

Taxonomic investigation of the *Xanthium strumarium* L. complex (Asteraceae) distributed in Iran inferred from morphological, palynological and molecular data

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Abstract. Noedoost F, Vaezi J, Nikzat Siahkolaee S. 2021. Taxonomic investigation of the *Xanthium strumarium* L. complex (Asteraceae) distributed in Iran inferred from morphological, palynological and molecular data. *Biodiversitas* 22: 1961-1974. Unclear species boundaries due to high morphological similarities lead to different opinions on *Xanthium* classification. To solve this problem, we conducted investigation on morphological, palynological and molecular (ITS sequencing) of three species of *Xanthium* (i.e., *X. strumarium*, *X. sibiricum*, and *X. brasilicum*) forming *Xanthium strumarium* complex in Iran. We randomly collected 110 plant individuals of the *Xanthium strumarium* complex as well as *X. spinosum* from 30 geographical populations in eight provinces of Iran. Pollen grains of 19 populations were studied by scanning electron microscope (SEM). Results obtained from the analysis of morphological characters showed that the three species, *X. strumarium*, *X. brasilicum* and *X. sibiricum* were placed nearly together specially *X. strumarium* and *X. brasilicum* are obviously overlapped. Palynological results indicated the pollen grains of section *Acanthoxanthium* (i.e., *X. spinosum*) and *Xanthium* (i.e., *X. strumarium*, *X. sibiricum*, and *X. brasilicum*) are clearly differing by their LM and SEM characters. However, no significant difference found between the pollen of *X. strumarium* and *X. brasilicum*. The Bayesian phylogenetic tree of the ITS region of rDNA sequences revealed that *X. strumarium*, *X. sibiricum* and *X. brasilicum* could not be discriminated as independent species. Overall, the morphological, palynological and molecular data provided evidence to synonymize *X. brasilicum* and *X. strumarium* with *X. orientale* and *X. sibiricum* with *X. strumarium*.

Keywords: Bayesian tree, complex, delimitation, heterogeneous habitat, morphometric, pollen

INTRODUCTION

Improvement in the utilization and conservation of biodiversity depends on the defensible species delimitation. Distinguishing species and establishing stable and consistent taxonomies is mostly important for many biological fields. The act of defining boundaries between species is on a comprehensive meaning species delimitation. It has been done in the past mostly relying on morphological traits, and on phenological and ecological evidence (Tomasello 2018). Plants growing in different habitats exhibit morphological and phenological differences. It is controversial to draw the obvious borders between putative taxa in their widely distributed areas (Moor et al. 2014). In this way, there are challenges about cryptic species and complexes, groups of organisms, or geographic areas that are poorly studied, and by differing opinions on species delimitation (Isaac et al. 2004; Mace 2004; Bickford et al. 2007).

Xanthium L. (Asteraceae) belongs to the subfamily Asteroideae, tribe Heliantheae and subtribe Ambrosiinae (Bentham 1873; Hoffmann 1890). It is a medicinal and weedy plant with global distribution (Alamet al. 2011) which may cause problems in sheep-grazing lands through its large spiny fruits and toxicity of fresh seedling (Cole et

al. 1980; Alorfi et al. 2020). Moreover, the members of the genus are reported as invasive weeds for many crops in 28 countries (Hocking and Liddle 1986). However, different medical uses of the genus are mentioned, including for treatment of rhinitis, malaria, rheumatism, tuberculosis, cancer and ulcers (Ma et al. 1998; Gautamet al. 2007; Chandelet al. 2012; Tong et al. 2020).

Uncertainty in species boundaries due to high morphological similarities lead to different opinions on *Xanthium* classification. Different taxonomic concepts have been suggested, comprising several species, and other groupings the different taxa in a few extremely polymorphic species. The number of species comprised in *Xanthium* varies greatly. While more than 20 species were classified in two sections (*Acanthoxanthium* DC. and *Euxanthium* DC.) by Widder (1923), some botanists (e.g., Löve and Dansereau 1959; Löve 1976; Franco 1984) introduced only two species for the genus: namely *X. spinosum* L. (= *Acanthoxanthium spinosum*) -- a homogenous species with spiny stems, and *X. strumarium* L. -- a highly variable and heterogeneous species. According to Weaver and Lechowich (1982) and Caius (1986) classifications, the genus contains 20 and 25 species, respectively. Also Strother (2006) accepted Löve and Dansereau's (1959) opinion in flora of North America.

Recently, Tomasello (2018) used plastid and nuclear markers and coalescent-based species delimitation methods (BP & P and STACEY) to clarify the taxonomy and phylogeny of *Xanthium* sensu lato. He concluded that the genus comprises five species, including *X. ambrosioides* Hook & Arn. (= *Acanthoxanthium ambrosioides* (Hook & Arn.) D. Löve), *X. spinosum* L. (= *Acanthoxanthium spinosum* (L.) Fourr.), *X. chinense* Mill., *X. orientale* L. and *X. strumarium* L.

Xanthium spinosum and the *X. strumarium* complex (including *X. strumarium*, *X. brasiliicum* and *X. sibiricum*) are distributed in ecologically heterogeneous landscapes in Iran, including in agricultural lands, ruderal and urban areas, seaside to high elevation, forest, temperate, arid and semi-arid regions (Rechinger 1989). This heterogeneous habitat could explain some of unclear overlapping traits in morphology and palynology. Distinguishable features applied in species separation of the complex are mostly including characters of fruits, e.g., size, form, pubescence, number and length of spines on the fruits, and hooks status of beaks and spines (Widder 1923; Löve and Dansereau 1959; Nadeau 1961; Hicks 1971). The great variation of fruits causes the species complex with many local ecotypes and forms (Löve and Dansereau 1959; Weaver and Lechowicz 1982). There are several surveys based on morphological traits to clarify species delimitation in different countries, such as Italy, North America, and Bangladesh (McMillan 1974; Hare 1980; Blais and Lechowicz 1989; Baldoni et al. 2000; Dinelli et al. 2003). However, few molecular studies were implied for declaring relationships within the species complex (Wallace et al. 2012; Zhao and Hu 2014; Tomasello and Heubl 2017; Tomasello 2018). Several studies have emphasized the importance of the pollen morphological data in understanding the systematics and evolution of the Asteraceae (e.g. Skvarla and Turner 1966; Wagenitz 1976; Skvarla 1977; Wortley et al. 2008, 2012; Punt and Hoen 2009; Pereira Coutinho and Dinis 2009; Blackmore et al. 2010; Pereira Coutinho et al. 2012, 2021; Tellería et al. 2013; Bordbar and Mirtadzhadini 2015).

However, Species delimitation in the *X. strumarium* complex encounters various difficulties because of extensive hybridization, great phenotypic plasticity, and morphological variation due to defensive mechanisms, resource availability, diverse latitudinal and habitat distribution, and colonization in disturbed habitats (McMillan 1974; Moran et al. 1981; Blais and Lechowicz 1989). In this study, we aim at resolving species delimitation in *Xanthium* species complex distributed in Iran, particularly between *X. brasiliicum* and *X. strumarium* using molecular tools as well as pollen and morphological data. The latter species do not have any obvious boundaries and form very intermediate biotypes.

MATERIALS AND METHODS

Plant materials

We sampled 110 plant individuals of the *Xanthium* species complex from 30 geographical populations in eight

provinces of Iran (Table 1). The taxonomic identification of *Xanthium* species was accomplished based on Flora Iranica (Dittrich 1989). The voucher specimens have been deposited in the herbarium of Shahid Beheshti University, Tehran, Iran (HSBU) and in the herbarium of the Museum of Natural History in Vienna, Austria (W) (Table 1).

Morphological studies

Three to five specimens were randomly collected from each location for morphological studies. In total, 32 quantitative and qualitative morphological characters were measured or scored (Table 2). The morphological characters were standardized (Mean = 0, Variance = 1) and used to establish the Euclidean distance among the pairs of specimens (Podani 2000; Sheidaei et al. 2014). The obtained distances were then used for clustering the samples using UPGMA (Un-weighted paired group using average), Neighbor-Joining (NJ), and PCoA (Principal coordinate analysis) (Podani 2000). The morphological differentiation among the species was investigated by applying ANOVA (Analysis of Variance) to identify the most variable morphological characters (Podani 2000). The Mann-Whitney U test was used to compare differences between two species. All univariate analyses were implemented using SPSS release 18.0.0 (SPSS Inc., Chicago, USA).

Palynological studies

Light microscopy and Scanning electron microscopy (SEM): Pollen grains of 19 populations were studied by using light microscopy (LM) and scanning electron microscope (SEM). The pollen samples were obtained mostly from herbarium materials. Five randomly collected specimens were investigated for each population. The pollen grains were prepared for LM using the standard methods described by Erdtman (1963). For LM, the pollen grains were photographed using an Olympus BX51 microscope with automatic camera, under (E40, 0.65) oil immersion (E100, 1.25), with a 10× eyepiece and measured with the software Image Tools Version 3.0. For SEM studies, pollen grains were attached to aluminum stubs with double-sided cellophane tape and coated with gold. The specimens were examined with a Phillips × L20 SEM. UTHSCSA Image Tool Version 3.0 was used to carry out required measurements. Ten to 30 fully developed pollen grains were randomly selected for analysis. Eighteen quantitative and two qualitative palynological features were used for multivariate analysis, including shape of pollen, polar axis (P), equatorial diameter (E), P/E ratio, interval between colpi (IC), colpi length (CL), number of echinae in 20×20 μm² area, interval between two echinae (IE), echinae length (EL), echinae width in base (EW), length of lines on the surface of echinae (LL), length of echinae top (LET), width of echinae top (WET), distance between the apices of two ectocolpi (d), equatorial diameter (D), apocolpium index (AI), pollen size (PS), exine thickness (ET), cavea thickness (CT) and type of echinae. Mean, standard deviations and ANOVA were calculated using IBM SPSS Statistics 19. The terminology used for the palynological descriptions followed by Barth and Melhem (1988) based on updates described by Punt et al. (2007) and Punt and

Hoer (2009) for the Asteraceae pollen grains. Statistical analyses including PCoA were performed using PAST software for plotting variation among populations and

species (Hammer et al. 2009). In order to group the species, the cluster analysis was performed using Neighbor-Joining (NJ) method.

Table 1. Populations of *Xanthium* species, their locality and voucher number included in the morphological (Mor.), palynological (P) and molecular (Mol.) studies collected in the present study

Pop no.	Taxa	Province	Locality	Voucher no.	Mor.	P	Mol.
1	<i>X. brasilicum</i>	West Azarbayejan	Khoy	Azizi2018600 (HSBU)	+	-	-
2		West Azarbayejan	Sardasht, Bioran	Azizi2018601 (HSBU)	+	-	-
3		Gilan	Masal, on road near the city center.	Nikzat2018602 (HSBU)	+	-	-
4		Razavi Khorasan	Do rahiAbghadferizi	Basiri2018603 (HSBU)	+	-	-
5		Razavi Khorasan	Andadvabghad, poshtHaronie	Basir2018604 (HSBU)	+	-	-
6		Razavi Khorasan	Pardis University of Mashhad	Basiri2018605 (HSBU)	+	-	+
7		Tehran	Karaj, Botany Garden	Nikzat2018606 (HSBU)	+	-	-
8		Tehran	Lavasan, Emamkhomeyni Bolívar	Nikzat2018607 (HSBU)	+	-	-
9		Mazandaran	Babol	Nikzat2018608 (HSBU)	+	+	-
10		Mazandaran	Haraz, in Road to Amol, near Ask.	Nikzat2018609 (HSBU)	+	-	-
11		Mazandaran	Savadkooh	Nikzat2018610 (HSBU)	+	-	-
12		Mazandaran	Kiasar	Nikzat2018611 (HSBU)	+	+	-
13		Mazandaran	Babolsar, Parking, near the beach	Nikzat2018612 (HSBU)	+	-	+
14		Gilan	Pahlavi, Ghazian	Mir kamali20141 (W)	+	-	+
15	<i>X. sibiricum</i>	North Khorasan	BeinShirvan-Ghochan	Rechinger11850 (W)	+	-	+
16		Mazandaran	Amol	Nikzat2018613 (HSBU)	+	-	-
17		Mazandaran	HezarJarib,	Nikzat2018614 (HSBU)	+	-	-
18		Mazandaran	Kiasar, in road to Sari	Nikzat2018615 (HSBU)	+	+	+
19		Mazandaran	Savadkooh, Doab, in road to Veresk	Nikzat2018616 (HSBU)	+	-	-
20	<i>X. spinosum</i>	Wardak	Maidantal, Reisfeld W Maidan	Breckle05110 (W)	+	-	+
21		Golestan	Aliabad, beginning city, in road	Nikzat2018617 (HSBU)	+	-	+
22		Mazandaran	HezarJarib, Neka region.	Nikzat2018618 (HSBU)	+	-	-
23		Mazandaran	Sari, in road to Ghaemshahr	Nikzat2018619 (HSBU)	+	+	-
24		Mazandaran	Kiasar	Nikzat2018620 (HSBU)	+	+	-
25	<i>X. strumarium</i>	Mazandaran	Babol to Babolsar highway, initiation of road.	Nikzat2018621 (HSBU)	+	-	-
26		Hamedan	Hamedan	Sabeti3859 (W)	+	-	+
27		Gilan	Masal	Nikzat2018622 (HSBU)	+	-	-
28		Mazandaran	Mahmood Abad to Amol. In road	Nikzat2018623 (HSBU)	+	-	-
29		Razavi Khorasan	Mashhad Ferizi	Basiri2018624 (HSBU)	+	+	-
30		North Khorasan	Bojnourd	Terme & Matine01877 (W)	+	-	+

Table 2. Morphological characters studied

No.	Abbrev.	Morphological characters	No.	Abbrev.	Morphological characters
1	SS	Spine in stem (absent = 1; present = 2)	17	FSH	Fruit shape (elliptic = 1; ovate = 2)
2	Lco	Leaf color on both sides (green = 1; two color = 2)	18	FB	Fruit base (pulled = 1; fasciculated = 2)
3	LB	Leaf base (cordate = 1; wedge = 2)	19	BL	Bur length (cm)
4	NLLo	Number of leaf lobes	20	BW	Bur width (cm)
5	LL	Leaf length (cm)	21	BLW	Bur length/ Bur width
6	LW	Leaf width (cm)	22	BeL	Beak length (mm)
7	PL	Petiole length (cm)	23	BS	Beak situation (curved = 1; right = 2)
8	PW	Petiole width (cm)	24	HS	Hook situation (parallel = 1; converge = 2; divergent = 3)
9	LLW	Leaf length/ Leaf width	25	Fspd	Fruit spine density (dense = 1; scattered = 2)
10	LLP	Leaf length/ Petiole length	26	FT	Fruit trichrome density (dense=1; scattered=2)
11	CoL	Corolla length (mm)	27	SpT	Spine tip in fruit
12	CoW	Corolla width (mm)	28	NSp	Number of spines on 5*5 mm surface of fruit
13	LCoL	Length of corolla lobes (mm)	29	SL	Spine length (mm)
14	CoLW	Corolla length/ Corolla width	30	SpWb	Spine width at base (mm)
15	AnL	Anther length (mm)	31	SpLW	Spine length/ Spine width
16	FL	Filament length (mm)	32	SpTd	Trichrome in spine (up to 1/3 = 1; up to 2/3 = 2; absent = 3)

Molecular study

Genomic DNA was extracted using the CTAB method (Doyle and Doyle 1987) method. The quality of extracted DNA was examined by running on 0.8% agarose gel. The internal transcribed regions (ITS) were amplified using the universal primers ITS4 and ITS5 described by White et al. (1990). The amplification reaction was performed in 25 µl volumes containing 10X PCR buffer (Fermentas, Lithuania), 2.5 µl MgCl₂ (25 mM, Fermentas, Lithuania), 0.2 mM of each dNTP, 2U of Taq polymerase, 100 µmol/L of the universal primers and ca. 200 ng genomic DNA. Amplifications were carried out under the following program: one initial denaturation step at 95 °C for 5 min followed by 35 cycles of 1 min for denaturation at 95 °C, annealing at 54 °C for 45s, and extension at 72 °C for 1 min. The reaction was terminated with a final extension for 3 min at 72°C. PCR products were visualized on 2.5% agarose gel through GelRed™ Nucleic Acid Gel Staining. Sequencing was conducted using Macrogen's sequencing service (Macrogen Inc., Korea). Furthermore, ITS sequences from 12 *Xanthium* species have been obtained from GenBank (*X. ambrosioides* KY215719, *X. spinosum* KY215702, *X. sibiricum* GU724274, *X. strumarium* KY215701, *X. abyssinicum* KY215720, *X. albinum* KY215706, *X. californicum* KY215724, *X. echinatum* KY215716, *X. orientale* KY215704, *X. pungens* KY215703, *X. saccharatum* KY215700, *X. indicum* KY215707) and were added to the molecular matrix. *Ambrosia artemisiifolia* (KY215732) was also used as outgroup.

Sequences were edited using Sequencher version 5.2.4 (Gene Codes Inc., Ann Arbor, Michigan) and aligned using Clustal W (Thompson et al. 1994) as performed in BioEdit Sequence Alignment Editor (Hall 1999). We subsequently used the "simple indel coding" method (Simmons and Ochoterena 2000) to code the indels (insertions/deletions) using SeqState ver. 1.25 (Müller 2005). In order to determine the best evolutionary model fitted for the dataset, the hierarchical likelihood ratio test was calculated using MrModeltest 2.2 (Nylander 2004). The ITS dataset was investigated using the Bayesian MCMC inference for one million generations, sampling every 100 generations. The convergence and burn-in phase were determined using Tracer ver. 1.4 (Rambaut and Drummond 2007). Trees were visualized using TreeView ver. 1.6.6 (Page 2001).

RESULTS AND DISCUSSION

Morphological analysis

Statistical analysis (ANOVA) indicated that the characters including the bur length to width ratio (BLW), fruit base (FB), beak length (BeL) and Hook situation (HS) could not be used to discriminate among the taxa under study (Table 3). Results of the statistical Mann-Whitney test showed that 22, 19 and 22 of 32 (68.7%, 59.3% and 69.7%) characters can significantly discriminate *X. spinosum* from *X. brasiliicum*, *X. strumarium* and *X.*

sibiricum, respectively. Of 32 morphological variables, 13 (40.6%) characters are appropriate to differentiate *X. brasiliicum* from *X. strumarium*. Likewise, 15 of 32 (47%) traits seem to be useful to discriminate *X. sibiricum* from *X. brasiliicum* and *X. strumarium* (Table 3). Moreover, there is no species delimitation in the foliar characteristics among the members of the *X. strumarium* complex (Table 3).

Results obtained from the PCoA (Figure 1) related to the 32 quality and quantity features illustrated that the three species under study as well as *X. spinosum* form two distinct groups. All individuals of *X. spinosum* located on the right side of the graph. The PCoA analysis did not support the taxonomic segregation of the studied species where the three species of the *X. strumarium* complex were placed nearly together on the left side of the graph.

The PCoA results, which represented the first principal component (PCo1) 31.63% of the total variations, the second (PCo2) 19.06%, the third (PCo3) 10.87%, the fourth (PCo4) 8.87%, and the fifth (PCo5) 6.79%, showed that features such as the ratio of leaf length to petiole length (0.465), the ratio of spine length to width (0.32818) and petiole length (0.301) in the first component (PCo1), width of corolla (0.60718) and the ratio of corolla length to width (0.425) in the PCo2, and bur width (0.413) in the PCo3, spine width at base (0.815) in PCo4 and anther length (0.88) in PCo5 were the most variable morphological characteristics (Table 4).

The NJ analysis (Figure 2) separated *X. spinosum* from the species complex. The three species under study slightly overlapped together and the use of morphological features is somewhat problematic. The leaf and fruit variations are obviously overlapped among the members of the *X. brasiliicum* and *X. strumarium* (Figures 3-4). For example, we observed two specimens of the same population of *X. brasiliicum* (Mashhad population, Figure 4, i) with differences in density and size of spines on fruits. *X. sibiricum*'s burs (Figure 4 j-m) are very distinct from the others of the complex. And looking at table 4, spine length/spine width (0.307) and spine width at the base (0.28) seem to be also somewhat responsible for scattering samples along axis 2.

Palynological studies

According to the terminology of Punt and Hoen (2009), pollen type of the species complex overall seems to be similar to that of *X. strumarium* type. Pollen grains are mainly 3-zonocolporate and rarely 4-zonocolporate (Figure 6) with very short, narrow and acutecolpus (Figure 5). Apocolpium index is large. Cavea is distinct (Figure 6) and sculpturing in the members of the complex is scabrate (echinate with spines <1 µm) (Figure 5c,f,i), acute at the top and very broad in base, while it is conical echinate with obtuse tip in *X. spinosum* (Figure 5l). Outlines of pollen in the equatorial view are oblate- spheroidal (Figure 5b), spheroidal (Figure 5e), subprolate (Figure 5h) and prolate-spheroidal (Figure 5k).

Table 3. Description and results of univariate analyses of the morphological characters

Character Abbrev.	ANOVA Sig.	Mean				Std. Dev.				Mann-Whitney test (<i>P</i> -value)					
		Spin	Bras	Stru	Sibr	Spin	Bras	Stru	Sibr	Spin/Bras	Spin/Stru	Spin/Sibr	Bras/Stru	Bras/Sibr	Stru/Sibr
SS	.	1	2	2	2	0	0	0	0	0	0	0	0.639	0.017	0.021
Lco	.	2	1	1	1	0	0	0	0	0	0	0	0.09	0.087	0.002
LB	.	1	2	2	2	0	0	0	0	0	0	0	0.048	0.076	0.002
NLLo	.000	3	4.98	5	4.9	0	0.135	0	0.308	0	0	0	0.019	0.815	0.006
LL	.000	4.16	8.09	8.50	6.91	0.666	1.844	1.980	1.446	0	0	0	0.002	0.243	0.105
LW	.000	1.68	7.41	8.67	6.53	0.393	2.096	1.950	1.285	0.022	0.246	0.003	0.516	0.857	0.174
PL	.000	1.06	7.73	9.28	6.35	0.668	2.787	2.800	1.557	0	0	0	1	1	1
PW	.138	0.17	0.22	0.20	0.23	0.047	0.122	0.061	0.101	0	0	0	1	1	1
LLW	.000	2.53	1.12	0.98	1.06	0.435	0.159	0.080	0.135	0	0	0	0.627	0.112	0.247
LLP	.000	5.22	1.11	0.98	1.12	2.430	0.251	0.326	0.228	0	0	0	1	1	1
CoL	.000	1.87	2.38	1.72	2.15	0.390	0.675	0.262	0.451	0	0.07	0.23	0	0.056	0.002
CoW	.000	1.08	3.09	0.57	0.59	0.096	3.228	0.103	0.199	0.664	0	0	0	0	0.65
LCoL	.000	0.37	0.58	0.49	0.25	0.092	0.269	0.086	0.076	0	0.002	0	0.624	0	0
CoLW	.000	1.73	1.76	3.13	3.99	0.356	1.158	0.769	1.476	0.782	0	0	0	0	0.089
AnL	.564	1.04	1.16	1.51	1.09	0.159	0.801	2.317	0.176	0.126	0.036	0.53	0.23	0.051	0.014
FL	.809	1.20	1.35	1.13	1.44	0.206	1.309	1.468	0.357	0.599	0	0.047	0.058	0.063	0.063
FSh	.000	1	1.47	1	1	0	0.504	0	0	0	1	1	0.002	0	1
FB	.574	1	1.04	1	1	0	0.189	0	0	0.368	1	1	0.488	0.391	1
BL	.000	1.42	2.67	1.82	1.53	0.160	0.577	0.636	0.226	0	0.148	0.081	0	0	0.251
BW	.000	0.82	1.41	1.06	0.79	0.217	0.673	0.519	0.191	0.003	0.236	0.507	0.055	0.001	0.308
BLW	.074	1.82	2.23	1.88	2.04	0.394	0.814	0.437	0.601	0.132	0.864	0.29	0.369	0.719	0.337
BeL	.000	1.63	5.17	3.65	2.41	0.443	0.993	1.224	0.569	1	1	1	1	1	1
BS	.	1	1	1	1	0	0	0	0	0	0	0	0	0.005	0
HS	.000	1	2.69	2	3	0	0.466	0	0	1	1	1	1	1	1
Fspd	.	1	1	1	2	0	0	0	0	1	1	0	1	0	0
FT	.000	1	1.02	1	2	0	0.135	0	0	0.527	1	0	0.627	0	0
SpT	.	2	2	2	2	0	0	0	0	0.001	0.655	0	0.002	0	0
NSp	.000	9.68	11.3	9.46	5.7	1.249	1.860	1.266	0.864	0	0.252	0	0	0	0.001
SL	.000	3.02	5.34	3.6	2.07	0.393	1.098	1.448	0.406	0	0	0	0.231	0.325	0.239
SpWb	.001	0.10	0.58	0.71	0.9	0.021	0.262	0.383	1.444	0	0	0	0.001	0	0.103

Table 4. Eigen vectors of the principal components axes

	PC 1	PC 2	PC 3	PC 4	PC 5
Leaf base	0.14879	0.016653	0.05697	0.02368	0.02072
Leaf color on both sides	0.21689	0.024275	0.083042	0.034512	0.030202
Spine in stem	0.14879	0.016653	0.05697	0.02368	0.02072
Fruit trichrome density	0.022179	0.18788	0.16887	0.060647	0.12273
Fruit shape	0.11352	0.1142	0.14319	0.039138	0.05594
Beak situation	5.66E-20	8.91E-18	3.49E-17	1.30E-16	5.33E-17
Spine tip	1.82E-20	4.65E-18	2.70E-17	9.15E-17	2.46E-17
Spine trichrome density	0.15143	0.12014	0.05703	0.08504	0.060302
Fruit base	0.005595	0.026075	0.04388	0.047672	0.008887
Fruit spine density	0.019727	0.19966	0.14659	0.042442	0.13817
Hook situation	0.18691	0.030209	0.03065	0.02612	0.0775
Leaf length	0.16817	0.035723	0.00388	0.11058	0.03765
Leaf width	0.26245	0.038588	0.0607	0.15249	0.07191
Leaf length/ Leaf width	0.27516	0.019637	0.12794	0.086023	0.067408
Petiole length	0.30106	0.0085361	0.02645	0.2179	0.01884
Leaf length/ Petiole length	0.46572	0.053468	0.19886	0.16505	0.10592
Bur length	0.15771	0.13248	0.18628	0.04139	0.029322
Bur width	0.16491	0.093639	0.41122	0.07297	0.06567
Bur length/ Bur width	0.01165	0.043222	0.21706	0.029977	0.01966
spine length	0.12745	0.2177	0.21144	0.02357	0.02936
Spine width at base	0.26443	0.28092	0.2015	0.81553	0.077424
spine length/ Spine width	0.32818	0.30736	0.054772	0.06698	0.13123
Number of leaf lobes	0.11782	0.0137	0.0358	0.02118	0.0097
Petiole width	0.10033	0.026643	0.05832	0.03993	0.040362
Beak length	0.22396	0.1896	0.095278	0.02548	0.046926
Corolla length	0.060419	0.041246	0.087608	0.054111	0.021166
Corolla width	0.12094	0.60718	0.37043	0.39219	0.18482
Length of corolla lobes	0.11049	0.16875	0.26388	0.038668	0.2268
Corolla length/ Corolla width	0.054727	0.42547	0.031198	0.09235	0.01706
Anther length	0.008051	0.017858	0.38901	0.0195	0.88031
Filament length	0.042	0.00011054	0.33179	0.11769	0.15312
Number of spines on 5×5 mm surface of fruit	0.033425	0.15014	0.14061	0.0336	0.1011

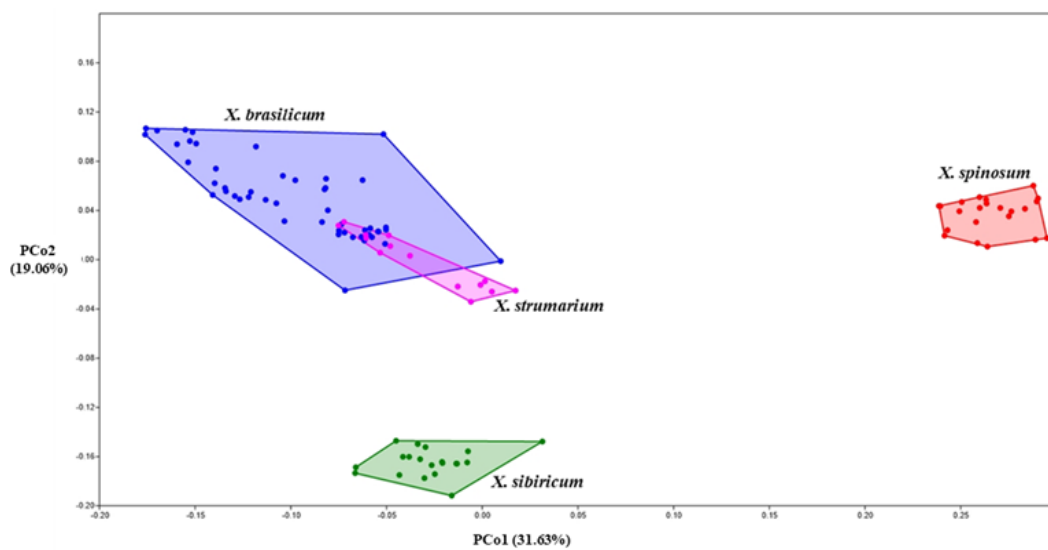


Figure 1. PCoA graph of morphological features based on Euclidian distance

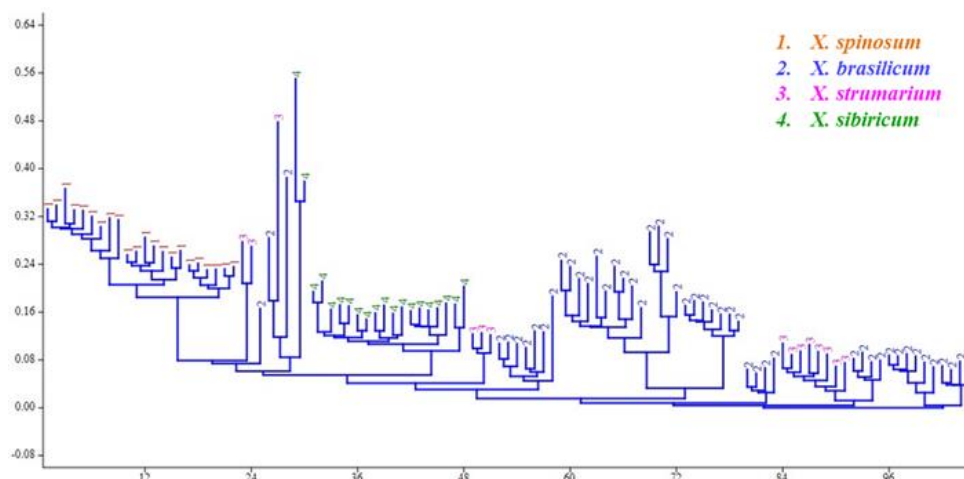


Figure 2. NJ plot of morphological features of *Xanthium* species

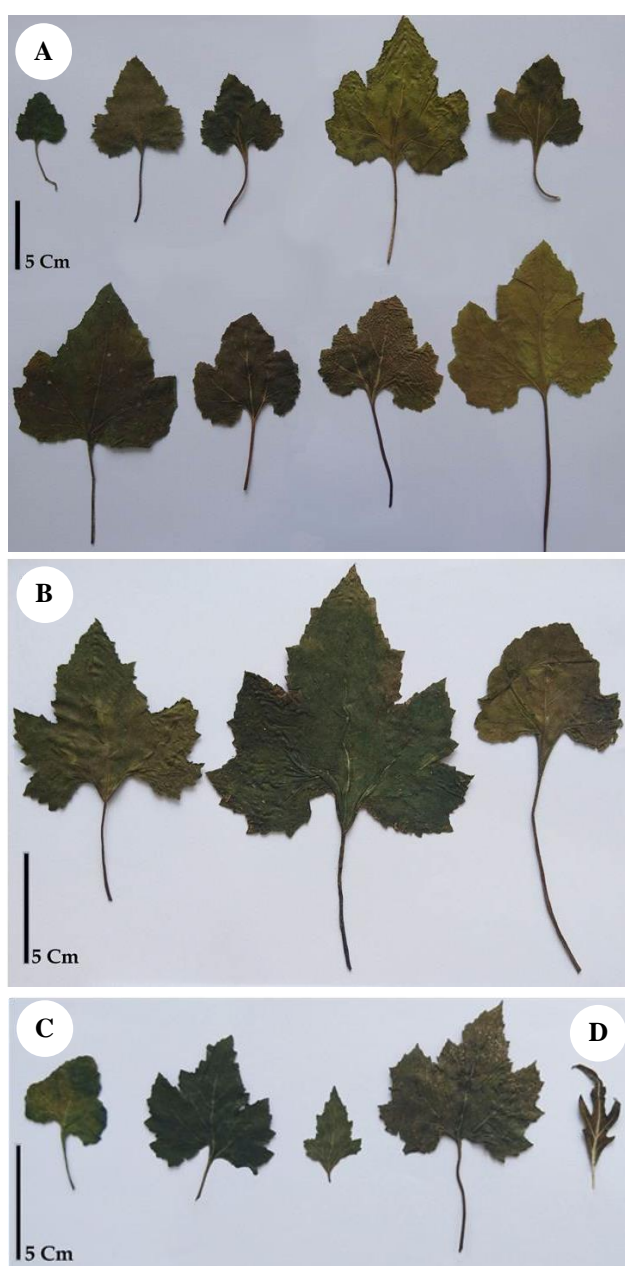


Figure 3. Diversity of leaves in different population of *Xanthium* species in terms of size and shape; a: *X. brasiliicum* (left to right in up: Sardasht, Karaj, Lavasan, Haraz road, Doab) In down: Babol, Abghad, Frizi, Mashhad). b: *X. strumarium* (left to right: Masal, Mahmoodabad, Frizi). c: *X. sibiricum* (left to right: Hezarjarib, Kiasar, Amol, Doab) d: *X. spinosum* (Dargaz road)

Mean length of the pollen grains varied from 23.99 μm (*X. brasiliicum*) to 28 μm (*X. strumarium*), while the width varied from 24.03 μm (*X. spinosum*) to 24.93 μm (*X. brasiliicum*). The main colpus length and the P/E ratio varied from 1.18 μm (*X. spinosum*) to 8.82 μm (*X. sibiricum*) and from 0.96 μm (*X. brasiliicum*) to 1.15 μm (*X. strumarium*), respectively. The main features of the pollen grains of the *Xanthium* species under study are summarized in Table 5.

The PCoA analysis of the pollen features clearly separated *X. spinosum* from the members of the *Xanthium* complex (Figure 7), whereas the individuals of the complex were placed together in the reduced-space graph. The PCo1 and PCo2 axes account for 94% of the total variation. The PCoA results showed that the features such as pollen size, length, density and tip of echinate, shape of pollen, exine thickness were the most variable pollen characteristics.

Molecular studies

A total of 712 nucleotides including coded indels related to 22 taxa were integrated into the Bayesian analysis. Of these, 623 characters were constant, 42 parsimony-uninformative and 47 characters were parsimony-informative. The ITS sequences were analyzed using Bayesian inference with the GTR+G nucleotide substitution model based on the Akaike information criterion generated a 50% majority-rule consensus tree (Figure 8). Except for *X. ambrosioides* and *X. spinosum* which occurred in a well-supported clade, the remaining *Xanthium* species included in the study formed a poorly resolved polytomous clade possibly resulting from relatively low sequence divergence. Within the latter clade, the members of the complex under study were placed inter-mixed with the other *Xanthium* species.

Discussion

Rechinger (1989) in Flora Iranica, separated *X. spinosum* from other *Xanthium* species with spiny stem and color of abaxial leaf surface. On the other hand, shape and density of fruit spines are used to differentiate between *X. brasiliicum* and *X. strumarium*, so that *X. brasiliicum* has ovate-subcircular fruit with high density of spines while *X. strumarium* and *X. sibiricum* have oval fruits with low density of spine, especially near the apex. Thickness of

spine at base and squeezing of fruits in base separated these two latter species from each other (Figure 4). However, in Flora of Turkey (Davis 1975), the genus consists of two species including, *X. spinosum* and *X. strumarium* with two subspecies: *X. strumarium* subsp. *strumarium* L. and *X. strumarium* subsp. *covanillesii* (Schouw) D. Löve & P.Dansereau (syn: *X. brasiliicum* Vellozo, *X.italicum* Moretti in Brugat).

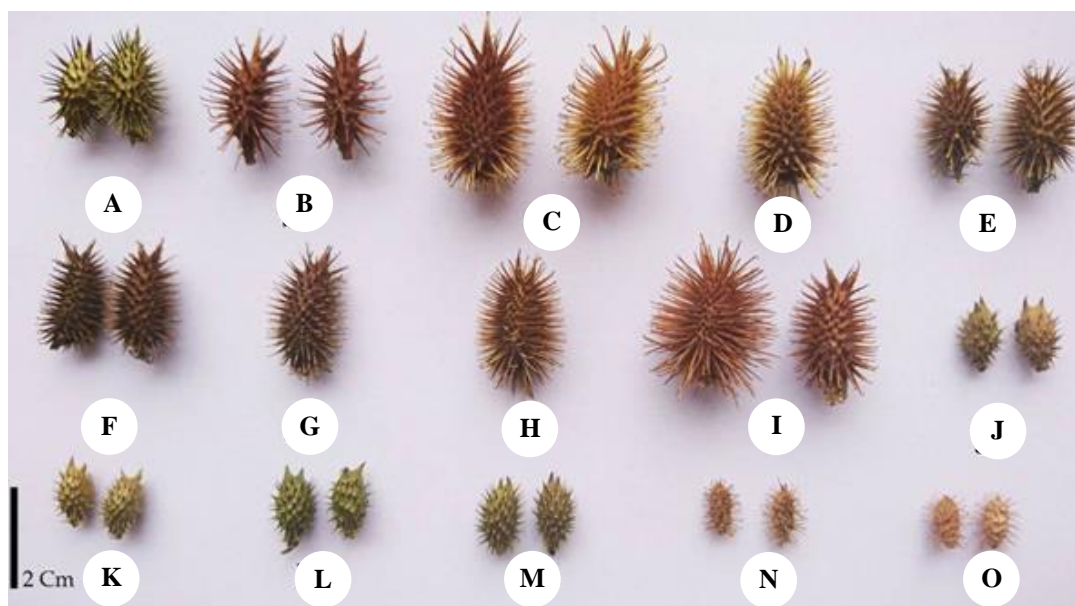


Figure 4. Diversity of fruits in different population of *Xanthium* species in terms of size, shape and spine density; *X. strumarium* (a, Mahmoodabad; b, Mashhad); *X. brasiliicum* (c, Sardasht; d, Lavasan; e, Karaj, f, Haraz road; g, Babol; h, Kiasar; i, Mashhad), *X. sibiricum* (j, Kiasar; k, Doab; l, Hezarjerib; m, Amol); *X. spinosum* (n, Sari; o, Mashhad)

Table 5. Details of examined characters of *Xanthium* sp. pollen specimens in this study

Specimen	SP	P(μ)	P/E	E(μ)	IC(μ)	CL(μ)	NE	IE(μ)	EL(μ)	EW(μ)
<i>X. brasiliicum</i>	Oblate-spheroidal	23.99±0.46	0.96	24.93±0.21	16.02±0.19	7.59±0.99	20±4	0.55±0.09	0.88±0.13	1.81±0.18
<i>X. strumarium</i>	Sunprolate	28±0.62	1.15	24.28±1	17.45±1.24	6.67±0.68	18±3	2±0.35	0.58±0.18	1.96±0.64
<i>X. sibiricum</i>	Prolate-spheroidal	25.57±2.08	1.05	24.26±0.77	10.72±0.35	8.82±1.29	20±4	0.57±0.09	0.7±0.14	1.83±0.12
<i>X. spinosum</i>	Prolate-spheroidal	25.25±0.24	1.05	24.03±1.14	12.28±1.17	1.18±0.18	9±2	1.39±0.31	1.18±0.18	2.18±0.17

Table 5. Details of examined characters of *Xanthium* sp. pollen specimens in this study (continued)

Specimen	LL(μ)	I	P	ι	WET(μ)	d(μ)	D(μ)	AI	PS(μ)	ET(μ)	CT(μ)	TE
<i>X. brasiliicum</i>	0.61±0.08	0.18±0.04	0.15±0.03	17.17±0.43	25.85±0.78	0.66	29.42±1.26	3.44±0.58	1.83±0.71	acute		
<i>X. strumarium</i>	0.55±0.13	0.18±0.04	0.14±0.04	16.99±0.62	26.28±0.47	0.65	30.75±1.29	4.01±0.79	2.19±0.41	acute		
<i>X. sibiricum</i>	0.51±0.12	0.12±0.04	0.09±0.04	17.8±0.96	88.48±0.79	0.63	29.81±3.36	4.42±0.44	2.14±0.23	acute		
<i>x. spinosum</i>	0.39±0.1	0	0	18.1±0.93	28.14±0.93	0.64	24.66±2.11	2.61±0.33	1.28±0.47	obtuse		

Note: SP, shape of pollen shape; P, polar axis; E, equatorial diameter; P/E ratio, interval between colpi; CL, colpi length; NE, number of echinae in 20*20 μm² area; IE, interval between two echinae; EL, echinae length; EW, echinae width in base; LL, length of lines on the surface of echinae; LET, length of echinae top; WET, width of echinae top; d, distance between the apices of two ectocolpi; D, equatorial diameter; AI, apocolpium index; PS, pollen size; ET, exine thickness; CT, cavea thickness; TE, type of echinae.

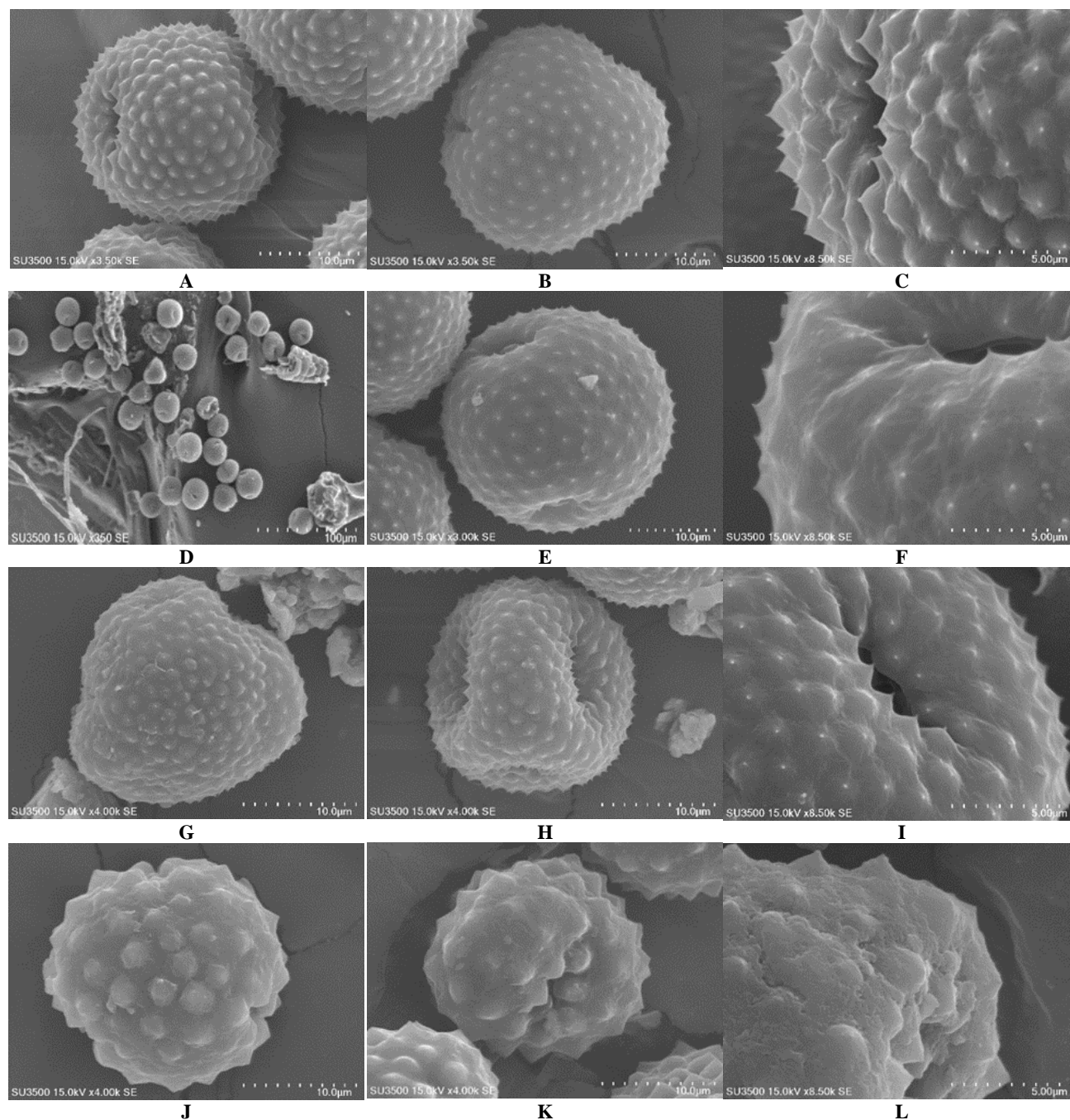


Figure 5. SEM micrograph of pollen grain in *Xanthium* L. A, B, C: *X. brasiliicum*; D, E, F. *X. strumarium*; G, H, I. *X. sibiricum*; J, K, L. *X. spinosum*

Density and situation of spines (\pm hook) and situation of beaks (parallel, divergent) determine their taxonomic boundaries. Furthermore, this taxonomic treatment is proposed in Flora of North America and Flora of Bolivia with only two species including *X. spinosum* and *X. strumarium* (Strother 2006; Hind 1993, 2011). On contrary, *X. spinosum* can be distinguished simply with obvious and characteristic characters in morphology and palynology, such as spiny stem, whitish at the beneath of leaves, leaf base and length, petiole length, ratio of leaf length to width, fruit shape, colpi length, number of echinae in $20 \times 20 \mu\text{m}^2$

area of pollen, echinae length, echinae width in base, length of echinae top, width of echinae top and type of echinae.

The multivariate analysis of morphological variation in the *X. strumarium* complex did not support separation of the complex into the three distinct species as described in Flora Iranica (Rechinger 1989). Results obtained from the PCoA analysis of morphological characters (Figure 1) showed that the three species, *X. strumarium*, *X. brasiliicum* and *X. sibiricum* were placed nearly together specially *X. strumarium* and *X. brasiliicum* are obviously overlapped in PCoA graph.

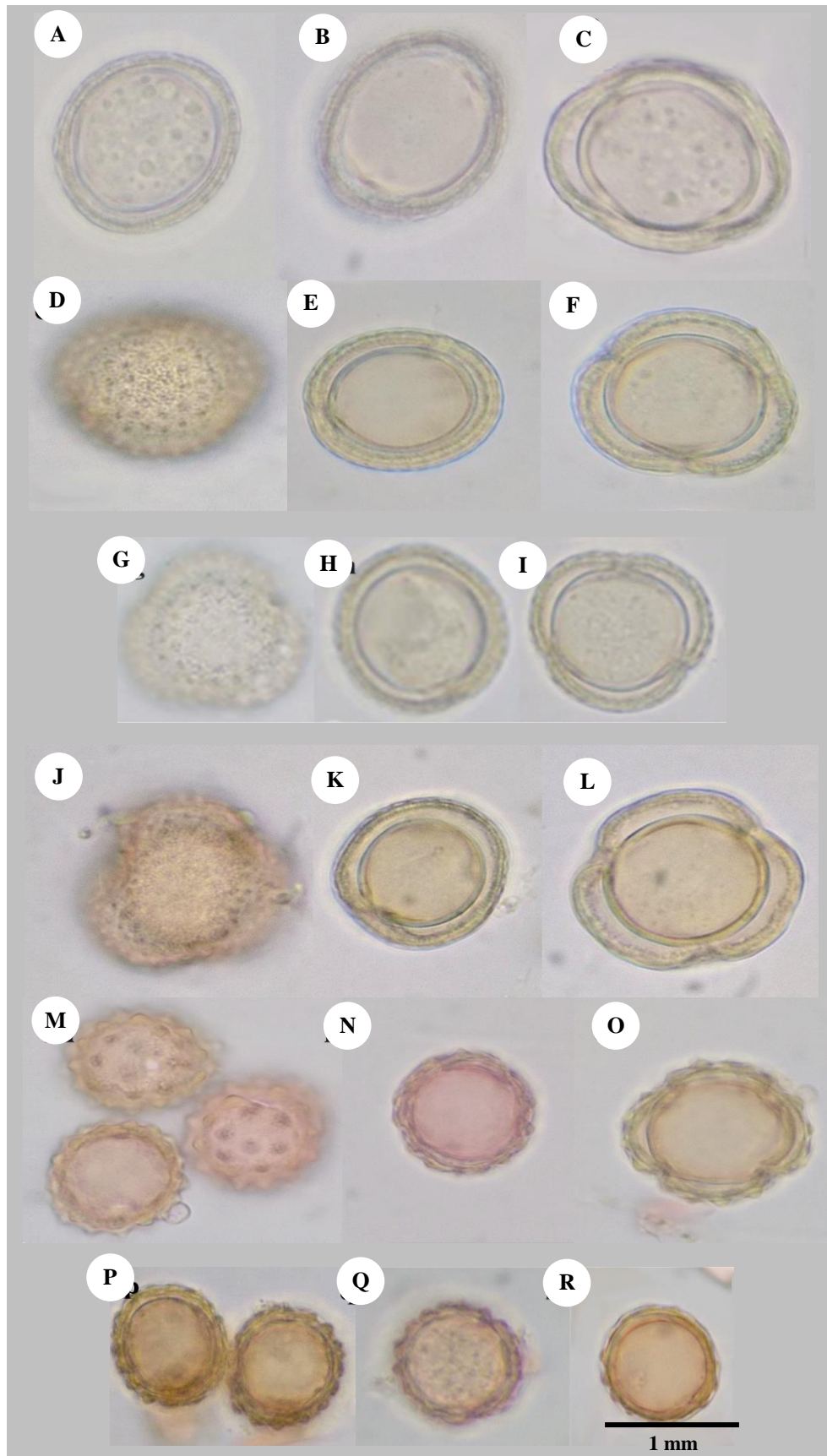


Figure 6. LM micrograph of pollen grain in *Xanthium* L.: A-C. *X. brasiliicum* (Babol), D-F. *X. brasiliicum* (Kiasar), G-I. *X. strumarium*, J-L. *X. sibiricum*, M-O. *X. spinosum* (Ghaemshahr), P-R. *X. spinosum* (Kiasar)

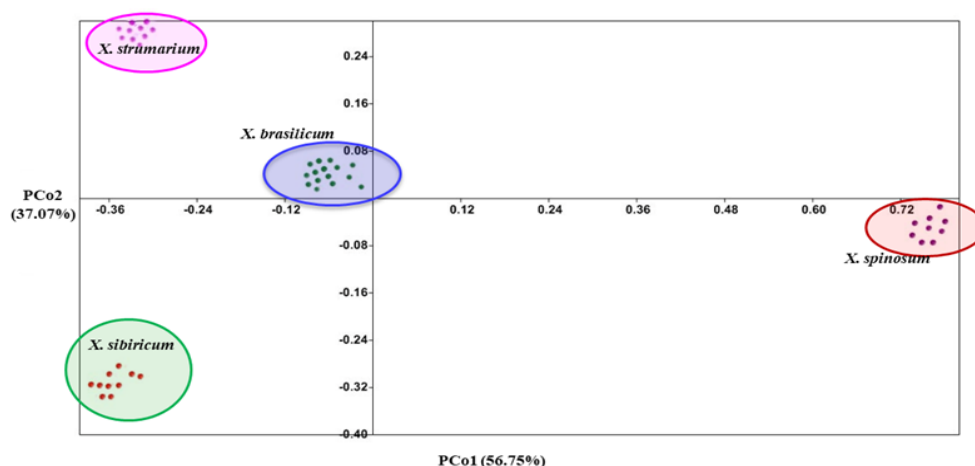


Figure 7. PCoA plot of pollen features of *Xanthium* sp.

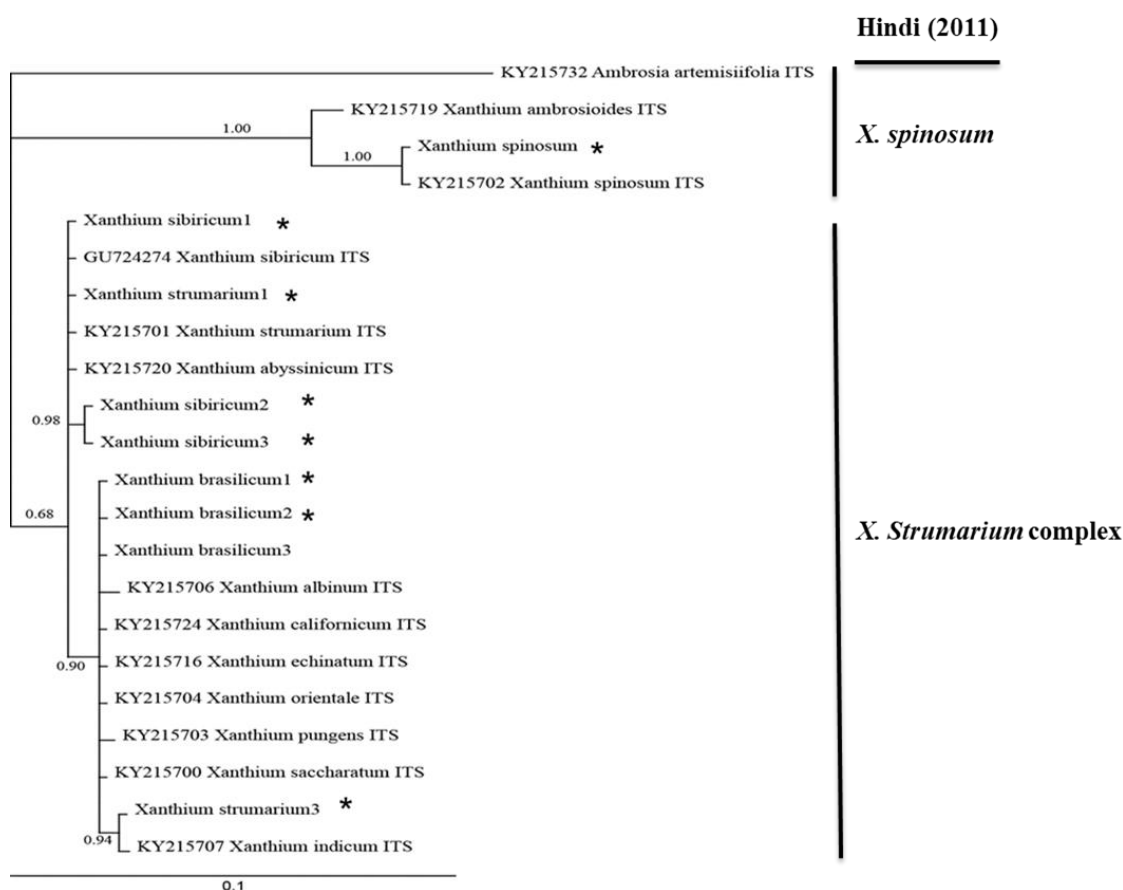


Figure 8. ITS phylogenetic tree resulting from Bayesian analysis of the taxa understudy, 12 *Xanthium* species and one outgroup. Numbers above or below branches are posterior probability supports

Based on the first and second axis (principal coordinates) where the greatest amount of variance (totally 50.69%) exists, there are no obvious boundaries separating *X. strumarium*, *X. brasiliicum* and *X. sibiricum*. The three species can be separated only by a few vegetative characters such as the width of corolla and the ratio of corolla length to width. Likewise, this close relationship is

also visible in the results of the PCoA of palynological characters (Figure 7) in which no differentiating traits could be identified to distinguish distinct species. Based on the first axis with the greatest amount of variance (56.75%), *X. spinosum* was isolated from the members of the complex. Moreover, the second axis of the multivariate analysis of the pollen features with the 37.07% variance

could not also separate the *Xanthium* species complex. The results of palynological analysis of this study are inconsistent with the results of Pereira Coutinho et al. (2021) that showed the pollen grains of section *Acanthoxanthium* and *Xanthium* are clearly differed by their LM, SEM and TEM characters. However, no significant difference found between the pollen of *X. strumarium* subsp. *strumarium* and of *X. strumarium* subsp. *brasilicum*.

Therefore, our results showed abundant phenotypic plasticity in the *X. strumarium* complex species including extensive variation in shape, size and margins of leaves, fruit size, number and length of spines on the burs even within an individual. This is in accordance with the results of Moran and Marshall (1978) that showed *Xanthium* species in Australia had sufficient phenotypic plasticity to cope successfully with fluctuating environments without being dependent on broad genetic variation. Dinelli et al. (2003) indicated the same relevant phenotypic plasticity in Italian *Xanthium* species. Because of these obstacles in the genus, taxonomic classification affronts various insights: classification in several species and condensation to few highly polymorphic species. Results of Tomasello and Heubl (2017) supported the latter opinion, in spite of sequencing of four marker regions. Nonetheless, the results improved the identification of *X. sibiricum* by preparing specific restriction pattern. Moreover, Wallace et al. (2012) implied ITS2 sequencing for seven *Xanthium* species and indicated low divergence among these species in terms of informative sequence.

Hind (2011) in Flora of Bolivia considered a set of species including *X. brasilicum*, *X. sibiricum*, *X. abyssinicum* Wallr., *X. californicum* Greene, *X. albinum* (Widder) Scholz & Sukopp, *X. orientale* L., *X. echinatum* Murray, *X. pungens* Wallr., *X. saccharatum* Wallr. and *X. indicum* DC. to be synonymous with *X. strumarium*. Similarly, *X. ambrosioides* Hook. & Arn. is synonymous with *X. spinosum*. In summary, according to this Flora, all species that were previously defined as *Xanthium* have been synonymous with two species: *X. strumarium* and *X. spinosum*. The species with petiole subtended by an axillary 3-fid spine, rhomboid leaves that are whitish at the beneath and bur without or with 1 beak are considered to be *X. spinosum* and spineless plants with triangular concolorous leaves, cordate at base and bur with 2 beaks are defined as *X. strumarium*. Tomasello (2018) evaluated 34 *Xanthium* individuals from 12 species with molecular markers and coalescent-based species delimitation methods (BP & P and STACEY) to determine species boundaries in *Xanthium*. He recognized five species in the genus *Xanthium* worldwide: *X. spinosum* L. And *X. ambrosioides* Hook & Arn. on the one hand, and *X. strumarium* L., *X. orientale* L. and *X. chinense* Mill. (*X. pungens*) on the other, and provided an identification key.

However, our results obtained from the morphological, palynological and molecular study are in agreement with those proposed by Hind (2011) and Tomasello (2018) in which strongly supported scenarios with a reduced number of species. Our findings are inconsistent with Tomasello (2018) which revealed that in species tree reconstruction

published by this author, the distance between the *Acanthoxanthium* and *Xanthium* section clades is always larger than the distances between the remaining clades (as shown in Fig1, 7 and 8), and Species in *Xanthium* section (*Xanthium chinense*, *X. orientale*, and *X. strumarium*) are traditionally characterized by their bur morphology but, as they frequently hybridize, an extensive number of transition morphotypes exist (Löve and Dansereau 1959; Pereira Coutinho and Paiva 2019). In summary, according to Tomasello (2018), two species of section *Xanthium* present in Iran (*X. orientale* and *X. strumarium*) that separate well by bur size and morphological features.

In conclusion, all species that were previously defined as *Xanthium* in Iran have been synonymous with three species: *X. spinosum*, *X. strumarium* and *X. orientale* (*X. brasilicum* and *X. strumarium* are considered to be synonymous with *X. orientale* and *X. sibiricum* are considered to be synonymous with *X. strumarium*). The species with petiole subtended by an axillary 3-fid spine, leaf blade lanceolate to ovate and 3-4 times longer than broader, burrs only lightly hairy that bearing 0-1 apical beak (if 2, then unequal) and erect to decumbent annual reaching 50-100 cm in height are considered to be *X. spinosum* and spineless plants with cordate to orbicular leaf blade and burrs bearing 2 equal beaks are defined as *X. strumarium* or *X. orientale* (*X. strumarium*: burrs glabrous and small, rarely exceeding 1 cm in length, with few, thin prickles and strait beaks and leaves to a large extent cordate; *X. orientale*: burrs generally hirsute, up to 3 cm long (including beaks), prickles uncinated and beaks straight or curved).

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