

Seed morphology of some *Plantago* (Plantaginaceae) species in Iran and its systematic and phylogenetic implications

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Abstract: *Plantago* is a cosmopolitan genus with approximately 200 species and various infrageneric classifications have been reported. In this study, the seed morphologies of 19 species of *Plantago* from Iran were studied and documented in detail using scanning electron microscopy and light microscopy. The studied species were grouped by using statistical analyses such as a minimum spherical cluster method based on Euclidean distance, principal coordinate analysis, and multidimensional scaling. We have described four types based on the seed shape and states of the inner side of the studied seeds: convex- angular type, convex-elliptic type, flat-elliptic type, and concave-elliptic type. In this study, three types of seed coat patterns were recognized: reticulate, scalariform to reticulate, and papillate. Regardless of the limited range taxa considered, our data convincingly support a close relationship between subgenera *Plantago* and *Coronopus*, and between *Albicans* and *Psyllium*. Consequently, we would suggest that the species of subgenus *Coronopus* should be placed within subgenus *Plantago* as Pilger's taxonomy, and the species of subgenus *Albicans* should be merged within subgenus *Psyllium* as Rønsted et al.'s taxonomy. The seed morphology revealed that subgenus *Plantago* and subgenus *Albicans* are paraphyletic. Moreover, the deformation of testa cells can be an indicator for finding different lineages at series to the subgenus level. The features of the arrangement of testa cells and anticlinal walls can be used at the species level, especially when the morphological diversity between species is low. We conclude that seed morphology provides useful and important information on phylogenetic relationships of *Plantago* species, and thus they have systematic significance.

Key words: Light microscopy, lineage, scanning electron microscopy, macromorphology, micromorphology, testa, taxonomy

1. Introduction

The genus *Plantago* L. is a cosmopolitan genus with more than 200 species, mostly distributed in the temperate and high-elevation tropical regions (Schwarzbach, 2004; Hassemer, 2017; Iwanycki Ahlstrand et al., 2019). Species of this genus are annual or perennial herbs, and shrubs with alternative and opposite leaves, cylindrical, and ovoid spikes with small hermaphrodite flowers (Kazmi, 1974; Rahn, 1996).

The seed of the most *Plantago* species has been used in both conventional and traditional systems of medicine throughout Asia, Europe, and North America. Furthermore, few species such as *P. afra* L. and *P. ovata* Forssk. are highly valued in nutraceutical, pharmaceutical, and cosmetic industries. The polysaccharides obtained from seed husks in these species can improve intestinal performance, as well as obesity, high cholesterol, colon cancer, constipation, and diabetes (Sarihan et al., 2005; Goncalves and Romano, 2016).

Taxonomy of the genus *Plantago* is controversial at the infrageneric level and the sectional position of a few species

is ambiguous in spite of multiple molecular phylogenetic studies (Hassemer et al., 2018, 2019). Different researchers subdivided this genus into 2-6 subgenera. Thus, Pilger (1937) assigned 260 species into 2 subgenera of *Euplantago* Harms (subgenus *Plantago*) and *Psyllium* (Juss.) Harms, while Rahn (1978) initially classified the genus *Plantago* into 3 subgenera of *Plantago*, *Coronopus* (Lam. et DC.) Rahn, and *Psyllium* Rahn (here is included subgenus *Psyllium* and 5 sections of subgenus *Euplantago* in the sense of Pilger, 1937). Subsequently, based on cladistic analysis of the combined morphological, embryological and chemical data, Rahn (1996) subdivided the genus into 6 subgenera of *Plantago*, *Coronopus*, *Albicans* Rahn, *Psyllium* Juss., *Bougueria* (Decne.) Rahn (= genus *Bougueria* Decne.), and *Littorella* (P.J.Bergius) Rahn (= genus *Littorella* P.J.Bergius). Moreover, based on nuclear ribosomal ITS and plastid trnL-F sequences, Rønsted et al. (2002) classified the genus into 5 subgenera of *Plantago*, *Coronopus*, *Psyllium* (Juss.) Harms & Reiche, *Bougueria* and, *Littorella*. However, based on ITS sequences, Hoggard et al. (2003) recognized the subgenus *Littorella* as a distinct

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genus, and also placed the subgenus *Bougueria* within the subgenus of *Albicans*. In a numerical taxonomy study of the genus *Plantago*, Shalabi and Abou-El-Enain (2013) showed that there is a high affinity between species of subgenera *Albicans* and *Psyllium*, and the evolutionary line of *P. major* L. is different from species of subgenera *Coronopus*, *Psyllium*, and *Albicans*. Recently, Iwanycki Ahlstrand et al. (2019) and Hassemmer et al. (2019) studied subgenus *Plantago* using ITS and plastid trnL-F, ndhF-rpl32, rpl32-trnL sequences, and high-throughput plastid genome sequencing, respectively. They obtained controversial results, especially in placing species of sections *Plantago* Rahn and *Mesembrynia* (Decne.) Rahn. For example, they placed *P. reniformis* Beck, *P. media* L., *P. maxima* Jacq., *P. canescens* Adams, *P. palmata* Hook.f., and *P. depressa* Willd. into different sections. Similarly, using a nuclear-encoding single-copy gene SUC1, Ishikawa et al. (2009) concluded that reticulate evolution and two independent long-distance dispersals between the northern and southern hemispheres are probably the reasons for taxonomic complexity in this subgenus. Therefore, the comprehensive study using all fields and combined analyses is necessary to investigate the interspecies relationships in the genus *Plantago*.

Both macro- and micromorphological features of seeds are of essential systematic significance within and among the genera of traditional Scrophulariaceae, Orobanchaceae, and Plantaginaceae (Ghimire et al., 2017). The morphology or micromorphology of seeds is often utilized for taxonomic purposes in different plants including Plantaginaceae (e.g., Munoz-Centeno et al., 2006; Yilmaz, 2013; Hassan and Abdel Khalik, 2014; Mazur et al., 2018) and related families, such as Scrophulariaceae or Gesneriaceae (e.g., Juan et al., 2000; Mendum et al., 2001; Ghimire et al., 2017). Seed morphology is valuable in taxonomy and evolution of the genus *Plantago* (Rezk, 1980; Liu et al., 1992; Klimko et al., 2004; Shehata and Loutfy, 2006; Hoghoughi et al., 2016; Verma et al., 2017). Rezk (1980), by examining the seed structure of 31 species of *Plantago*, showed 4 hypotheses of evolutionary lines in the genus *Plantago* including 1) change from closed triquetrous seed shape to the open plano-convex type to the open concavo-convex type, 2) change from transversely oriented cotyledons to vertically oriented cotyledons to obliquely oriented cotyledons, 3) crushing of the cellular coloring layer resulting in declined sensitivity to light in germination, 4) increase of seed size from 0.9 mm to 3.4 mm. Moreover, he mentioned that *P. major* would be regarded as an ancient species based on the correlation of seed structure, phytogeographic distribution, and evolutionary lines. By studying the seed morphology of 17 *Plantago* species in China, Liu et al. (1992) concluded that seed morphology classification is in

agreement with Pilger's system (1937). Based on the seed coat patterns using SEM of 8 *Plantago* species of Poland, Klimko et al. (2004) concluded that Seed structure is a good marker for identifying interspecific relationships and *P. maritima* L. should not be placed in the section *Coronopus* DC. Shehata and Loutfy (2006), using SEM of the seed coat of several genera of Plantaginaceae, inferred that subgenus *Albicans* may be paraphyletic. By examining pollen and seed morphology of several *Plantago* species, Hoghoughi et al. (2016) showed that despite similar pollen in the studied *Plantago* species, seed features can help to determine the studied species. Thus, the seed morphology can help improve our understanding of the systematic and phylogenetic relationships of *Plantago* species.

The genus *Plantago* includes 23 species from Iran, which are distributed across different geographical regions. According to the classification of Pilger (1937), Rahn (1996), and Rønsted et al. (2002), Iranian *Plantago* species are placed into 2 subgenera (based on morphological data), 4 subgenera (based on morphological, embryological, and chemical data), and 3 subgenera (based on molecular data), respectively. Unlike Rahn's opinion, Rønsted et al. (2002) have shown that *Plantago* subgen. *Albicans* is paraphyletic and should be merged into the *Plantago* subgen. *Psyllium* to get a monophyletic clade. Rahn (1978) separated the Mediterranean species of subgenus *Euplantago* and placed them into subgenus *Coronopus* but Taskova et al. (2002) using iridoid patterns in the genus *Plantago* concluded that Rahn's decision was not correct. Although there have been several molecular studies in the genus *Plantago*, the taxonomic position of some species has not been determined, or there is no agreement between the molecular and morphological classifications. Therefore, comprehensive studies using all fields and combined analyses are necessary to investigate the interspecies relationships in the genus *Plantago*. The aims of the present paper are to: 1) conduct a comprehensive study of the seed macro- and micromorphology of the *Plantago* distributed in Iran, 2) demonstrate and document the variability of seed morphology within the genus *Plantago* and to provide qualitative and quantitative data for a future phylogenetic reassessment of the whole genus, 3) infer different lineages based on seed morphology data, and 4) investigate the application of seed morphology in systematics of examined *Plantago* taxa particularly in infrageneric classification.

2. Material and methods

2.1. Plant material

In this study, the investigated taxa included 19 species (representatively belong to 7 sections of 8 sections of three subgenera including *Plantago*, *Coronopus*, *Albicans* plus two species of subgenus *Psyllium* according to the most comprehensive infrageneric classification of Rahn,

1996; note that subgenus *Psyllium* has no section in this classification) of *Plantago* in Iran flora which were collected from natural habitats in Iran. The voucher specimens were deposited in the Herbarium Shahid Beheshti University (HSBU), Herbarium Ministerii Iranici Agriculturae (IRAN), and Ferdowsi University of Mashhad Herbarium (FUMH) (Table 1, Appendix 1). The *Plantago* species were identified using different references (Patzak and Rechinger, 1965; Kazmi, 1974; Sell, 2010). The seed morphology data of the collected species were used for statistical analyses.

2.2. Seed observations and measurements

For each species, at least 5 individuals were studied, 20–30 mature seeds were examined under light microscope (LM) for macromorphological study, and at least 5 intact seeds were examined for micromorphological study via scanning electron microscopy (SEM). For SEM observations, dried seeds were mounted on aluminum stubs and sputter-coated with gold and further examined using a Hitachi SU3500 (Japan) scanning electron microscope at 15–20 kV. The seed morphology was analyzed for 17 qualitative and quantitative characters (Table 2, Appendix 2). For quantitative characters, the arithmetic mean and standard deviation were calculated. The seed morphology was

described following Barthlott (1981), Stearn (1983), and Rahn (1996) terminology.

2.3. Statistical analyses

WARD clustering (a minimum spherical cluster method) based on Euclidean distance after 100 times bootstrapping, principal coordinate analysis (PCoA) and multidimensional scaling (MDS) were performed to group the plant specimens according to qualitative and quantitative characters of the seed morphology (Podani, 2000). The seed morphology data were standardized (mean = 0, variance = 1) for these analyses (Podani, 2000). Data analysis was performed via PAST ver. 2.17 software (Hammer et al., 2012).

3. Results

3.1. Morphological variation within the studied *Plantago* species

The species studied are annual (for example, *P. loeflingii* L.) or perennial herbs (*P. atrata* subsp. *spadicea* Pilg.), acaulescent (*P. major*), or infrequently stemmed (*P. indica* L.). The leaves are simple in basal rosettes (in subgenus *Plantago*), alternate (in subgenera *Coronopus*, and *Albicans*), or opposite on branched stems (in subgenus *Psyllium*), and the leaf blade

Table 1. List of localities of the studied *Plantago* species.

No	Taxa	Subgenus/ section Rahn (1996)	Locality	Voucher number
1	<i>P. major</i> L.	<i>Plantago/Plantago</i>	Tehran, Darakeh	HSBU-2018950
2	<i>P. gentianoides</i> subsp. <i>griffithii</i> Rech. f.	<i>Plantago/Plantago</i>	Khorassan, Mashhad	FUMH-46166
3	<i>P. coronopus</i> subsp. <i>coronopus</i> L.	<i>Coronopus/Coronopus</i>	Fars, Kazerun	HSBU-2018951
4	<i>P. coronopus</i> subsp. <i>commutata</i> Pilg.	<i>Coronopus/Coronopus</i>	Bushehr, Dalaki	HSBU-2018952
5	<i>P. amplexicaulis</i> subsp. <i>bauphula</i> Rech. f.	<i>Albicans/Bauphula</i>	Hormozgan, Bandarabbas	HSBU-2018953
6	<i>P. lanceolata</i> L.	<i>Albicans/ Lanceifolia</i>	Tehran, Darakeh	HSBU-2018954
7	<i>P. lagopus</i> L.	<i>Albicans/ Lanceifolia</i>	Fars, Kazerun	HSBU-2018955
8	<i>P. atrata</i> subsp. <i>spadicea</i> Pilg.	<i>Albicans/Montana</i>	Azerbaijan, Arasbaran	HSBU-83798
9	<i>P. loeflingii</i> L.	<i>Albicans/Montana</i>	Fars, Kazerun	HSBU-2018956
10	<i>P. notata</i> subsp. <i>haussknechtii</i> Rech. f.	<i>Albicans/Montana</i>	Khuzestan, Ahvaz	IRAN-32614
11	<i>P. boissieri</i> var. <i>boissieri</i> Hausskn. & Bornm.	<i>Albicans/Albicans</i>	Bushehr, Borazjan	HSBU-2018957
12	<i>P. ciliata</i> subsp. <i>lanata</i> Rech. f.	<i>Albicans/Albicans</i>	Kerman, Jiroft	HSBU-2018958
13	<i>P. cylindrica</i> Forssk.	<i>Albicans/Albicans</i>	Hormozgan, Bandarabbas	IRAN-32617
14	<i>P. evacina</i> Boiss.	<i>Albicans/Albicans</i>	Khorassan, Mashhad	FUMH-17188
15	<i>P. orzuensis</i> Mohsenz. et al.	<i>Albicans/Albicans</i>	Kerman, Orzuia	HSBU-2018959
16	<i>P. ovata</i> Forssk.	<i>Albicans/Albicans</i>	Fars, Kazerun	HSBU-2018400
17	<i>P. stocksii</i> Boiss.	<i>Albicans/Albicans</i>	Semnan, Shahroud	IRAN-54272
18	<i>P. bellardii</i> subsp. <i>deflexa</i> Rech. f.	<i>Albicans/Hymenopsyllium</i>	Fars, Kazerun	HSBU-2018960
19	<i>P. indica</i> L.	<i>Psyllium / -</i>	Mazandaran, Miankaleh	HSBU-87204
20	<i>P. afra</i> L.	<i>Psyllium / -</i>	Bushehr, Dalaki	HSBU-2018411

Table 2. Evaluated seed morphology characters.

No	Seed morphology characters		Character-states
1	Shape		1- Angular, 2- Broadly elliptic, 3-Elliptic, 4- Narrowly elliptic
2	Color		1- Darkish brown, 2- Grayish brown, 3- Yellowish brown, 4- Brown, 5- Light brown, 6- Brown with mucilage coating
3	Testa thickness		1- Thick, 2- Thin
4	Inner side		1- Convex, 2- Flat, 3- Concave
5	Transverse furrow at middle		1. Absent, 2. Presence
6	Hilum		1- Superficial, 2- On septum, 3- In cavity
7	Seed coat pattern		1- Reticulate, 2- Scalariform to reticulate, 3- Papillate
8	Cell shape		1- Angular, 2- Rounded
9	Anticlinal walls	1) Shape	1- Straight, 2- Undulate, 3- Bi-armed, 4- Curved or slightly curved, 5- slightly curved
		2) Thickness degree	1- Thin, 2- Mediate, 3- Thick
		3) Level	1- Raised, 2- Channeled
10	Periclinal walls	1) Level	1- ±Flat, 2- concave, 3- Flat, 4- convex
		2) Texture	1- Striate, 2- Ruminant, 3- Scalariform, 4- Granulate, 5- Rugose, 6- Reticulate, 7- Microreticulate
11	Seed length (mm)		
12	Width seed (mm)		
13	Length/width		
14	Cell size (µm)		

is ovate (*P. gentianoides* subsp. *griffithii* Rech. f.), lanceolate (*P. lanceolata* L.), linear (*P. afra*), or linear-lanceolate (*P. cylindrica* Forssk.); pilose (*P. ciliata* subsp. *lanata* Rech. f.) or glabrous (*P. major*) with simple (*P. stocksii* Boiss.) or glandular (*P. afra*) hairs, margin entire (*P. stocksii*), toothed (*P. ovata*), rarely pinnately or palmately cleft (*P. coronopus* subsp. *coronopus* L. and subsp. *commutata* Pilg.). The flowers are 4-merous, hermaphrodite, polygamous, usually in many-flowered cylindrical (*P. major*) or ovate (*P. evacina* Boiss.) spikes. Bracts are ovate (*P. bellardii* subsp. *deflexa* Rech. f.) to boat-shaped (*P. atrata* subsp. *spadicea* Pilg.), rarely dissimilar (*P. indica*). Calyx lobes are usually subequal, ovate (*P. evacina*) or ovate-lanceolate (*P. indica*). Corolla tube is cylindrical, glabrous (*P. notata* subsp. *haussknechtii* Rech. f.) or pilose (*P. coronopus* subsp. *coronopus* and subsp. *commutata*), lobes 4, ovate, equal, glabrous (*P. lagopus* L.) or Pilose (*P. ciliata* subsp. *lanata*). Stamens 4 alternate with corolla lobes; anthers free with an apical appendage. Pistil consisting of 2 carpels; ovary superior, 2-3 loculed; style 1, filamentous. Fruit a pyxis, with 2 to multiple seeds. Seeds are minute, and testa is mucilaginous when wet.

3.2. Macromorphological features of seeds

The seeds of the studied *Plantago* species varied in their size (length = 0.9–6.1 mm, width = 0.45–3.2 mm) (Table3).

The seeds of the studied species had two basic angular and elliptic shapes. The angular shape occurred only in *P. major* (subgenus *Plantago*), while the elliptical shape was subdivided into three states: broadly elliptic, elliptic, and narrowly elliptic. The base of the seed color in the studied species was brown and they were visible in darkish brown, grayish brown, yellowish brown, brown, light brown, and brown with white mucilage coating. The inner side of the *Plantago* seeds was convex, flat, and concave. Moreover, the testa thickness of the studied seeds was considered. The testa thickness of the studied seeds was observed in thin and thick states, with the former observed only in *P. amplexicaulis* (subgenus *Albicans*), while the latter found in the other studied species. Finally, we observed three states of hilum position on the inner surface of the studied seeds: superficial, on septum, and in cavity (Figure1).

3.3. Micromorphological features of seeds

The characteristics of the testa surface of seeds of the 19 species of *Plantago* are summarized in Table 4 and Figures 2 and 3. We found that among species, the most important differences were in the cell size, coat pattern, shape of testa cells, and the anticlinal plus periclinal walls' features. Furthermore, these seed characters were constant within species. The testa cells of the studied

Table 3. Seed macromorphological features of the studied *Plantago* species. C, color [darkish brown: DB, grayish brown: GB, yellowish brown: YB, brown: B, light brown: LB, brown with white mucilage coating: BMC]; L, seed length [range; mean values \pm standard deviation]; W, seed width [range, mean values \pm standard deviation]; L/W, length/width. TT, testa thickness [thick: T, thin: Th]; TFM, transverse furrow at middle [Absent: -, Presence: +]; H, Hilum [superficial: Su, on septum: OS, in cavity: IC].

Taxa	Shape	C	L (mm), mean \pm SD	W (mm), mean \pm SD	L/W ratio	TT	Inner side	TFM	H
<i>P. major</i>	Angular	DB	0.9–1.3 1.07 \pm 0.1	0.45–0.74 0.59 \pm 0.07	1.84 \pm 0.3	T	Convex	-	Su
<i>P. gentianoides</i> subsp. <i>griffithii</i>	Elliptic	DB	1.15–1.8 1.44 \pm 0.2	0.54–1 0.73 \pm 0.1	2.01 \pm 0.3	T	Convex	-	Su
<i>P. coronopus</i> subsp. <i>coronopus</i>	Elliptic	GB	1.2–1.54 1.35 \pm 0.1	0.72–0.9 0.84 \pm 0.06	1.68 \pm 0.1	T	Flat	+	Su
<i>P. coronopus</i> subsp. <i>commutata</i>	Elliptic	GB	1.2–1.5 1.32 \pm 0.08	0.66–0.9 0.8 \pm 0.08	1.65 \pm 0.2	T	Flat	+	Su
<i>P. amplexicaulis</i> subsp. <i>bauphula</i>	Broadly elliptic	YB	5–6.1 5.47 \pm 0.4	2.3–3.2 2.98 \pm 0.3	1.57 \pm 0.7	Th	Concave	-	OS
<i>P. lanceolata</i>	Narrowly elliptic	B	1.9–2.3 2.13 \pm 0.1	0.94–1.2 1.02 \pm 0.05	2.09 \pm 0.28	T	Concave	-	IC
<i>P. lagopus</i>	Narrowly elliptic	LB	1.2–1.8 1.54 \pm 0.1	0.5–0.76 0.65 \pm 0.1	2.4 \pm 0.2	T	Concave	-	IC
<i>P. atrata</i> subsp. <i>spadicea</i>	Narrowly elliptic	LB	3.28–4.7 4.12 \pm 0.34	1.76–2.2 1.93 \pm 0.15	2.15 \pm 0.2	T	Concave	-	IC
<i>P. loeflingii</i>	Narrowly elliptic	B	2.06–2.65 2.33 \pm 0.14	0.73–1.13 0.93 \pm 0.1	2.53 \pm 0.2	T	Concave	-	IC
<i>P. notata</i>	Narrowly elliptic	B	1.5–2.04 1.72 \pm 0.11	0.63–0.84 0.73 \pm 0.1	2.35 \pm 0.1	T	Concave	-	IC
<i>P. boissieri</i> var. <i>boissieri</i>	Narrowly elliptic	LB	1.88–2.22 2.08 \pm 0.09	0.98–1.34 1.14 \pm 0.1	1.84 \pm 0.15	T	Concave	-	IC
<i>P. ciliata</i> subsp. <i>lanata</i>	Elliptic	BMC	2.1–2.36 2.2 \pm 0.08	1.18–1.4 1.26 \pm 0.08	1.74 \pm 0.1	T	Concave	-	IC
<i>P. cylindrica</i>	Narrowly elliptic	LB	1.67–2.3 2.06 \pm 0.2	0.92–1.2 1.03 \pm 0.1	1.99 \pm 0.16	T	Concave	-	IC
<i>P. evacina</i>	Elliptic	B	2.3–2.81 2.53 \pm 0.2	1.2–1.65 1.4 \pm 0.13	1.81 \pm 0.15	T	Concave	-	IC
<i>P. orzuiensis</i>	Elliptic	BMC	2.6–3.1 2.77 \pm 0.14	1.36–1.8 1.59 \pm 0.12	1.75 \pm 0.1	T	Concave	-	IC
<i>P. ovata</i>	Elliptic	BMC	2.12–2.8 2.5 \pm 0.2	1.25–1.6 1.39 \pm 0.08	1.8 \pm 0.14	T	Concave	-	IC
<i>P. stocksii</i>	Narrowly elliptic	LB	3.6–4.76 4.12 \pm 0.5	1.5–1.85 1.66 \pm 0.12	2.5 \pm 0.36	T	Concave	-	IC
<i>P. bellardii</i> subsp. <i>deflexa</i>	Elliptic	B	1.76–2.4 1.99 \pm 0.2	0.93–1.3 1.14 \pm 0.1	1.76 \pm 0.1	T	Concave	+	IC
<i>P. indica</i>	Narrowly elliptic	B	1.98–2.56 2.27 \pm 0.18	0.85–1.18 1.07 \pm 0.11	2.35 \pm 0.2	T	Concave	-	IC
<i>P. afra</i>	Narrowly elliptic	B	1.96–2.96 2.46 \pm 0.26	0.69–0.96 0.85 \pm 0.07	2.92 \pm 0.	T	Concave	-	IC

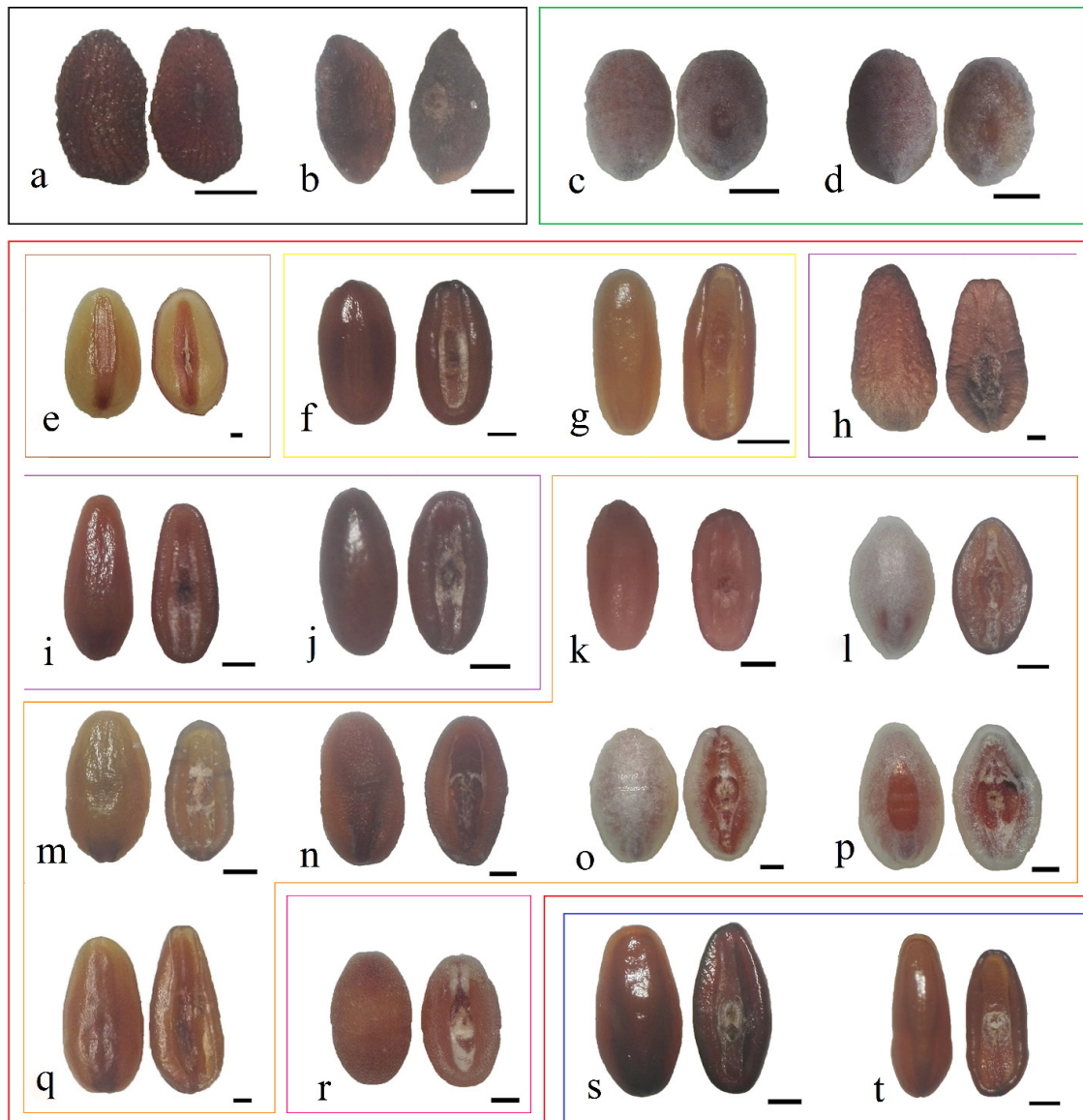


Figure 1. Seed macromorphological characteristics of the studied *Plantago* species. Subgenus *Plantago*/section *Plantago* (black box): *P. major* (a), *P. gentianoides* subsp. *griffithii* (b); subgenus *Coronopus*/section *Coronopus* (green box): *P. coronopus* subsp. *coronopus* (c), *P. coronopus* subsp. *commutata* (d); subgenus *Albicans* (red box): section *Bauphula* (brown box), *P. amplexicaulis* subsp. *bauphula* (e); section *Lanceifolia* (yellow box), *P. lanceolata* (f), *P. lagopus* (g); section *Montana* (purple box), *P. atrata* subsp. *spadicea* (h), *P. loeflingii* (i), *P. notata* subsp. *haussknechtii* (j); section *Albicans* (orange box), *P. boissieri* var. *boissieri* (k), *P. ciliata* subsp. *lanata* (l), *P. cylindrica* (m), *P. evacina* (n), *P. orzuensis* (o), *P. ovata* (p), *P. stocksii* (q); section *Hymenopsyllium* (pink box), *P. bellardii* subsp. *deflexa* (r); Subgenus *Psyllium* (blue box): *P. indica* (s), *P. afra* (t). Scale bars—0.5 mm.

Plantago species varied in their size (18.75 μm –159.45 μm). The lowest mean size of the testa cells (23.37 \pm 2.47 μm) occurred in *Plantago gentianoides* subsp. *griffithii* Rech. f. (subgenus *Plantago*) (Figure 2), while the highest mean value (112.68 \pm 22.79 μm) was observed in *Plantago amplexicaulis* subsp. *bauphula* Rech. f. (subgenus *Albicans* Barn.) (Figure 3). The shapes of the testa cells were angular or rounded (Figure 3). The seed coat pattern of testa cells was observed in three types:

reticulate, scalariform to reticulate, and papillate. The papillate coat pattern was observed only in *P. evacina* (section *Albicans*) of subgenus *Albicans*. A transverse furrow at middle seeds occurred only in *P. bellardii* subsp. *deflexa* (subgenus *Albicans*) and *P. coronopus* subsp. *coronopus* and subsp. *commutata* (subgenus *coronopus*). There were great variations in the cell walls. The anticlinal walls of testa cells were straight, slightly curved, undulate, curved, and biarmed in different

Table 4. Seed micromorphological features of the studied *Plantago* species

Taxa	Overall seed coat pattern	Cell shape	Cell size (µm)	Anticlinal walls					Periclinal walls
				Shape	Thickness degree	Level	Level	Texture	
<i>P. major</i>	Reticulate	Angular	46.33–66.1 53.19 ± 4.96	Straight	Thin	Raised	±Flat		Striate
<i>P. gentianoides</i> subsp. <i>griffithii</i>	Reticulate	Rounded	18.75–29.66 23.37 ± 2.47	Undulate	Mediate	Raised	±Flat		Ruminate
<i>P. coronopus</i> subsp. <i>coronopus</i>	Reticulate	Rounded	22–30.44 26.17 ± 2.71	Undulate	Mediate	Raised	Concave		Ruminate
<i>P. coronopus</i> subsp. <i>commutata</i>	Reticulate	Rounded	23.37–39.13 29.66 ± 4.52	Undulate	Mediate	Raised	Concave		Ruminate
<i>P. amplexicaulis</i> subsp. <i>bauphula</i>	Scalariform to reticulate	Angular	68.33–159.45 112.68 ± 22.8	Straight	Thin	Channeled	Flat		Scalariform
<i>P. lanceolata</i>	Scalariform to reticulate	Angular	26.981–48.42 35.92 ± 5.2	Straight	Thin	Raised	Flat		Granulate
				Shape	Thickness degree	Level	Level	Texture	
<i>P. lagopus</i>	Scalariform to reticulate	Angular	21.43–42.51 32.17 ± 5.54	Straight	Thin	Channeled	Flat	Granulate	
<i>P. atrata</i> subsp. <i>spadicea</i>	Reticulate	Rounded	29.28–44.57 36.91 ± 4.34	Bi-armed	Thick	Raised	Concave	Striate	
<i>P. loeflingii</i>	Scalariform to reticulate	Angular	69.34–119.8 92.63 ± 15.24	Straight	Thin	Channeled	Flat	Granulate	
<i>P. notata</i>	Scalariform to reticulate	Angular	52.54–67.63 62.66 ± 5.74	Straight	Thin	Channeled	Flat	Rugose	
<i>P. boissieri</i> var. <i>boissieri</i>	Scalariform to reticulate	Angular	37.1–65.72 49.36 ± 7.14	Straight	Thin	Raised	Flat	Granulate	
<i>P. ciliata</i> subsp. <i>lanata</i>	Reticulate	Angular	50.25–64.95 57.1 ± 4.68	Straight	Thin	Channeled	Convex	Granulate	
				Shape	Thickness degree	Level	Level	Texture	
<i>P. cylindrica</i>	Scalariform to reticulate	Angular	43.4–61.11 51.39 ± 6.01	Straight	Thin	Raised	Convex		Rugose
<i>P. evacina</i>	Papillate	Rounded	49.78–68.51 58.47 ± 4.88	Curved or slightly curved	Thick	Channeled	Flat		Microreticulate
<i>P. orzuiensis</i>	Reticulate	Angular	68.34–102.18 86.3 ± 9.35	Straight	Thin	Channeled	Flat		Rugose
<i>P. ovata</i>	Scalariform to reticulate	Angular	41.32–67.4 52.46 ± 6.6	Straight	Thin	Raised	Convex		Rugose
<i>P. stocksii</i>	Scalariform to reticulate	Angular	49.8–66.7 60.64 ± 6.51	Straight	Thin	Channeled	Flat		Granulate
<i>P. bellardii</i> subsp. <i>deflexa</i>	Reticulate	Rounded	73.88–88.66 80.13 ± 5.11	Slightly curved	Thick	Raised	Concave		Reticulate

Table 4. (Continued).

				Shape	Thickness degree	Level	Level	Texture	
<i>P. indica</i>	Scalariform to reticulate	Angular	52.3–81.76 66.63 ± 8.5	Straight	Thin	Channeled	Flat	Granulate	
<i>P. afra</i>	Scalariform to reticulate	Angular	53.68–85.63 68.19 ± 7.38	Straight	Thin	Channeled	Flat	Granulate	

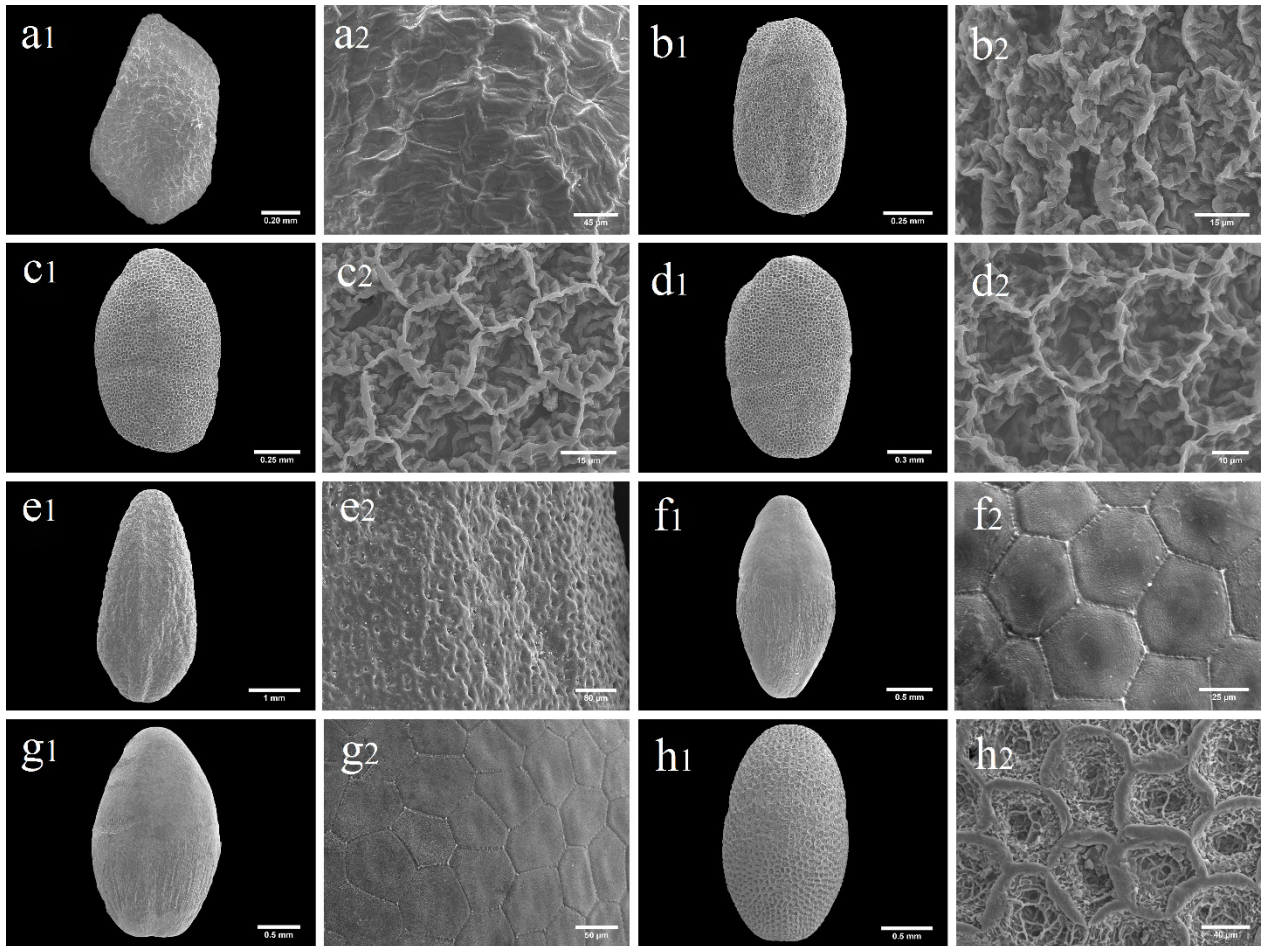


Figure 2. Seed micromorphological characteristics of the studied *Plantago* species. a-h, reticulate type; a, *P. major*; b, *P. gentianoides* subsp. *griffithii*; c, *P. coronopus* subsp. *coronopus*; d, *P. coronopus* subsp. *commutata*; e, *P. atrata* subsp. *spadicea*; f, *P. ciliata* subsp. *lanata*; g, *P. orzuensis*; h, *P. bellardii* subsp. *deflexa*.

degrees of thickness and they were observed with levels: raised and channeled. The periclinal walls ranged from ± flat and flat to convex or concave, and there were striate, ruminant, scalariform, granulate, rugose, microreticulate, and reticulate ornamental sculpturing on the testa cells.

3.4. Taxonomic implications

The MDS plot (Figure 4) of macro- and micromorphological data of the seeds showed that the seed morphological

data is insufficient to provide clear infrageneric grouping especially at sectional level sensu Rahn (1996).

PCoA plot (Figure 5) of macro- and micromorphological data of the seeds indicated that there is high affinity between subgenus *Plantago* and subgenus *Coronopus*, and between subgenus *Albicans* and subgenus *Psyllium*.

WARD tree (Figure 6) of the macro- and micromorphological data of the studied seeds showed two

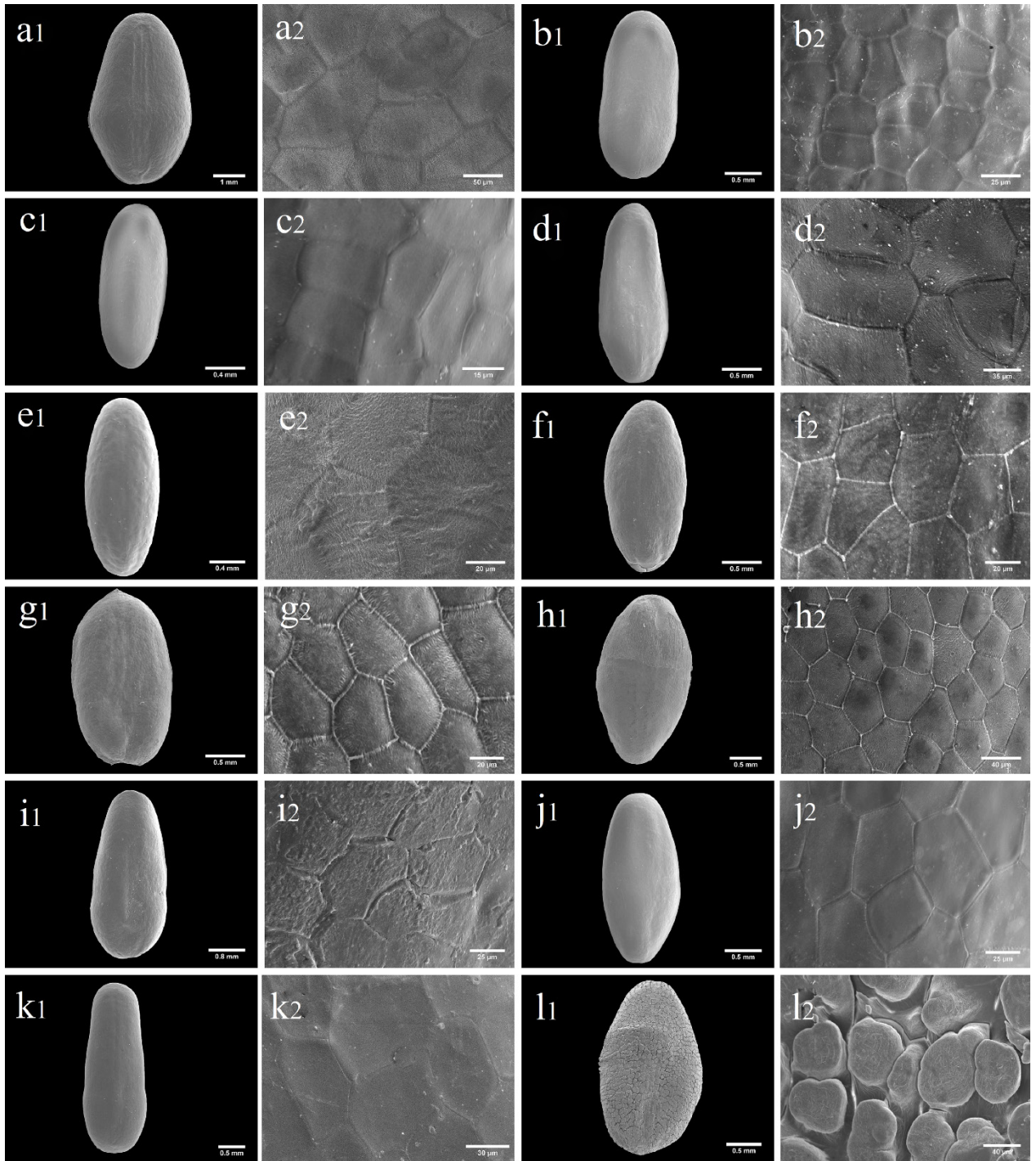


Figure 3. Seed micromorphological characteristics of the studied *Plantago* species. a-k, scalariform to reticulate type; a, *P. amplexicaulis* subsp. *bauphula*; b, *P. lanceolata*; c, *P. lagopus*; d, *P. loeflingii*; e, *P. notata* subsp. *haussknechtii*; f, *P. boissieri* var. *boissieri*; g, *P. cylindrica*; h, *P. ovata*; i, *P. stocksii*; j, *P. indica*; k, *P. afra*; papillate type, l, *P. evacina*.

major clades with several subclades. Species of subgenus *Psyllium* and most species of subgenus *Albicans* fell into the first clade. The second clade had two major subclades: the first subclade included three species of subgenus

Albicans, while the second subclade included the species of subgenus *Plantago* and subgenus *Coronopus*.

Section *Plantago* of subgenus *Plantago* contained two species including *P. major* and *P. gentianoides* subsp.

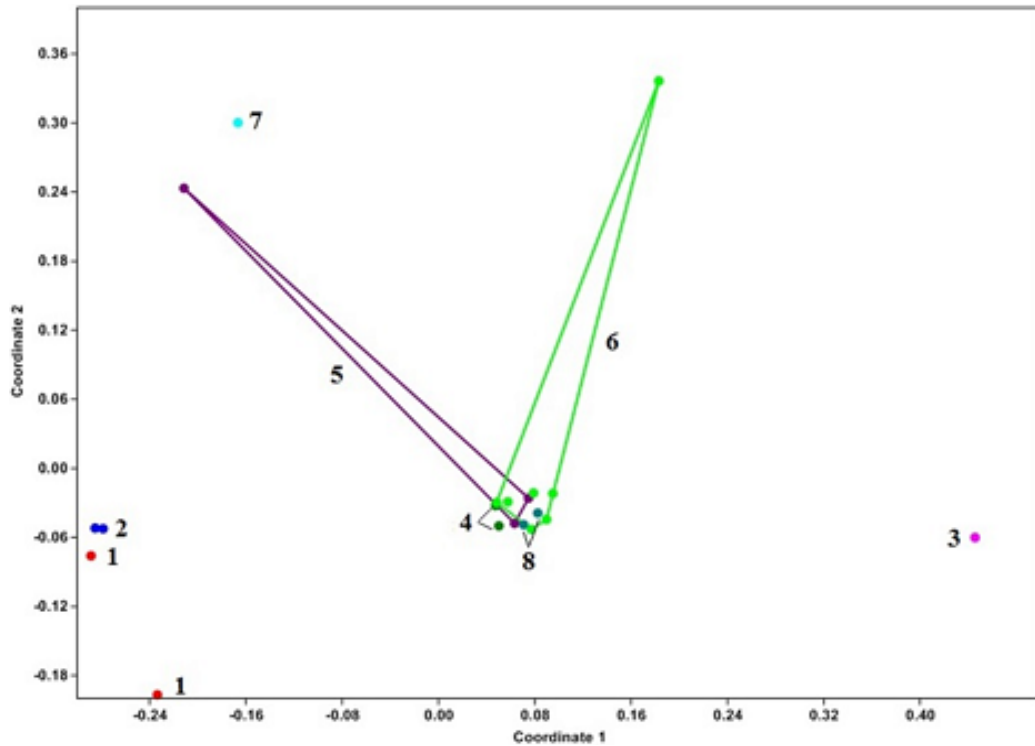


Figure 4. MDS plot of *Plantago* sections studied based on seed morphological characteristics (section *Plantago* Rahn (1); section *Coronopus* Rahn (2); section *Bauphula* Decne. (3); section *Lanceifolia* Barn. (4); section *Montana* Barn. (5); section *Albicans* Barn. (6); section *Hymenopsyllium* Pilg. (7); subgenus *Psyllium* Juss. (8); based on classification Rahn (1996).

griffithii in our analysis. These two species were different in shape of seeds, position of hilum, cell shape of testa cells, shape and thickness of anticlinal walls, and texture of periclinal walls, but they had similar seed coat patterns.

In this study, there was only one species of subgenus *Coronopus*. This species was *P. coronopus* of section *Coronopus* with two subspecies including subsp. *coronopus* L. and subsp. *commutata* Pilg. These two subspecies showed high affinity in seed features.

Different species of subgenus *Albicans* fell into two major clades: *P. amplexicaulis* subsp. *bauphula*, *P. lanceolata*, *P. lagopus*, *P. loeflingii*, *P. notata* subsp. *haussknechtii*, *P. boissieri* var. *boissieri* Hausskn. & Bornm., *P. ciliata* subsp. *lanata*, *P. cylindrica*, *P. orzuiensis* Mohsenzadeh et al., *P. ovata*, and *P. stocksii* plus two species of subgenus *Psyllium* including *P. indica* and *P. afra* in the first major clade, while three species including *P. evacina*, *P. atrata* subsp. *spadicea*, and *P. bellardii* subsp. *deflexa* in a subclade of the second major clade.

P. amplexicaulis subsp. *bauphula*, the only species of section *Bauphula*, was distinguished from the other studied species by macromorphological features (shape, color, size, testa thickness, and position of hilum on the septum) of the seeds.

Section *Albicans* is a heterogeneous section. Three species including *P. stocksii*, *P. ciliata*, and *P. orzuiensis* of this section were placed in a subclade. Anticlinal wall features and cell shape were similar in them. Furthermore, three species of this section including *P. boissieri* var. *boissieri*, *P. ovata* and *P. cylindrica* were placed in another subclade but there was greater affinity between *P. ovata* and *P. cylindrica* due to high affinity in micromorphological features of seeds including seed coat pattern, testa cell shape, and features of anticlinal and periclinal walls. Finally, *P. evacina* was different among the studied species of this section and placed in the other clade.

The perennial species *P. lanceolata* and the annual species *P. lagopus* of section *Lanceifolia* Barn. were different regarding the level of anticlinal walls, color, and size of seeds, and fell into two subclades. On the other hand, *P. lagopus* and two perennials including *P. lanceolata* and *P. leiopetala* Lowe fell into two subclades of a clade in the molecular study (Rønsted et al., 2002). Thus, it seems that the perennial species and the annual species in this section are genetically different.

The two annual species including *P. loeflingii*, and *P. notata* subsp. *haussknechtii*, and the perennial species *P. atrata* subsp. *spadicea* of section *Montana* Barn. were

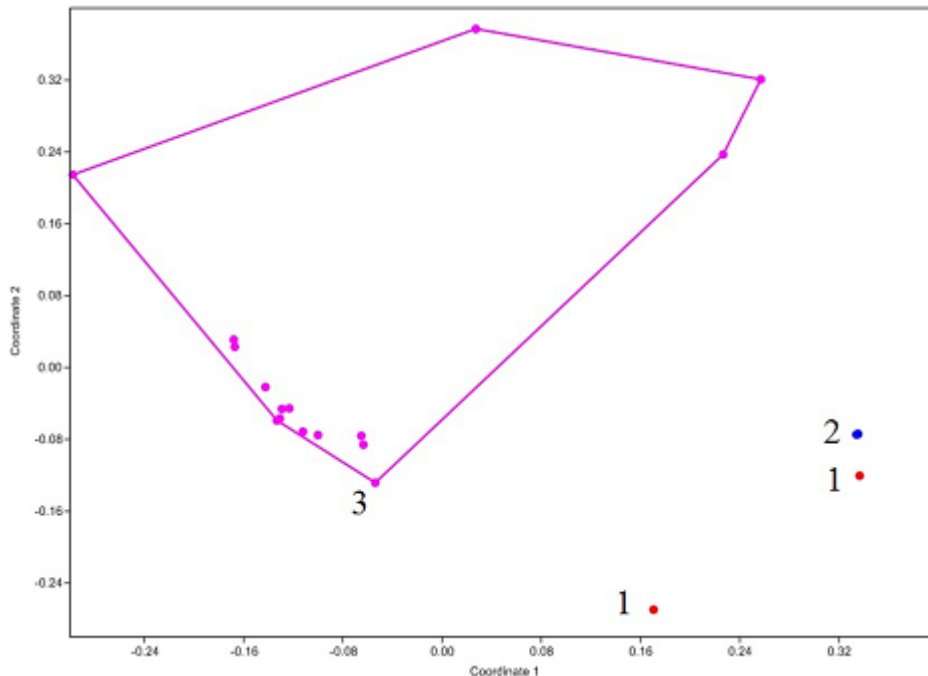


Figure 5. PCoA plot of *Plantago* subgenera studied based on seed morphological characteristics (subgenus *Plantago* Harms (1); subgenus *Coronopus* Rahn (2); two subgenera *Albicans* Rahn & *Psyllium* Juss. (3); based on classification Rønsted et al. (2002).

different in seed features. *Plantago loeflingii* and *P. notata* subsp. *haussknechtii* had more affinity and placed in a subclade, but *P. atrata* subsp. *spadicea* was placed in a different subclade.

Like the results of the molecular study by Rønsted et al. (2002), there was more close relationship between *P. indica* and *P. afra* of subgenus *Psyllium* than the other studied species because of their high similarity in macro- and micromorphological features of seeds such as seed shape, seed coat pattern, as well as features of anticlinal and periclinal walls.

4. Discussion

4.1. Macromorphological variations: shapes, types, seed size, hilum position, testa thickness

Different shapes of seeds have been distinguished in the genus *Plantago* by different researchers. For example, Liu et al. (1992) distinguished four shapes including variously angular, recti-circular, navicular, and long-ovoid. Klimko et al. (2004) distinguished four shapes including elliptic, peltate, angular, and ovoid, while Shehata and Louty (2006) distinguished variable shapes (oblong, cymbiform, ovate, fusiform, lenticular, rounded, angular, ellipsoid, and reniform) of seeds in Plantaginaceae. On the other hand, here in this study, we distinguished angular, broadly elliptic, elliptic, and narrowly elliptic shapes of seeds in the studied species.

We observed two basic shapes in the studied seeds: angular (in *P. major*) and elliptic (in the other studied species). We also found three states of inner side of the studied seeds: convex (in *P. major* and *P. gentianoides* subsp. *griffithii*), flat (in *P. coronopus* subsp. *coronopus* and subsp. *commutata*), and concave (in species of two subgenera *Albicans* and *Psyllium*). Here, we describe four types based on these two features: convex-angular type, convex-elliptic type, flat-elliptic type, and concave-elliptic type. Although Rezk (1980) described three types based on shapes and dimensions including irregular type, more regular type, and oval type.

Rezk (1980) considered the increasing size of seeds from 0.9 mm to 3.4 mm as an evolutionary line. Rahn (1996) considered three categories: 1) seeds shorter than 2 mm, 2) seeds 2-3 mm, 3) seeds longer than 3 mm. We observed the size of seeds as 0.9-6.1 mm in this study. Based on the seed size, *P. major* (0.9-1.3 mm) is a primitive species while *P. amplexicaulis* subsp. *bauphula* (5-6.1 mm) is an advanced species. However, we cannot describe a clear evolutionary line of the subgenera based on the seed size due to the overlap in the size range of the seeds of the studied species.

We observed three states of hilum position on the inner side of the studied seeds: 1) superficial in species of subgenera *Plantago* and *Coronopus*, 2) in cavity in species of subgenera *Albicans* and *Psyllium*, 3) on septum

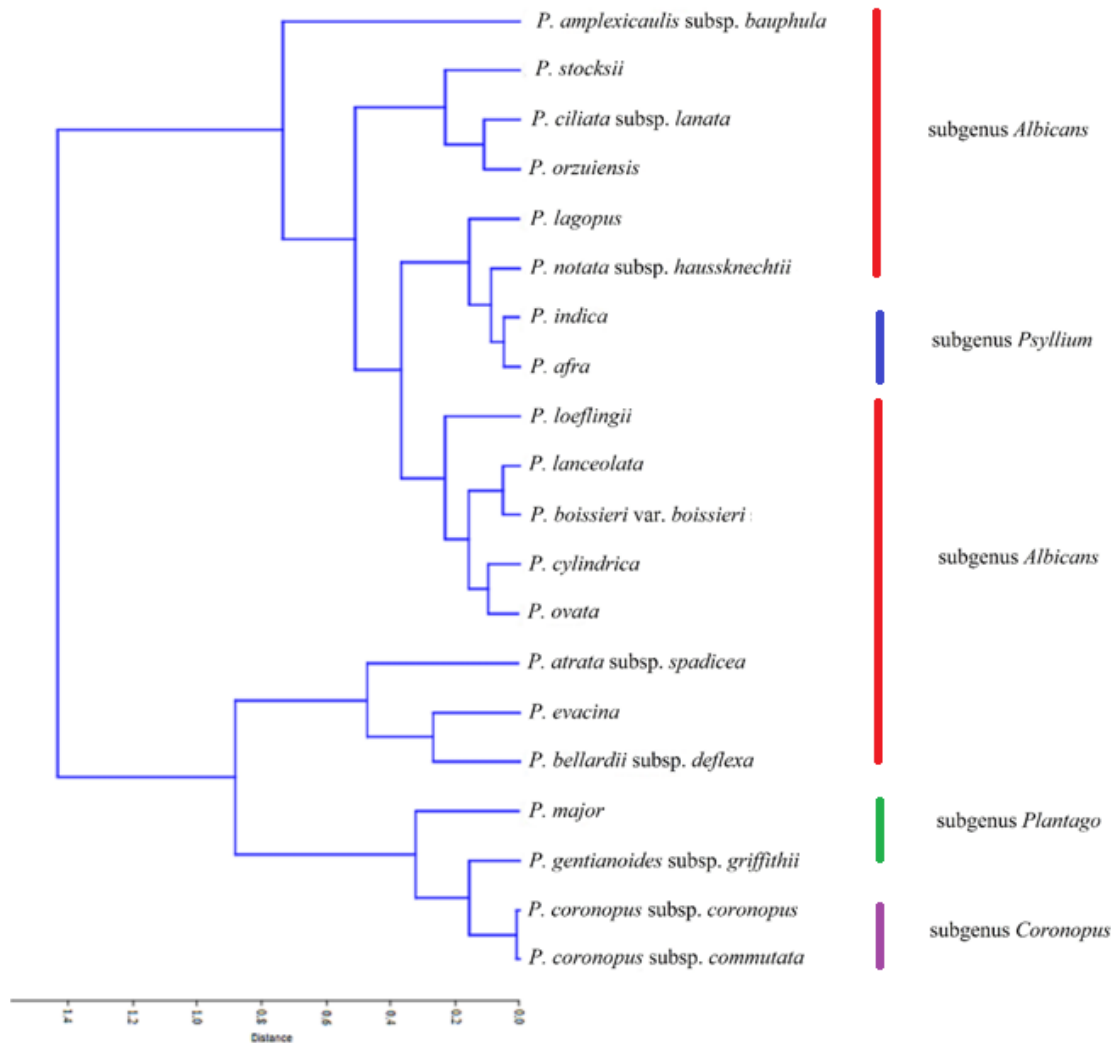


Figure 6. WARD tree of *Plantago* species based on seed morphological characteristics.

in *P. amplexicaulis* subsp. *bauphula* of subgenus *Albicans*. Furthermore, there were two states of testa thickness in the studied seeds including thick and thin. The thin testa of seeds was observed only in *P. amplexicaulis* subsp. *bauphula*, while the seeds in the other studied species had thick testa. Thus, we introduced two characters for *P. amplexicaulis* subsp. *bauphula* including hilum position on septum and thin testa where this species had the largest seed in the genus *Plantago*.

4.2. Micromorphology: seed coat pattern and its significance in taxonomy

Liu et al. (1992) described four types with using macro- and micromorphology of seeds in genus *Plantago*, including 1) multiangular type, 2) navicular type, 3) ovoid type, 4) recti-circular type. Moreover, based on several SEM studies, some seed coat patterns were reported in the genus *Plantago* including tuberculate, scalariform to reticulate,

scalariform, reticulate to scalariform, reticulate, reticulate to papillate, papillate, and areolate (see Liu et al., 1992; Klimko et al., 2004; Shehata and Loutfy, 2006; Hoghoughi et al., 2016; Verma et al. 2017). In this present study, based on the seed coat patterns using SEM, we described three types, including 1) reticulate type, 2) scalariform to reticulate type, 3) papillate type. The seed coat pattern should be constant for a species, though we observed differences in our results as compared with previous reports for a few species. For example, a reticulate type (in this study; Shehata and Loutfy, 2006), tuberculate type (Verma et al., 2017), and areolate type (Hoghoughi et al., 2016) were reported for *P. major*. Furthermore, we reported scalariform to reticulate type for *P. ovata* while Shehata and Loutfy (2006) reported a scalariform type for this species. Differences in the reporting of seed coat patterns for a species may be because of damages to testa cells, causing error in identification,

or existence of a cryptic species, especially in subgenus *Plantago*, which has low morphological diversity.

4.3. Seed morphological data and its significance in taxonomy

Micromorphological data of seed plants can offer invaluable information about their evolution and classification. These data also play an important role in the modern synthetic systems of angiosperms (Barthlott, 1981). Hair and seed characters are the most important features for estimating the phylogeny of the infrageneric taxa within *Plantago* (Rahn, 1996). The features of arrangement of cells and anticlinal walls can be of high systematic significance, usually for determining taxa between species and at the genus level (Barthlott, 1981). Based on the results of this study, seed features can be used as an indicator for finding the paraphyletic groups and for delimitation at the infrageneric level. Furthermore, the features of arrangement of testa cells and anticlinal walls can be used at species level, especially when the morphological diversity between species is low.

4.4. Role of seed morphology in identification of paraphyly

We observed that *P. gentianoides* subsp. *griffithii* is more closely related to *P. coronopus* subsp. *coronopus* and subsp. *commutata* than *P. major*. In spite of *P. gentianoides* subsp. *griffithii* and *P. major* are both members of the subgenus *Plantago*. *P. gentianoides* subsp. *griffithii* differed in the seed shape, size and testa cells shape, anticlinal walls features, and texture of periclinal walls with *P. major*. However, *P. gentianoides* subsp. *griffithii* shows similarities in the seed morphology, especially in micromorphology including seed coat pattern, testa cells shape, anticlinal wall features, and texture of periclinal walls with *P. coronopus* subsp. *coronopus* and subsp. *commutata*. In addition, they also showed consistently three mature seeds per capsule. Normally, *Plantago* species has a large number of seeds per capsule (Schwarzbach, 2004). However, here we found that the capsule of *P. major* always bear more than ten seeds while only two seeds per capsule matured from subgenera *Albicans* and *Psyllium*. Based on the number of seeds per capsule of the studied *Plantago*, the evolutionary line can range from ten seeds or more (in *P. major*) to three seeds (in *P. gentianoides* subsp. *griffithii* and *P. coronopus* subsp. *coronopus* and subsp. *commutata*) to two seeds (in species of subgenera *Albicans* and *Psyllium*). Thus, the evolutionary line in *P. gentianoides* subsp. *griffithii* of section *Plantago* is closer to that of *P. coronopus* subsp. *coronopus* and subsp. *commutata* of section *Coronopus* than *P. major* of section *Plantago*. Hence, we suggest that there were two different lineages within section *Plantago* in this study. Furthermore, Iwanycki Ahlstrand et al. (2019) studied subgenus *Plantago* using ITS and plastid trnL-F, ndhF-rpl32, rpl32-trnL sequences. They suggested that

there are several lineages in subgenus *Plantago* on oceanic islands.

We observed that three species of subgenus *Albicans* including *P. evacina*, *P. atrata* subsp. *spadicea*, and *P. bellardii* subsp. *deflexa* were separated from the other species of this subgenus in WARD tree and placed near the species of subgenera *Plantago* and *Coronopus*. These three species have two common characters including round shape of testa cells and thick anticlinal walls unlike the other studied species of subgenus *Albicans*. In addition, we observed slightly curved (in *P. bellardii* subsp. *deflexa*), curved or slightly curved (in *P. evacina*), and bi-armed (in *P. atrata* subsp. *spadicea*) shapes of anticlinal walls in them, while the shape of anticlinal walls in the other studied species of subgenus *Albicans* was straight. On the other hand, we observed a round shape of testa cells in *P. coronopus* subsp. *coronopus* and subsp. *commutata* of subgenus *Coronopus*, and *P. gentianoides* subsp. *griffithii* of subgenus *Plantago*. Moreover, we observed a papillate coat pattern only in *P. evacina* of the studied species, while this coat pattern was previously reported in subgenus *Plantago* by Liu et al. (1992). Thus, it suggests that more than one lineage within subgenus *Albicans* was successful in dispersion and speciation at least in Iran. Note that the deformation of testa cells can indicate different lineages within a group, and we can identify the paraphyletic group at series to the subgenus level in genus *Plantago*.

4.5. Significance of seed morphology at subgeneric classification

The morphological study of seeds revealed that there is a close affinity between subgenus *Albicans* and subgenus *Psyllium* as well as between subgenus *Plantago* and subgenus *Coronopus*.

Rahn (1978) separated the section *Coronopus* of subgenus *Euplantago* of Pilger (1937) classification and assumes the status subgenus for this section and divides into section *Coronopus* Lam. et DC. and section *Maritima* Rahn, but Dietrich (1980) distinguished it in the frame of subgenus *Plantago* section *Coronopus* DC and section *Maritima* Dietrich. Moreover, Taskova et al. (2002) using iridoid patterns in genus *Plantago* concluded that two sections *Coronopus* and *Maritima* must be placed in subgenus *Plantago*. In this study, we observed that species of subgenus *Plantago* and subgenus *Coronopus* were similar in their seed morphological features. These are consistent with Taskova et al. (2002). Thus, we suggest that the species of subgenus *Coronopus* should be placed within subgenus *Plantago* as with Dietrich (1980) decision.

Rahn (1978) separated five sections including *Leucopsyllium*, *Lanceifolia*, *Bauphula*, *Oreades*, and *Hymenopsyllium* of subgenus *Euplantago* and placed them in subgenus *Psyllium*, but he has been criticized by Andrzejewska-Golec and Swietoslowski, 1989a, 1989b,

1993 based on their hairs studies. Then, he (Rahn, 1996,) using 91 characters of morphological, embryological, and chemical data, separated them again and placed them in a new subgenus *Albicans* and introduced it as a monophyletic group. However, Rønsted et al. (2002), using sequence data of nuclear ribosomal ITS and plastid trnL-F, revealed that subgenus *Albicans* is paraphyletic and should be merged in subgenus *Psyllium* to obtain a monophyletic clade. In this study, the results of the seed morphology study are in agreement with Rønsted et al.'s (2002) decision.

We concluded that species of subgenus *Coronopus* should be placed into subgenus *Plantago* as Pilger (1937) classification, while species of subgenus *Albicans* should be incorporated into subgenus *Psyllium* as Rønsted et al. (2002) classification.

4.6. Species and subspecies relationships based on seed morphological characteristics and comparing the results with previous studies

The sectional position of *P. gentianoides* subsp. *griffithii* is controversial. Rahn (1996) separated this species of section *Gentianoides* Pilg., and placed it in section *Plantago* and introduced it paraphyletic. Recently, Hassemmer et al. (2019) via high-throughput plastid genome sequencing placed this species in section *Eremopsyllium* Pilg. Based on our results, it is clear that the seed morphology in this species has not great affinity with *P. major* of section *Plantago* L. Thus, it can be assumed that Rahn's decision on placing this species in section *Plantago* is incorrect.

Rahn (1996) placed *P. minuta* Pall., *P. stocksii* and *P. evacina* into series *Minutae* Rahn and introduced it as paraphyletic. Note that Rahn (1996) considered *P. evacina* as synonymous with *P. lachnantha* Bunge, but Patzak and Rechinger (1965) considered them as two separate species (see also Mohsenzadeh et al., 2007). There were two species including *P. evacina* and *P. stocksii* of this series in this study. These two species were different in shape and size of seeds, seed coat pattern, and cell shape, and they were placed into two different clades. The morphology study showed that these two species are not closely related, so they should not be in a series. Rønsted et al. (2002), using sequence data of nuclear ribosomal ITS and plastid trnL-F, showed that *P. stocksii* had close affinity to *P. amplexicaulis* than the other species of subgenus *Albicans*, but Rahn mentioned that *P. amplexicaulis* subsp. *bauphula* has many autapomorphies. Based on our results, these two species were different in macromorphology features such as shape, color, hilum position, and testa thickness of seeds, and they were placed into different subclades of a major clade. We suggest that further study is required to determine the taxonomic status of series *Minutae* species.

Note that there are three perennial species (*P. atrata*, *P. nivalis* Boiss., and *P. monosperma* Pourr.) and three annual species (*P. cafra* Decne., *P. loeflingii*, and *P. notata*) in

section *Montana*. Pilger (1937) separated three perennial species and placed them in section *Oreades* Decne., while he classified three annual species in section *Leucopsyllium* Pilg. (in Rahn's classification 1978; 1996 including sections *Albicans* and *Gnaphaloides* Barn.). According to our results, it is better to place *P. atrata* into the section *Oreades* Decne., and *P. loeflingii* and *P. notata* into the section *Leucopsyllium* rather than place them in the section *Montana*. Nevertheless, more studies are required for considering the affinity between them.

We observed that there is more affinity among *P. bellardii* subsp. *deflexa* of section, *P. evacina* of section *Albicans*, and *P. atrata* subsp. *spadicea* as compared to the other studied species of subgenus *Albicans*. The most important common feature among them is the rounded shape of testa cells. Rønsted et al. (2002) revealed that species of section *Hymenopsyllium* Pilg. were more closely related to species of section *Gnaphaloides* (American section) than species of section *Albicans* (Mediterranean section). In this study, although no species of section *Gnaphaloides* were observed, we found *P. bellardii* subsp. *deflexa* was separated from most species of section *Albicans*. Thus, the seed morphology study can be in agreement with the results of Rønsted et al. (2002).

Finally, Shalabi and Abou-El-Enain (2013), using 72 characters of morphological data in genus *Plantago*, showed that there is a high affinity between species of subgenera *Albicans* and *Psyllium*, like our results. Moreover, they revealed that the evolutionary line of *P. major* is different from species of subgenera *Coronopus*, *Psyllium*, and *Albicans*. Unlike their results, we showed that species of subgenus *Plantago* are more closely related to species of subgenus *Coronopus* than species of subgenus *Albicans* and *Psyllium*.

Therefore, the further comprehensive study of *Plantago* including seed morphology seems to be necessary for showing phylogenetic relationships in *Plantago* species and constructing a more satisfactory infrageneric classification.

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Conflicts of interest

We declare that we have no conflicts of interest.

Author contribution

Saeed Mohsenzadeh data collection, lab work, and writing the paper; Fahimeh Koohdar: data collection, lab work, and writing the paper; Masood Sheidai; conceptualization of the project.

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Appendix 1. Collection data of the studied *Plantago* species.

No	Species	Collection data
1	<i>P. major</i>	Iran: Tehran, Darakeh, 1820 m, 9/10/2018, Mohsenzadeh et al.
2	<i>P. gentianoides</i>	Iran: Khorassan, Mashhad, 1950 m, 5/2/2015, Joharchi
3	<i>P. coronopus</i> subsp. <i>coronopus</i>	Iran: Fars, Kazerun, Aboali village, 820 m, 4/6/2017, Mohsenzadeh et al.
4	<i>P. coronopus</i> subsp. <i>commutata</i>	Iran: Bushehr, Dalaki, 90 m, 3/12/2018, Mohsenzadeh et al.
5	<i>P. amplexicaulis</i>	Iran: Hormozgan, Bandarabbas, Geno mountain, 1080 m, 3/21/2018, Mohsenzadeh et al.
6	<i>P. lanceolata</i>	Iran: Tehran, Darakeh, 1900 m, 7/21/2018, Mohsenzadeh et al.
7	<i>P. lagopus</i>	Iran: Fars, Kazerun, 870 m, 4/5/2017, Mohsenzadeh et al.
8	<i>P. atrata</i>	Iran: Azerbaijan, Arasbaran, 2900 m, 4/8/1984, Zehzad
9	<i>P. loeflingii</i>	Iran: Fars, Kazerun, 890 m, 4/5/2017, Mohsenzadeh et al.
10	<i>P. notata</i>	Iran: Khuzestan, Ahvaz, 32 m, 2/23/1992, Iranshar & Termeh
11	<i>P. boissieri</i>	Iran: Bushehr, Borazjan, 25 m, 3/12/2018, Mohsenzadeh et al.
12	<i>P. ciliata</i>	Iran: Kerman, Jiroft, 790 m, 2/28/2015, Ghanbari
13	<i>P. cylindrica</i>	Iran: Hormozgan, Bandarabbas, 10 m, 3/21/2018, Mohsenzadeh et al.
14	<i>P. evacina</i>	Iran: Khorassan, Mashhad, 1040 m, 3/2/2016, Joharchi
15	<i>P. orzuiensis</i>	Iran: Kerman, Orzuia, 1300, 3/20/2009, Mohsenzadeh
16	<i>P. ovata</i>	Iran: Fars, Kazerun, Taleghanei mountain, 956 m, 4/5/2017, Mohsenzadeh et al.
17	<i>P. stocksii</i>	Iran: Semnan, Shahroud, 1250 m, 4/7/1993, Termeh, Moussavi & Tehrani
18	<i>P. bellardii</i>	Iran: Fars, Kazerun, Seifabad village, 760 m, 4/6/2017, Mohsenzadeh et al.
19	<i>P. indica</i>	Iran: Mazandaran, Miankaleh, 5/20/1986, Zehzad
20	<i>P. afra</i>	Iran: Bushehr, Dalaki, 83 m, 3/12/2018, Mohsenzadeh et al.

Appendix 2. Comparative recording of the [17] characters listed in Table 2.

No	Species/characters	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	<i>P. major</i>	1	1	1	1	1	1	1	1	1	1	1	1	1	1.07	0.59	1.84	53.19
2	<i>P. gentianoides</i>	3	1	1	1	1	1	1	2	2	2	1	1	2	1.44	0.73	2.01	23.37
3	<i>P. coronopus</i> subsp. <i>coronopus</i>	3	2	1	2	2	1	1	2	2	2	1	2	2	1.35	0.84	1.68	26.17
4	<i>P. coronopus</i> subsp. <i>commutata</i>	3	2	1	2	2	1	1	2	2	2	1	2	2	1.32	0.8	1.65	29.66
5	<i>P. amplexicaulis</i>	2	3	2	3	1	2	2	1	1	1	2	3	3	5.47	2.98	1.57	112.68
6	<i>P. lanceolata</i>	4	4	1	3	1	3	2	1	1	1	1	3	4	2.13	1.02	2.09	35.92
7	<i>P. lagopus</i>	4	5	1	3	1	3	2	1	1	1	2	3	4	1.54	0.65	2.4	32.17
8	<i>P. atrata</i>	4	5	1	3	1	2	1	2	3	3	1	2	1	4.12	1.93	2.15	36.91
9	<i>P. loeflingii</i>	4	4	1	3	1	3	2	1	1	1	1	3	4	2.33	0.93	2.53	92.63
10	<i>P. notata</i>	4	4	1	3	1	3	2	1	1	1	2	3	5	1.72	0.73	2.35	62.66
11	<i>P. boissieri</i>	4	5	1	3	1	3	2	1	1	1	1	3	4	2.08	1.14	1.84	49.36
12	<i>P. ciliata</i>	3	6	1	3	1	3	1	1	1	1	2	4	4	2.2	1.26	1.74	57.1
13	<i>P. cylindrica</i>	4	5	1	3	1	3	2	1	1	1	1	4	5	2.06	1.03	1.99	51.39
14	<i>P. evacina</i>	3	4	1	3	1	3	3	2	4	3	2	3	7	2.53	1.4	1.81	58.47
15	<i>P. orzuiensis</i>	3	6	1	3	1	3	1	1	1	1	2	3	5	2.77	1.59	1.75	86.3
16	<i>P. ovata</i>	3	6	1	3	1	3	2	1	1	1	1	4	5	2.5	1.39	1.8	52.46
17	<i>P. stocksii</i>	4	5	1	3	1	3	2	1	1	1	2	3	4	4.12	1.66	2.5	60.64
18	<i>P. bellardii</i>	3	4	1	3	2	3	1	2	5	3	1	2	6	1.99	1.14	1.76	80.13
19	<i>P. indica</i>	4	4	1	3	1	3	2	1	1	1	2	3	4	2.27	1.07	2.35	66.63
20	<i>P. afra</i>	4	4	1	3	1	3	2	1	1	1	2	3	4	2.46	0.85	2.92	68.19