



Crystal types and their distribution in the bark of African genistoid legumes (Fabaceae tribes Sophoreae, Podalyriaceae, Crotalarieae and Genisteae)

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The occurrence and distribution of seven crystal types in 114 bark samples from 25 genera and 91 species, representing all four tribes of African genistoid legumes, are reported. The seven types are prismatic, druse (including irregular crystal clusters), navicular (including truncated navicular, here reported for the first time), spherical crystal cluster, styloid, crystal sand and acicular crystals in sheaf-like aggregates. Unlike most studies, the elemental composition of the crystals was examined using X-ray microanalyses. With the exception of acicular crystals, all crystals showed the typical peaks of calcium (sometimes accompanied by small amounts of magnesium). Acicular crystals in sheaf-like aggregates were composed only of carbon and oxygen, indicating that they are organic and precipitate during drying or after fixation in alcohol. These crystals are found only in the two early-diverging lineages of Podalyriaceae (*Cyclopia* and *Virgilia*+*Calpurnia* clades), consistent with the phylogenetic pattern in the tribe (indicating a secondary loss). Navicular crystals are restricted to Podalyriaceae and Crotalarieae. Prismatic crystals in bark are proposed to be the ancestral condition, with multiple losses (or reversals) in Podalyriaceae, Crotalarieae and Genisteae. © 2015 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2015, 178, 620–632.

ADDITIONAL KEYWORDS: calcium oxalate – elemental composition – magnesium oxalate – organic crystals – systematics.

INTRODUCTION

Crystals are present in higher plant families and can be found in different plant tissues, including leaves, stems, roots, seeds and floral structures (Franceschi & Nakata, 2005). Crystals are products of plant metabolism and their chemical composition, shape, size and distribution in a plant depend on a combination of genetic and environmental factors (Franceschi & Horner, 1980; Metcalfe & Chalk, 1983; Franceschi & Nakata, 2005). The number of crystals in a plant depends on the seasons and life cycle of the plant, which are not constant (Marcati & Angyalossy, 2005).

The shape and distribution of crystals in a plant vary between species and have taxonomic significance (Prychid & Rudall, 1999; Cervantes-Martinez *et al.*, 2005; Lersten & Horner, 2009, 2011; Horner, Wanke, & Samain, 2009, 2012; Horner *et al.*, 2015). Crystals in plants are usually called mineral crystals because they contain calcium (in the form of calcium oxalate), the most abundant biomineral in living organisms (Arnott, 1982), but sometimes also magnesium, sulphur, strontium or barium (He *et al.*, 2012a, b). In contrast, organic crystals do not contain any minerals but are artefacts resulting from precipitation. According to Metcalfe & Chalk (1983), the products of plant metabolism such as fructans (inulin), glycosides (myrosin) and flavones (hesperidin and diosmin) are in solution in the

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living plant. They precipitate out as crystals only when the material is fixed in alcohol or when it is dried to prepare herbarium specimens. Information about the shape of organic crystals is scarce. It is only known that inulin can precipitate as globose aggregates, often composed of needles, and hesperidin as needle-shaped, plumose or dendritic crystals (Borodin, 1883; Metcalfe & Chalk, 1983; Isejima, Figueiredo-Ribeiro & Zaidan, 1991). Analyses to determine the chemical nature of crystals are rarely part of comparative anatomical studies focused on taxonomy.

The diversity of crystals in Fabaceae has attracted the attention of researchers since Solereder (1908). Crystals occur in all three subfamilies. They can be variously shaped and may be located in different parts of the plant (Metcalfe & Chalk, 1950; Leelavathi, Prabhakar & Ramayya, 1984; Zindler-Frank, 1987; Cervantes-Martinez *et al.*, 2005). Investigation of the bark anatomy of *Virgilia* Lam. (Kotina *et al.*, 2013) and the wood anatomy of several members of tribes Podalyrieae and Crotalariaeae (Stepanova *et al.*, 2013; Oskolski *et al.*, 2014) revealed a diversity of crystals warranting more detailed study. The aim of this study is to describe the different crystal types and their distribution in the bark of African genistoid legumes (Fabaceae tribes Sophoreae, Podalyrieae, Crotalariaeae and Genisteae) and to evaluate the systematic value of the data.

MATERIAL AND METHODS

More than 114 bark samples from 91 species and 25 genera of African genistoid legumes (tribes Sophoreae, Podalyrieae, Crotalariaeae and Genisteae) were examined. Most of the plants are woody shrubs. The material includes herbarium voucher specimens and materials preserved in formaldehyde–acetic acid–alcohol (FAA) (Johansen, 1940) collected over more than 30 years as part of taxonomic revisions of southern African legume genera. Species sampled and voucher specimens (all in JRAU) are as follows (abbreviations: ALS = A. L. Schutte; ANM = A. N. Moteetee; AS = A. Stepanova; AdC = A. de Castro; BEVW = B.-E. van Wyk; BE & MVW = B.-E. & C. M. van Wyk; JSB = J. S. Boatwright; GJC = G. J. Campbell; KK = E. L. Kotina; MLR = M. M. le Roux; TP = T. Phago; PMT = P. M. Tilney; Vlok *et al.* = Vlok, Schutte & Van Wyk): **Tribe Sophoreae:** *Sophora inhambanensis* Klotzsch (BEVW *s.n.*); **Tribe Podalyrieae:** *Amphithalea ericifolia* (L.) Eckl. & Zeyh. (ALS 617), *A. rostrata* A.L.Schutte & B.-E.van Wyk (ALS 629), *A. tomentosa* (Thunb.) Granby (ALS 632), *A. vlokii* (A.L.Schutte & B.-E.van Wyk) A.L.Schutte (ALS 743); *Cadia purpurea* (G.Piccioli) Aiton (KK 33-11); *Calpurnia aurea* Benth. (KK 39-11), *C. intrusa* E.Mey. (BEVW 3249c), *C. sericea* Harv. (KK 35-11); *Cyclopia*

burtonii Hofmeyr & E.Phillips (ALS 643), *C. burtonii* (ALS 641), *C. buxifolia* (Burm.f.) Kies (ALS 544), *C. buxifolia* (ALS 602), *C. galioides* E.Mey. ex Benth. (ALS 550, 789), *C. genistoides* Sieber ex C.Presl (ALS 615), *C. intermedia* E.Mey. (ALS 679, 680), *C. maculata* (Andrews) Kies (ALS 528, 636), *C. plicata* Kies (ALS 517, 670a), *C. subternata* Vogel (KK 43-11, JSB 596); *Liparia hirsuta* Moench (JSB 595, BEVW 2961), *L. myrtifolia* Thunb. (ALS 727a, 727 b), *L. racemosa* A.L.Schutte (ALS 642), *L. splendens* (Burm.f.) Bos & de Wit (BEVW 3147); *Podalyria calyprata* Willd. (KK 03-10, BEVW 3141, ALS 709b), *P. hirsuta* Willd. (BEVW 3144), *P. lanceolata* Poir. (ALS *s.n.*), *P. leipoldtii* L.Bolus ex A.L.Schutte (ALS 596), *P. myrtifolia* D.Dietr. (ALS 630, AS & BEVW 166), *P. rotundifolia* (P.J.Bergius) A.L.Schutte (ALS *s.n.*); *Stirtonanthus chrysanthus* (Adamson) B.-E.van Wyk & A.L.Schutte (BEVW & ALS 3329a), *S. insignis* (Compton) B.-E.van Wyk & A.L.Schutte (BEVW 3184, BEVW 3331), *S. taylorianus* (L.Bolus) B.-E.van Wyk & A.L.Schutte (JSB 591), *S. taylorianus* (BEVW 3169); *Xiphotheca canescens* (Thunb.) A.L.Schutte & B.-E.van Wyk (JSB 127, ALS 595a), *X. phyllicoides* A.L.Schutte & B.-E.van Wyk (ALS 648), *X. tecta* (Thunb.) A.L.Schutte & B.-E.van Wyk (ALS 597, ALS 600); *Virgilia divaricata* Adamson (KK 02-10), *V. oroboides* (P.J.Bergius) T.M.Salter subsp. *oroboides* (KK 01-10), *V. oroboides* (P.J.Bergius) T.M.Salter subsp. *ferruginea* B.-E.van Wyk (KK 44-11); **Tribe Crotalariaeae:** *Aspalathus excelsa* R.Dahlgren (AS & BEVW168), *A. linearis* (Burm.f.) R.Dahlgren (KK 48-11, KK 50-11, KK 51-11, KK 56-11), *A. nivea* Willd. ex Walp. (KK 45-11), *A. vulnerans* Thunb. (KK 49-11); *Calobota cinerea* (E.Mey.) Boatwr. & B.-E.van Wyk (JSB 136), *C. cuspidosa* (Burch.) Boatwr. & B.-E.van Wyk (KK 46-11, BEVW 2534), *C. cytisoides* Eckl. & Zeyh. (JSB *et al.* 114), *C. halenbergensis* (Merxm. & A.Schreib.) Boatwr. & B.-E.van Wyk (JSB 146), *C. multiflora* Benth. (JSB 138), *C. psiloloba* (E.Mey.) Boatwr. & B.-E.van Wyk (JSB 107), *C. sericea* (Thunb.) Boatwr. & B.-E.van Wyk (JSB 140, JSB 141), *C. spinescens* (Harv.) Boatwr. & B.-E.van Wyk (BEVW 2849); *Crotalaria agatiflora* Schweinf. (TP *s.n.*), *C. capensis* Baker (KK 40-11), *C. pallida* Aiton (BEVW 5833); *Euchlora serpens* Eckl. & Zeyh. (ALS 255); *Lebeckia ambigua* E.Mey. (BEVW & PMT *s.n.*), *L. brevicarpa* M.M.le Roux & B.-E.van Wyk (BEVW 3123), *L. carnosa* Druce (Vlok *et al.* *s.n.*); *Leobordea adpressa* (N.E.Br.) B.-E.van Wyk & Boatwr. (BEVW 1916), *L. arida* (Dümmer) B.-E.van Wyk & Boatwr. (BEVW 1825), *L. benthamiana* (Dümmer) B.-E.van Wyk & Boatwr. (BEVW 2539), *L. magnifica* (B.-E.van Wyk) B.-E.van Wyk & Boatwr. (BEVW 2422), *L. mollis* (E.Mey.) B.-E.van Wyk & Boatwr. (BEVW 3105), *L. mucronata* (Conrath) B.-E.van Wyk & Boatwr. (BEVW 2618), *L. procumbens* (Bolus) B.-E.van Wyk &

Boatwr. (BEVW 2508); *Listia heterophylla* E.Mey. (BEVW 2474); *Lotononis densa* Harv. subsp. *leuoclada* (Schltr.) B.-E.van Wyk (BEVW 3122), *L. nutans* B.-E.van Wyk (BEVW 3442), *L. pulchella* (E.Mey.) B.-E.van Wyk (BEVW 1332), *L. sericophylla* Benth. (KK 36-11), *L. trichodes* (E.Mey.) B.-E.van Wyk (BEVW 1682), *L. umbellata* Benth. (AS & BEVW 163/2, KK 61-11); *Rafnia amplexicaulis* Thunb. (KK 55-11), *R. angulata* Thunb. (KK 59-11), *R. capensis* (L.) Schinz subsp. *pedicellata* C.J.Campb. & B.-E.van Wyk (GJC & AdC 85); *Wiborgia monoptera* E. Mey. (JSB 152), *W. obcordata* (P.J.Bergius) Thunb. (KK 52-11); *Wiborgiella bowieana* (Benth.) Boatwr. & B.-E.van Wyk (BEVW 2106), *W. leipoldtiana* (Schltr. ex R.Dahlgren) Boatwr. & B.-E.van Wyk (JSB *et al.* 123); **Tribe Genisteae:** *Argyrolobium argenteum* Eckl. & Zeyh. (BEVW *s.n.*, Carlton Heights), *A. crassifolium* Eckl. & Zeyh. (BE & MVW 1838), *A. lotoides* Harv. (ALS 364), *A. transvaalense* Schinz (BEVW 3038); *Dichilus gracilis* Eckl. & Zeyh. (ALS 194b), *D. reflexus* (N.E.Br.) A.L.Schutte (ALS 169), *D. reflexus* (ALS 171), *D. strictus* E.Mey (ALS 155); *Melolobium aethiopicum* Druce (BEVW 4040), *M. candicans* Eckl. & Zeyh. (JSB 601), *M. microphyllum* (L.f.) Eckl. & Zeyh. (ANM & BEVW 6), *M. stipulatum* Harv. (BEVW 4037); *Polhillia brevicalyx* (C.H.Stirt.) B.-E.van Wyk & A.L.Schutte (ALS 228), *P. canescens* C.H.Stirt. (BEVW 2092), *P. involucratum* (Thunb.) B.-E.van Wyk & A.L.Schutte (JSB 602), *P. obsoleta* (Harv.) B.-E.van Wyk (AS & BEVW 154).

We used the thickest portions of above-ground stems from the FAA collection or, in the case of unfixed dry material, the pieces of bark were soaked in hot water before sectioning. Sections were made by hand or with a freezing microtome. The sections were left unstained or were stained in a mixture of safranin and alcian blue, after which they were mounted in glycerol or euparal. The shape and distribution of crystals were studied using transmitted normal and polarized light. Digital images were taken using an Olympus ColorView Soft Imaging System. We also examined fresh, i.e. unfixed, samples of the bark of *Virgilia divaricata*. The morphology and chemical composition of the crystals were investigated by scanning electron microscopy (SEM; TESCAN, soft – VegaTS and Mira 3 LMU) at the central analytical facility (called Spectrum) of the University of Johannesburg and at the research resource centre (known as Molecular and Cell Technologies) of St. Petersburg State University. For SEM examination, the FAA-fixed samples were freeze-dried, mounted on aluminium stubs with double-sided carbon tape and coated with gold or carbon for additional analyses. Backscattered electron (BSE) analysis was done on carbon-coated samples, and energy-dispersive X-ray spectroscopy (EDS) was done on both

gold-coated and carbon-coated samples. The BSE signal is strongly related to the atomic mass of the studied specimen: if crystals contain compounds heavier than carbon, then they have a brighter appearance than the surrounding organic tissues. The BSE analysis was performed to determine the distribution and localization of minerals (e.g. calcium and magnesium contained in the crystals). Elemental and chemical analyses (EDS) of a sample by SEM were made by Oxford Instruments with AZtec software. The voltages for the BSE and EDS analyses were 8 and 20 kV, respectively.

Evolutionary pathways for bark features were reconstructed by mapping their character states onto a subsample of the strict consensus tree for the Bayesian inference (BI) analysis of the internal transcribed spacer (ITS) and *rbcL* data for Crotalariaeae (modified from Boatwright *et al.*, 2008; Boatwright, Tilney & Van Wyk, 2009). Evolutionary traces of the character states along the tree branches were visualized by the parsimony reconstruction method using the Character History Tracing option in the Mesquite 2.5 package (Maddison & Maddison, 2008).

RESULTS

CLASSIFICATION OF CRYSTALS AND THEIR TAXONOMIC DISTRIBUTION

Crystals of seven types, i.e. prismatic (including cubic), navicular (including truncated), druse (including irregular crystal clusters), styloid, spherical crystal clusters, crystal sand and acicular crystals in sheaf-like aggregates (Fig. 1A–I), were found in the bark of South African genistoid Fabaceae (Table 1). Five of these crystal types were distinguished according to the classification system proposed by the IAWA Committee (1989). The spherical crystal clusters as found in this study (e.g. Fig. 1G) have not yet been defined in the literature. Acicular crystals in sheaf-like aggregates also do not fit into the IAWA classification system. These organic crystals occur as numerous acicular crystals radiating from one point to form a sheaf-like aggregate, appearing fan-shaped in transverse section. All crystal types occurred in cortical parenchyma and phloem rays and all except spherical crystal clusters and styloids were found in axial parenchyma. All crystals showed birefringence under polarized light, but this effect was weaker in acicular crystals in sheaf-like aggregates than in all the other types of crystals. The taxonomic and tissue distributions of the crystal types are shown in Table 1.

Prismatic crystals were the only crystal type that occurred in all four tribes. They are 5–20 µm in diameter and are relatively common in Crotalariaeae, occur-

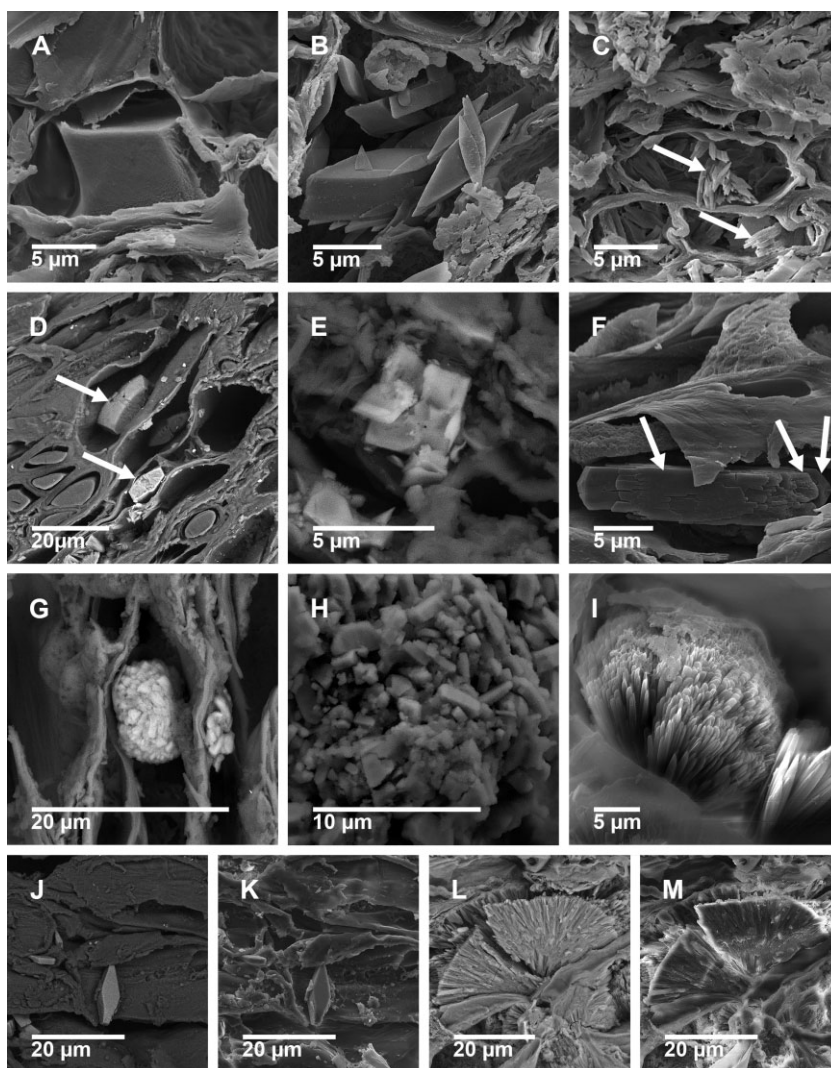


Figure 1. Scanning electron micrographs showing morphology of crystals in bark of some members of tribes Sophoreae, Crotonariae, Genisteae and Podalyrieae. A–C, F, I, coated with gold; D, E, G, H, J–M, coated with carbon. A, prismatic crystal in a ray cell of *Sophora inhambanensis*. B, large and small navicular crystals in ray cell of *Stirtonanthus insignis*. Large navicular crystal is delaminated on lower surface; C, small navicular crystals in aggregates (arrows) in ray cells of *Amphithalea ericifolia*. D, Truncated navicular crystals (arrows) in ray cells of *Lotononis trichodes*. E, druse (irregular crystal cluster) in cortical parenchyma cell of *Calobota multiflora*. F, styloids (arrows) in cortical parenchyma cell of *Melolobium aethiopicum*. G, spherical crystal cluster in cortical parenchyma cell of *Polhillia obsoleta*. H, crystal sand in cortex of *Stirtonanthus insignis*. I, acicular crystals in sheaf-like aggregates in axial parenchyma of *Virgilia oroboides* subsp. *oroboides*. J–M, BSE analysis. J, L, BSE detector. K, M, normal SEM. J, K, navicular crystals with truncated ends in *Cadia purpurea*. L, M, acicular crystals in sheaf-like aggregates in *Calpurnia intrusa*.

ring in some species of *Aspalathus* L., *Calobota* Eckl. & Zeyh., *Lebeckia* Thunb. and *Lotononis* (DC.) Eckl. & Zeyh. They were found in only two genera (*Cadia* Forssk. and *Calpurnia* E.Mey.) of Podalyrieae and a single species (*Melolobium aethiopicum*) of Genisteae.

Navicular crystals were found in 35 species (38% of the total number of species) belonging to all genera of Podalyrieae except *Cyclopia* Vent. and to *Aspalathus*, *Calobota*, *Lebeckia* and *Lotononis* in Crotonariae.

These crystals are 2–10 μm long and are referred to as diamond-shaped or lozenge-shaped crystals by some authors. Crystals of this type were not observed, however, in Genisteae and Sophoreae. In four species (*Cadia purpurea*, *Aspalathus linearis*, *Calobota cuspidosa*, *Lotononis trichodes*), somewhat larger navicular crystals (8–20 μm long) with truncated ends co-occurred with ordinary navicular crystals. Some navicular crystals showed a layered structure,

Table 1. Distribution of crystal types in African genistoid legumes (tribes Sophoreae, Podalyriaceae, Crotalariaeae and Genisteae)

Tribe/species	Type of crystals								SEM	
	Prismatic	Navicular	Truncated navicular	Druse	Spherical crystal clusters	Styloids	Sand	Acicular (in sheaf-like aggregates)	BSE	EDS
Sophoreae										
<i>Sophora inhambanensis</i>	AP,R	–	–	–	–	–	–	–	+	+
Podalyriaceae										
<i>Amphithalea ericifolia</i>	–	C,AP,R	–	C,R	–	–	–	–	+	–
<i>Amphithalea rostrata</i>	–	C,AP,R	–	–	–	–	C,R	–	+	–
<i>Amphithalea tomentosa</i>	–	R (rare)	–	R (rare)	–	–	–	–	–	–
<i>Amphithalea vlokii</i>	–	AP,R	–	–	–	–	–	–	–	–
<i>Cadia purpurea</i>	AP,R	AP,R	AP,R*	–	–	–	–	–	+	+
<i>Calpurnia aurea</i>	C,AP,R	–	–	–	–	–	–	–	–	–
<i>Calpurnia intrusa</i>	C,AP,R	C,AP,R*	–	C,AP,R	–	–	–	C,AP,R*	+	+
<i>Calpurnia sericea</i>	AP,R	–	–	–	–	–	–	–	–	–
<i>Cyclopia burtonii</i>	–	–	–	–	–	–	–	C,AP,R	–	–
<i>Cyclopia buxifolia</i>	–	–	–	–	–	–	–	C,AP,R	–	–
<i>Cyclopia galioides</i>	–	–	–	–	–	–	–	C,AP,R	+	–
<i>Cyclopia genistoides</i>	–	–	–	–	–	–	–	C,AP,R	–	–
<i>Cyclopia intermedia</i>	–	–	–	–	–	–	–	C,AP,R*	+	+
<i>Cyclopia maculata</i>	–	–	–	–	–	–	–	C,AP,R	–	–
<i>Cyclopia plicata</i>	–	–	–	–	–	–	–	C,AP,R	+	–
<i>Cyclopia subternata</i>	–	–	–	–	–	–	–	C,AP,R	–	–
<i>Liparia hirsuta</i>	–	C,R	–	–	–	–	–	–	–	–
<i>Liparia myrtifolia</i>	–	R	–	–	–	–	–	–	–	–
<i>Liparia racemosa</i>	–	C,AP,R	–	–	–	–	–	–	–	–
<i>Liparia splendens</i>	–	AP,R	–	–	–	–	C	–	–	–
<i>Podalyria calyptrata</i>	–	R	–	–	–	–	–	–	–	–
<i>Podalyria hirsuta</i>	–	–	–	–	–	–	–	–	–	–
<i>Podalyria lanceolata</i>	–	–	–	–	–	–	–	–	–	–
<i>Podalyria leipoldtii</i>	–	–	–	–	–	–	AP,R	–	–	–
<i>Podalyria myrtifolia</i>	–	–	–	–	–	–	–	–	–	–
<i>Podalyria rotundifolia</i>	–	–	–	–	–	–	–	–	–	–
<i>Stirtonanthus chrysanthus</i>	–	AP,R	–	C,R	–	–	–	–	–	–
<i>Stirtonanthus insignis</i>	–	C,AP,R*	–	C,R	–	–	C,R	–	+	–
<i>Stirtonanthus taylorianus</i>	–	C,AP,R*	–	C,AP,R*	–	–	–	–	+	+
<i>Xiphosoma canescens</i>	–	C,R	–	C,R	–	–	–	–	–	–
<i>Xiphosoma phyllicoides</i>	–	–	–	R	–	–	–	–	–	–
<i>Xiphosoma tecta</i>	–	R	–	–	–	–	–	–	–	–
<i>Virgilia divaricata</i>	–	R	–	–	–	–	–	C,AP,R	–	–
<i>Virgilia oroboides</i> subsp. <i>oroboides</i>	–	R	–	–	–	–	–	C,AP,R	+	–
<i>Virgilia oroboides</i> subsp. <i>ferruginea</i>	–	R	–	–	–	–	–	C,AP,R	–	–
Crotalariaeae										
<i>Aspalathus excelsa</i>	–	R	–	R	–	–	–	–	–	–
<i>Aspalathus linearis</i>	C,R	R*	C,R*	C,R*	C,R	R*	C,R*	–	+	+
<i>Aspalathus nivea</i>	C,AP,R	C,AP,R	–	C,R	–	–	–	–	+	–
<i>Aspalathus vulnerans</i>	–	R	–	R	–	–	–	–	–	–
<i>Calobota cinerea</i>	R	C,AP,R	–	–	–	–	–	–	–	–
<i>Calobota cuspidosa</i>	–	C,AP,R*	C,AP,R	C,AP,R	–	–	–	–	+	+
<i>Calobota cytisoides</i>	–	AP,R*	–	–	–	–	–	–	+	–
<i>Calobota halenbergensis</i>	–	C,AP,R	–	–	–	–	–	–	–	–
<i>Calobota multiflora</i>	R	C,AP,R*	–	C,AP,R*	–	–	–	–	+	+
<i>Calobota psiloloba</i>	C,AP,R	C,AP,R	–	C,AP,R	–	–	–	–	–	–
<i>Calobota sericea</i>	C,AP,R	C,AP,R	–	AP,R	–	–	–	–	+	–
<i>Calobota spinescens</i>	AP,R	AP,R	–	–	–	–	–	–	–	–
<i>Crotalaria agatiflora</i>	–	–	–	–	–	–	–	–	–	–
<i>Crotalaria capensis</i>	–	–	–	–	–	–	–	–	–	–
<i>Crotalaria pallida</i>	–	–	–	–	–	–	–	–	–	–
<i>Euclora serpens</i>	–	–	–	–	–	–	–	–	–	–
<i>Lebeckia ambigua</i>	–	C,R	–	C,R	–	–	–	–	–	–
<i>Lebeckia brevicarpa</i>	C,R	R	–	–	–	–	–	–	–	–

Table 1. Continued

Tribe/species	Type of crystals								SEM	
	Prismatic	Navicular	Truncated navicular	Druse	Spherical crystal clusters	Styloids	Sand	Acicular (in sheaf-like aggregates)	BSE	EDS
<i>Lebeckia carnososa</i>	–	–	–	–	R	–	–	–	–	–
<i>Leobordea adpressa</i>	–	–	–	–	–	–	–	–	–	–
<i>Leobordea arida</i>	–	–	–	–	–	–	–	–	–	–
<i>Leobordea benthamiana</i>	–	–	–	–	–	–	–	–	–	–
<i>Leobordea magnifica</i>	–	–	–	–	–	–	–	–	–	–
<i>Leobordea mollis</i>	–	–	–	–	–	–	–	–	–	–
<i>Leobordea mucronata</i>	–	–	–	–	–	–	–	–	–	–
<i>Leobordea procumbens</i>	–	–	–	–	–	–	–	–	–	–
<i>Listia heterophylla</i>	–	–	–	–	–	–	–	–	–	–
<i>Lotononis densa</i> subsp. <i>leucoclada</i>	–	–	–	–	–	–	–	–	–	–
<i>Lotononis nutans</i>	–	–	–	–	–	–	–	–	–	–
<i>Lotononis pulchella</i>	C,AP,R	C,R	–	C,R	–	–	–	–	+	–
<i>Lotononis sericophylla</i>	–	–	–	–	–	–	–	–	–	–
<i>Lotononis trichodes</i>	C,R	C,R	C,R*	C,R*	–	–	–	–	+	+
<i>Lotononis umbellata</i>	–	–	–	–	–	–	–	–	–	–
<i>Rafnia amplexicaulis</i>	–	–	–	–	–	C	–	–	+	–
<i>Rafnia angulata</i>	–	–	–	–	–	C	–	–	+	–
<i>Rafnia capensis</i> subsp. <i>pedicellata</i>	–	–	–	–	–	–	–	–	–	–
<i>Wiborgia monoptera</i>	–	–	–	–	–	–	–	–	–	–
<i>Wiborgia obcordata</i>	–	–	–	–	–	–	–	–	–	–
<i>Wiborgiella bowieana</i>	–	–	–	–	–	–	–	–	–	–
<i>Wiborgiella leipoldtiana</i>	–	–	–	–	–	–	–	–	–	–
Genisteae										
<i>Argyrolobium argenteum</i>	–	–	–	–	–	–	–	–	–	–
<i>Argyrolobium crassifolium</i>	–	–	–	–	–	–	–	–	–	–
<i>Argyrolobium lotoides</i>	–	–	–	–	–	–	–	–	–	–
<i>Argyrolobium transvaalense</i>	–	–	–	–	–	–	–	–	–	–
<i>Dichilus gracilis</i>	–	–	–	–	–	–	–	–	–	–
<i>Dichilus reflexus</i>	–	–	–	–	–	–	–	–	–	–
<i>Dichilus strictus</i>	–	–	–	–	–	–	–	–	–	–
<i>Melolobium aethiopicum</i>	C,R	–	–	–	–	C,R*	–	–	+	+
<i>Melolobium candicans</i>	–	–	–	–	–	–	–	–	–	–
<i>Melolobium microphyllum</i>	–	–	–	–	–	–	–	–	–	–
<i>Melolobium stipulatum</i>	–	–	–	–	–	–	–	–	–	–
<i>Polhillia brevicalyx</i>	–	–	–	–	–	–	–	–	–	–
<i>Polhillia canescens</i>	–	–	–	–	C,R	–	–	–	–	–
<i>Polhillia involucreatum</i>	–	–	–	–	–	–	–	–	–	–
<i>Polhillia obsoleta</i>	–	–	–	–	C,R*	–	–	–	+	+

C, in cortex; AP, in axial parenchyma; R, in rays.

*Type of crystals were studied by X-ray microanalyses (EDS).

forming thin sheets (Fig. 1B). Navicular crystals can easily be confused with prismatic crystals when viewed directly from the side.

Druses were observed only in 19 (21%) species. They were restricted to Podalyrieae (*Stirtonanthus* B.E.van Wyk & A.L.Schutte and some species of *Amphithalea* Eckl. & Zeyh., *Calpurnia* and *Xiphotheca* Eckl. & Zeyh.) and Crotalariaeae (*Aspalathus*, some species of *Calobota*, one species of *Lebeckia* and two species of *Lotononis*). Their size varied from 5 to 15 µm.

Acicular crystals in sheaf-like aggregates were found only in 12 (13%) species of three genera of

Podalyrieae, *Calpurnia*, *Cyclopia* and *Virgilia*. These aggregates could be distinguished from ordinary crystal bundles by the divergent (not parallel) orientation of the needle-like crystals. These acicular crystals were 5–20 µm long.

Crystals of other types were rare. Styloids were found only in *Aspalathus linearis*, *Rafnia amplexicaulis*, *R. angulata* (Crotalariaeae) and *Melolobium aethiopicum* (Genisteae). These aggregates consist of styloids with blunt ends, arranged nearly parallel to one another. Spherical crystal clusters of 5–15 µm in diameter were found only in two species of Crotalariaeae (*Aspalathus linearis*, *Lebeckia carnososa*) and in

two species of Genisteae (*Polhillia canescens*, *P. obsoleta*). Crystal sand was observed in four species of Podalyrieae (*Amphithalea rostrata*, *Liparia splendens*, *Podalyria leipoldtii* and *Stirtonanthus insignis*) and only one species of Crotalariaeae (*Aspalathus linearis*).

CHEMICAL COMPOSITION OF CRYSTALS

The bark crystals of a representative selection of 23 species were examined by SEM using a BSE detector (Fig. 1J, L). The results are summarized in Table 1. As expected, crystals containing minerals appear much lighter than the background because the BSE detector shows up atoms with atomic masses that exceed those of carbon and oxygen (Fig. 1J). Under normal SEM, the crystals and the background are similar (Fig. 1K). In contrast, acicular crystals in sheaf-like aggregates (as present in *Calpurnia intrusa*, *Cyclopia intermedia*, *C. genistoides* and *C. plicata*) do not appear lighter than the background because of the absence of minerals (Fig. 1L, M). These results suggest that crystals of this type contain no atoms with an atomic mass exceeding those of carbon and oxygen, i.e. that they consist of organic molecules and do not contain calcium or magnesium. No nitrogen was detected.

To confirm these preliminary observations, the elemental composition of the crystals was also studied by X-ray microanalyses (EDS) (Fig. 2A–J). This technique shows the elements that are present and their relative proportions (but it should be kept in mind that the method is qualitative only and that relative proportions may be misleading, especially for biological samples). The results are shown in Table 1. The result for a gold-coated prismatic crystal is shown in Figure 2B (Fig. 2A shows the background result for the same sample). The results for carbon-coated samples are shown in Figure 2C–J. These samples show a weaker background noise level than the gold-coated ones due to the lower atomic mass of carbon. All the crystals (except Fig. 2J) showed peaks for calcium, as can be expected for calcium oxalate crystals. The truncated navicular crystals of *Lotononis trichodes* (Fig. 2E) and spherical crystal clusters of *Polhillia obsoleta* (Fig. 1H) also showed distinctive peaks for magnesium, indicating the co-occurrence of calcium oxalate and magnesium oxalate in their composition. Magnesium was absent in these two types of crystals in other species (*Aspalathus linearis* and *Cadia purpurea*).

For the acicular crystals in sheaf-like aggregates, EDS analysis confirmed the results obtained by BSE analysis, namely that these crystals contain only carbon and oxygen, with no minerals or other heavier elements (Fig. 2J). Additional tests were performed

on fresh bark material of *Virgilia divaricata*. Acicular crystals in sheaf-like aggregates were not found in fresh bark, but we observed their precipitation after fixation of the samples in FAA or in 70% alcohol.

CHARACTER EVOLUTION WITHIN THE GENISTOID FABACEAE

To explore possible evolutionary trends, we chose the two most common crystal types (prismatic crystals and navicular crystals) and plotted their presence or absence on an ultrametric tree. Prismatic crystals were chosen because they are widely distributed (in many legumes and indeed in many other plant families), whereas navicular crystals were chosen because of their limited occurrence (apparently limited to African genistoid legumes and Lauraceae, see below). The ultrametric tree was reconstructed from ITS data for the 'core' genistoids (Boatwright *et al.*, 2008) corrected following the combined simultaneous parsimony analysis of ITS and *rbcL* data for Podalyrieae (Boatwright *et al.*, 2008) and Crotalariaeae (Boatwright *et al.*, 2009). As the reconstruction of evolutionary trends shows (Fig. 3), prismatic crystals are an ancestral condition for genistoid legumes. This feature is present in Sophoreae and in two early diverging lineages of Podalyrieae (*Cadia* and *Calpurina+Virgilia*), but was lost in other clades. It appears that prismatic crystals arose secondarily in several lineages of Crotalariaeae and Genisteae. These sporadic reversals were found only in some genera and some species (especially common in *Calobota*). In contrast, navicular crystals (Fig. 4) appear to be a derived condition that arose independently in Podalyrieae and a few clades of Crotalariaeae, such as *Aspalathus* and *Calobota*.

DISCUSSION

Our results show considerable diversity of crystals in bark tissues of South African genistoid legumes. We found seven crystal types (prismatic, navicular, druse, spherical crystal clusters, styloids, crystal sand and acicular crystals in sheaf-like aggregates). These crystals, with the exception of navicular crystals, have previously been reported for vegetative organs of other members of Fabaceae. Crystal types previously reported for Fabaceae but not found in our sample of species include raphides, platy and bladed aggregation clusters, columnar, granular, cubic and elongate crystals (Metcalf & Chalk, 1950; Leelavathi *et al.*, 1984; Zindler-Frank, 1987; Fujii *et al.*, 1994; Gasson, 1994, 2000; Gasson, Wray & Schrire, 2004; He *et al.*, 2012a, b), (rod-shaped) and kinked crystals (Zindler-Frank, 1987; He *et al.*, 2012a, b). Four of the seven crystal types occurring in the bark of South

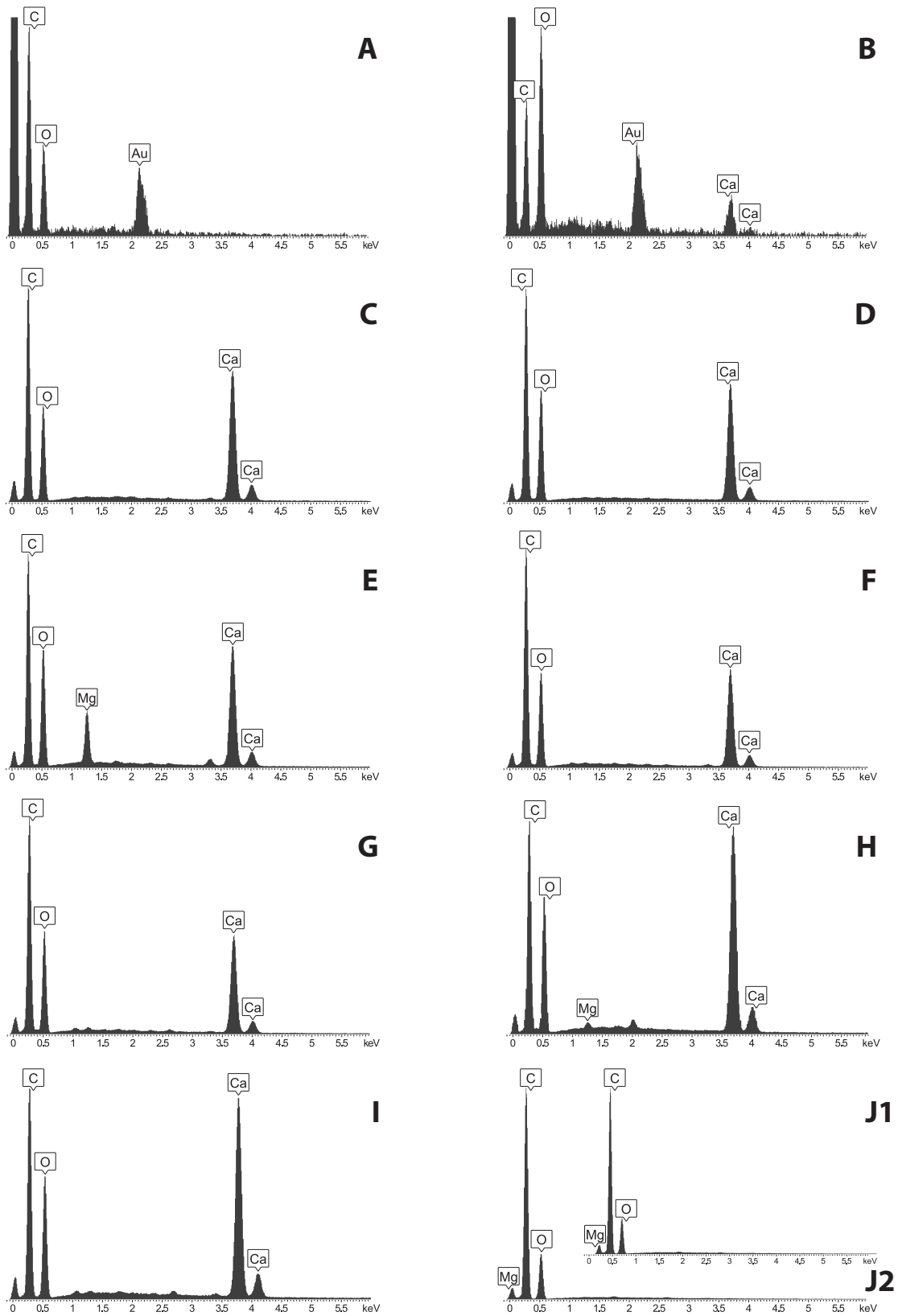


Figure 2. See caption on next page.

Figure 2. Elemental analysis of crystals in bark of African genistoid legumes performed by energy-dispersive X-ray spectroscopy (EDS). A, B, gold-coated samples of *Sophora inhambanensis* [large peak to left of carbon (C) peak is background noise]. A, EDS spectrum showing only carbon (C) and oxygen (O) peaks of cells without crystals. B, EDS spectrum showing calcium (Ca), carbon (C) and oxygen (O) peaks of prismatic crystals. C–J, carbon-coated samples [C–I spectra showing calcium (Ca), oxygen (O) and carbon (C) peaks]. C, *Calobota cytisoides*, navicular crystal. D, *Cadia purpurea*, truncated navicular crystal. E, *Lotononis trichodes*, truncated navicular crystal [note magnesium (Mg) peak]. F, *Calobota multiflora*, druse crystal. G, *Aspalathus linearis*, styloid. H, *Polhillia obsoleta*, spherical crystal cluster [note magnesium (Mg) peak]. I, *Aspalathus linearis*, crystal sand. J, acicular crystal in sheaf-like aggregate [spectrum shows oxygen (O) and large carbon (C) peaks but no Ca or Mg peaks]. J1, *Cyclopia intermedia*. J2, *Calpurnia intrusa*.

African genistoid legumes (prismatic and navicular crystals, acicular crystals in sheaf-like aggregates and crystal sand) have also previously been found in wood. The crystals were observed in ray cells of the secondary xylem of a few members of Podalyriaceae and Crotalariaeae (Stepanova *et al.*, 2013; Oskolski *et al.*, 2014). In these legumes, crystals are therefore more common in bark than in wood. A diversity of crystal types has also been reported in other families for which detailed studies are available: six types in Lauraceae (Richter, 1980), five in Oleaceae (Lersten & Horner, 2009) and five in Piperales (Horner *et al.*, 2015). Other families should be examined to allow broader comparisons at ordinal level.

It is noteworthy, however, that navicular crystals occur the bark of many South African genistoids. They were recently found in wood ray cells of two *Amphithalea* species (Stepanova *et al.*, 2013), but they have not been recorded for any other Fabaceae. Among other families, navicular crystals occur in bark of some Lauraceae [the ‘spindelförmige Kristalle’ of Richter (1980)] and in wood of some Rubiaceae (Jansen *et al.*, 1997). This crystal habit is typical for whewellite, i.e. the monohydrate form of calcium oxalate (McNair, 1932; Khan *et al.*, 2002; De Yoreo, Qiu & Hoyer, 2006).

In our results, navicular crystals are restricted to Crotalariaeae and Podalyriaceae (Fig. 4). Their presence is seemingly an ancestral trait for Podalyriaceae that was secondarily lost in *Cyclopia* and in most *Calpurnia* and *Podalyria* species. In Crotalariaeae, this trait is derived, arising independently in *Aspalathus*, *Calobota* and some *Lebeckia* and *Lotononis* species. Navicular crystals commonly occur in the same species and bark tissues as druses; just *Cadia*, *Liparia*, *Virgilia* and a few *Amphithalea* and *Xiphotheca* species have navicular crystals only, whereas *Xiphotheca phyllicoides* shows only druses, without any other crystal types. This tendency for the co-occurrence of two crystal types seems to suggest a common condition for their formation. Thus, we can expect that druses found in the bark of South African genistoids represent whewellite, the same form as in

navicular crystals. The druse habit has been reported both for the monohydrate form of calcium oxalate crystals (whewellite) and for their dihydrate form, i.e. weddellite (Franceschi & Horner, 1980; Arnott, 1982; Monje & Baran, 2002).

Prismatic crystals are common in wood of Sophoreae (Fujii *et al.*, 1994; Gasson, 1994, 2000), but they are rare in wood of Podalyriaceae and Crotalariaeae, and they are not reported in Genisteae (Stepanova *et al.*, 2013; Oskolski *et al.*, 2014). The taxonomic distribution of these crystals in bark shows a similar pattern (Fig. 3). The mapping of prismatic crystals on the phylogenetic tree suggests that their presence in bark may be confidently interpreted as the ancestral condition for genistoid legumes, with losses and multiple reversals in the crown clades corresponding to tribes Genisteae, Crotalariaeae and Podalyriaceae.

The observations using a BSE detector and X-ray microanalyses (EDS) revealed the presence of calcium (sometimes combined with magnesium) in all types of crystals except the acicular crystals in sheaf-like aggregates. Their composition of calcium oxalate is confirmed by these data. Noteworthy is the detection of magnesium in truncated navicular crystals of *Lotononis trichodes* and spherical crystal clusters of *Polhillia obsoleta*, suggesting a co-occurrence of calcium oxalate and magnesium oxalate. Magnesium oxalate is a rare component of crystals in plant cells that has been reported only on the surface of fungal lesions in leaves of *Coffea* L. (Rao & Tewari, 1989) and in spherical crystals observed by He *et al.* (2012a) in phyllodes and branchlets of *Acacia roborum* Maslin. Our observations show no relationship, however, between the types of crystals and the presence of magnesium: this element was not detected in truncated navicular crystals of *Cadia purpurea* and *Aspalathus linearis*, and also not in spherical crystal clusters of *Aspalathus linearis*. The sporadic presence of magnesium in crystals was shown to be a pathological effect in *Coffea arabica* L. (Rao & Tewari, 1989).

The occurrence of acicular crystals in sheaf-like aggregates in plant cells is an artefact. As our obser-

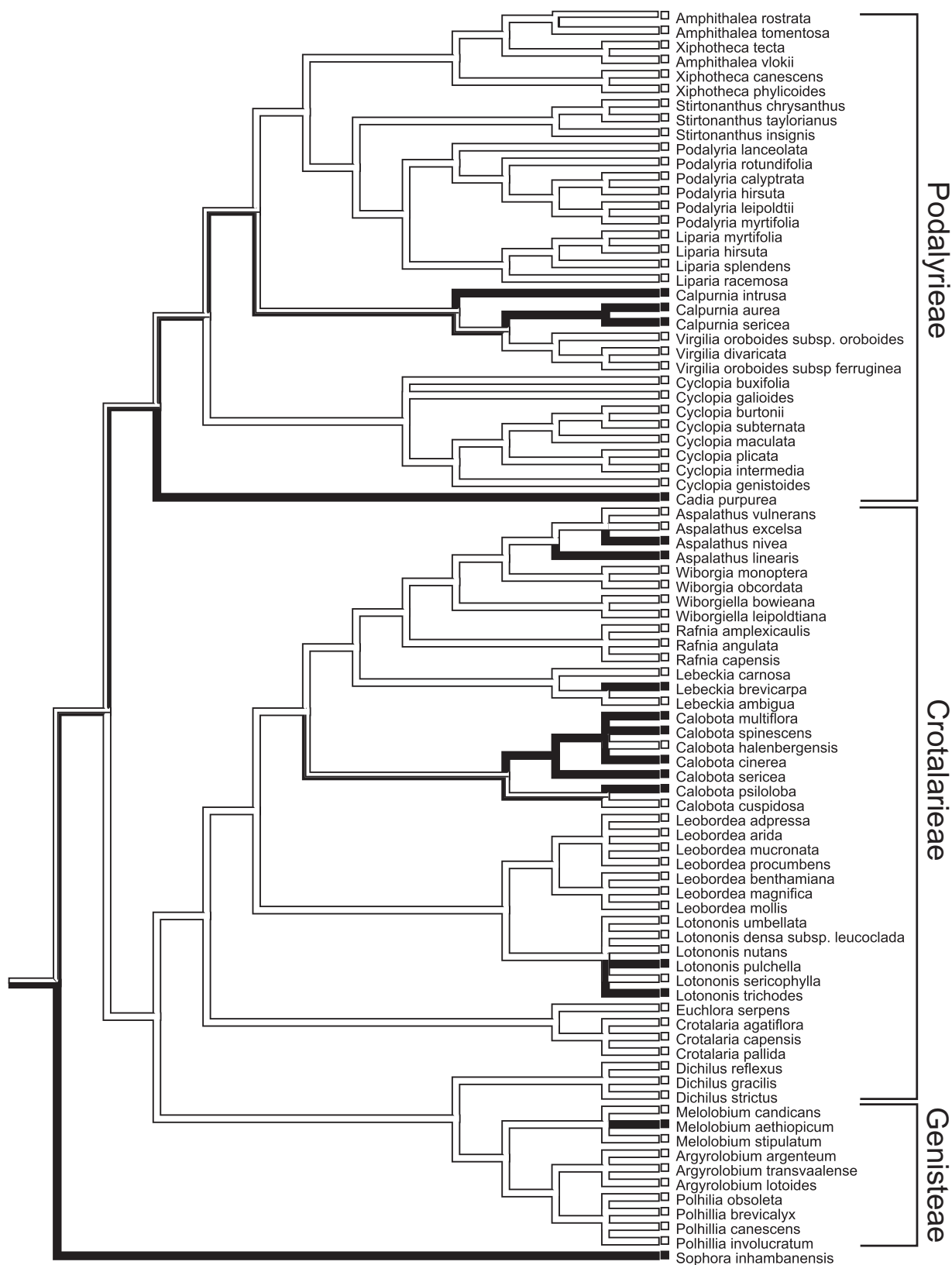


Figure 3. Prismatic crystals (black lines) in bark, mapped onto phylogenetic tree of African genistoid tribes [subsample of strict consensus tree for BI analysis of ITS and *rbcL* data, modified from Boatwright *et al.* (2008) and Boatwright, Tilney & Van Wyk (2009)].

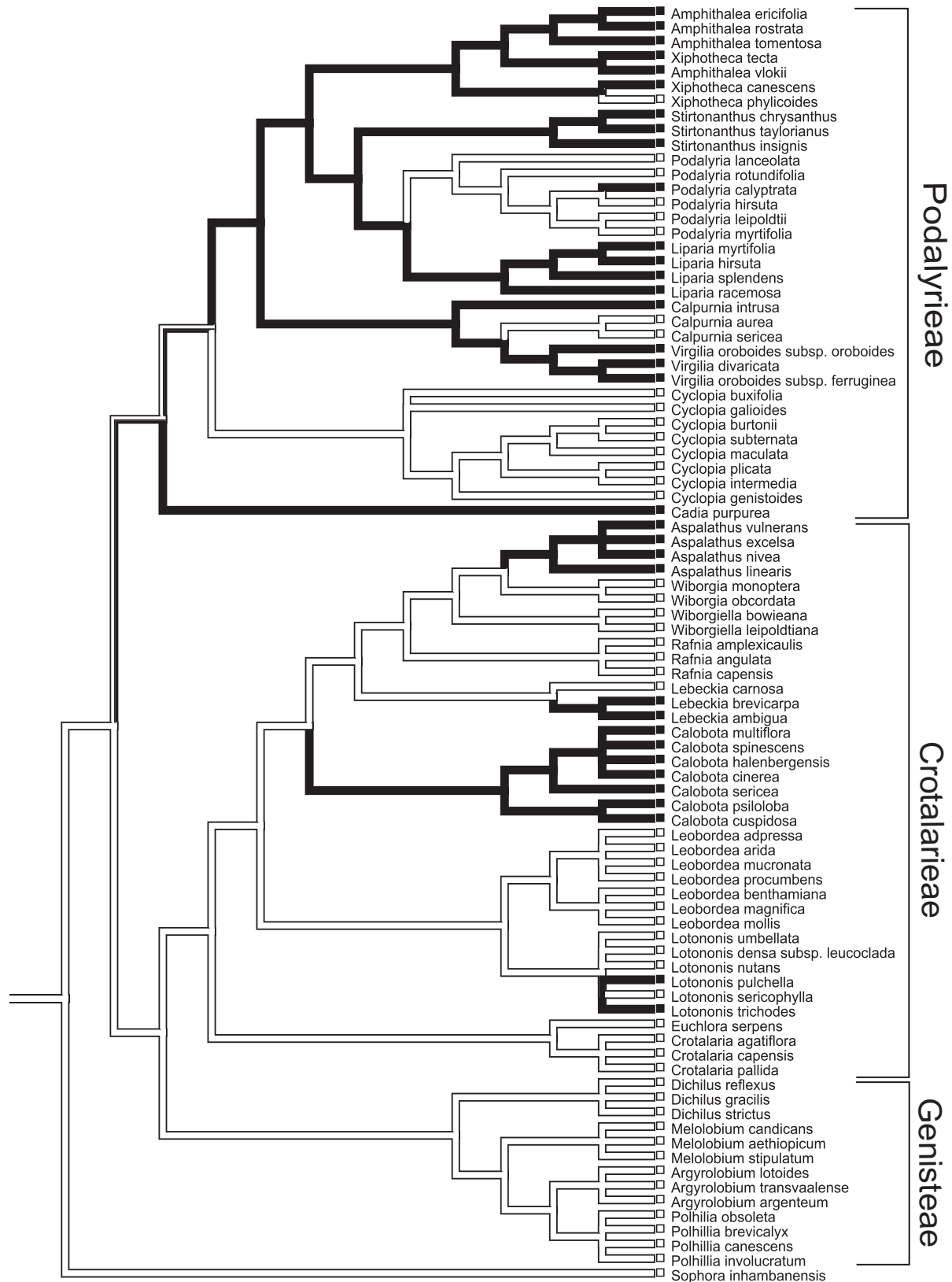


Figure 4. Navicular crystals (black lines) in bark, mapped onto phylogenetic tree of African genistoid tribes [subsample of strict consensus tree for BI analysis of ITS and *rbcL* data, modified from Boatwright *et al.* (2008) and Boatwright, Tilney & Van Wyk (2009)].

vations show, these crystals are absent in living bark tissues, and their precipitation occurs during drying of the plant material and after fixation in alcohol. It therefore seems likely that the acicular crystals reported by Kotina *et al.* (2013) in bark of *Virgilia* and by Stepanova *et al.* (2013) inside the wood vessels of some species of Podalyrieae and in the chambers of intervessel pits of *Cyclopia intermedia* are also artefacts. Nevertheless, the taxonomic distribution of this trait is consistent with the phylogenetic pattern of tribe Podalyrieae (Table 1) where acicular crystals in sheaf-like aggregates occur in two early diverged lineages, the *Cyclopia* clade and the *Virgilia*+*Calpurnia* clade (Boatwright *et al.*, 2008).

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