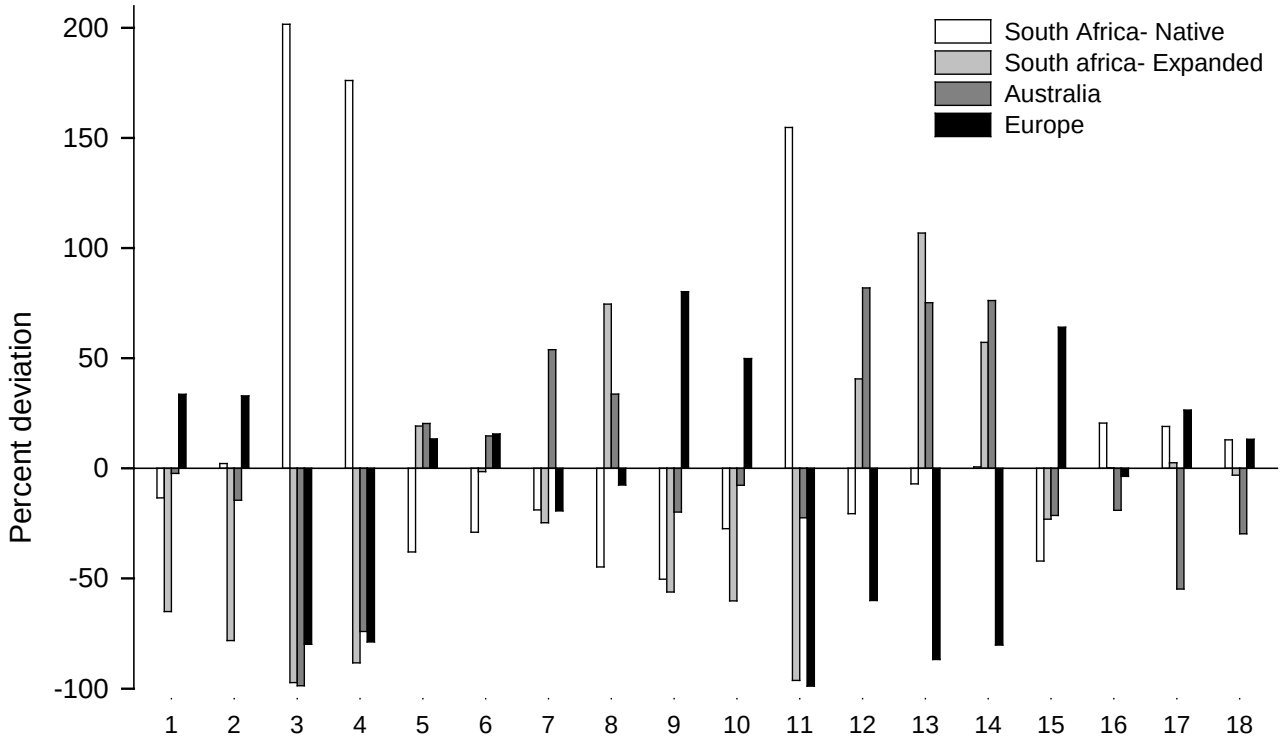
Figure 4


South Africa- Native
South africa- Expanded
Australia
Europe
Percent deviation
200
150
100
50
0
-50
-100
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18