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NEW INSIGHT INTO THE MOLECULAR AND MICROMORPHOLOGICAL CHARACTERISTICS OF POTENTILLA INDICA AND POTENTILLA REPTANS (ROSACEAE)

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The present study investigates the molecular phylogeny and micromorphological characteristics of *Potentilla indica* (Andrews) Th. Wolf (=*Duchesnea indica* (Andrews) Focke), and *P. reptans* L.. For the phylogenetic study, the nrDNA ITS, *trn*H-*psb*A, and combined sequence datasets were analyzed using maximum Parsimony (MP), Bayesian inference (BI), and maximum likelihood (ML) methods. For the micromorphological study, epicalyx, calyx, and pedicle characters were examined. Individual and combined analyses of ITS and *trn*H-*psb*A data revealed a phylogenetic divergence in the Reptans clade, corresponding to *P. reptans* and *P. indica* (groups L and M) in ITS trees, *P. reptans*, *P. hebiichigo* Yonek. & H.Ohashi (group O) and *P. indica* (group N) in plastid and combined trees. In all obtained trees, *P. indica* showed a sister group relationship with *P. reptans*. The micromorphological characters of the epicalyx, calyx, and pedicel were found to be taxonomically effective for species separation. Furthermore, the results supported the phylogenetic relationships among the species.

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Keywords: Epicalyx; calyx; pedicle; *trn*H-*psb*A; ITS; phylogeny

بینش نوین در ویژگی‌های مولکولی و ریزریخت‌شناسی Potentilla indica و Potentilla reptans از تیره گل‌سرخیان

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در مطالعه‌ی حاضر، فیلوژنی مولکولی و صفات ریزریخت‌شناسی Potentilla indica (Andrews) Th. Wolf (=Duchesnea indica (Andrews) Focke) و P. reptans L. مورد بررسی قرار گرفت. برای تحلیل‌های فیلوژنتیکی، اطلاعات حاصل از توالی‌های nrDNA ITS، trnH-psbA به‌طور مجزا و ترکیبی با استفاده از روش بیشینه‌ی پارسیمونی، تحلیل بایزین و روش بیشینه‌ی درست‌نمایی انجام شد. ریزریخت‌شناسی، صفات کاسبرگ فرعی، کاسبرگ اصلی و دمگل مورد بررسی قرار گرفت. آنالیز داده‌های ITS، trnH-psbA و ترکیبی، جدایی فیلوژنتیکی در کلاد Reptans، شامل P.

P. indica و reptans P. (گروه‌های L و M) روی درخت ITS، P. reptans L.، P. hebiichigo Yonek. & H.Ohashi (گروه O) و P. indica (گروه N) روی درختان پلاستیدی و ترکیبی را نشان داد. روی همه درخت‌ها، P. indica به‌عنوان گروه خواهری P. reptans قرار گرفت. مشخص شد صفات ریزریخت‌شناسی کاسبرگ فرعی، کاسبرگ اصلی و دمگل دارای ارزش تاکسونومیکی و برای جداسازی گونه ها موثر می‌باشند. همچنین نتایج حاصل، روابط فیلوژنتیک بین گونه ها را مورد حمایت قرار داد.

INTRODUCTION

Potentilla indica (Andrews) Th. Wolf (Syn.: *Duchesnea indica* (Andrews) Focke) and *Potentilla reptans* L. are two stoloniferous species, having bostryx-like cymose inflorescences with exceptionally long petioles (Wolf 1908), yellow petal, terminal style, anther with two thecae, achene fruit (Schonbeck-Temesy 1969, Soják 2004 and 2012, Faghir & al. 2018). In general, common features can be used as an important classification tool, but it is not always simple as we found many contradictions and ambiguities in the case of *Potentilla indica.* Smith (1810) placed this species in the distinct genus *Duchesnea* Sm., based on its false fruits similar to strawberries; but, in Wolf's monograph (1908), this species was placed in the genus *Potentilla* L. and in the group "Tormentillae"(with *P. reptans* and *P. erecta* (L.) Raeusch.). Since then, some authors (e.g. Schulze-Mentz 1964, Schonbeck-Temesy 1969, Soják 2004, 2012) followed Smith´s (1810) classification, while, others used Wolf's classification (e.g. Kalkman 1988, Panigrahi & Dikshit 1987). Bentham & Hooker (1865) merged *Potentilla* in the genus *Fragaria* (Syn.: *Fragaria indica* Andrews, *F. malayana* Roxb.). In addition, it also appeared as *Duchesnea indica*, in the Flora of the former USSR, Flora Iranica (Juzepchuk 1941, Schonbeck-Temesy 1969), Flora of China, East Asia (Lee 1996, Li & al. 2003, Naruhashi 2001) and several papers (Naruhashi 2001, Boufford & al. 2003, Li & al. 2003, Soják 2004). However, Kalkman (1988 & 2004) considered it in the genus *Potentilla* and according to Soják (2008), it is more convenient to classify *Potentilla indica* and *P. reptans* within the *Potentilla*, Sect. *Potentilla* (Schiman-Czeika 1969, Soják 1987, Ertter and Attar 2007). This idea was confirmed by molecular phylogenetic analyses in which *Duchesnea* was nested in the Reptans clade among *Potentilla* species (Eriksson & al. 1998, 2003). Subsequent research (Dobeš & Paule 2010, Topel & al. 2011, Feng & al. 2017) and recent low-copy nuclear marker analysis (Persson & al. 2020) confirmed the inclusion of *P. indica* in the genus *Potentilla.* This large taxonomically complicated genus comprises ca. 485 species and several hybrids, distributed in the arctic and temperate regions of Eurasia and North America (Eriksson & al. 1998, Sojak 2008, 2012a). Iranian species of *Potentilla* grow in Hyrcanian (Caspian), and Zagros floristic provinces and in different altitudes ranging from 100 m to 4600 m (Faghir & al. 2011). They are composed of *ca.* 42 species, with *ca.* 22 endemics arranged in 10 sections (Faghir & al. 2014, Naqinezhad & Faghir 2019). Among them, Sect. *Potentilla* is recognized by its perennial habit, with long creeping flowering stem, rooting in nodes, basal leaves 3-7 lobed, and style shorter or equaling the carpel. In several previous studies, *P. reptans* was introduced as the only representative of the Sect. *Potentilla* in Iran (Schiman-Czeika 1969, Khatamsaz 1993, Faghir & al. 2010. 2011, 2012, 2014, 2018, Sadeghi & al. 2021). So far, the phylogenetic and micromorphological characteristics of the species of Sect. *Potentilla* have not been studied in Iran. The main purpose of this study was to investigate the phylogenetic relationship between *P. indica* and *P. reptans* using nrDNA ITS, cpDNA *trn*H-*psb*A, and combined sequence data. In addition, the micromorphological characteristics of the epicalyx, calyx, and pedicle were discussed based on the results of the phylogenetic analysis.

MATERIALS AND METHODS

Taxon Sampling

The plant list used for phylogenetic and micromorphological analysis is given in Table 1. In the current analysis, a total number of four newly sequenced plus 30 sequences from Genbank were involved (Table 1). *Rosa persica* Michx. ex J. F. Gmel. and *Filipendula vulgaris* Moench were used as out-group species based on previous studies (Eriksson & al. 1998, 2003, Dobeš & Paule 2010, Faghir & al. 2014). We used *P. hebiichigo* Yonek. & H. Ohashi (Syn.: *Duchesnea. chrysantha* (Zoll. & Moritzi) Miq.) as a closely related species in the Reptans clade, which has a chromosome number of 2n=14 (Naruhashi & al. 1999), reported from Japan, China, India, Korea, Taiwan, Philippines, and Indonesia, and collected from Iran (Table 1).

DNA extraction

In this study, 16, 17, and 20 taxa were included in nrDNA ITS, *trn*H-*psb*A, and combined datasets, respectively (Table 1). Total DNA was extracted from 10 mg dried leaves of freshly collected specimens (during 2017-2018) deposited in the Guilan (GUH) and Tehran (TUH) Universities Herbaria and herbarium of Guilan Natural Resources Research Center (GILAN) using the CTAB procedure of Doyle & Doyle (1987). For identification purposes, we used the following references: Juzepczuk (1941), Schiman-Czeika (1969), Schönbech-Temesy (1969), and Khatamsaz (1993).

PCR and DNA sequencing

The PCR was performed in a 20 µL volume, containing 8 µl deionized water, 10 mL of Taq DNA polymerase 2x Master Mix RED (Amplicon, Cat No. 180301), 10 µmol/L of each dNTP, 0.5 µl of each primer (10 pmol/µl), one unit of Taq DNA polymerase and 1 µl template DNA (20–70 ng). The *trn*H–*psb*A intergenic spacer region was amplified using the primers *psb*A (Sang & al. 1997) and *trn*H (Tate & Simpson 2003). The PCR was performed in a 25 µl volume, containing 0.5 µl deionized water, 2X PCR Buffer with 10 mmol/L MgCl2, 10 µmol/L of each dNTP, 0.5 µl of each primer (10 pmol/ µl), one unit of Taq DNA polymerase and 2 µl template DNA (20 –50 ng). PCR procedures include 5min at 94 °C for pre-denaturation, followed by 35 cycles of 94 °C for 45 seconds, 56 °C for 50 seconds, and 72 °C for 50 seconds plus a final extension of 72 °C for 5 seconds.

The internal transcribed spacer of the nuclear ribosomal DNA (nrDNA ITS) region was amplified using primers ITS5m (Sang & al., 1995) and ITS4 (White & al., 1990). PCR procedures for the nrDNA ITS were 5 min at 94°C for predenaturation, followed by 30 cycles of 94°C for 45 seconds, 56°C for 50 seconds, and 72°C for 45 seconds plus a final extension of 72°C for 5 min. The quality of PCR products was checked by electrophoresis on a 1% (w/v) agarose gel. Sequencing was performed on an ABI 3730xl capillary sequencer through Pishgam Co.

Sequence alignment

Sequences of *trn*H-*psb*A and nrDNA ITS datasets were edited by BioEdit ver. 7.0.9.0 (Hall 2001). The alignment was carried out using Muscle v4.0 (Edgar 2004), and manual adjustment. The alignment of regions was examined and the insertions and deletions (indels) were identified as missing data. The length of the indels varied from a single up to 10 (in positions 495–505) base pairs in *trn*H-*psb*A data.

Phylogenetic analyses

Phylogenetic reconstruction was performed using maximum Parsimony, Bayesian Inference (BI), and maximum likelihood (ML) analyses. In the Maximum Parsimony method, we used the heuristic search with Tree Bisection-Reconnection (TBR), Branch Swapping, Simple addition sequence, 100 replications of random addition sequence, and the maximum number of trees was set to 20,000. Rescaled Consistency Index (RC), Consistency Index (CI), and Retention Index (RI) are also included in the PAUP* 4.b10 software (Swofford 2002) that was implemented. Bayesian analysis was run with MrBayes version 3.2 (Ronquist & al. 2012) as implemented in the CIPRES Science Gateway (http://www. phylo.org/, Miller & al. 2010), with the following settings: Four Markov chain Monte Carlo heuristic searches of 10 million generations were performed in two independent runs for the nuclear, plastid and combined datasets. The results and effective sample size sufficiency were checked using Tracer version 1.6 (Drummond & Rambaut 2007).

The first 25% of trees were discarded as burn-in. Posterior probabilities (PP) were used to illustrate the support of nodes. The model of sequence evolution was determined using the Akaike Information Criterion (AIC) (Posada & Buckley 2004) as implemented in the MrModeltest 2.2 (Nylander 2004). Based on this analysis, TVM+F+I, GTR+F+G4, GTR+F+G4 were identified as the best model for the plastid, nuclear, and combined datasets, respectively. The mean distances between sequences were calculated on a p-distance matrix with complete deletion of gaps, using MEGA version 7 (Kumar & al. 2016). The maximum likelihood (ML) analysis was conducted using the online phylogenetic software W-IQ-TREE (Trifnopoulos & al. 2016) available at http://iqtree.cibiv.univie.ac.at. Node supports were calculated via rapid bootstrap analyses with 1000 replicates.

Epicalyx, calyx, and pedicle micromorphology

For scanning electron microscopy (SEM), dry samples were mounted on the stubs with double-sided cellophane tape and coated in a sputter coater with 25nm of gold-palladium at an accelerating voltage of 10–15 kv. The samples were observed and photographed on a scanning electron microscope Tescan SEM Vega. The terminology used was adopted from that of Barthlott & al. (1998), Ergen Akin & al. (2013), and Kumar & Murugan (2015) with some modifications.

Table 1. Samples used for phylogenetic and micromorphological analysis. * shows newly sequenced taxa, indicates sample used for the micromorphological study.

Species	**Collection Data**	**Genbank Accession no.**
1. Subtribe *Fragariinae*		
1.1. *Fragaria*		
1.1.1 *Fragaria viridis* West.		
*trn*H-*psb*A	Ardabil Province: Sardabeh, 10. 5.2005, Ghahreman & Attar, TUH 35314	*LC632301
ITS	Lundberg 16 (S)	FJ356166-
1.1. 2 *F. vesca* L.		
*trn*H-*psb*A	Guilan Province: Fuman, ghale Rudkhan, 17.5.2017, Pourebrahim, GUH 5792	*LC632300
ITS	Eriksson and Smedmark 43 (SBT) Sweden, Uppland	AJ511771
1.1.3 *F. virginiana* Mill.		
ITS	Eriksson s.n. (SBT) Canada, Nova Scotia USA	AJ511772
*psb*A–*trn*H	Njuguna, W., Bassil, N.V. and Hummer, K.E., 2009 USA	GQ476772.1
1.2. *Alchemilla*		
1.2.1 *Alchemilla mollis* (Buser) Rothm.		
*trn*H-*psb*A		-
ITS	Eriksson & al. 1998 Sweden, Torne Lappmark	AJ511769
1.2.2 *A. alpina* L.		
*trn*H-*psb*A		-
ITS	Eriksson & al. 1998 Sweden, Torne Lappmark	U90816
1.3. *Comarum palustris* L.		
*trn*H-*psb*A	Guglielmo, F., Poggio, L. and Tutino, 11020, Italy	MF543678.1
ITS	Eriksson 659 (GH, S) Sweden	AJ511777
2. Subtribe *Potentillinae*		
2.1. *Potentilla*		
Sect. *Potentilla*		
2.1.1 *P. indica* (Jacks.) Th. Wolf		
*trn*H-*psb*A	Erickson, D., Kuzmina, M., Kress, J. and Mcshea, W. DC 20013-7012, USA	KP6435141
	Erickson, D., Kuzmina, M., Kress, J. and Mcshea,W., DC 20013-7012, USA	KP643621.1
	Zuniga, J.D., Mulcahy, D.G. and Coddington, J., DC 20560, USA	MF786018.1
	Guilan Province: Pirbazar, 29.6.2015, Ashouri, GUH 5793	*LC632299
	Guilan Province: Rudbar, 5.6.2011, Assadi, GILAN 4085	*LC632298
2.1.2 *P. indica* (=former *Duchesnea indica)*		
ITS	Eriksson s.n. (GH, SBT) HB; China, Gansu	AJ511775
	Feng, T., Michael, M.J. and Wang, H., 430074, China	KP875300.1

Table 1 continued:

2.1.3 *P. hebiichigo* (=former *P. chrysantha)*		
ITS	Yuan,C.I., Hsieh,Y.C. and Chiang, M.Y., Taichung Hsien	FN430803.1
	Heo,K.-I. and Kim,S.-C, Gyeonggido 440-746, South Korea	FN421385.1
	Tehran province: Tehran University, Hadizadeh & Rahmani 46799 (TUH)	-
2.1.4 *Potentilla reptans* L.		
ITS	Guilan Province: Siahkal, Faghir, 16.6.2006, 36639 (TUH)	AB894161
	Mazandaran Province: Youshbaladeh, 18.5.2006	AB894162
	Topel, M., Lundberg, M., Eriksson, T. and Eriksen, B. University of Gothenburg	FN430815.1
*trn*H-*psb*A	Bruni, I., De Mattia, F., Martellos, S., Galimberti, A., & al., 20126, Italy	HE966756.1
	James, K.E., Rumsey, F., Spencer, M., Carine, & al., SW7 5BD, UK	FJ395525.1
	Dobes, C. and Paule, J., University of Vienna, Althanstrasse 14, 1090, Austria	GQ384955.1
	Dobes & al. 2009, University of Vienna, Althanstrasse 14, Vienna 1090, Austria	GQ476781.1
2.2. Sect. *Aureae* (Rydb.) Juz. 2.2.1 *P. gelida* C.A. Mey.		
*trn*H-*psb*A	Dobes & al. 2009, University of Vienna, Althanstrasse 14, Vienna 1090, Austria	GQ476781.1
ITS		-
2.2.2 *P. crantzii* (Crantz) Beck ex Fritsch		
*trn*H-*psb*A	Dobes & al. 2009, University of Vienna, Althanstrasse 14, Vienna 1090, Austria	GQ476764.1
ITS	Mazandaran Province: Ramsar, Samamous mountain, Faghir, 22.7.06	AB894165
2. 3. Sect. *Terminales* (Döll) Gren. & Godr. 2. 3. 1 *P. argentea* Rydb.		
*trn*H-*psb*A	Dobes & al. 2009, University of Vienna, Althanstrasse 14, Vienna 1090, Austria	GQ476779.1
ITS	Guilan Province: Siahkal, Faghir, 16.6.06, 36585 (TUH)	AB894151
3. *Rosa persica* Michx. ex Juss		
ITS	Eriksson, T., Hibbs, M.S., Donoghue, M.J., Yoder, A.D., Sweden	AJ416468.1
*trn*H-*psb*A	Bruneau, A., Starr, J.R. and Joly, S., H1X 2B2, Canada	DQ778799.1
4. *Filipendula vulgaris* Moench		
*trn*H-*psb*A	Bruni, 2012, University of Milano Bicocca, 20126, Italy	GQ384982
ITS	Eriksson 821 (SBT) Sweden	AJ416467

RESULTS

*trn*H-*psb*A sequence data

The *trn*H-*psb*A datasets contained 17 taxa and 474 aligned DNA characters. Parsimony analysis consisted of 250 constant, 102 parsimony-uninformative, and 122 parsimony-informative characters (Table 2). The phylogenetic analyses led to the formation of topologically similar trees in maximum Parsimony (MP), Bayesian (BI), and maximum Likelihood (ML) methods (Fig. 1), and only the Bayesian tree has been shown. All plastid trees show the separation of representatives of *Potentilla* in clade A (with PP= 97% in MP tree, BP= 68 in ML tree, PP= 0.98 in BI tree) from its related genera in clade B (with PP= 99% in MP tree, BP= 67 in ML tree, PP= 0.96 in BI tree). Clade A is divided into two small clades: C including *P. gelida* C.A. Meyer, *P. crantzii* (Crantz) Beck and *P. argentea* L. (with BP= 100 in MP tree, BP= 100 in ML tree, PP= 1 in BI tree) and Reptans (with BP= 97 in MP tree, BP= 68 in ML tree, PP= 0.98 in BI tree). The Reptans clade consists of two subgroups: 1) subgroup L including three populations of *P. reptans* (with BP= 100 in MP tree, BP= 100 in ML tree, PP= 1 in BI tree) and 2) subgroup M including three populations of *P. indica* (with BP= 75 in MP tree, BP= 93 in ML tree, PP= 1 in BI tree) plus two taxa of *P. indica* in independent branches (newly sequenced taxa, Table 1). While clade B comprises four representatives of the subtribe *Fragariinae* as *Comarum palustris* L., *Fragaria vesca* L., *F. viridis*, and *F. virginiana* Mill. The first two species are found in isolated branches, while the latter two form a small monophyletic group.

nrDNA ITS sequence data

The nrDNA ITS datasets contained 16 taxa and 655 aligned DNA characters. Based on maximum Parsimony analysis, the final alignment encompassed 366 constant characters,133 parsimony-uninformative, and 156 parsimony-informative characters (Table 2). The phylogenetic analysis led to the formation of topologically similar trees in maximum Parsimony (MP), Bayesian (BI), and maximum Likelihood (ML) analyses (Fig. 2). These trees are also similar to plastid trees in terms of topology and composition, except for clade M containing *P. hebiichigo* within Reptans clade. All obtained nuclear trees, show the representatives of *Potentilla* in clade A (with BP= 75% in MP tree, BP = 76 in ML tree, PP= 0.99 in BI tree), and its related genera in clade B (with BP= 100% in MP tree, BP= 73 in ML tree, PP= 0.94 in BI tree). Clade A is divided into two clades; including C with *P. crantzii* (Crantz) Beck ex Fritsch and *P. argentea* (with BP= 100 in MP tree, BP = 100 in ML tree PP= 1 in BI tree), and Reptans clade (with BP= 100 in MP tree, BP = 95 in ML tree PP= 0.99 in BI tree). The Reptans clade contains three subclades:1) Subclade L containing *P. reptans* (with BP= 100 in MP tree, BP= 99 in ML tree, PP= 1 in BI tree), 2) Subclade M containing *P. hebiichigo* (with BP= 97 in MP tree, BP = 87 in ML tree, PP= 0.99 in BI tree), and 3) Subclade N containing *P. indica* populations (with BP= 100 in MP tree, BP= 100 in ML tree, PP= 1 in BI tree) plus two populations of *P. indica* in independent branches (newly sequenced taxa, Table 1). Clade B consists of four representatives of the subtribe *Fragariinae* as shown in the plastid trees.

Table 2. DNA sequence characteristics and statistics for each data partition.

	ITS	***trn*H-*psb*A**	**ITS+*trn*H-*psb*A**
Number of sequences	16	17	20
Number of characters	655	474	1229
Number of parsimony-informative characters	133	102	200
CI	0.7679	0.8840	0.8127
RI	0.2321	0.1160	0.1873
The evolutionary model selected (under AIC)	GTR+I+G4	TVM+F+I	GTR+F+G4

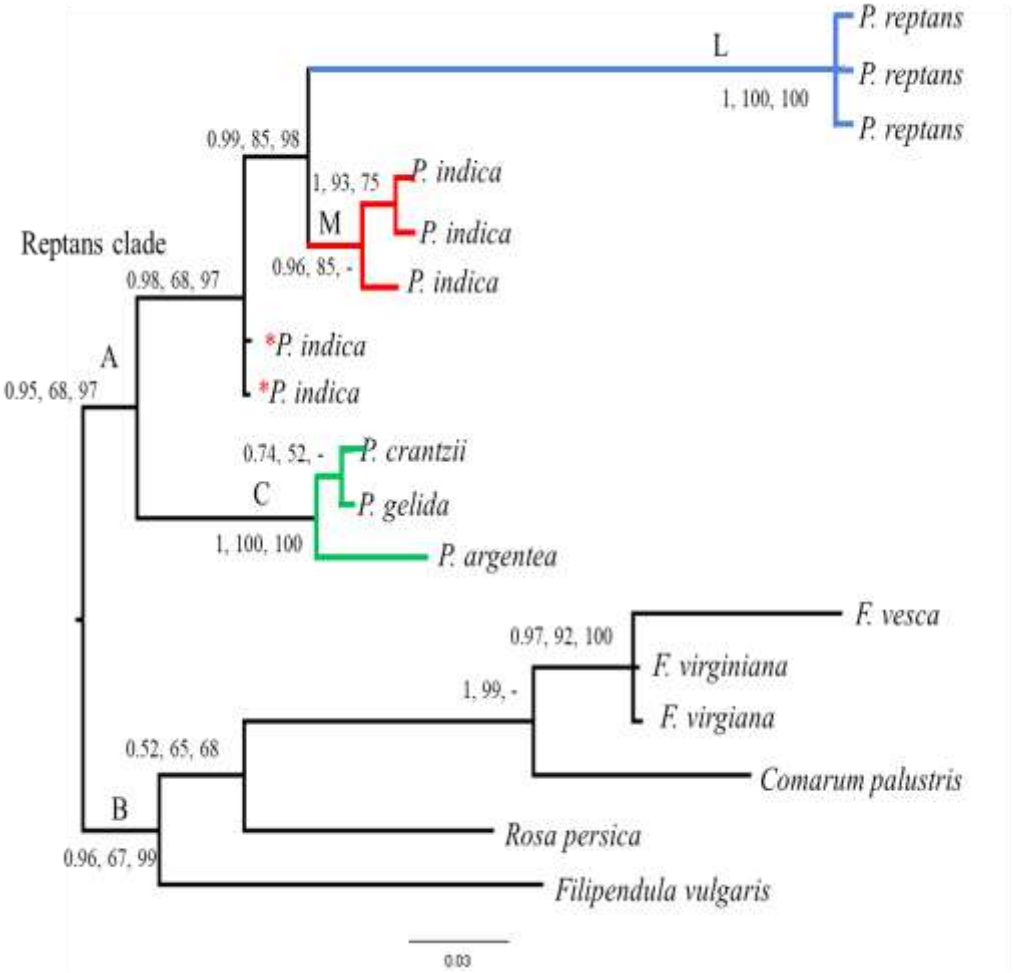

Fig. 1. Bayesian 50% majority-rule consensus tree of *Potentilla indica*, *P. reptans*, and related species based on the *trn*H-*psb*A sequences.* indicates two newly sequenced taxa of *P. indica*. The numbers above branches are maximum Parsimony/Maximum Likelihood bootstraps values/Bayesian posterior probabilities. Consistency index (CI)= 0.8840, Homoplasy index (HI)= 0.1160, CI excluding uninformative characters= 0.8201, HI excluding uninformative characters= 0.1799, Retention index (RI)= 0.8840, Rescaled consistency index (RC)= 0.7814 in maximum parsimony analysis.

Combined sequence data

The combined (ITS+*trn*H-*psb*A) datasets contained 20 taxa and 1129 aligned DNA characters. Based on the maximum parsimony analysis, the final concatenated alignment consists of 626 constant, 200 parsimony-uninformative, and 303 parsimony-informative characters (Table 2). The phylogenetic analyses led to the formation of topologically similar trees in maximum Parsimony (MP), Bayesian (BI), and maximum Likelihood (ML) analyses (Fig. 3). Only the Bayesian tree has been shown. All combined trees, show the representatives of *Potentilla* in clade A (with PP= 100% in MP tree, BP=90 in ML tree, PP= 0.99 in BI tree), and its related genera in clade B (with BP= 99% in MP tree, BP= 67 in ML tree, PP= 0.59 in BI tree). Clade A is divided into clade C (including *P. gelida*, *P. crantzii*, and *P. argentea*, with BP= 99 in the MP tree, BP= 100 in the ML tree PP= 1 in the BI tree), and Reptans clade

(with BP= 100 in MP tree, BP = 100 in ML tree PP= 1 in BI tree). The Reptans clade is further divided into two subclades, P and P1. The P subclade contains two populations of *P. indica* (newly sequenced taxa, Table 1) that formed a small group. Whereas subclade P is derived into three small subclades (L, O, and N). Subclade L contains a small group containing *P. reptans* taxa (with BP= 100 in the MP tree, BP= 99 in the ML tree, and PP= 1 in the BI tree), subclade O includes two *P. hebiichigo* taxa (in isolated branches), and subclade N contains three *P. indica* taxa (with BP= 100 in MP tree, BP= 99 in ML tree, PP= 1 in BI tree).

Clade B consists of four representatives of the subtribe *Fragariinae* as shown in the plastid and nrDNA ITS trees (Fig. 3).

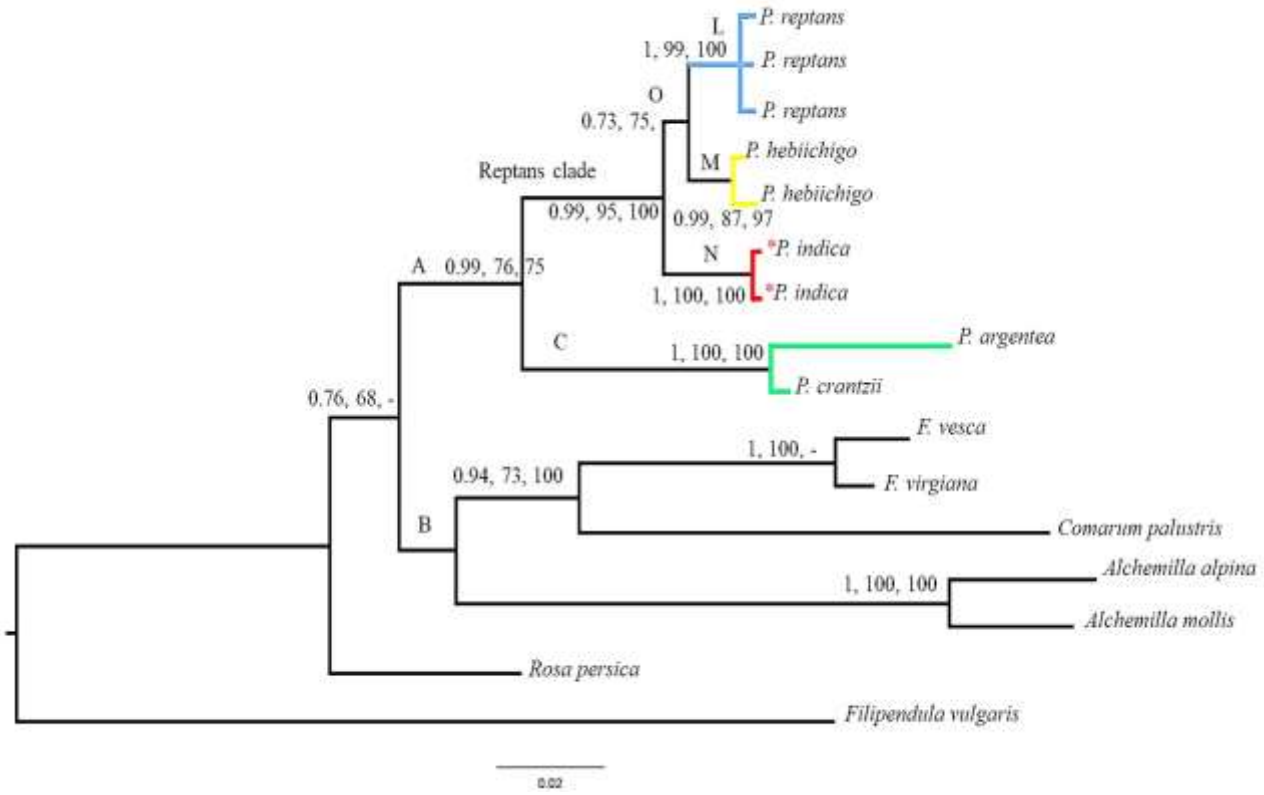

Fig. 2. Bayesian 50% majority-rule consensus tree of *Potentilla indica*, *P. reptans*, and related species based on nrDNA ITS sequence. * indicates two newly sequenced taxa of *P. indica.* The numbers above branches are maximum Parsimony / Maximum Likelihood bootstraps values/Bayesian posterior probabilities. The parsimony tree includes Consistency index (CI)= 0.7679, Homoplasy index (HI)= 0.2321, CI excluding uninformative characters= 0.6899, HI excluding uninformative characters= 0.3101, Retention index (RI)= 0.7494, Rescaled consistency index (RC)= 0.5755.

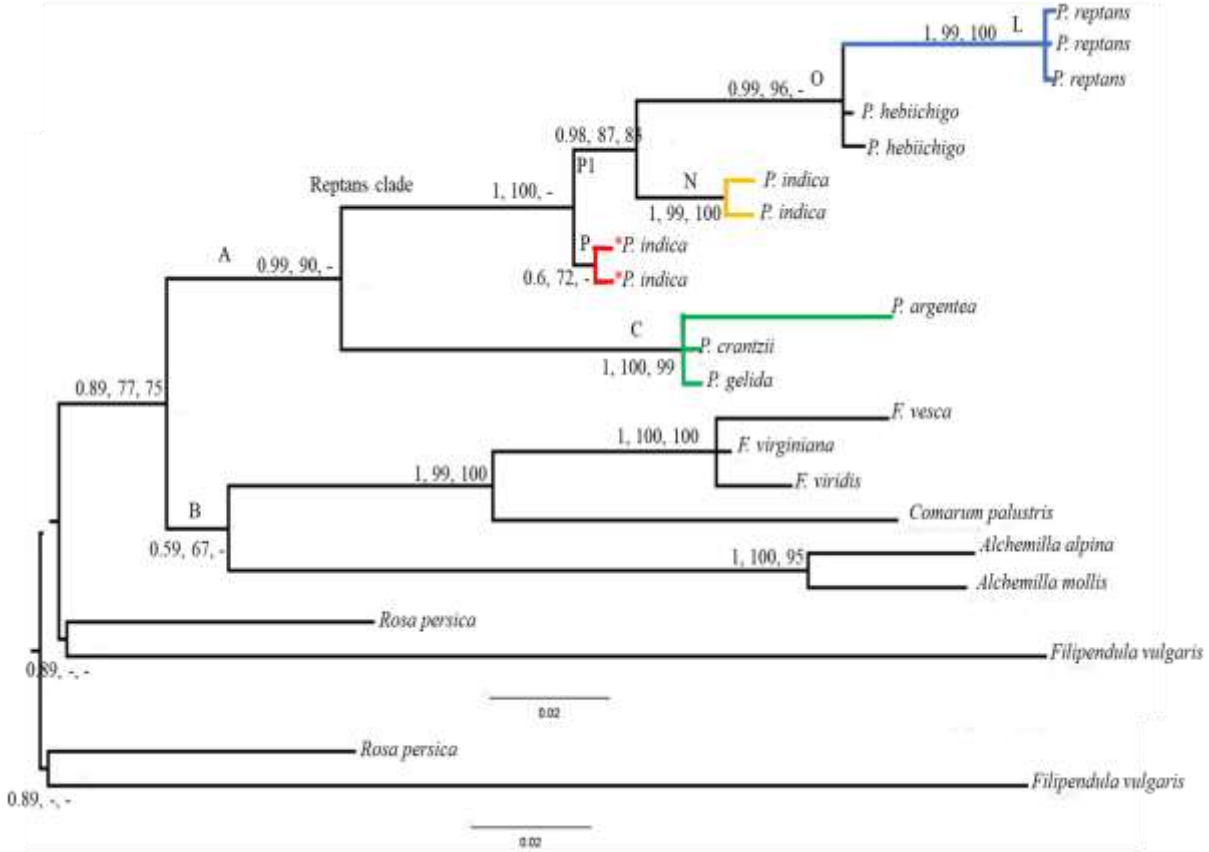

Fig. 3. Bayesian 50% majority-rule consensus tree of *Potentilla indica*, *P. reptans*, and related species based on *trn*H-*psb*A and nrDNA ITS combined sequences. * indicates two newly sequenced taxa of *P. indica*. The numbers above branches are maximum parsimony/maximum likelihood bootstraps values/Bayesian posterior probabilities. *psb*A-*trn*H and nrDNA ITS combined sequences fifty percent majority consensus tree showed Consistency index (CI)= 0.8127, Homoplasy index (HI)= 0.1873, CI excluding uninformative characters= 0.7437, HI excluding uninformative characters= 0.2563, Retention index (RI)= 0.7968, Rescaled consistency index (RC)= 0.6476.

Epicalyx, calyx, and pedicle micromorphology

In the present survey, 34 micromorphological characteristics of the studied species were examined (Table 3 and Figs. 4-7). The results show two types of epicalyx hair position (on the adaxial/abaxial surfaces), including: only on the upper half (in *P. indica*), (Fig. 4A), on the whole surface (in *P. indica*, *P.hebiichigo*, and *P. reptans*), (Figs. 4B, E-F, I-J); three types of calyx hair position on the adaxial/abaxial surfaces: including on the surface (*P. indica* and *P. reptans*), (Figs. 4C-D, K-L), only at the margin (Fig. 4G), and in the central part (*P. hebiichigo*), (Fig. 4H); three types of epicalyx hair density adaxial/abaxial surfaces: hairy (in *P. indica* and *P. reptans*) with few scattered hairs (*P. hebiichigo*) and densely hairy (in *P. indica*); two types of calyx hair density on adaxial/abaxial surfaces (*P. indica*, *P. hebiichigo*, and *P. reptans*), densely hair (*P. indica*). The studied species have similar hair types (straight-flexuous) on the epicalyx adaxial/abaxial surfaces, two types of calyx hair type: straight-flexuous (in *P. indica* and *P. reptans*) and straight (in *P. hebiichigo*). Glandular hairs are present on the epicalyx and calyx surfaces of the three species (Fig. 4). Stomata are present on both epicalyx and calyx surfaces. Glandular hairs are stalked capitate to cylindrical heads in *P. indica* and *P. hebiichigo*. Epicalyx hair position in

relation to epidermal cells adaxial/abaxial surfaces changes from sub appressed (in *P. indica* and *P. reptans*), (Figs. 4A-D, 4I-L) to appressed (in *P. hebiichigo*), (Figs. 4E-H). *Potentilla indica*, *P. hebiichigo,* and *P. reptans* have sub appressed calyx hair position in relation to epidermal cells. Epicalyx and calyx trichome have a similar wax type (i.e., Platelets/Granulate-Platelets) in *P. indica* (Figs. 5A-D) and *P. hebiichigo* (Figs. 5E-H). The wax type consists of film and crystalloid in either epicalyx or calyx in the three studied species. Three types of epicalyx epicuticular wax ornamentation were observed; this includes striate, irregular platelets-granule/membranous platelets, (in *D. indica*), (Figs. 6A-B), crust, scattered platelets (in *P. hebiichigo*), (Figs. 6E-F) and crust, granulate, platelets (in *P. reptans*), (Figs. 6I-J). Calyx epicuticular wax ornamentation also differed in the three studied species: Irregular platelets/membranous platelets (in *D. indica*), (Figs. 6C-D), crust, scattered platelets/crust, scattered platelets, granulate (in *P. hebiichigo*), (Figs. 6G-H). and striate/crust, granulate, and platelets (in *P. reptans*), (Figs. 6I-L). Stomata are present in both epicalyx and calyx on the surfaces of the three studied species. These species show similar wax distribution patterns on the epicalyx and calyx surfaces. Two types of epicalyx and calyx outer stomata rim, including overlapping/raised (in *P. hebiichigo* and *P. reptans*), (Figs. 6E-F, I-J) and overlapping/overlapping (in *D. indica*), (Figs. 6A-B), two types of epicalyx and calyx peristomatal rim: overlapping/overlapping (in *P. indica*) and overlapping/stout (in *P. hebiichigo* and *P. reptans*), two types of epicalyx inner stomata rim: sinuolate/sinuolate (in *P. hebiichigo* and *P. indica*), sinuolate–erose/sinuolate–erose (in *P. reptans*), two types of calyx inner stomata rim sinuolate/sinuolate (in *P. hebiichigo* and *P. indica*) and sinuolate–erose/sinuolate–erose (in *P. reptans*) were identified. Pedicles are hairy in *P. indica* (Fig. 7A) and *P. hebiichigo* (Fig. 7D) and glabrous in *P. reptans* (Figs. 7G-I). However, the hair types differ from straight, sub-appressed in *P. indica* (Fig. 7C) to flexuous–straight, sub appressed-erect in *P. hebiichigo* (Fig. 7D). Hair is heavily granulate-platelets in *P. indica* (Fig. 7B) and slightly granulate in *P. hebiichigo* (Fig. 7E). Epicalyx and calyx epicuticular wax ornamentation is the crust and granulate in all three species studied. Stomata surrounded by epicuticular wax chimneys are recorded in *P. reptans* (Fig. 7I)

Table 3. Epicalyx, calyx, and pedicle micromorphological characters of adaxial/abaxial surfaces of *P. indica*, *P. hebiichigo*, and *P. reptans*.

Characters	***P. indica*** ad/ab surfaces	***P. hebiichigo*** ad/ab surfaces	***P. reptans*** ad/ab surfaces
Epicalyx hair position	Only in the upper half/ On the surface	On the surface/ On the surface	On the surface/ On the surface
Calyx hair position	On the surface/On the surface	only at margin/ only in the central part	On the surface/ On the surface
Epicalyx hair density	Hairy/Densely hairy	Few scattered hairs/ Few scattered hairs	Hairy/Hairy
Calyx hair density	Hairy/Densely hairy	Hairy/Hairy	Hairy/Hairy
Epicalyx hair type	Straight-Flexuous/Straight-Flexuous	Straight-Flexuous/ Straight-Flexuous	Straight-Flexuous/Straight-Flexuous
Calyx hair type	Straight-Flexuous/Straight-Flexuous	Straight/Straight	Straight-Flexuous/Straight-Flexuous
Epicalyx glandular hair	+/+	+/+	+/+
Calyx glandular hair	+/+	+/+	+/+
Epicalyx gland shape	Stalked, Capitate-cylindrical head	Stalked, Capitate-cylindrical head	Stalked, Capitate-cylindrical head
Calyx gland shape	Stalked, Capitate- cylindrical head	Stalked, Capitate-cylindrical head	Stalked, Capitate- cylindrical head
Epicalyx hair position in relation to epidermal cells	Sub appressed/Sub appressed	Appressed/Appressed	Sub appressed/Sub appressed

Table 3 continued

Calyx hair position in relation to epidermal cells	Sub appressed/Sub appressed	Sub appressed/Sub appressed	Sub appressed/Sub appressed
Epicalyx trichome wax type	Platelets/Granulate-Platelets	Platelets/Granulate-Platelets	Platelets/Granulate-Platelets
Calyx trichome wax type	Granulate-Platelets/Granulate-Platelets	Smooth/Granulate-Platelets	Platelets/Granulate-Platelets
Epicalyx wax type	Film and crystalloid	Film and crystalloid	Film and crystalloid
Calyx wax type	Film and crystalloid	Film and crystalloid	Film and crystalloid
Epicalyx epicuticular wax ornamentation types	Striate, Irregular platelets-granule/ Membranous platelets	Crust, Scattered platelets/Crust Scattered platelets	Crust, Granulate, platelets/Crust, Granulate, Platelets
Calyx epicuticular wax ornamentation type on ad/ab surfaces	Irregular platelets/ Membranousn platelets	Crust, Scattered platelets/ Crust, Scattered platelets, Granulate	Striate/ Crust, Granulate, platelets
Epicalyx stomata	+/+	+/+	+/+
Calyx stomata	+/+	+/+	+/+
Epicalyx stomata wax distribution pattern	Epidermal cell, stomata rims covered by wax, and pore-free on both sides	Epidermal cell, stomata rims covered by wax, and pore-free on both sides	Epidermal cell, stomata rims covered by wax, and pore-free on both sides
Calyx stomata wax distribution pattern	Epidermal cell, stomata rims covered by wax, and pore-free on both sides	Epidermal cell, stomata rims covered by wax, and pore-free on both sides	Epidermal cell, stomata rims covered by wax, and pore-free on both sides
Epicalyx outer stomata rim	Overlapping/Overlapping	Overlapping/Raised	Overlapping/Raised
Calyx outer stomata rim	Overlapping/Overlapping	Overlapping/Raised	Overlapping/Raised
Epicalyx peristomatal rim	Overlapping/Overlapping	Overlapping/Stout	Overlapping/Stout
Calyx peristomatal rim	Overlapping/Overlapping	Overlapping/Stout	Overlapping/Stout
Epicalyx inner stomata rim	Sinuolate/Sinuolate	Sinuolate/Sinuolate	Sinuolate–Erose/Sinuolate–Erose
Calyx inner stomata rim	Sinuolate/Sinuolate	Sinuolate/Sinuolate	Sinuolate–Erose/Sinuolate–Erose
Pedicle hair type	Straight	Flexuous-Straight	-
Pedicle hair position in relation to epidermis	Sub appressed	Sub appressed-Erect	-
Pedicle hair wax type	Heavily granulate-Platelets	Slightly granulate	-
Pedicle wax type	Film and crystalloid	Film and crystalloid	Film and crystalloid
Pedicle wax ornamentation	Crust with a few granules	Crust-granule	Irregular platelets and granules
Stomata with chimney	-	-	+

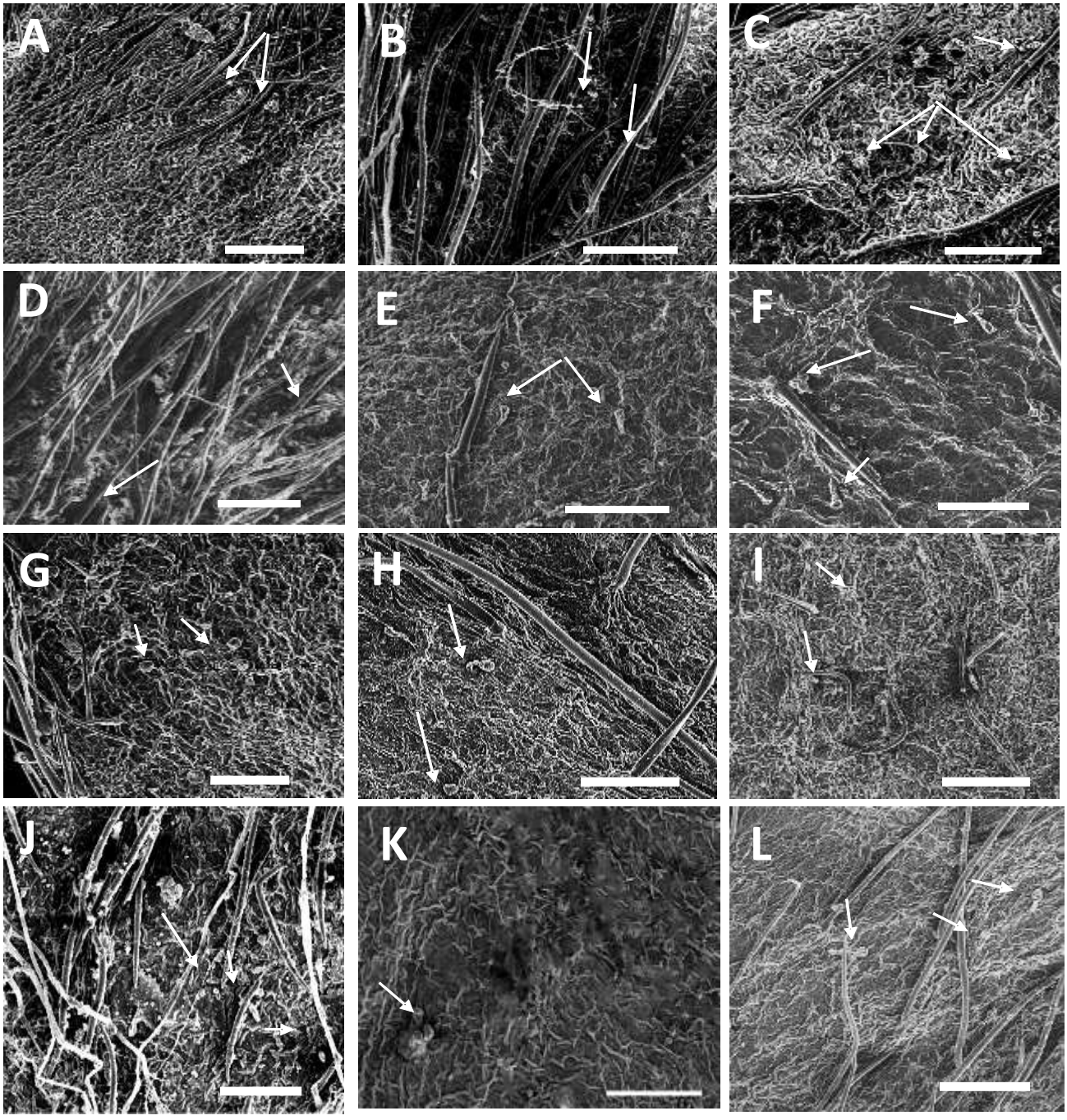

Fig. 4. SEM micrographs showing epicalyx and calyx surfaces. A-D, *P. indica*; E-H, *P. hebiichigo*; I-L, *P. reptans* (scale bare= 25µm), arrow indicates the glands (A, C, E, G, I and K adaxial surface; B, D, F, H, J, and L abaxial surface).

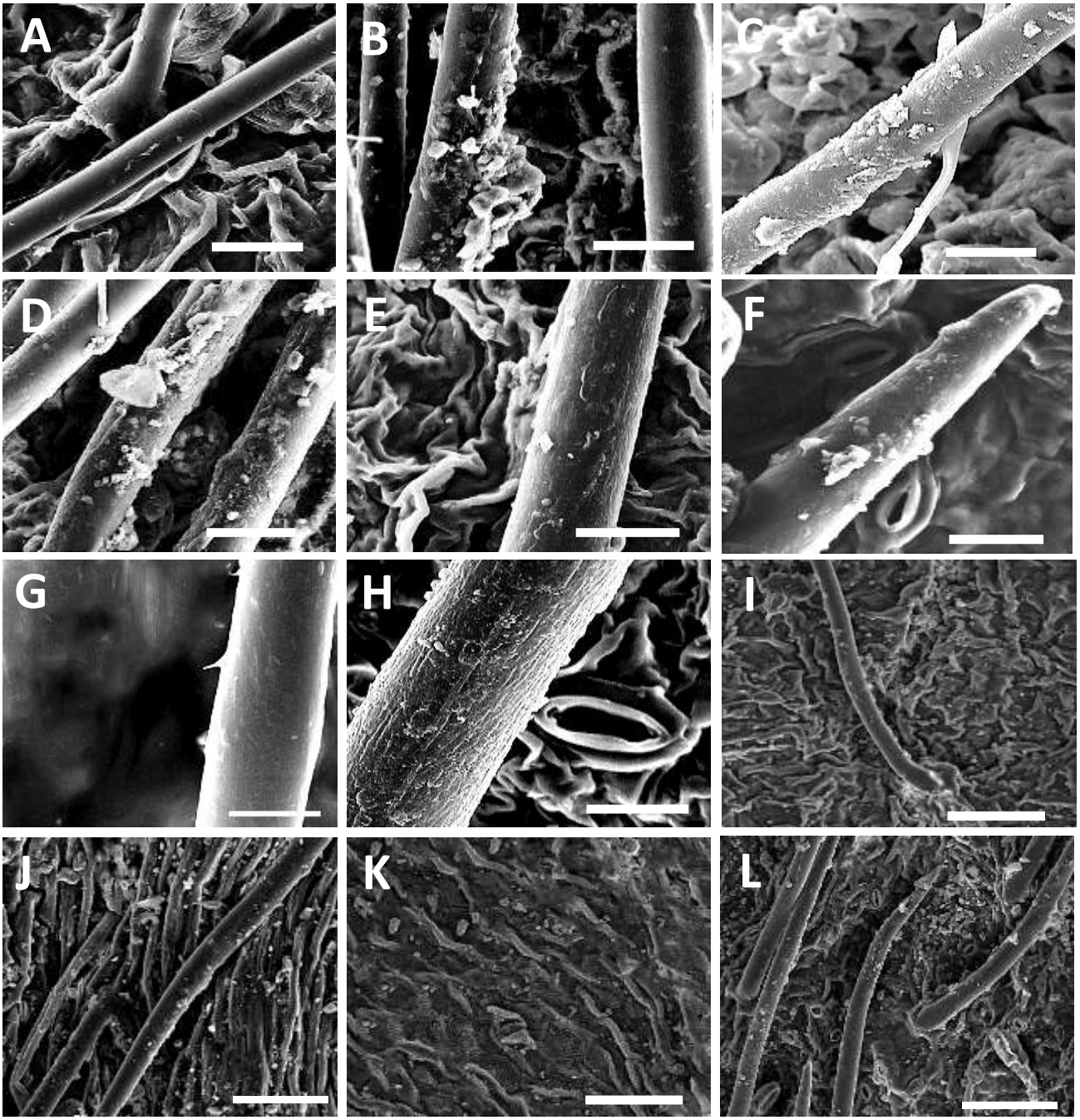

Fig. 5. SEM micrographs showing hair types on epicalyx and calyx adaxial and abaxial surfaces. A-D, *P. indica*; E-H, *P. hebiichigo*; I-L, *P. reptans* (scale bare= 25µm), (A, C, E, G, I and K adaxial surface; B, D, F, H, J and L abaxial surface).

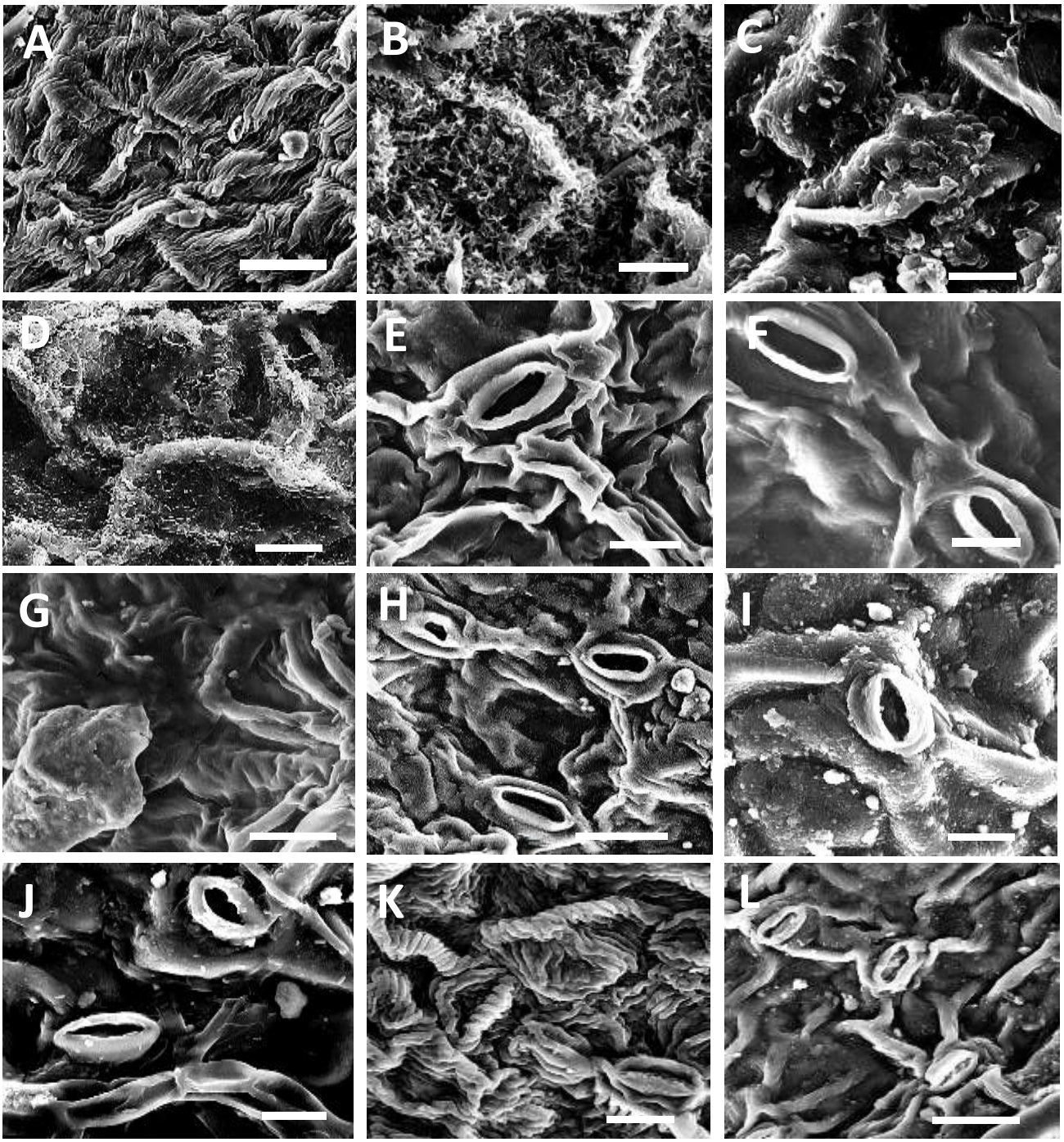

Fig. 6. SEM micrographs showing sculpturing types on epicalyx and calyx adaxial and abaxial surfaces. A-D, *P. indica*; E-H, *P. hebiichigo*; I-K, *P. reptans* (A, B, G, H, and L scale bare= 20µm. C, D, B, F, I, J, and K scale bare= 10µm), (A, C, E, G, I, and K adaxial surface; B, D, F, H, J, and L abaxial surface).

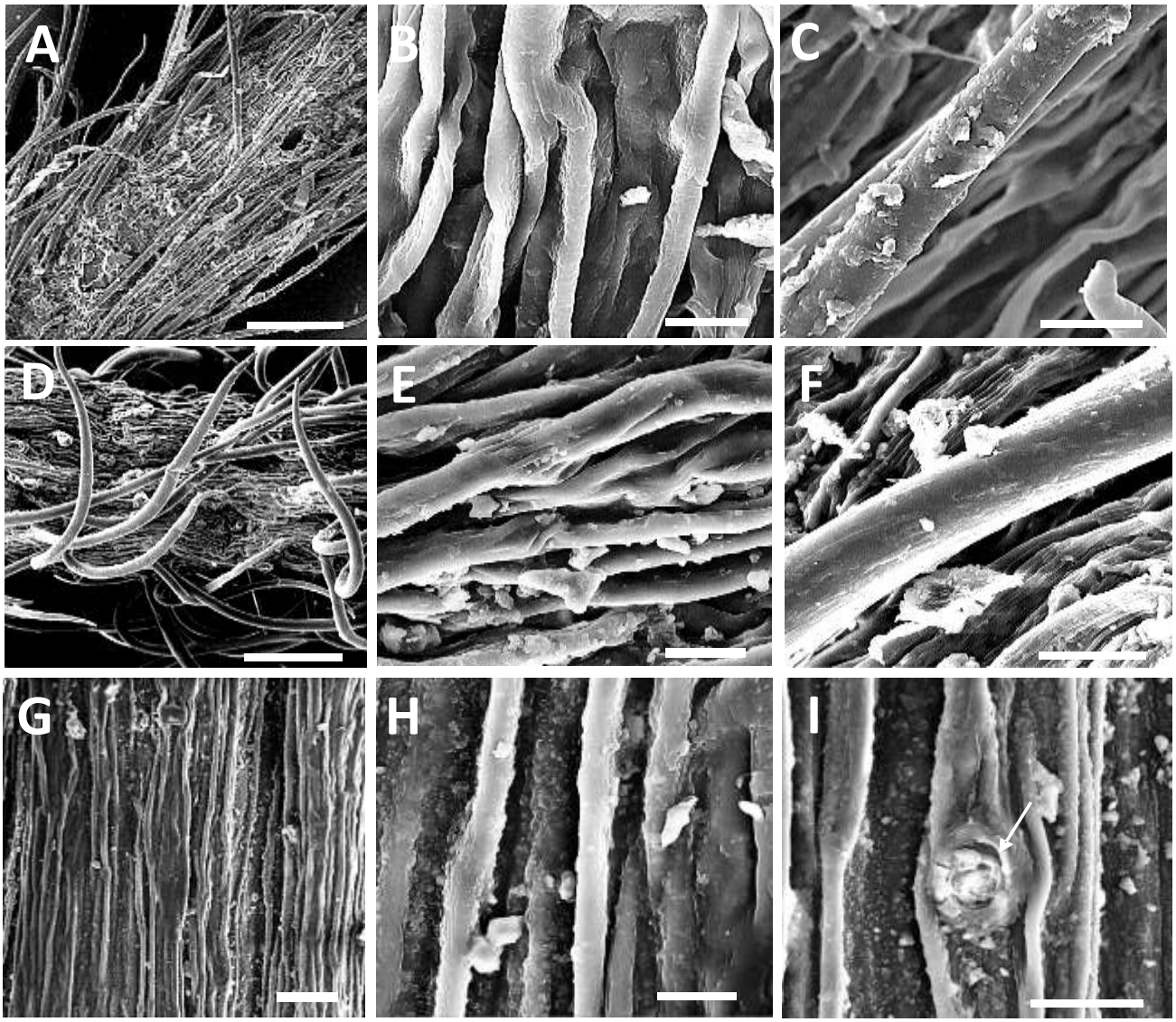

Fig. 7. SEM micrographs showing pedicle surface: A-C, *P. indica;* D-F, *P. hebiichigo;* G-I, *P. reptans* (scale bar A= 200µm, B, H= 10 µm, C, D, E, F, and I= 20 µm, G=50 µm), arrow indicates the chimney stomata.

DISCUSSION

In the present analyses, as well as in preliminary studies (Eriksson & al. 1998, 2003, Kurtto & Eriksson 2003, Töpel & al. 2011), *P. indica* is placed with *P. reptans* and *P. hebiichigo* in a well-supported clade of Reptans (Figs. 1-3). In addition, minimal nucleotide sequence divergence (0.01-0.02 %) of the nrDNA ITS was recorded within the Reptans clade (while it increased to 0.06 between *P. indica, P. hebiichigo,* and other species of *Potentilla*, and reached its maximum (0.11) between *P. indica, P. hebiichigo*, and *Fragaria*). This result is also confirmed by their morphological similarities, such as anthers with two thecae and terminal to subterminal style, according to the classification proposed by previous authors (Dobeš & Paule 2010; Topel & al. 2011; Feng & al. 2017; Persson & al. 2020). However, the members of the Reptans clade including *P. reptans* (section *Potentilla*), *P. indica,* and,

P. hebiichigo are identified by their diagnostic features such as the number of leaflets and their shape, the shape of episepals and the receptacle types (Faghir & al. 2018).

The results also showed a phylogenetic split in the Reptans clade, corresponding to *P. reptans* and *P. indica* (L and M groups) in the ITS trees (Fig. 1), *P. reptans, P. hebiichigo* (group O), and *P. indica* (group N) in the plastid and combined trees (Figs. 2 & 3).

The ITS, plastid, and combined sequence data grouped three *P. reptans* populations into a single clade, but their relationship collapsed into a tritomy. *Potentilla reptans* is characterized by some traits such as smooth internodes of the stolons, the shape of stipules, the number and shape of teeth of the middle leaf, flower diameter, width of episepals (Soják 2012), and shape of achenes (Sadeghi & al. 2021). This species is the most widespread *Potentilla* species in Iran (Fahgir & al. 2011), showing morphological differences, and is involved in the formation of several natural and experimentally produced hybrids (Wolf 1908, Leht & Paal 2004).

Two specimens of *P. hebiichigo* formed a monophyletic group in the ITS and in the combined trees. This species is characterized by its pink fruiting receptacle, obcordate petals, yellowish-green leaves, slender, broadly ovate and obtuse middle leaflet, and brownish, rugose or tuberculate achenes (Soják 2012). Based on previous studies (Kurtto & Eriksson 2003, Yonekuraet 2008, Ohashi & Ohashi 2008), *P. hebiichigo* was found to be close to *P. indica* and nested in *Potentilla* clade (Park & al. 2019). However, *P. hebiichigo* differs from *P. indica* in the shape and color of the leaves, the shape of the petals, the color of the receptacle, and the surface of the achenes (Chaoluan & al. 2003, Heo & al. 2019, Soják 2012).

Based on the present findings, *P. indica* is the sister to *P. hebiichigo* and *P. reptans*. The narrowly obovate petals, the green, rather thick leaves, the rhombic-oblong and acute central leaflet, the red fruiting receptacle, and the red and almost smooth achene surface are other diagnostic characteristics of *P. indica* (Soják 2012). In his monograph, Wolf (1908) classified *Potentilla indica* as a species with a high morphological polymorphism and different varieties such as *P. indica* var. *serrulata* Th. W., *P. indica* var. *wallichii* Th. W. (or *D. chrysantha*) and *P. indica* × reptans Th. W. in Asch. & Gr. These varieties differ mainly in the size, shape, and color of the leaves, the height of the stem, and the size of the flowers.

Potentilla indica is native to Asia (India) (Naruhashi & al. 1999), distributed in northern Iran (Zare & al. 2007, Pourebrahim & al. 2018), with 2n=84 (dodecaploid) (Naruhashi & Sagimoto 1996). It has a higher ploidy level and may have undergone additional periods of autoploidy (Persson & al. 2020). This may be the reason for the isolation of two newly sequenced taxa from the three Genbank taxa on all resulting trees, which requires further research at the population level and adding of more samples.

Phylogenetic utility of micromorphological traits

Based on the results, synapomorphies of the Reptans clade are followed as straight-flexuous epicalyx hair; stalked, capitate-cylindrical head, and sub appressed calyx hair; film, and crystalloid epicalyx and calyx wax type; the presence of stomata on epicalyx and calyx; epidermal cell; stomata rims covered by wax, and pore-free on both sides of epicalyx' stomata; pedicle wax type and distribution pattern. In general, the characteristics of the hairs and glandular trichomes of these three species obviously coincide with the characteristic features of the genus *Potentilla* (Faghir & al. 2010; Faghir & al. 2011). The results showed that the three species *P. indica, P. hebiichigo, P. reptans* have many similar micromorphological traits (Table 2), (19 traits, about 55.88% of the total number of traits studied), which demonstrates a close relationship between the three species and supports the phylogenetic results. The present phylogenetic analyses also show the early divergence of *P. indica*, with its micromorphological characters of calyx and epicalyx, and pedicle as plesiomorphic stages. Based on Cho (2012), in the ML tree the branch length is directly related to the number of changes accumulated along that branch, and the longer the branches, the more they derive from the ancestral state. According to the present molecular analyses, *P. reptans* was found on the terminal (in all trees) and on long branches (in ML trees). The results showed that the presence of a stomatal chimney, a simulate–erose inner stomatal rim on the epicalyx and calyx, the presence of a glabrous pedicle, the presence of irregular platelets and wax ornamentation on the pedicel are synapomorphies of *P. reptans.* Furthermore, the results revealed that wax ornamentation and characters of the stomata of the epicalyx and calyx, and the types of pedicel hairs and wax ornamentation are the most important characters that can be used for species identification.

Identification key based on the micromorphological features of the epicalyx, calyx, and pedice:

1- Inner stomata rim sinuolate on both sides; pedicle wax ornamentation crust-granule 2
- Inner stomata rim sinuolate–erose on both sides, pedicle wax ornamentation irregular platelets and granule ..... *P. reptans*

2- Epicalyx epicuticular wax ornamentation striate, irregular platelets-granule on adaxial and membranous platelets on the abaxial side, calyx epicuticular wax ornamentation irregular platelets on the adaxial and membranous platelets on the abaxial side, epicalyx, and calyx outer stomata rim overlapping on both surfaces .... *P. indica*
- Epicalyx and calyx epicuticular wax ornamentation crust, scattered platelets with granules on both sides, epicalyx, and calyx outer stomata rim overlapping and raised on adaxial and abaxial surfaces. Respectively .... *P. hebiichigo*

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