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Anatomy of Balkan *Amphoricarpos* Vis. (Cardueae, *Asteraceae*) taxa

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Abstract

Anatomical analysis of root, rhizome, leaf, peduncle and inflorescences has been conducted on Balkan *Amphoricarpos* taxa, *A. neumayerianus* (Vis.) Greuter subsp. *neumayerianus*, *A. neumayerianus* subsp. *murbeckii* Bošnjak, *A. autariatus* Blečić & Mayer subsp. *autariatus* and *A. autariatus* subsp. *bertisceus* Blečić & Mayer using light microscopy (LM) and scanning electron microscopy (SEM), in order to examine the anatomical traits of this genus for the first time. All taxa show similar features. Young adventitious roots share a typical structure. Sclerenchyma fibers are present in the center of older root. On the rhizome cross sections, secondary tissues are noticed with wide parenchyma rays which interrupt a-well developed xylem. Rhizomes show eccentric growth. The leaf blade is amphistomatous, with dorsiventral structure. Crystal druses are found in leaf epidermal and mesophyll cells. The peduncle cross sections is characterized by more or less polygonal shape with medullary collateral vascular bundles arranged in a circle, and a few of them outside of the circle, toward to cortex region. Secretory canals are absent. Involucral bracts and paleae are characterised by the presence of multilayer sclerenchyma in the mesophyll. Inflorescence anatomy shows structures commonly described for *Asteraceae* members. Densely distributed vermiform (lanate) and glandular biseriate trichomes are present on the peduncle and on both leaf sides, but much more on the abaxial. Anatomical uniformity indicates very close relationships between examined taxa regarding conserve nature of the genome of the genus. Obtained characters contribute to the knowledge of the genus *Amphoricarpos* anatomy.

Keywords Inflorescences · Leaf · Peduncle · Rhizome · Root

Introduction

The family *Asteraceae* comprises around 1600 – 2000 genera and 24,000 – 30,000 species with global distribution (Funk et al. 2005; Funk and Robinson 2005; Anderberg et al. 2007; Hind 2007). Members of this family are mostly annual or perennial herbaceous plants. Metcalfe and Chalk (1957) noted some particular anatomical traits, which showed to have taxonomic importance within the family, e.g., presence of

secretory and laticiferous canals, types of nonglandular and glandular trichomes, occurrence of medullar and cortical vascular bundles and presence of anomalous secondary thickening.

The genus *Amphoricarpos* Vis. is a member of subtribe Xarantheminae from tribe Cardueae (Herrando-Moraira et al. 2019). The Xarantheminae include unarmed annual or perennial herbs, rarely dwarf shrubs with entire leaves. Besides *Amphoricarpos*, four genera are included in the subtribe, namely *Chardinia* Desf., *Siebera* J. Gay, *Xeranthemum* L. and *Shangwua* Yu J. Wang, Raab-Straube, Susanna & J. Quan Liu (Wang et al. 2013; Herrando-Moraira et al. 2019). Molecular-phylogenetic analysis placed *Shangwua* and *Amphoricarpos* as successive sister to the rest of genera, while *Chardinia* was placed as sister to *Siebera* and *Xeranthemum* (Wang et al. 2013). *Amphoricarpos* is formed by heterocarpic perennial mountain chasmophytic plants from the eastern Mediterranean in the Balkans, Anatolia and the Caucasus (Susanna and Garcia-Jacas 2007). Such disjunct distribution indicates that *Amphoricarpos* taxa most likely belong to the orophytic flora of Tertiary age and thus represents part of an

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ancient flora which connect Balkans, Asia Minor and Caucasus (Turril 1929).

Amphoricarpos is a genus of complicated taxonomy. Most authors consider that the genus includes five species (Euro+ Med Plantbase, 2019 http://ww2.bgbm.org/EuroPlusMed/ PTaxonDetail.asp?NameCache=Amphoricarpos&PTRefFk= 7000000): *Amphoricarpos autariatus* Blečić & E. Mayer, *A. elegans* Albov, *A. exsul* O. Schwarz, *A. neumayerianus* (Vis.) Greuter (Greuter 2003) and *A. praedictus* Ayasligil & Grierson. In the Balkan Peninsula, populations of *Amphoricarpos* taxa inhabit crevices and fissures of limestone from central Bosnia to north-west Greece (Blečić and Mayer 1967). The type species *A. neumayerianus* (Vis.) Greuter from Mt. Orjen was described by Visiani (1844). Murbeck (1891) described a variety with broad leaves from Herzegovina as var. *velezenzis* Murbeck. Later, Bošnjak (1936) redescribed this taxon as subsp. *murbeckii* Bošnjak. According to Blečić and Mayer (1967), there are two species distributed on the Balkan Peninsula: *A. neumayerianus* with long narrow acuminate leaves distributed on the mountain ranges Orjen, Bijela Gora, and Lovćen; and broad-leafed *A. autariatus* with wider distribution range. They noted that both taxa can be additionally separated by the shape of cypselae, the cypselae wings width and the involucral bracts, especially by the nature of the apex of the involucral bracts. Moreover, Blečić and Mayer (1967) recognized two subspecies within *A. autariatus* subsp. *autariatus* Blečić & Mayer with northwestern distribution (Bosnia and Herzegovina and Montenegro) and subsp. *bertisceus* Blečić & Mayer with a more southeastern range (Montenegro, Macedonia, Kosovo, Albania and northern Greece). On the other hand, some authors have suggested that Balkan *Amphoricarpos* should be treated as a single species, *A. neumayerianus* (Caković et al. 2015). For this study, we accept four taxa in the Balkan *Amphoricarpos* (Bošnjak 1936; Blečić and Mayer 1967).

Some Balkan *Amphoricarpos* have been the subject of phytochemical (Djordjević et al. 2004, 2006; Cvetković et al. 2014, 2018), biological activity (Atrrog et al. 2008; Jadranin et al. 2013; Gavrilović et al. 2016), morphological (Petit 1997) and molecular and taxonomic studies (Caković et al. 2015).

To our knowledge, there are no anatomical investigations of *Amphoricarpos* from the Balkan complex. Also, there is only one study on the morpho-anatomy of *A. elegans* (Petit 1997), making *Amphoricarpos* almost unexplored from an anatomical point of view. In this regard, the main aim of the present study was to conduct anatomical analysis of root, rhizome, leaf, peduncle and inflorescences of *A. neumayerianus* subsp. *neumayerianus*, *A. neumayerianus* subsp. *murbeckii*, *A. autariatus* subsp. *autariatus* and *A. autariatus* subsp. *bertisceus*, in order to examine the anatomy of these taxa, as well as to explore important anatomical traits which will contribute to the anatomy of the genus.

Material and methods

Plant material

We examined Balkan *Amphoricarpos* taxa (Table 1). Parts of root, rhizome, leaf, peduncle and inflorescences from five individuals per taxon were collected from natural populations in Montenegro and Bosnia and Herzegovina during the flowering period (2013) and kept in FAA. Voucher specimens are deposited in the Herbarium (BEOU) of University of Belgrade, Faculty of Biology, Institute of Botany and Botanical Garden "Jevremovac" (Table 1).

Anatomical analysis

Temporary and permanent slides of mature roots, rhizomes, leaves, peduncles and inflorescences of adult plants were prepared. Plant parts were sectioned fresh or fixed (FAA) before preparation for a standard paraffin method (Ruzin 1999). Fresh plant material was hand sectioned with a razor blade and stained with toluidine blue (0.05%, *w/v*, aqueous) (O'Brien et al. 1964), phloroglucinol-HCl to detect lignified cell walls (Jensen 1962), and Lugol to visualise starch (Johansen 1940). In addition, parts of leaves were cleared by keeping in a mixture of glacial acetic acid and 30% hydrogen peroxide (1:1 *v/v*) at 60 °C (1 – 5 days). Leaf-blade epidermal

Table 1 List of the examined *Amphoricarpos* taxa

Taxon	Locality	Coordinates	Date	Voucher numbers (BEOU)
Amphoricarpos neumayerianus (Vis.) Greuter subsp. *neumayerianus*	Mt. Orjen (Montenegro)	N 42.55992 E 18.5514407	2013	16915
A. neumayerianus subsp. *murbeckii* Bošnjak	Mt. Čvrsnica (Bosnia and Herzegovina)	N 43.60229 E 18.16318	2013	16913
A. autariatus Blečić & Mayer subsp. *autariatus*	Tara River Canyon (Montenegro)	N 43.214097 E 19.059158	2013	16883
A. autariatus subsp. *bertisceus* Blečić & Mayer	Mt. Visitor (Montenegro)	N 42.6230556 E 19.8849999	2013	16918

prints were made using transparent nail polish and adhesive tape. Paraffin method was applied for preparing cross-sections (8 – 10 μm thick) of middle parts of mature roots, rhizome, leaf, peduncle and inflorescences, as well as longitudinal sections of inflorescenes (8 – 10 μm thick). Sections were double stained in Safranin O (1 %, w/v, 50 % ethanol) and Alcian blue (1 %, w/v, aqueous) and mounted using Canada balsam. The permanent slides are storaged in the Department of Morphology and Systematics of Plants, University of Belgrade, Faculty of Biology. Observations of the obtained microslides were performed on a light microscope Leica DM2000 equipped with a digital camera Leica DFC320 and a computer with the imaging software Leica IM 1000.

Micromorphological methods

Micromorphological analysis was carried out using scanning electron microscopy (SEM). Small parts of dry leaves were sputter-coated with gold for 180 s at 30 mA (BAL-TEC SCD 005), and observed using a JEOL JSM-6460LV electron microscope at an acceleration voltage of 20 kV.

Results

Root

Amphoricarpos taxa are rhizomatous perennial herbs and develop adventitious roots. The cross section is rounded in outline (Fig. 1a–d). Roots show typical structure, with rhizodermis on its surface and parenchyma cortex below, which is a dominant part of the root (Fig. 1a–d). Exodermis, first layer of cortex, is noticed. Parenchyma cells are rich in starch (Fig. 1a, b, d). In the center of the young root parenchyma cells are noticed (Fig. 1b, c), surrounded with xylem, while in the center of the older root sclerenchyma fibers, surrounded with xylem, are visible (Fig. 1a, d). In later stage, roots show secondary structure. Roots lack secretory canals (Fig. 1a–d).

Rhizome

On cross-section, rhizomes are more or less round (Fig. 2a, c, e), or irregular in shape (Fig. 2g). On the rhizome cross-
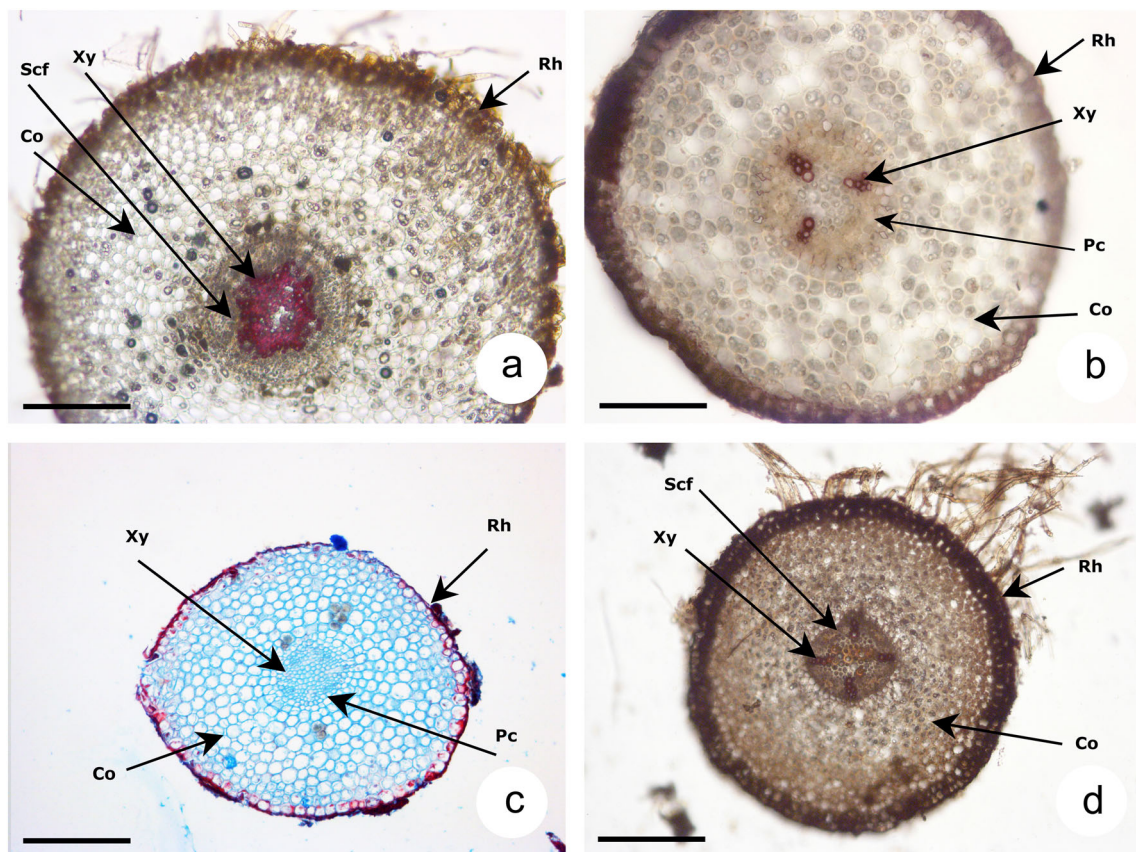
Fig. 1 Cross-sestions of the adventitious roots of *A. neumayerianus* subsp. *neumayerianus* (**a**), *A. autariatus* subsp. *autariatus* (**b**), *A. autariatus* subsp. *bertisceus* (**c**) and *A. neumayerianus* subsp. *murbeckii* (**d**). **b**, **c**. Young root with parenchyma cells in the center. **a**-**d**. Older root with sclerenchyma fibers in the center. Abbreviations: Co = cortex; Pc = parenchyma cells; Rh = rhizodermis; Scf = sclerenchyma fibers; Xy = xylem. *Bar* = 200 μm

Fig. 2 Cross-sections of the rhizomes *A. neumayerianus* subsp. *neumayerianus* (**a-b**), *A. autariatus* subsp. *autariatus* (**c-d**), *A. autariatus* subsp. *bertisceus* (**e-f**) and *A. neumayerianus* subsp. *murbeckii* (**g-h**). **a**, **c**, **e**, **g** General rhizome anatomy showing secondary structures and pith. **b**, **d**, **f**, **h** Detail of the rhizome anatomy showing periderm, parenchyma cortex, secondary phloem, well developed xylem and wide parenchyma rays. Abbreviations: Co = cortex; Pe = periderm; Pi = pith; Pr = parenchyma rays; Sph = secondary phloem; Sxy = secondary xylem. *Bar* = 500 μm in a, c, e and g; 200 μm in b, d, f and h
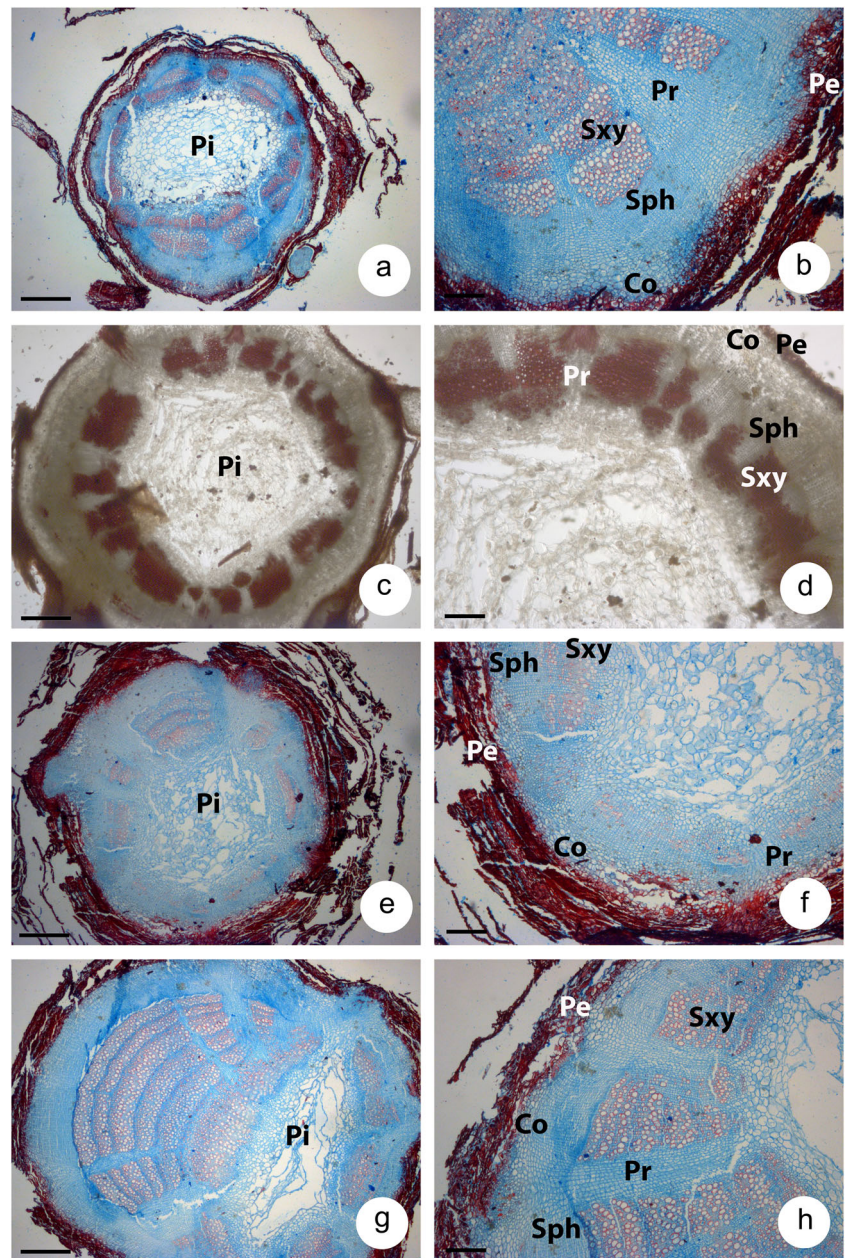
sections secondary tissues are noticed (Fig. 2a, c, g, e) with well developed periderm on its surface and a narrow zone of parenchyma cortex below (Fig. 2b, d, f, h). In the central cylinder, well developed xylem is noticed (Fig. 2b, d, f, h) interrupted by wide parenchyma rays (Fig. 2b, d, f, h). Rhizomes show eccentric growth (uneven growth of xylem rings) (Fig. 2g). The vascular rays are homocellular and mostly multiseriate. A pith, composed of very large parenchyma cells, is noticed in the central region (Fig. 2a, c, e, g). Rhizomes lack secretory canals.

Leaf

On the leaf cross section, the main vein is heart-shaped with two ribs (Fig. 3a, c, e, g). Adaxial epidermis is one-layered and covered with thick cuticle (Fig. 3b, d, f, h, i). The cells of adaxial epidermis are visibly larger compared to the cells of abaxial epidermis (Fig. 3b, d, f, h). Epidermal cells are irregularly polygonal in shape (Fig. 4a–d). Outer periclinal cell walls are convex and smooth (Fig. 4a–d). Anticlinal cell walls are straight (Fig. 4a–d). The leaf blades are amphistomatous (Fig. 3b, d,

f, h). Raised stomata could be noticed, predominantly on abaxial side (Fig. 3b, d, f, h), while their presence on the adaxial side is extremely rare (Fig. 3b). The leaf blade has dorsiventral structure (Fig. 3b, d, f, h). Below adaxial epidermis, two-layered palisade tissue, consisted of large cells rich in chloroplasts, and spongy tissue of several layers of polygonal cells and large intercellular spaces is visible (Fig. 3b, d, f, h). Collateral closed vascular bundles, surrounded by parenchyma tissue, arranged in a row, are noticed in the central leaf blade plane (Fig. 3b, d, f, h). One vascular bundle, or one large and two small vascular bundles are present in the main vein, with a surrounding parenchyma sheath which extended to both epidermises (Fig. 3a, c, e, g). On the adaxial side, subepidermal collenchyma is noticed, whereas on the abaxial side it alternated with chlorenchyma (Fig. 3a, c, e, g). The abaxial epidermis is one-layered and covered with a thinner cuticle compared to adaxial epidermis. Leaves lack of secretory canals.

Druses (CaOx crystals) of irregular shape can be noticed in both adaxial and abaxial epidermal cells of the leaves (Fig. 3i, j). These crystals are especially visible inside the adaxial epidermal cells (Fig. 3j). They are also observed in some leaf mesophyll cells (Fig. 3i).

Curly, nonglandular trichomes, cover both leaf surfaces, but much more so abaxial side, forming a velvety indumentum (Fig. 5a, b). These trichomes are vermiform (lanate). Glandular trichomes, seen as glandular dots, are also evident on both leaf surfaces, but much more so on the abaxial side (Fig. 5a, b). Glandular trichomes are of the biseriate type (Fig. 6a–c).

Peduncle

Peduncle cross section has irregular polygonal shape (Fig. 7a, c, e, g). One-layered epidermis consists of oval to isodiametric cells is on the surface (Fig. 7b, d, f, h). Below epidermis there is a cortex made up of collenchyma and chlorenchyma, alternately arranged. Prominent ribs contain collenchyma tissue, while chlorenchyma occurs between the ribs (Fig. 7b, d, f, h). The vascular bundles are collateral and arranged in a circle (Fig. 7a, c, e, g) separated from one another by a parenchyma tissue (Fig. 7b, d, f, h). Well-lignified sclerenchyma tissue surrounds each vascular bundle. A few vascular bundles ("cortical" vascular bundles) are positioned outside the circle, near cortex (Fig. 7b, c, f, h). A clearly visible endodermis layer, which contains starch, separates cortex from the central cylinder (Fig. 7h). There is a pith, composed of large parenchyma cells, in the central region of the peduncle (Fig. 7b, d, f, h). Peduncles lack secretory canals.

Inflorescence

The cross and longitudinal sections of the inflorescence show different stages of flowers and fruits development (Fig. 8a–h). Involucral bract has one-layered epidermis, below a one-layer hypodermis, or hypodermis is absent, and a highly developed multilayer sclerenchyma tissue (Fig. 8b, d, f). Below sclerenchyma, a parenchyma tissue with vascular bundles is present. Receptacular bracts or paleae show similar anatomy to involucral bract anatomy (Fig. 8b, d, f). In the early stages of ovule development, stamens with polen grains are observed (Fig. 8g). Ovary is inferior, bicarpelar, syncarpous, and unilocular (Fig. 8b, f, g, h). The ovule is anatropous, unitegmic, and tenuinucellate with basal placentation (Fig. 8g). The outer and inner pericarp epidermis is uniseriated, whereas two regions could be seen in the mesophyll (Fig. 8g).

Amphoricarpos taxa are heterocarpic plants: immature inner cypselae, derived from central hermaphrodite florets (Fig. 8b, d, f), and immature outer cypselae, derived from peripheral female florets, can be observed (Fig. 8f, h). Immature fruit contains a pericarp layer, composed of uniseriated epidermis, groups of sclerenchyma fibers and parenchyma cells (Fig. 8d), and testa layer, composed of testa epidermis with lignified cells and crashed cells below (Fig. 8d). The cells of the pericarp parenchyma have more or less the same structure throughout the pericarp and in the mature state they are rich in intercellular space (Fig. 8d). Phytomelanin layer is not observed. The endosperm, which surrounds the embryo, is composed of one to three cell layers (Fig. 8a, d). The mature embryo is axial and occupies the whole seminal chamber (Fig. 8a, d). The embryo axis is straight, and the plumule is poorly differentiated (Fig. 8a). Two plano-convex cotyledons are noticed (Fig. 8a).

Discussion

Amphoricarpos taxa show thickened underground organs, a rhizome, which produces adventitious roots. In general, anatomical studies which included roots and rhizomes were mainly focused on those species that possess some secretory structures and have potential application in pharmacy. Cury and Appezzato-da-Glória (2009) thoroughly analyzed and documented different secretory structures in thickened underground organs (root, xylopodium, underground stem) of six *Asteraceae* species belonging to the different tribes. Anatomical investigations of underground organs of Cardueae are scarce (Fritz and Saukel 2011a, b), and most studies deal with South American species, especially those which contain secretory ducts and cavities (Melo-de-Pinna and Menezes 2002; Appezzato-da-Glória et al. 2008). Ginko

Fig. 3 Cross-sestions of the leaves of *A. neumayerianus* subsp. *neumayerianus* (**a**, **b**), *A. autariatus* subsp. *autariatus* (**c**, **d**), *A. autariatus* subsp. *bertisceus* (**e**, **f**) and *A. neumayerianus* subsp. *murbeckii* (**g**–**j**). **a**, **c**, **e**, **g**, **i** Heart-shaped main vein, with one, or three vascular bundles, and two ribs. **b**, **d**, **f**, **h** Detail of the leaf anatomy showing amphistomatous structure and raised stomata (*arrowheads*). **i**, **j** Crystals of irregular shape (*arrowheads*). Abbreviations: Abe = abaxial epidermis; Ade = adaxial epidermis; Pp = palisade parenchyma; Sp = spongy parenchyma; Vb = vascular bundle. *Bar* = 200 μm in a-b-c-d-e-f-g-h; 100 μm in i-j
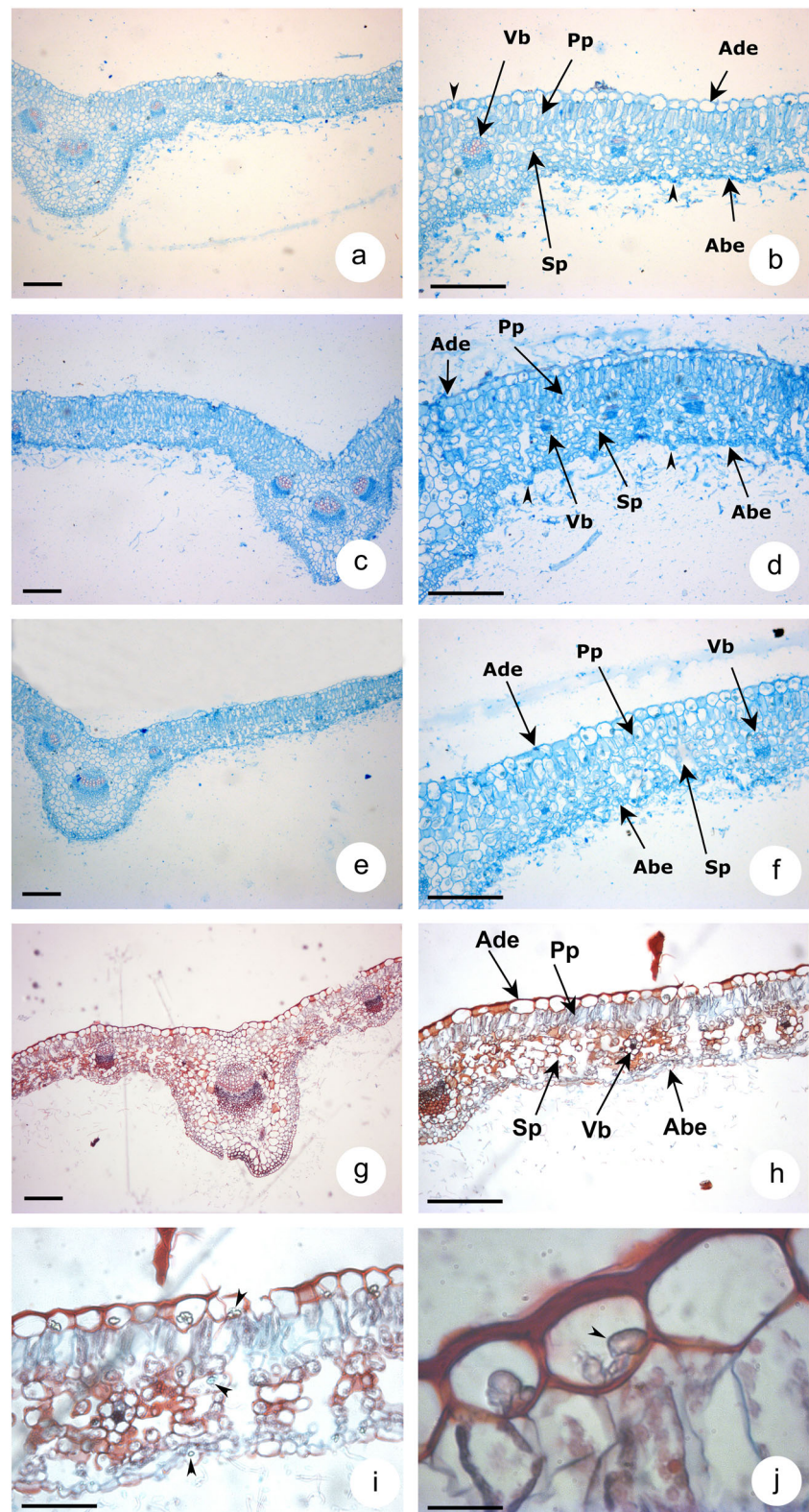
et al. ([2016](#)) tested suitability of root and rhizome anatomical characters for taxonomic classification and phylogenetic reconstruction of 59 species from tribes Cardueae and Cichorieae, although selection of taxa was based on pharmaceutical importance. They concluded that most anatomical features showed at least some degree of homoplastic evolution, thus limiting their suitability as phylogenetically informative characters. Fritz and Saukel

Fig. 4 Light micrographs of the adaxial epidermis of *A. neumayerianus* subsp. *neumayerianus* (**a**), *A. autariatus* subsp. *autariatus* (**b**), *A. autariatus* subsp. *bertisceus* (**c**) and *A. neumayerianus* subsp. *murbeckii* (**d**). Frontal view of adaxial epidermis showing striaght anticlinal walls. *Bar* = 100 μm
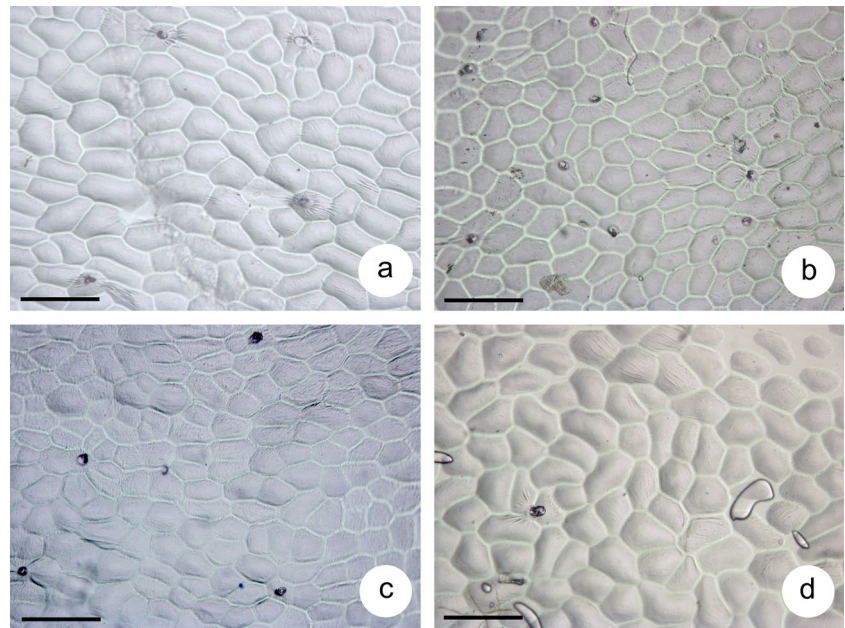
(2011c) documented the presence of interxylary cork in the roots of two *Saussurea* DC. species. Interxylary cork originates in the secondary xylem and involves the development of a periderm which separates the originally xylem cylinder. Thereafter, the root is splitting into various strands (Fritz and Saukel 2011c). However, more comparative taxonomic studies of the root/rhizome anatomy of other Cardueae species are needed.

Presence of meristematic endodermis and secretory canals is related to adventitious roots in *Asteraceae* (Williams 1947; Melo-de-Pinna and Menezes 2002). According to Williams (1947), who studied a number of different plant species, the meristematic endodermis produces tissues between the endodermis and the hypodermis. It was documented that in roots of *Ianthopappus corymbosus* (Less.) Roque & D.J.N.Hind (Mutisieae, *Asteraceae*), the meristematic endodermis forms more than 2/3 of the cortex, while the hypodermis, at early differentiation stages, produces the external part of the cortex (Melo-de-Pinna and Menezes 2002). Likewise, in the species of *Richterago* (Melo-de-Pinna 2000), meristematic endodermis only forms the inner cortex. Also, in *Ianthopappus*

corymbosus, the meristematic endodermis has a main role in the formation of secretory canals (Melo-de-Pinna and Menezes 2002). Moreover, epithelial organization of the secretory canals cells of adventitious roots support the exclusion of *I. corymbosus* from the genus *Richterago* (Melo-de-Pinna and Menezes 2002). In the present work, we didn't notice meristematic endodermis, as we used older adventitious roots for sectioning, where cortex is already developed. Although root secretory canals segregating lipophilic substances are common in *Asteraceae* (Fahn 1979; Cury and Appezzato-da-Glória 2009; Janaćković et al. 2019), as well as in some Cardueae species (Metcalfe and Chalk 1950; Williams 1947), *Amphoricarpos* lacks secretory canals in the roots. The same character, lack of root secretory canals, was documented for two related *Xeranthemum* species (Gavrilović et al. 2019a).

Rhizome, although showing secondary structures, retained primary cortex in the examined *Amphoricarpos* taxa. Rhizome anatomy could be considered as typical, except for observed eccentric growth. Evans et al. (2012) reported the interesting phenomenon of eccentricity in the stems of *Artemisia tridentata* Nutt. They showed that some

Fig. 5 Scanning electron micrographs of the abaxial leaf blade epidermis of *A. autariatus* subsp. *bertisceus* (**a**, **b**) showing densely distributed vermiform (lanate) trichomes and glandular trichomes, seen as glandular dots (*arrows*)
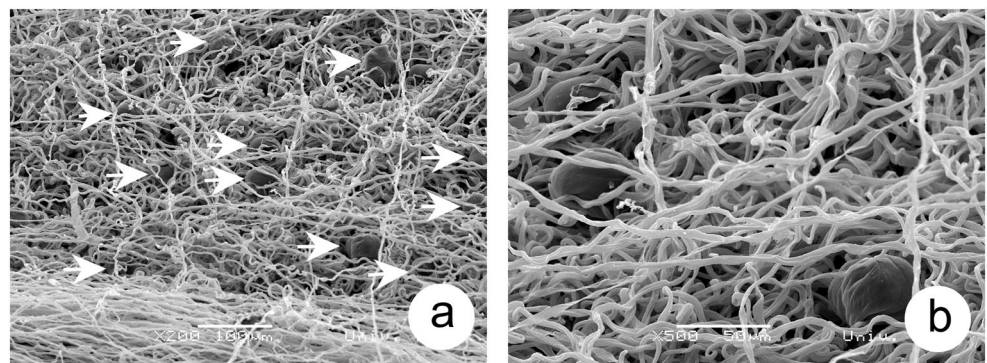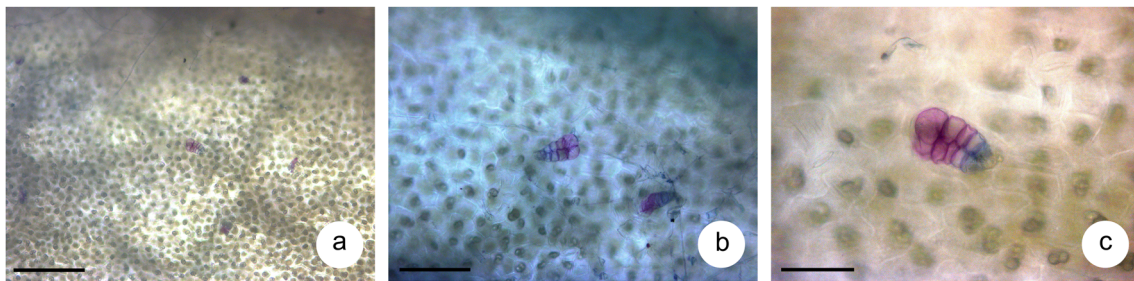
Fig. 6 Adaxial leaf blade epidermis of *A. autariatus* subsp. *bertisceus* (**a–c**) showing crystals inside the cells and biseriate glandular trichomes on the surface. *Bar* = 200 μm in a; 100 μm in b; 50 μm in c

determinate flowering branches growth causes the death of the vascular cambium which surrounds their attachment points on the main stem. This death results in the eccentric growth of the

stem. Furthermore, they suggested that this peculiar eccentric growth phenomenon, which is not associated with interxylary cork, supports the hypothesis that *A. tridentata* descends from

Fig. 7 Cross-sestions of the peduncles of *A. neumayerianus* subsp. *neumayerianus* (**a**, **b**), *A. autariatus* subsp. *autariatus* (**c**, **d**), *A. autariatus* subsp. *bertisceus* (**e**, **f**) and *A. neumayerianus* subsp. *murbeckii* (**g**, **h**). **a**, **c**, **e**, **g**. General peduncle anatomy. **b**, **d**, **f**, **h** Detail of the peduncle anatomy showing epidermis, cortex, medullary vascular bundles and a few "cortical" vascular bundles (*arrows*). **h** Endodermis layer with starch separated central cylinder from cortex (*arrowhead*). Abbreviations: Chl = chlorenchyma; Col = collenchyma; Ep = epidermis; Pi = pith; Vb = vascular bundles. *Bar* = 200 μm
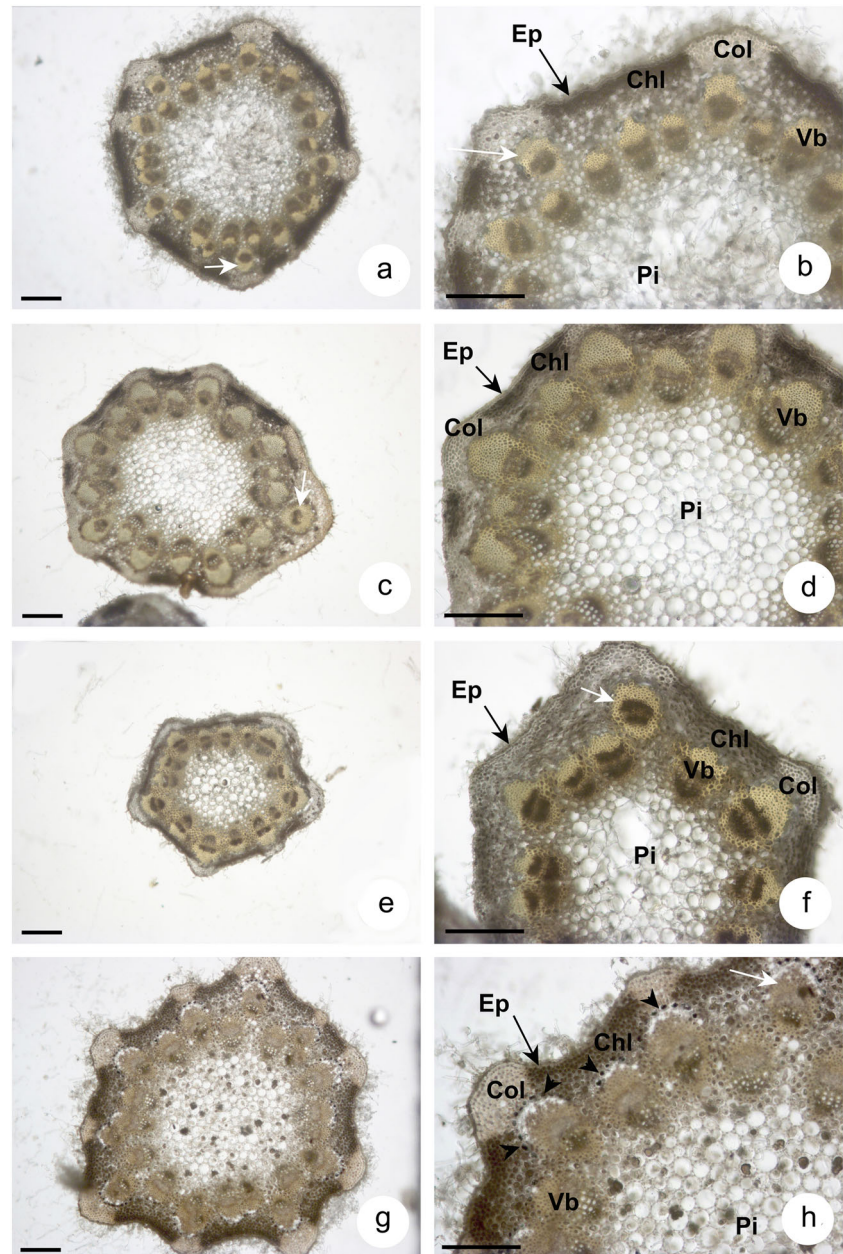
Fig. 8 Inflorescence cross sections (**a**, **c**, **e**, **g**) and longitudinal sections (**b**, **d**, **f**, **h**) of *A. neumayerianus* subsp. *neumayerianus* (**a**, **b**), *A. autariatus* subsp. *autariatus* (**c**, **d**), *A. autariatus* subsp. *bertisceus* (**e**, **f**) and *A. neumayerianus* subsp. *murbeckii* (**g**, **h**). Abbreviations: Cr = crashed cells; En = endosperm; Ep = pericarp epidermis; Et = testa epidermis; Ib = involucral bracts; IC = inner cypsela; OC = outer cypsela; Ova = ovary; Ovu = ovule; Pa = Palea; Pc = Parenchyma cells; Re = receptaculum; Sf = sclerenchyma fibers; Tvb = testa vascular bundles. *Bar* = 500 μm in a,c,e and g; 200 μm in b,d,f and h
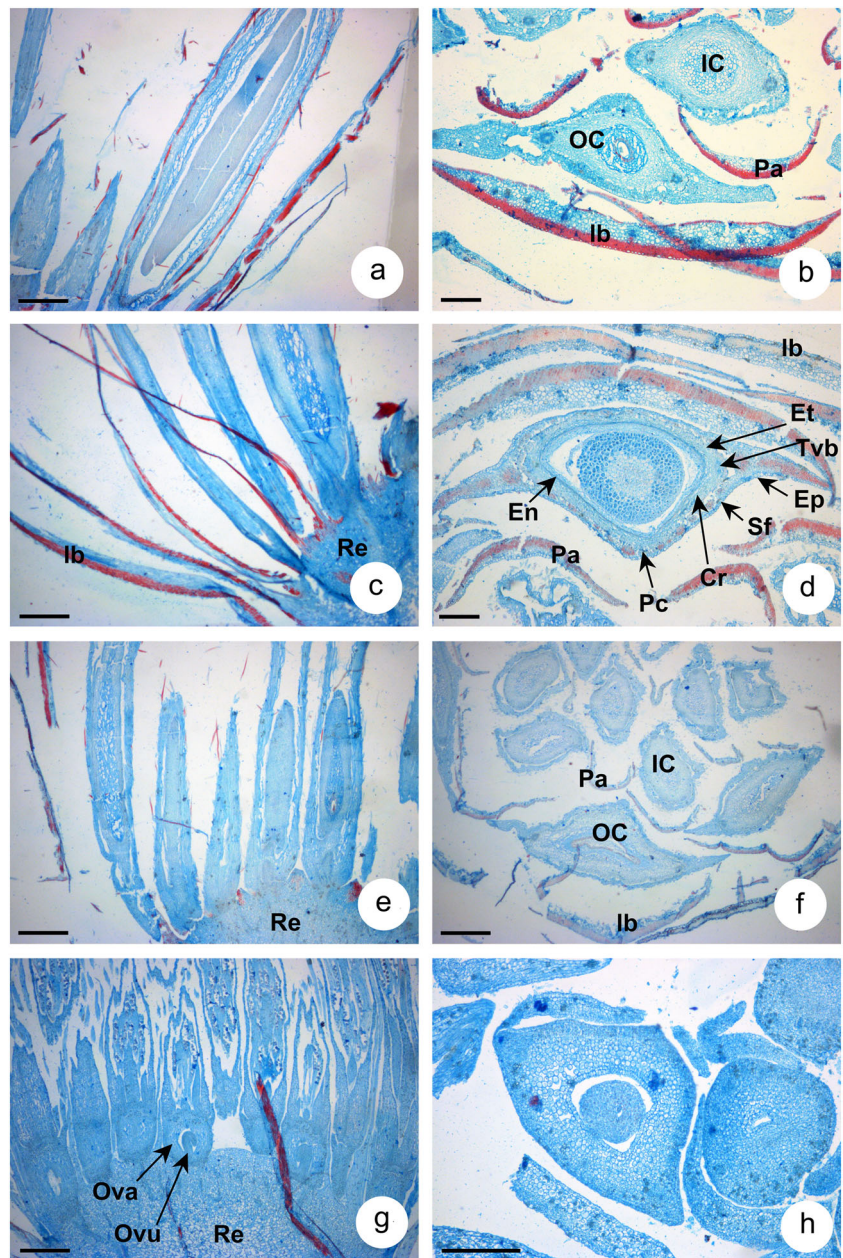
an herbaceous ancestor and has evolved secondarily imperfect woodiness. However, eccentric growth documented in rhizomes of *Amphoricarpos* do not agree with that kind of scenario. Namely, in the Xerantheminae, our favored hypothesis, also based on other results (Garnatje et al. 2004; Barres et al. 2013; Gavrilović et al. 2019a, b), is that the annual habit in *Xeranthemum*, *Chardinia* and *Siebera* is a secondary adaptation to arid climates from mesophilous perennial ancestors. Another evidence came from Omer and Moseley (1981) who studied the vegetative anatomy of *Jaumea carnosa* (Less.) A. Gray and found very broad parenchymatous areas that develop in the secondary tissues of the rhizome. Omer and Moseley (1981) connected these parenchymatous areas to a slower activity of vascular cambial cells, which forms

depressions in the axis. Broad parenchymatous areas were also recorded in examined *Amphoricarpos* taxa. It was also concluded that adventitious roots in the rhizomes develop from ground tissues near to the primary xylem and from interfascicular regions close to the primary phloem (Omer and Moseley 1981).

Peduncle anatomy of the examined taxa was typical one described for the *Asteraceae* (Metcalfe and Chalk 1957) and related *Xeranthemum* species (Gavrilović et al. 2019a). We observed several so called "cortical" vascular bundles outside the circle consisted of medullary vascular bundles. According to Metcalfe and Chalk (1950), the presence of medullar and cortical bundles is important for taxonomy. Makbul et al. (2012) found cortical vascular bundles in the stem of

Scorzonera ahmet-duranii Makbul & Coşkunç, but not in the stem of related *S. semicana* DC., reinforcing the taxonomic importance of these bundles. In another study, Makbul et al. (2016) found small cortical bundles among the main large vascular bundles of Turkish species of *Scorzonera*. However, they stated that there is no correlation between this feature and the subgeneric or sectional taxonomy (Makbul et al. 2016). Occurrence of cortical and medullar vascular bundles is also documented in *Centaurea sadleriana* Janka (Luković et al. 2013), *Ianthopappus corymbosus* (Melo-de-Pinna and Menezes 2002) and *Xeranthemum annuum* L. (Gavrilović et al. 2019a). Melo-de-Pinna and Menezes (2002) distinguished cortical vascular bundles from leaf traces regarding xylem and phloem position; in the cortical vascular bundles the xylem is outside the phloem, whereas in leaf traces xylem is oriented to the organ axis. But, are these cortical vascular bundles really positioned in the cortex of examined *Amphoricarpos* taxa? In a simple histochemical test, we confirmed that endodermis, which cells are rich in starch, surrounds all vascular bundles. Moreover, in these bundles, xylem is oriented to the organ axis. Thus, one should check xylem/phloem orientation in the bundles and use term "cortical" with caution, as these bundles are within central cylinder, just outside the circle, although term cortical was freely used throughout literature. Anyhow, presence of these bundles might have taxonomic value, but other related taxa from subtribe Xerantheminae should be examined anatomically.

All the studied taxa lack secretory organs in the peduncle, as was also documented for related *Xeranthemum* species (Gavrilović et al. 2019a). Most genera of Cardueae have only laticifers or have no secretory organs in the aerial parts, e.g. *Xeranthemum*, *Siebera*, *Chardinia*, *Cardopatium* Juss. etc. (Dittrich 1996; Gavrilović et al. 2019a). Thus, lack of secretory organs inside the plant body connect *Amphoricarpos* and *Xeranthemum* taxa.

The taxonomic value of leaf epidermal characters is very well documented in *Asteraceae* (Barthlott 1981; Adedeji and Jewoola 2008; da Silva et al. 2014; Karanović et al. 2015; Gavrilović et al. 2019b). Epidermal cells in examined taxa lack ribbed thickenings of outer periclinal cell walls, as was also documented for *Amphoricarpos exsul* and *Shangwua denticulata* (DC.) Raab-Straube & Yu J. Wang (Gavrilović et al. 2019b). This feature is most likely associated to the mesophytic, mountane habitats of these species. In all the studied taxa, epidermal cells are polygonal in shape, as also documented for *A. exsul*. Anticlinal epidermal cell walls in the examined taxa are straight, while those in *A. exsul* are slightly sinuate (Gavrilović et al. 2019b) and sinuate in *A. elegans* (Gavrilović et al. 2018).

Although leaf anatomical features are often related with the environment, they are under genetic control and thus have taxonomic value (Anderson and Creech 1975). Presence of thick cuticle, which we documented on the adaxial surface

of all examined taxa, might indicate xeromorphy (Anderson and Creech 1975). On the other hand, leaves of studied taxa have dorsiventral leaf structure, which is common in *Asteraceae* members (Duarte et al. 2011; Oliveira et al. 2011). This trait is particularly important for the phylogeny of the Xerantheminae, as it is conserved in two *Xeranthemum* species (Gavrilović et al. 2019a) that grow at open, arid habitats. The hypothesis that *Xeranthemum* species may have originated from mesophylous ancestors is probably true.

Morphology and distribution of crystals are considered genetically controlled by the cell (Meric 2009) and could be useful in taxonomic evaluation of certain taxa. According to Prychid and Rudall (1999) druses are common in dicotyledons. Meric (2008) documented druses in leaves (epidermis and mesophyll) of *Conyza canadensis* (L.) Cronquist and *C. bonariensis* (L.) Cronquist. Crystals were found in the epidermal cells of *Gleditsia triacanthos* L. (Borchert 1984) and in the leaflets of *Stylosanthes guianensis* (Aubl.) Sw. (Brubaker and Horner 1989). Wu and Kuo-Huang (1997) also recorded druses in the leaf epidermal cells of *Artocarpus altilis* (Parkinson ex F.A.Zorn) Fosberg and in the mesophyll cells of *A. altilis*, *Cudrania cochinchinensis* (Lour.) Yakuro Kudo & Masam., *Ficus virgata* Reinw. ex Blume and *Morus australis* Poir. Meric (2009) found druses in the leaf mesophyll layers of *Aster squamatus* (Spreng.) Hieron. but not in the leaf epidermal cells. In addition, Lersten and Horner (2000) reported that druses in the leaves of *Prunus* L. species can be useful for the vague taxonomy of this genus. Kuo-Huang et al. (2007) mentioned that druses from the palisade cells of *Peperomia glabella* (Sw.) A.Dietr. are involved in the photosynthetic process and that the diameter of the crystals was correlate to the light intensity. Moreover, druses provide structural support to the tissues and are involved in Ca regulation (Nakata 2003). In the leaves of related *Xeranthemum* species, we did not found these crystals (Gavrilović et al. 2019a). Thus, further anatomical investigations of other *Amphoricarpos* and related taxa from subtribe Xerantheminae are needed for understanding and evaluating taxonomic importance of druses.

Indumentum traits represent valuable characters in taxonomy (Hayat et al. 2009). We have shown here that all of the examined taxa possess hairy peduncles and a woolly indumentum on both leaf surfaces, but it much more densely on the abaxial side. It was shown that most Carduinae taxa possess a woolly indumentum (Häffner 2000). Trichomes could be categorized as unicellular or multicellular, composed of one to a few basal cells and a long filiform terminal cell; rarely, trichomes are large, uniseriate and multicellular (Häffner 2000). Capitate glandular trichomes are found on peduncles and on leaves of all of the studied species, but they are much more numerous on the abaxial leaf surface. In *Asteraceae*, a widely distributed feature on stems and leaves is short-stalked capitate glandular trichomes, seen as glandular

dots (Robinson 2009). Glandular trichomes are often formed by a biseriate peduncle, usually formed by five pairs of cells (Ciccarelli et al. 2007), and a head, formed from one to many cells. Development of glandular trichomes was described in *Artemisia annua* L. (Duke and Paul 1993; Duke et al. 1994) and in *A. campestris* subsp. *maritima* (DC.) Arcang. (Ascensão and Pais 1987). Analysing glandular trichomes of *Grindelia pulchella* Dunal, Bartoli et al. (2011) recorded that the secretion product is accumulated between the cell wall and the cuticle, which breaks and releases the secretion. It is documented that the secretion is composed of different specialized metabolites, e.g. essential oils, sesquiterpene lactones, pectin-like substances, flavonoids, etc. (Ascensão and Pais 1987, 1988; Pagni et al. 2003; Andreucci et al. 2008; Lusa et al. 2016; Gavrilović et al. 2018). However, glands are absent in some Carduinae s. l. species (Häffner 2000). A dense glandular indument, interpreted as an ancient adaptation to xeric habitats, was found in the genus *Phalacrachena* Iljin from Siberia (Susanna et al. 2011). On the leaf surfaces of the *A. elegans* both nonglandular as well as rare glandular capitate trichomes are recorded (Gavrilović et al. 2018).

Some floral anatomical and morphological traits, e.g., pappus form, branchs of the style, morpho-anatomy of the corolla and anthers, are useful for the classification of the taxa within the tribes of *Asteraceae* (Judd et al. 2002). However, historically *Asteraceae* taxa represents difficulties for ontogenetic studies (Dadpour et al. 2012), and the papers regarding floral and inflorescence anatomy is rare. Batista and De Souza (2017) examined the floral ontogeny of ten *Asteraceae* species and showed that flower characters are useful in distinguishing species. Also, Franca et al. (2015) investigated embryology of two *Ageratum* L. species and confirmed heterogeneity of embryological processes within the family. According to Palser (1975) and Stuessy (2009) embryological characters could be important in perceiving taxonomic relationships among families, genera and taxa. Regarding Xerantheminae, Dadpour et al. (2012) analyzed flower morphology of *X. squarrosum* Boiss. and found substantial differences in development between female and perfect florets. Also, Harris (1995) in his comprehensive micromorphological work on florets and inflorescences development of 39 *Asteraceae* taxa, included *X. annuum*. A well-developed multilayer sclerenchyma present in bracts and palea of examined *Amphoricarpos* taxa might be considered as a protection layer for the inflorescence. The same bract and palea anatomy was found in *Xeranthemum* species (Gavrilović et al. 2019a), except for crystals, which were not found on bracts surface of *Amphoricarpos*. In the Cardueae, the receptacle is often bristly, and numerous bristles are scattered over the receptacle (Bremer 1994). In *Amphoricarpos* the true bracts form a laciniate keeled tongue, which is more narrow in *A. elegans*, while in *A. neumayerianus* it is wider and usually irregularly split lengthwise (Petit 1997). *Amphoricarpos* are heterocarpic plants, thus produce two types

of cypselae, the inner ones from central florets and outer ones from peripheral florets (Bremer 1994). We documented several sclerenchyma fascicles in the pericarp of immature outer cypsela, which is characteristic for Xerantheminae (Lavialle 1912; Petit 1997). We observed radially lengthened and sclerified walls of testa epidermal cells, which were also mentioned by Lavialle (1912) and Petit (1997). Regarding ovary, as earlier documented for the family (Davis 1966; Johri et al. 1992; Gavrilović et al. 2019a), examined taxa have an anatropous ovule with basal placentation. Phytomelanin was observed in some tribes of *Asteraceae* (Pandey and Singh 1982), although we did not observe such layer in the pericarp. Embryo, cotyledons and endosperm layer are in accordance with description in Häffner (2000). Our data contribute to the knowledge of the inflorescence anatomy of the genus, but further analysis of related species from subtribe Xerantheminae should be carried out.

All examined taxa show similar anatomical features. Adventitious young root shows typical structure. Sclerenchyma fibers are present in the center of older root. On the rhizome cross sections secondary tissues are noticed, with wide parenchyma rays which interrupted well developed xylem. Rhizomes show eccentric growth. The leaf blade is amphistomatous, with dorsiventral structure. Crystals druses were found in leaf epidermal and mesophyll cells. The peduncle cross section is characterized by more or less polygonal shape with medullary collateral vascular bundles arranged in a circle, and a few "cortical" vascular bundles. Secretory canals were not found. Multilayer sclerenchyma is present in the mesophyll of involucral bract and palea. Inflorescence anatomy show similar structures with those described for *Asteraceae* members. Densely distributed vermiform (lanate) trichomes as well as biseriate glandular trichomes are present on the peduncle and on both leaf sides, but much more so on the abaxial. Anatomical uniformity indicates very close relationships between examined taxa regarding conserve nature of the genome of the genus. However, taxonomic treatments of these taxa remain unclear (Blečić and Mayer 1967; Caković et al. 2015; Cvetković et al. 2018). Our results reveal qualitative characters which contribute to the general anatomy of the genus *Amphoricarpos*. Some of the described anatomical traits could be a guideline for future investigations of other taxa of the Xerantheminae.

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