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ANATOMICAL AND GENETIC CHARACTERIZATION OF THE GENUS *Xanthium* SPECIES FROM BOSNIA AND HERZEGOVINA

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ABSTRACT

Taxonomy of *Xanthium* genus is very problematic because of numerous described taxa and low morphological distinction among species. In this study anatomical and genetic variability of *Xanthium* genus in the region of Bosnia and Herzegovina was analyzed. Based upon morphological characteristics analyzed plant material two species *X. spinosum* and *X. orientale* were determined. Within species *X. orientale* two infraspecific taxa *X. orientale* subsp. *italicum* and *X. orientale* subsp. *riparium* were identified. The anatomical differences were observed at the level of species, but they were not ascertained at subspecies level. The genetic results based on ITS2 sequences were in accordance with anatomical analyses. Regarding anatomical and genetic analysis two species were confirmed but there were no differences at the subspecies level.

Keywords: *Asteraceae*, anatomy, genetics, species delimitation, ITS2.

INTRODUCTION

Genus *Xanthium* L. (Asteraceae) was the subject of description and classification by a great number of authors. Löve (1976) accepted presence of two species from genus *Xanthium* in Europe: *X. strumarium* L. [with two subspecies subsp. *strumarium* and subsp. *italicum* (Moretti) D. Löve] and *X. spinosum* L. Species *X. strumarium* is an extremely variable species in the section *Xanthium*, and it is a complex of species, that contains a great number of intermediate forms (Löve and Dansereau, 1959; Baldoni et al., 2000; Wassom et al., 2002), all of which are interfertile tetraploids, with a chromosome number of 2n = 36 (Dogan and Kiran, 2017). More homogeneous species is *X. spinosum*, in the section *Acanthoxanthium* DC. Nowadays, according to the global database of The Plant List (Plant List, 2020) there are eleven accepted species of genus *Xanthium*, with a great number of subspecies and numerous described, taxonomically unresolved or invalid names.

On the territory of Bosnia and Herzegovina (Slišković, 1983), Serbia (Gajić, 1975) and Slovenia (Martinčič et al., 2007) three species of the named genus are determined: *X. spinosum, X. strumarium* and *X. italicum*. In Croatia (Domac, 1994) four species are present: *X. spinosum, X. strumarium, X. italicum* and *X. brasilicum*. In Italy determined species are following: *X. ambrosioides* Hook. & Arn., *X. spinosum* L., *X. italicum* Moretti, *X. orientale* L., *X. strumarium* L. subsp. *brasilicum* (Vell.) O. Bolòs & Vigo and *X. strumarium* L. subsp. *strumarium* (Pignatti, 1982; Bartolucci et al., 2018). While in Hungary Király (2009) recorded five species: *X. spinosum*, *X. strumarium*, *X. albinum* subsp. *riparium*, *X. italicum* and *X. saccharatum*. Also they have invasive status in some of the countries (Lohmeyer and Sukopp, 1992; Sobrino et al., 2002; Pál, 2004; Tabacchi and Planty-Tabacchi, 2005; Stefanović et al., 2006; Verloove, 2006; Anastasiu et al., 2007; Boršić et al., 2008; Anačkov et al. 2013; Galanos, 2015). Thanks to photoperiodic adaptation through

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hybridization, outcrossing occurs in about 12% of cases (Löve and Dansereau, 1959), *Xanthium* species have a great capacity for growth and colonization of diverse latitudes and habitats (McMillan, 1974).

Species of of *Xanthium* genus are annual herbs. The cortex of the root has been surrounded by the layer of epidermis. The cortex is large and collenchymatous with compactly arranged cells. Endodermis is well developed. The vascular bundles are distributed near the periphery of vascular cylinder (Ullah Khan et al., 2013). Species have a primary type of stem anatomy typical for dicotyledonous species which includes the epidermis, cortex and central cylinder (Sârbu and Smarandache, 2013). Epidermis is persistent. Endodermis of stem cortex is well defined and with well-marked casparian thickenings. Medullary bundles are usually collateral (Metcalfe and Chalk, 1950). Ducti are visible in regular intervals in cortex zone (Ghorbanil et al., 2010) and extending through the petiole to the lamina of the leaf (Metcalfe and Chalk, 1950). The leaf lamina is amphystomatic and has a dorsiventral structure, consisting of the adaxial epidermis, mesophyll and abaxial epidermis (Reeta et al., 2010). Secretory ducti occurs in the root, stem, and leaf generally situated in the region of the endodermis (Metcalfe and Chalk, 1950). Non-glandular hairs are uniseriate, consisting of uniform cells apart from modifications of the terminal and basal cell, glandular hairs having a multiseriate stalk of varying length and a unicellular head.

Genetic relationships and classification within *Xanthium* genus have been investigated by different methods. Baldoni et al. (2000) in the analysis of seed reserve proteins demonstrated presence of the three different biotypes of the *X. strumarium* complex. Tranel and Wassom (2001) among the 217 *X. strumarium* accessions identified 135 unique genotypes grouped into two main clusters by the 24 ISSR markers. Applying isozyme analysis, genetic diversity of the genus *Xanthium* from Italy was examined at the species and population levels (Dinelli et al., 2003). Regarding total gene diversity at population level a low isozyme diversity within the three analysed *Xanthium* species (*X. italicum, X. strumarium, X. orientale*) was evidenced. On contrary, the considerable interspecies genetic differentiation at several loci was detected, revealing *X. orientale* as the most divergent compared with *X. italicum* and *X. strumarium* which showed as closely related species. Recently, ITS2 region, as a nuclear ribosomal internal transcribed spacer region, has become one of the most frequently utilized region in plant DNA barcoding (Coleman, 2007; Chen et al., 2010; Yao et al., 2010). Although ITS2 showed slightly lower performance in identification of some vascular plants (Braukmann et al., 2017), for samples of Asteraceae family the correct identification rates of this nuclear region were significantly higher compared to the plastid markers (rbcL, matK, psbA-tmH) (Gao et al., 2010). Considering the short length, the ITS2 sequences have two important advantages: suitability for high-throughput screening (HTS)-based applications and it is readily recovered from diverse taxa (Braukmann et al., 2017), including old specimens and medicinal plant materials (Han et al., 2013). Furthermore, for plant generic and infrageneric phylogenetic inference, ITS (ITS1 and ITS2) region is the most commonly used locus (Alvarez and Wendel, 2003). Regarding the genus *Xanthium*, the named nuclear region was successfully applied to distinguish and identify *Xanthium* species (Zhao and Hu, 2014; Wang et al., 2014; Tomasello and Heubl, 2017). In the study of *Xanthium* genus conducted by Tomasello and Heubl (2017) ITS2 region was proved to be the most variable among the other analysed nuclear and chloroplast regions.

Due to numerous described taxa of genus *Xanthium* and low morphological distinction among them it was difficult to clearly define the species. In the present study, we made an attempt to exceed this continuous problem on the territory of Bosnia and Herzegovina by two different approaches: characterization of anatomical structure with aim to highlight taxonomically important parameters, and using nuclear ITS2 marker to resolve the belonging of sampled accessions to the

particular species and to determine species boundaries.

MATERIAL AND METHODS

Study area

Territory of Bosnia and Herzegovina (B&H) is located in the western part of the Balkan Peninsula and covers an area of 52129 km². Northern part, south of the Sava river is southern edge of Pannonian plain, belonging to the peripannonian region, while central and southern part lies on Dinaric mountain range, and descends towards the coast of the Adriatic Sea in the southernmost part. Plant material analysed in this study was collected from 20 localities on the territory of Bosnia and Herzegovina (on arable land and ruderal area) (Table 1) during the fruiting season.

Morphological determination

Plant material was collected as mature fruits-burs. Herbarium exsiccates were identified and deposited in Herbarium BUNS, at the University of Novi Sad, Faculty of Sciences, Department of Biology and Ecology (Kiraly, 2009; The Plant List, 2019). For each exsiccate voucher number is assigned (Table 1).

Table 1. Information of analysed *Xanthium* samples on the territory of research

Species	Locality	Code	Genetic ID	Latitude (N)	Longitude (E)	Elevation (m.a.s.l.)	Habitat	Voch Nº
X. orientale L. subsp. *italicum*	Petrovo polje	Pep	XN1-XN5	42°39'54,2"	18°19'33,0"	270	Vineyard	2-1498
X. orientale L. subsp. *italicum*	Velino Selo	Ves	XN6-XN10	44°53'17,6"	19°19'28,2"	82	Maize field	2-1492
X. orientale L. subsp. *italicum*	Aleksandrovac 2	Ale2	XN11-XN15	44°58'09,7"	17°18'25,9"	120	Roadside	2-1497
X. orientale L. subsp. *italicum*	Lončari	Lon	XN16-XN20	44°56'44,7"	18°39'50,7"	85	Maize field	2-1500
X. orientale L. subsp. *italicum*	Bardača	Bar	XN21-XN25	45°05'22,5"	17°26'32,4"	81	Ditch	2-1493
X. orientale L. subsp. *italicum*	Gorica	Gor	XN26-XN30	42°42'54,8"	18°21'05,6"	285	Riverside	2-1494
X. orientale L. subsp. *italicum*	Seferovci	Sef	XN31-XN35	44°59'52,9"	17°20'59,9"	106	Wasteland	2-1495
X. orientale L. subsp. *italicum*	Aleksandrovac 1	Ale1	XN36-XN40	44°58'09,7"	17°18'25,9"	120	Roadside	2-1496
X. orientale L. subsp. *italicum*	Cerovljani	Cer	XN41-XN45	45°02'29,4"	17°15'24,1"	103	Maize field	2-1499
X. orientale L. subsp. *italicum*	Agrofin	Agf	XN46-XN50	42°40'14,3"	18°19'38,0"	272	Vineyard	2-1501
X. orientale L. subsp. *italicum*	Balatun	Bal	XN51-XN55	44°52'57,1"	19°19'56,9"	81	Roadside	2-1505
X. orientale L. subsp. *italicum*	Vilusi	Vil	XN56-XN60	45°00'30,0"	17°16'53,0"	108	Maize field	2-1490
X. orientale L. subsp. *italicum*	Berek	Ber	XN61-XN65	45°02'35,5"	17°14'30,6"	104	Maize field	2-1491
X. orientale L. subsp. *italicum*	Lukavac	Luk	XN66-XN70	45°04'19,1"	17°12'44,0"	104	Maize field	2-1502
X. orientale L. subsp. *italicum*	Domanovići	Dom	XN71-XN75	43°08'13,2"	17°47'01,5"	144	Vineyard	2-1503
X. orientale L. subsp. *italicum*	Dolgodi 2	Dol2	XN76-XN80	43°51'41,5"	18°17'41,4"	483	Maize field	2-1508
X. orientale L. subsp. *italicum*	Volujac	Vol	XN81-XN85	42°41'06,9"	18°19'25,0"	269	Vineyard	2-1504
X. spinosum	Trebinje	Tre	XN86-XN90	42°41'06,9"	18°19'25,0"	269	Vineyard	2-1506
X. orientale L. subsp. *riparium*	Dolgodi 1	Dol1	XN91-XN95	43°52'03,3"	18°17'01,5"	486	Wasteland	2-1507
X. orientale L. subsp. *italicum*	Mašići	Maš	XN96-XN100	45°01'43,6"	17°15'57,6"	103	Maize field	2-1509

Anatomical analyses

Plants reproduced from collected fruits were used for anatomical analysis. All plants were grown in the same conditions, in order to minimize impact of ecological factors. Five plants per locality were analyzed. The analyses were conducted on transverse cross sections in the central part of root, stem and lamina (on the median nervure). Microscopic preparations were made by modifided paraffin technique (Blaženčić, 1988; Mićić, 2018). Paraffin molds were sectioned by microtome Leica RM 2135, and stained with Delafield′s Haematoxylin. The analyses of the microscopic slides and their micro-photography were conducted in microscope Carl Zeiss AxioImagerA.2 and stereomicroscope Leica M205C, equiped with LAS V4.11 software for image analyses. On the root cross sections secondary wood-xylem, cambium, protective tissue and total cross section were analyzed. On stem cross section bundles, parenchyma of central cylinder and cortex and total cross section, as well. Leaf cross section measurements included the thickness of palisade shaped cell and thickness of spongy shaped cell. The presence secretory structures of plant was ascertained by using the optical magnifier Leica S6D. A raw data matrix for the numerical analysis was created by using the average measurements and observations. Analysed characteristics are listed in Table 2.

The results were processed by Statistica 13.0 software. To determine whether differences among studied anatomical parameters of the analyzed samples were statistically significant, one-way ANOVA was applied, followed by Tukey HSD *post hoc* test for significance level of $p \leq 0.05$.

Table 2. List of characters used in numerical analysis

Root			Stem			Leaf		
Sym.	Characters	Unit	Sym.	Characters	Unit	Sym.	Characters	Unit
R01	area of secondary wood-xylem	mm^2	**S01**	area of parenchyma of central cylinder	mm^2	**L01**	thickness of upper epidermis	µm
R02	area of cambium	mm^2	**S02**	area of bundles with sclerenchyma	mm^2	**L02**	thickness of lower epidermis	µm
R03	area of cortex	mm^2	**S03**	area of cortex parenchyma	mm^2	**L03**	thickness of palisade tissue	µm
R04	area of protective tissue	mm^2	**S04**	area of cortex collenchyma	mm^2	**L04**	thickness of spongy tissue	µm
R05	cross-section area of root	mm^2	**S05**	cross-section area of stem	mm^2	**L05**	thickness of cross-section of leaf area	µm
R06	contribution of secondary wood-xylem and sectional area of root	%	**S06**	contribution of central cylinder parenchyma and sectional area of stem	%	**L06**	number of palisade cells/100 µm²	num.
R07	contribution of cambium and sectional area of root	%	**S07**	contribution of bundles with sclerenchyma and sectional area of stem	%			
R08	contribution of cortex and sectional area of root	%	**S08**	contribution of cortex parenchyma and sectional area of stem	%			
R09	contribution of protective tissue and sectional area of root	%	**S09**	contribution of cortex collenchyma and sectional area of stem	%			

Genetic analysis

Germinated plants on the stage of two to four leaves were used for genetic analysis. We examined five plants per locality, in total 100 samples of *Xanthium* genus from B&H (Table 1). Total genomic DNA was extracted from fresh leaf tissue using a modified CTAB method (Padmalatha and Prasad, 2006). Concentrations and quality of the extracted genomic DNA were determined by Bio-Spec spectrophotometer (Shimadzu). DNA concentrations of the extracts were adjusted to 30 ng/µl. PCR reaction was performed in Applied Biosystems Verity thermal cycler in 25 µl reaction mixtures containing approximately 30 ng of genomic DNA template, 1 x Taq Buffer with $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.1 mM of both primer (synthesized by Invitrogen by Thermo Scientific) and 1.0 U Taq DNA Polymerase (recombinant; Thermo Scientific). The primer pair S2F 5′-ATGCGATACTTGGTGTGAAT-3′ and S3R 5′-GACGCTTCTCCAGACTACAAT-3′ (Chen et al., 2010) was used to amplify the ITS2 region. The PCR amplification was started with initial denaturation at 94ºC for 5 min, followed by 40 cycles of 30 sec at 94ºC, 30 sec at 56ºC and 45 sec at 72ºC. After the last cycle, reactions were incubated 10 min at 72ºC. Amplified PCR products were separated on 2% agarose gels for visual inspection. The products were purified by Exonuclease I and Shrimp Alkaline Phosphatase enzymes (Thermo Scientific, Lithuania) according to the manufacturer's protocol. The fragments were sequenced on the Applied Biosystems 3730XL sequencer in Macrogen Europe, Amsterdam, Netherlands. GenBank accession numbers for obtained sequences are listed in Table 1.

The ITS2 sequences were aligned using Clustal W (Thompson et al., 1994) as implemented in BioEdit version 7.2.5. (Hall, 1999), and then checked manually. Four species from the related genera (fam. Asteraceae, tribe Heliantheae): *Parthenium hysterophorus* L. (GenBank accession no. GU24333), *Polymnia canadensis* L. (GenBank accession no. KF607079), *Ambrosia artemisiifolia* L. (GenBank accession no. KY215732) and *Iva xanthiifolia* Nutt. (GenBank accession no. KY215730) were used as outgroups. Additionally, in the order to classify our samples into particular species and perform cluster analysis, sequences of *X. orientale* L. (GenBank accession no. KY215704), *X. orientale* L. subsp. *riparium* (GenBank accession no. KY215705), and *X. spinosum* L. (GenBank accession no. KY215702, MG218395) were retrieved from the GenBank and included into the analysis. Furthermore, sequences of *X. sibiricum* Widd. (GenBank accession no. KY215731, GU724337, KJ437623) were included in the analysis, with the aim to contribute to the revealing relationships within and between *Xanthium* sections. The sequences were clustered using Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses. Parsimony analysis was run by NONA (Goloboff, 1999) spawn with the aid of Winclada (Nixon, 2002), using the heuristic search algorithm with 1000 random addition replicates (mult*1000), holding 100 trees per round (hold/100), maxtrees set to 100 000 and applying TBR branch swapping. ML tree was constructed in MEGA version 7.0.21 (Kumar et al., 2016) under the Kimura 2-parameter model (Kimura, 1980) and a discrete Gamma distribution (+G) with 5 rate categories which was defined as the best evolutionary model in the same programme. Nodal supports for the both trees (MP and ML) were assessed using non-parametric bootstrapping with 1000 replicates. The obtained trees were rooted with *Polymnia canadensis* L.

RESULTS AND DISCUSSION

Identification of analyzed plant material based upon morphological characteristics only, regarding regional flora descriptions and on the basis of modern systematic principles, reveals presence of only two species in the investigated region: *X. spinosum* L. and *X. orientale* L. Within species *X. orientale* L. two subspecies *X. orientale* L. subsp. *italicum*

(Moretti) Greuter and *X. orientale* L. subsp. *riparium* (Čelak.) Greuter were determined (Table 1).

Anatomical results

All detailed measurements related to plant cross sections are given in the Appendix. Root anatomic features of the examined taxa based of transverse sections are given in Figure 1 A and 1 B. Root cross section shows that the species of genus *Xanthium* produce secondary tissue. Cortex parenchyma which is rich with air chambers is under protective tissue. Secretory ducti are poorly visible and present at the interface of endodermis and cortex parenchyma. Cambium forms a layer of secondary phloem and secondary xylem which at this stage comprised tracheary elements and xylem parenchyma only. Most of the root volume was occupied by the secondary xylem rich in xylem fibres that in time became separated by parenchymatous rays into delimited vascular bundles.

Results of Tukey HSD test showed significantly different values among analysed localities and divided plants in four to thirteen homogeneous groups. *X. spinosum* had the lowest value for all measured parameters of root (Figure 2). In contribution of individual tissues, at the cross-section of root, there were no statistical differences between species *X. spinosum* and *X.orientale* except in contribution of cambium (Appendix).

Stem anatomic features of the examined taxa based of transverse sections are given in Figure 1 C and 1 D. The epidermal tissue consists of a single row isodiametric cells covered by a ridged cuticule. The outer cortex is collenchymatous (with five to six rows of cells, for both species) and the internal is parenchymatous. The last layer of parenchyma forms an amiliferous sheath. The vascular bundles, interconected with an intravascular sclerenchyma, formed one ring in the stem. Secretory ducti are located very close to the floem tissue and irregularly distributed in cortex (Sc) (Figure 1 C and 1 D). Sclerenchymatous cells are present in vascular bundle. The pith is large and parenchymatous. On stem cross-section of all studied species of *Xanthium* genus possessed secretory ducts in cortex layer as well as tector hairs on epidermis. Secretory hairs were observed in *X. orientale* species, but it was not noticed in *X. spinosum*.

Results of Tukey HSD test showed significantly different values among analysed localities and divided plants in seven to ten homogeneous groups. *X. spinosum* has the lowest value for all measured parameters of stem (Figure 3), but the contribution of individual tissues were not statistically significant between these and *X. orientale* species except in contribution of cortex parenchyma and collenchyma (Appendix).

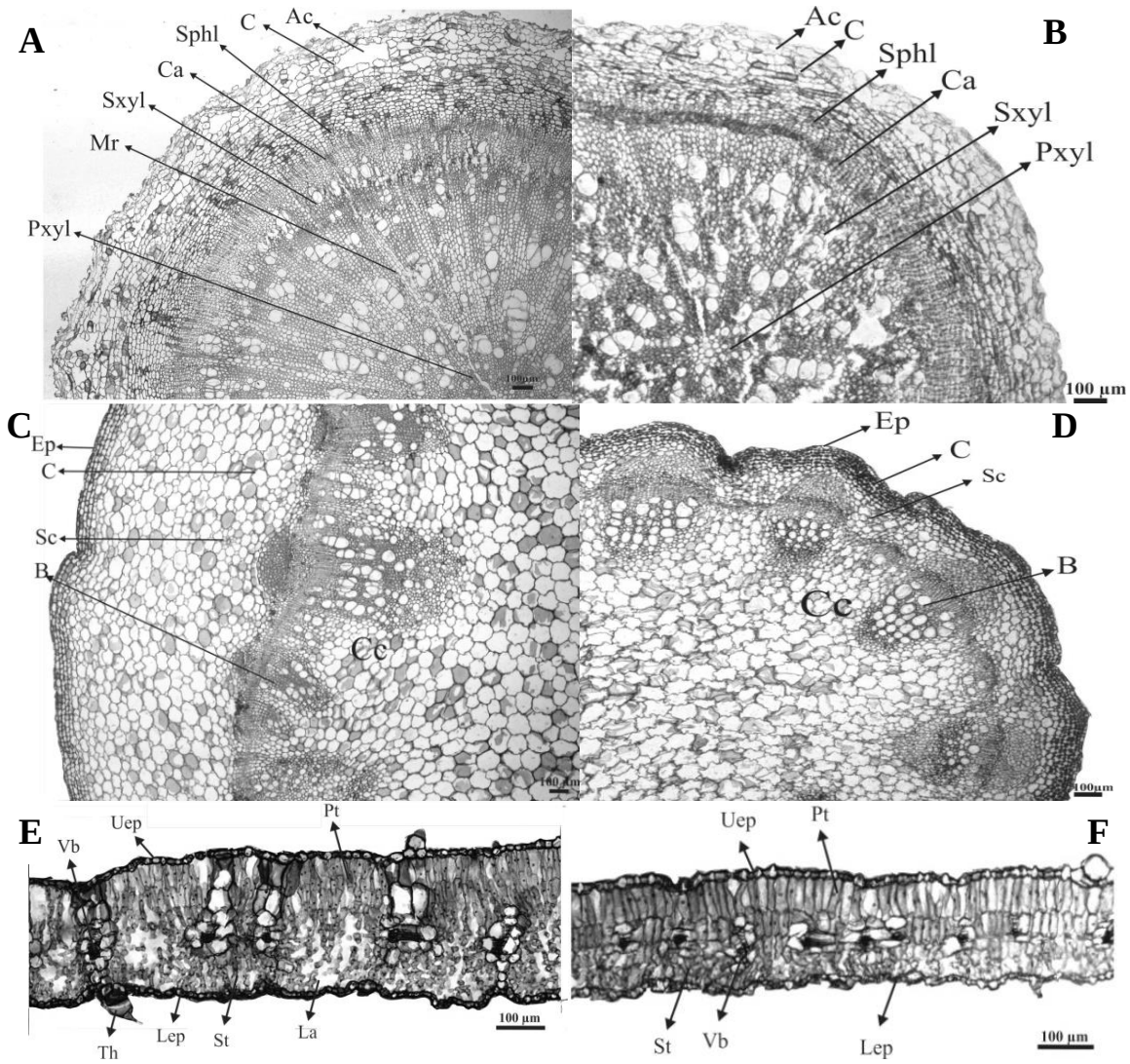

Figure 1. Optical micrographs of the plants cross-sections
Root cross-section of *X. orientale* (**A**) and *X. spinosum* (**B**), C - cortex, Ac - air chambers, Sphl - secondary phloem, Ca - cambium, Sxyl - secondary xylem, Pxyl - primary xylem, Mr - medullary rays.
Stem cross-section of *X. orientale* (**C**) and *X. spinosum* (**D**), Ep - epidermis, C - cortex, Cc - central cylinder, B - bundle.
Leaf cross-section of *X. orientale* (**E**) and *X. spinosum* (**F**), Uep - upper epidermis, Pt - palisade tissue, St - spongy tissue, vascular Vb - bundles, Lep - lower epidermis, Th - tector hair, La - lacuna (intercellular space).

Leaf anatomic features of the examined taxa based of transverse sections are given in Figure 1 E and 1 F. Analysis of leaf cross-section revealed that the leaf is amphistomatic with anamocytic stomata. Mesophyll is clearly divided into palisade and spongy tissue. Palisade tissue, as heliophytes, is well developed. Leaf upper epidermis was followed by two layers of palisade parenchyma in *X. spinosum* species, while three layers of palisade parenchyma in *X. orientale* species were detected. Palisade cells were replaced with spongy mesophyll riched with lacuna- intercellular space. Vein bundles are surrounded by non-chlorophyllose mechanical tissue. Regarding *X. orientale* species tector and massive secretory hairs were identified (Figure 1 E), while on *X. spinosum* species only tector hairs were identified.

Results of Tukey HSD test of leaf parameters showed significantly different values among analysed localities and divided plants in two to seven homogeneous groups. According to analyzed parameters, except number of palisade cells/100 µm² (Figure 4), there is no statistically significant difference between samples determined as *X. spinosum* and *X. orientale* (Appendix).

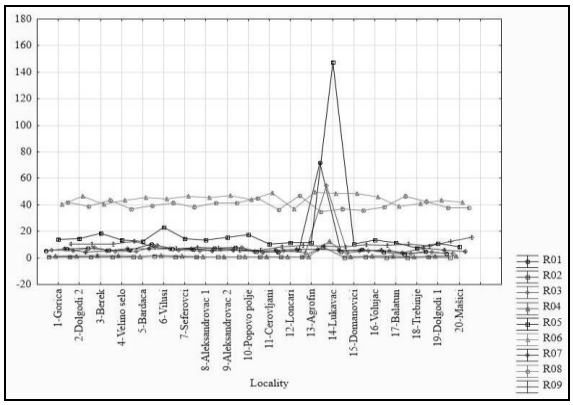

Figure 2. Mean values of root parameters for the analysed localities

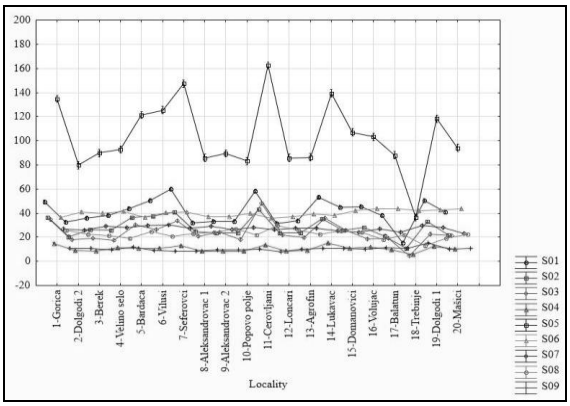

Figure 3. Mean values of stem parameters for the analysed localities

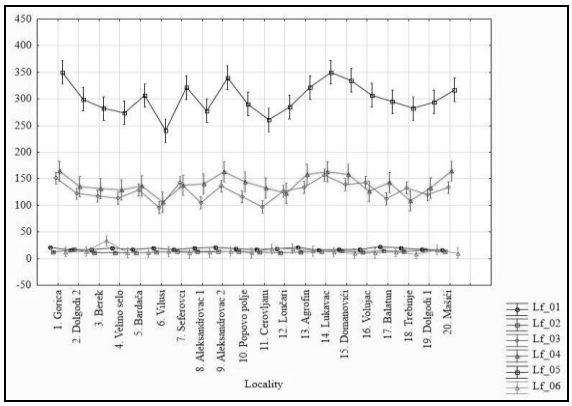

Figure 4. Mean values of leaf parameters for the analysed localities

Genetic analysis

The amplification of the analysed ITS2 region for the fresh plant material of *Xanthium* samples was successful in all samples (100%). The obtained aligned and pruned ITS2 data matrix comprised 234 nucleotide characters for in total 111 specimens (100 *Xanthium* samples from B&H + 7 *Xanthium* samples retrieved from GenBank + 4 outgroups). The number of parsimony-informative characters was 34 for the Figotal matrix. The strict consensus MP tree (Figure 5) recovered *Xanthium* genus as monophyletic entity. Five analysed accessions previously morphologically determined as *X. spinosum*, coinciding with sequences from GenBank of the same species, formed a unique cluster strongly separated from the other analysed species (bootstrap support 100).

All the other analysed B&H accessions (95; previously morphologically determined as *X. orientale* species) resolved as separate branch. These samples coincided with sequences from GenBank belonging to *X. orientale* and *X. orientale* subsp. *riparium*. The *X. orientale* clade was distinguished by 17 mutation steps from *X. spinosum* species, and by two from *X. sibiricum.* Three sequences of *X. sibiricum* species retrieved from GenBank formed one branch which clustered together with *X. orientale* clade. Based on these molecular data the recognition of subspecies in *X. orientale* clade was not supported. Maximum Likelihood (ML) analysis revealed similar topology to respective MP tree, supporting distinguishing of the *Xanthium* accessions from B&H on the two species (*X. orientale* and *X. spinosum*) and without resolving intraspecific structuring within *X. orientale* clade (Figure 6).

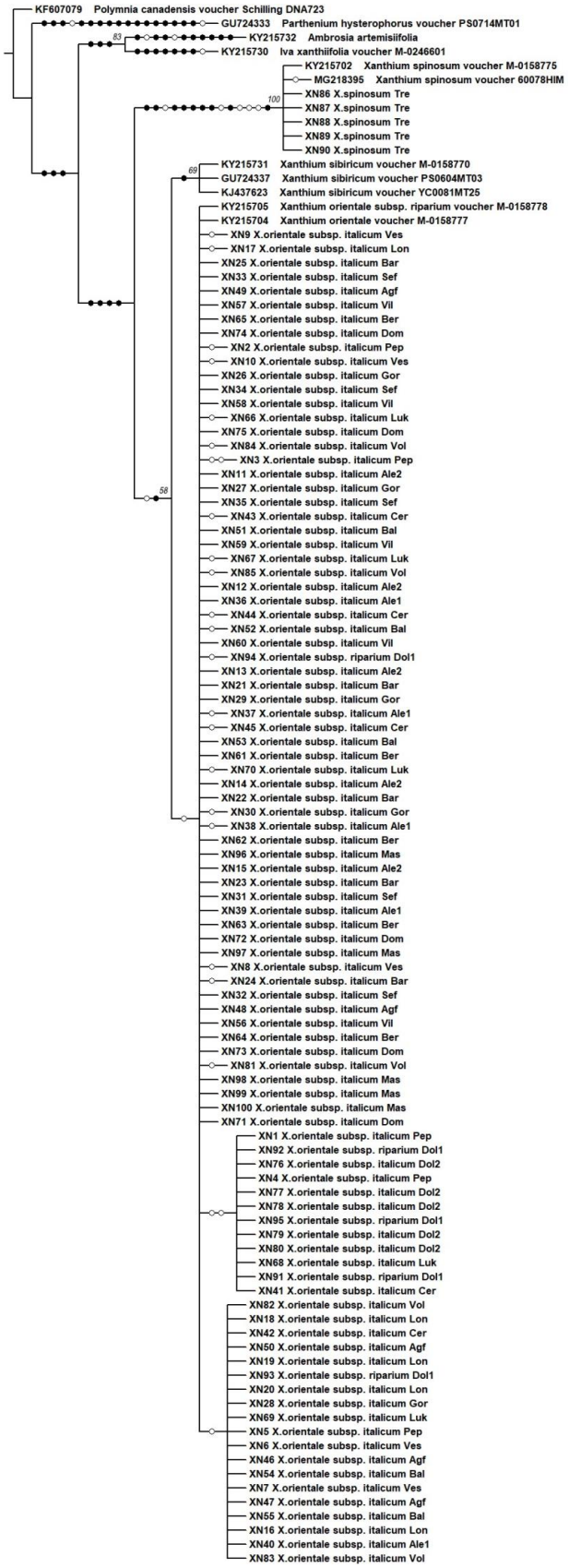

Figure 5. Strict consensus tree based on 16 equally parsimonious trees from the analysis of the ITS2 region. Length=139 steps, Consistency index (CI)=0.71, Retention index (RI)=0.81. Bootstrap values (≥50%) are indicated near nodes. Filled circles denote unique changes, open circles non-unique.

Figure 6. Maximum-likelihood tree of the ITS2 sequences. Bootstrap values (≥50%) are indicated near nodes.

In the study, we conducted anatomical and genetic analyses of selected *Xanthium* species in Bosnia and Herzegovina. According to morphological determination two species *X. spinosum* and *X. orientale* were *a priori* determined. Two subspecies *X. orientale* subsp. *italicum* and *X. orientale* subsp. *riparium* were identified within species *X. orientale*. Although *X. strumarium* and *X. italicum* are described often and in detail, they were not recorded at examined localities.

Root anatomy of *Xanthium* species was examined by numerous authors. For *X. orientale* as well as *X. strumarium (*section *Xanthium)* on root cross-sections it was defined secondary growth (Ullah Khan et al., 2013; Wolski et al., 2016). Cortex is well developed with air chambers. Secretory ducti were visible in cortex of root as in root of *X. strumarium* (Ghorbanil et al., 2010; Wolski et al., 2016). The largest values of root tissue recorded at the locality Luk can be explained by the influence of different habitat of the mother plant, which is in accordance to the Metcalfe and Chalk (1950). Analysing *X. spinosum* species (section *Acanthoxanthium* DC.) secondary growth of root was also defined with the same histological elements as species of section *Xanthium* DC.

A variability has been shown on the stem cross-section from different locations. The same stem anatomy as within species *X. italicum* (Sârbu and Smarandache, 2013) and *X. strumarium* (Ghorbanil et al., 2010; Wolski et al., 2016) were observed at *X. orientale*. Secretory ducti were visible in stem cortex as in *X. strumarium* and *X. italicum*. The important differences between species were noticed in cortex of *X. spinosum*, which has a smaller contribution of parenchyma and greater contribution of collenchyma compared to *X. orientale*. A presence of secretory ducti was not noticed in stem cross-section of *X. spinosum*.

X. orientale species has the same leaf anatomy as in *X. strumarium* (Bhogaonkar and Ahmad, 2012; Wolski et al., 2016) and *X. italicum* (Sârbu and Smarandache, 2013) species. In study of Reeta et al. (2010) from one to two palisade layers were observed in species *X. strumarium*, while three layers of palisade cells at *X. orientale* species were noticed in our research. Two layers of palisade cells were noticed at *X. spinosum*. The difference between *X. orientale* and *X. spinosum* species were noticed in thickness of palisade cells.

Tector hairs (trichomes) present on the plant (their shape, size and structure) are specific and often contributing to the plants identification (Metcalfe and Chalk, 1950). Tector hairs on the stem and leaf of *X. orientale* and *X. spinosum*, as in *X. italicum* (Sârbu and Smarandache, 2013), are pluricellular, uniseriate and have various shapes and sizes: with a sharp, pungent apical cell; with a rounded apical cell or with a slightly uncinate apical cell. Secretory hairs were observed in studied populations of *X. orientale* species, as in *X. italicum* (Sârbu and Smarandache, 2013) and *X. strumarium* (Ghorbanil et al., 2010; Ullah Khan et al., 2013; Wolski et al., 2016), while they were not identified on *X. spinosum* species.

Genetic analysis of *Xanthium* species based on ITS2 region revealed two clearly distinguished species of *Xanthium* genus from the territory of B&H, consistent with the results of morphological determination. On the both constructed trees (Maximum Parsimony and Maximum Likelihood) genus *Xanthium* was found to be monophyletic, consisted of two main clusters, one including *X. spinosum* species belonging to the section *Acanthoxanthium* and the other, with species belonging to the section *Xanthium* (*X. orientale* and *X. sibiricum*). This result was in accordance with data published by Tomasello and Heubl (2017). *X. sibiricum* clustered together with *X. orientale* species, but was clearly defined as separated branch, which is also in congruence with the study of Tomasello and Heubl (2017). Unlike these observations, in the phylogeny published by Zhao and Hu (2014) based on ITS2 sequences and including seven *Xanthium* species, *X. spinosum* was found to be closely related to *Xanthium* species as *X. sibiricum, X. pensylvanicum, X. brasilicum* and *X. occidentale*.

Obtained phylogenetic relationships within the *X. orientale* clade were less clear indicated that the ITS2 region was not sufficiently

informative to separate taxa on the subspecies level. Nevertheless, *Xanthium* accessions from B&H previously morphologically determined as *X. orientale,* clustered together with the sequences of the same species retrieved from GenBank, confirmed the affiliation to this taxon. The obtained results seem to support the concept of two species and generally acceptation of reduced number of species in *Xanthium* genus followed by e.g. Löve and Dansereau (1959), Sell and Murrell (2006), Strother (2006), Tomasello and Heubl (2017).

CONCLUSIONS

This study represents the first detailed analysis of the *Xanthium* genus on the territory of Bosnia and Herzegovina. According to our research, in the studied area, the presence of two species of the *Xanthium* genus *X. spinosum* (section *Acanthoxanthium*) and *X. orientale* (section *Xanthium*) has been confirmed on the basis of modern systematic principles, anatomical and genetic analyses. By morphological determination within species *X. orientale* two subspecies *X. orientale* subsp. *italicum* and *X. orientale* subsp. *riparium* were identified. The infraspecific differentiation was not supported by anatomical and molecular analyses. The anatomical differences were observed at the species level, but they were not ascertained at subspecies level. According to anatomical analysis difference in the stem parameters between two species was detected, due to better developed parenchym cell layer in *X. orientale* species. Furthermore, leaf upper epidermis followed by two layers of palisade parenchyma at *X. spinosum* species was observed, while three layers of palisade parenchyma were found at *X. orientale*. Also, secretory hairs were determined in *X. orientale*, unlike *X. spinosum* where we did not observe their presence on the leaf surface. Similarly, our genetic results have shown that ITS2 marker is a useful tool for evaluating taxonomic species delimitation of the genus *Xanthium*, clearly distinguishing *X. spinosum* and *X. orientale* clades, but not sufficiently informative to separate taxa on the subspecies level. Taking into account the prior researches and our results, we encourage the use of additional molecular markers, as well as application of integrative approach to increase taxonomic and phylogenetic resolution in the study of the named genus. In the present research we provide information for better understanding the diversity of the genus *Xanthium* on the territory of B&H, with the final aim to control and reduce harmful effects of *Xanthium* species as potential invasive species in the studied area.

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