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ACHENE MORPHOLOGY AND PERICARP ANATOMY OF *ANEMONE*, *HEPATICA*, AND *PULSATILLA* (ANEMONINAE, RANUNCULACEAE)

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The achene morphology and pericarp anatomy of 12 taxa representing three genera (*Anemone*, *Hepatica*, and *Pulsatilla*) of the subtribe Anemoninae were investigated using microtome and light microscopy to evaluate the taxonomic implications of achene characters. The achenes of *Anemone* were elliptical or obovoid and beaked, whereas the achene of *Hepatica* and *Pulsatilla* were obovoid and elliptical, respectively. Noticeable variations in both quantitative and qualitative features of achenes were observed among the species of the three genera. One-way analysis of variance indicated that the quantitative achene variables among the species were highly significant ($P<0.001$). Pearson's correlation coefficient also showed a significant correlation between different achene variables. The pericarp structure, particularly the number of cell layers and cell forms in the exocarp and endocarp, seems to be very useful for species delimitation in *Anemone* and *Hepatica*. The nature of the endotesta could provide substantial proof for sub-generic classification in *Anemone*. Unweighted paired group analysis showed the utility of achene features for taxonomic groupings of the species within the studied genera. Although the specimen samples represented a limited range of taxa, the achene features and pericarp anatomy provided a reasonable source for the taxonomic treatment of the studied genera within the subtribe.

Keywords: achene, Anemoninae, pericarp, Ranunculaceae, taxonomy

INTRODUCTION

The Ranunculaceae comprises 2,377 accepted species within 65 genera and is one of the largest basal families within Eudicots (Soltis et al., 2005; Simpson, 2006; Heywood et al., 2007; The Plant List, 2013). The plant is distributed worldwide and exhibits wide variations in morphological characters, especially in its fruit type and floral organization (Emadzade et al., 2010). Several classifications have been proposed based on its morphological characters, molecular data, and combined morphological and molecular dataset (Hutchinson, 1923; Janchen, 1949; Johansson and Jansen, 1993; Tamura, 1995; Jansen et al., 1995; Ro et al., 1997; Wang et al., 2009). Based on the basic chromosome number and carpel and fruit types Tamura (1995) divided the family into three subfamilies: Helleboroideae with four tribes, Ranunculoideae with three tribes, and Isopyroideae with four tribes.

Anemoneae is one of the three tribes of the subfamily Ranunculoideae which is traditionally divided into three subtribes: Kingdoniinae, Anemoninae, and Clematidinae. However, in contemporary classifications, based on morphology and molecular sequence data, the subtribe Kingdoniinae, with only one species, *Kingdonia uniflora*, has been excluded from Anemoneae, even from Ranunculaceae, and is treated as its own family Kingdoniaceae or included within the family Circaeasteraceae (Ren et al., 2004; Wang et al., 2009; APG IV 2016). In addition, Anemoneae, with the remaining two tribes, is strongly inferred as the monophyletic group in phylogenetic analyses (Jansen et al., 1995; Ro et al., 1997; Wang et al., 2009; Jiang, 2017).

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The subtribe Anemoninae consists of eight genera: *Anemoneclema* (Franch.) W. T. Wang, *Anemone* L., *Barneoudia* C. Gray, *Hepatica* Miller, *Knowltonia* Salisb, *Metanemone* W. T. Wang, *Oreithales* Schldl., and *Pulsatilla* Mill (Wang, 1979; Tamura, 1995). Among them, *Anemone* is the largest one with more than 150 species whereas *Anemoneclema*, *Metanemone*, and *Barneoudia* are monotypic. Molecular phylogenetic studies revealed that *Hepatica*, *Pulsatilla*, and *Knowltonia* are nested within *Anemone* (Hoot et al., 1994; Barniske, 2009; Meyer et al., 2010; Hoot et al., 2012). In addition, the phylogenetic relationship and taxonomic status of *Anemoneclema* in the subtribe has remained unclear and recent molecular studies suggested the transfer of the monotypic genus to Clematidinae from Anemoninae (Zhang et al., 2015; Lehtonen et al., 2016).

Fruit and seed morphologies have provided important phylogenetic sources, which are frequently used to differentiate the taxa in different taxonomic ranks. The surface sculpture of fruits, seeds or a combination of both are particularly used for phylogenetic and/or systematic studies (Fukuhara, 1999; Juan et al., 2000; Xu, 2003; Zhang et al., 2005; Amini et al., 2011; Ghimire et al., 2015, 2016a). There have been several studies that emphasize the taxonomic utility of fruit and seed morphological characteristics of different Ranunuculaceae taxa (Cappelletti and Poldini, 1984; Heiss et al., 2011; Ghimire et al., 2015, 2016b; Hadidchi et al., 2019; Ghimire et al., 2020).

The achene anatomy of *Anemone* has had a long history and proved to be of great value for the infrageneric classification of the genus (Starodubtsev, 1991; Tamura, 1995; Zeman et al., 2008). Zeman et al. (2008) extensively studied the anatomy of 110 species of *Anemone* and found as many as 25 achene features with potential diagnostic value. Before that Chaudhary and Trifonova (1988) examined the usefulness of fruit anatomy in the systematic position of 11 Nepalese *Anemone*. Maciejewska-Rutkowska and Antkowska (2013) demonstrated the importance of achene morphology and anatomy in four Polish *Anemone*. However, in the literature there is a lack of a comparative study of the achenes of *Anemone* species with other genera of the subtribe Anemoninae. Jung and Heo (2017) listed a few achene anatomical characters of three *Anemone*, three *Hepatica*, and two *Pulsatilla* species in their study but did not discuss the utility of the features within the Anemoninae.

In the present study, we investigated the achene anatomy of 12 species belonging to three genera of the subtribe Anemoninae. The primary objectives of this study were (1) to demonstrate the achene morphological and anatomical features of the included taxa and (2) to understand the taxonomic usefulness of achene features within and between the genera of the subtribe Anemoninae.

MATERIALS AND METHODS

SPECIMENS

More than 240 achenes from 12 taxa representing three genera (*Anemone*, *Hepatica*, and *Pulsatilla*) of the subtribe Anemoninae originating from the seed bank at the Korea National Arboretum, Pocheon, Korea were investigated. Names of the investigated species with their voucher numbers are shown in Table 1.

TABLE 1. Name of taxa with voucher number.

Taxon	**Voucher No.**
Anemone koraiensis Nakai	L10335
Anemone raddeana Regel	L2030
Anemone reflexa Steph. Ex. Willd	L8045
Anemone narcissiflora L.	L14911
Anemone narcissiflora subsp. *crinita* (Juz.) Kitag	L10766
Hepatica asiatica Nakai	L3363
Hepatica insularis Nakai	L9859
Hepatica maxima (Nakai) Nakai	2015UL001
Pulsatilla dahurica (Fisch. Ex DC.) Spreng	L12332
Pulsatilla cernua (Thunb.) Bercht. ex J. Presl	L13107
Pulsatilla koreana Nakai	L12512
Pulsatilla tongkangensis Y. N.Lee & T.C.Lee	L12843

LIGHT MICROSCOPY

At least three to five achenes of each taxa were considered for microtome sectioning. Microtome sections were prepared using the following proce-

dure. Mature achenes were passed through ethanol series (50, 70, 80, 90, 95 and 100%) for dehydration. After complete dehydration, the achenes were infiltered in alcohol/Technovit combinations (3:1, 1:1, 1:3, and 100% Technovit) and then embedded in Technovit 7100 resin. The embedded materials were cut into serial sections of 4–6 μm thickness using a Leica RM2255 rotary microtome (Leica Microsystems GmbH, Germany) with disposable blades, stuck onto a slide glass, and dried using an electric slide warmer for 12 h. The dried slides were stained with 0.1% Toluidine blue 'O' for 60–90 s, rinsed with water and again dried in slide warmer for more than 6 h to remove water. The stained slides were then mounted with Entellan (Merck Co., Germany) and permanent slides were prepared. The permanent slides were examined under an AXIO Imager A1 light microscope (Carl Zeiss, Germany). Photomicrographs were captured with an AxioCam MRc5 attached camera system, and pericarp measurements were made using AxioVision software for Windows (release 4.7, 2008). Multiple image alignment was performed using Photoshop CS for Windows 2010. None of the image-alteration facilities of Photoshop were used to change the original images captured by the camera.

MORPHOMETRY AND DATA ANALYSIS

A total of approximately 240 achenes were used for morphometric measurement. Digital images of whole achenes were captured with a Leica DFC420 C multifocal camera attached to a Leica MZ16 FA microscope (Leica Microsystems, Germany). The length and width of 20 seeds from each taxon were measured using Leica LAS V3.8 software for Windows. The biometric data were analyzed statistically. For each seed variable, one-factor analysis of variance (ANOVA) was used to examine the differences in means among the included species. Pearson's correlation coefficients were used to estimate the relationship among the achene length, width, pericarp and endocarp thickness, diameter cross section, and their relative ratios. All statistical analyses were performed using the SPSS statistical program (IBM SPSS Statistics for Windows Version 20.0., IBM Corp., Armonk, USA). Six quantitative and 11 qualitative characters were categorized and coded with binary and/or multistate coding. Cluster analysis using the unweighted paired group method with arithmetic mean (UPGMA) was also performed to verify achene features and it allowed grouping of the species by using the statistical program PAST ver. 3.25 (Hammer et al., 2001). Character states and their codes are shown in Supplementary File S1.

RESULTS

Selected stereomicroscopic images of a single achene and light microscopic images of the pericarp structure are shown in Figures 1–6. A comprehensive description of the morphological features of achenes and anatomical features of the pericarp is presented below.

ANEMONE L.

Altogether, five taxa of *Anemone* were investigated in this study. The achenes are elliptical or obovoid, brown to pale yellow in color, and flattened (Figs. 1a–e; Table 2). The largest achene was measured in *Anemone narcissiflora* (7.75±0.82 × 5.58±0.66 mm), followed by *A. narcissiflora* subsp. *crinita* (7.24±0.51 × 4.49±0.63 mm), whereas the smallest achene was measured in *A. raddeana* (2.26±0.18 × 1.18±0.11 mm), followed by *A. reflexa* (2.42±0.24 × 0.84±0.09 mm) (Table 3). Out of the five taxa, three (*A. koraiensis*, *A. raddeana*, and *A. reflexa*) have hair on their achene surface and narrow wings, but the remaining

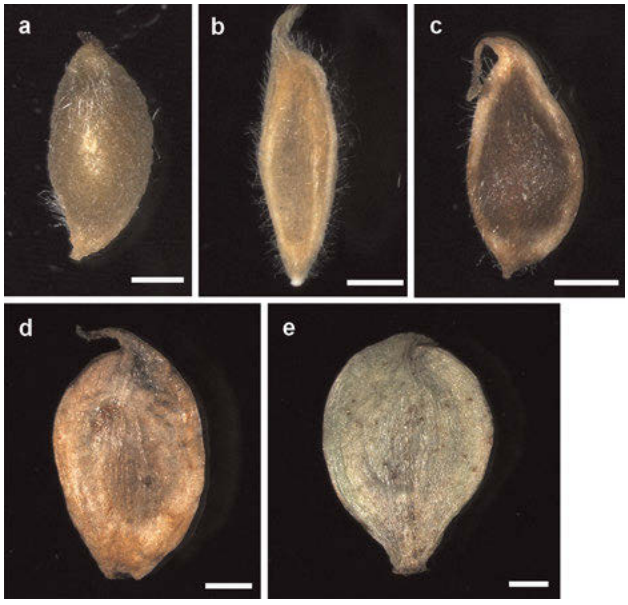

Fig. 1. Achenes of *Anemone* under stereomicroscope (**a**) *A. koraiensis* (**b**) *A. reflexa* (**c**) *A. raddeana* (**d**) *A. narcissiflora* subsp. *crinita* (**e**) *A. narcissiflora* Scale bars: **a**,**b**,**c** = 0.5 mm; **d**,**e** = 1 mm

TABLE 2. Morphological and anatomical features of achene of Anemoninae.

Taxon	Shape	Color	Surface	Wings	Style	Shape in CS	Exocarp	Mesocarp	Endocarp	Seed coat
A. koraiensis	Elliptical	Pale yellow to brown	Hairy	Very narrow	Hooked	Oval	Cutinized, rectangular cells	Well represented, 7-10 cells thick, isobilateral/elongated/ irregular parenchymatous cells with wavy walls	Thin layer of thick-walled rectangular cells	Thick and well represented, exotesta single layered, mesotesta 4-5 layered and endotesta single layered
A. reflexa	Elliptical	Pale yellow to brown	Hairy	Narrow	Hooked	Oval	Cutinized, rectangle to tangentially elongated	Well represented, 3-5 cells thick, elongated parenchymatous cells with straight or wavy walls	Thick layer of highly lignified palisade like cells	Either represented by parenchymatous layers or crushed. Exotesta single layered elongated cells, mesotesta 2-3 layers of thin walled cells, endotesta single layered thick walled
A. raddeana	Elliptical	Brown	Hairy	Narrow	Hooked	Oval	Cutinized, cell crushed	Represented by crushed cells	Thick layer of highly lignified palisade like cells	Represented by crushed layers
A. narcissiflora	Obovoid, flattened	Pale yellow to brown	Glabrous	Wide	Hooked	Elliptical	Cutinized, mostly crushed, if present tangentially elongated	Well represented, 3-5 cells thick, elongated parenchymatous cells with straight or wavy walls	2 to 3 layers, rarely single layer of highly lignified sclereid cells	3-4 layers of degenerating cells
A. narcissiflora subsp. *crinita*	Obovoid, flattened	brown	Glabrous	Wide	Hooked	Elliptical	Cutinized, cell crushed	Represented by crushed cells	Thick layer of highly lignified sclereid cells	Represented by crushed layers
H. asiatica	Obovoid	Pale yellow to brown	Hairy	Narrow	Slender	Circular	Cutinized, tangentially elongated	1-2 layers of elongated parenchyma cells.	2 to 3 layers, rarely single layer of highly lignified palisade cells	2-3 layers of degenerating cells
H. insularis	Obovoid	Pale yellow to brown	Hairy	Very narrow	Slender	Circular	Cutinized, tangentially elongated	1-2 layers of elongated parenchyma cells	2 to 3 layers of highly lignified palisade cells	2-3 layers of degenerating cells
H. maxima	Obovoid	Dark brown to black	Glabrous	Narrow	Slender	Circular	Cutinized, rectangular cells	Well represented, 3-5 cells thick, elongated parenchymatous cells	2 to 4 layers of highly lignified sclereid cells	2-3 layers of degenerating cells

Taxon	Shape	Color	Surface	Wings	Style	Shape in CS	Exocarp	Mesocarp	Endocarp	Seed coat
								with wavy walls		
P. dahurica	Elliptical	Brown	Hairy	Narrow	Slender	Circular	Crushed	Represented by crushed cells	Thick layer of highly lignified sclereid cells	Represented by crushed layers
P. cernua	Narrow elliptical	Brown to black	Hairy	Narrow	Slender	Circular	Crushed	Represented by crushed cells	Thick layer of highly lignified sclereid cells	Represented by crushed layers
P. koreana	Narrow elliptical	Brown to black	Hairy	Narrow	Slender	Circular	Crushed	Represented by crushed cells	Thick layer of highly lignified sclereid cells	Represented by crushed layers
P. tongkangensis	Narrow elliptical	Brown to black	Hairy	Narrow	Slender	Circular	Crushed	Represented by crushed cells	Thick layer of highly lignified sclereid cells	2-3 layers of elongated cells with thickened walls

TABLE 3. Achene characters and their measurements in Anemoninae species (mean and standard deviation). L/W = length and width ratio, P/E = pericarp and endocarp ratio, CSD1 = diameter parallel to cotyledon, CSD2 = diameter perpendicular to cotyledon, D1/D2 = CSD1 and CSD2 ratio.

Taxa	Length (mm)	Width (mm)	L/W	Pericarp (μm)	Endocarp (μm)	P/E	CSD1 (mm)	CSD2 (mm)	D1/D2
A. koraiensis	3.34±0.22	1.74±18	1.93±0.2	206.78±17.47	9.79±1.99	21.75±3.82	1.97±0.16	1.51±0.13	1.31±0.06
A. reflexa	2.42±0.24	0.84±0.09	2.9±0.34	115.1±22.86	25.4±4.01	4.57±0.66	1.8±0.07	1.17±0.1	1.55±0.07
A. raddeana	2.26±0.18	1.18±0.11	1.94±0.2	56.65±13.89	25.49±5.25	2.27±0.55	1.75±0.08	1.45±0.09	1.21±0.06
A. narcissiflora	7.75±0.82	5.58±0.66	1.4±0.17	149.27±15.3	29.84±4.62	5.11±0.97	3.91±0.15	1.82±0.06	2.15±0.03
A. narcissiflora subsp. *crinita*	7.24±0.51	4.49±0.63	1.58±0.24	43.53±6.03	18.29±2.93	2.42±0.37	3.13±0.11	1.44±0.09	2.17±0.12
H. asiatica	2.11±0.23	1.32±0.15	1.62±0.21	99.04±8.35	64.77±5.9	1.53±0.13	2.01±0.1	1.94±0.04	1.04±0.04
H. insularis	2.43±0.19	1.41±0.11	1.73±0.16	126.85±13.57	79.83±12.06	1.61±0.15	2.02±0.18	2.89±0.12	1.07±0.06
H. maxima	3.35±0.32	1.9±0.29	1.83±0.4	274.38±27.83	80.41±6.84	3.43±0.43	2.98±0.23	2.86±0.25	1.04±0.1
P. dahurica	2.7±0.3	0.87±0.08	3.12±0.4	37.31±4.32	25.33±3.88	1.48±0.11	0.86±0.11	0.85±0.11	1.02±0.6
P. cernua	3.6±0.19	1.05±0.07	3.42±0.33	32.11±5.78	18.24±3.41	1.78±0.25	0.91±0.07	0.85±0.13	1.12±0.14
P. koreana	3.2±0.33	1.11±0.17	2.93±0.38	34.55±9.78	18.59±3.23	1.78±0.43	1.05±0.05	1.02±0.08	1.03±0.04
P. tongkangensis	4.07±0.24	1.17±0.51	3.46±0.42	58.63±8.199	32.84±3.35	1.78±0.07	1.07±0.09	1.03±0.08	1.01±0.05
ANOVA	**F=451.9, P<0.001**	**F=424.64, P<0.001**	**F=101.07, P<0.001**	**F=400.55, P<0.001**	**F=300.14, P<0.001**	**F=334.31, P<0.001**	**F=244.34, P<0.001**	**F=206.1, P<0.001**	**F=115.18, P<0.001**

two (*A. narcissiflora* subsp. *crinita* and *A. narcissiflora*) have glabrous achene surfaces and wide wings. All the species have a hooked persistent style.

As outlined in the cross-section, ellipsoidal achenes are oval, whereas obovoid achenes are elliptical. The cross-section diameter corresponds with the length and width of achenes. In the cross-section, the thickest pericarp was found in *A. koraiensis* (206.78±17.47 μm), followed by *A. narcissiflora* (149.27±15.3 μm), and the thinnest pericarp measured was in *A. narcissiflora* subsp. *crinita* (43.53±6.03 μm), followed by *A. raddeana* (56.65±13.89 μm). Interestingly, *A. koraiensis*, which has the thickest pericarp, exceptionally bears the thinnest endocarp (9.79±1.99 μm). The exocarp in *Anemone* taxa is highly cutinized and the cells are usually crushed except *A. koraiensis* and *A. reflexa*, which have rectangular or tangentially elongated exotesta (Figs. 2a-j). The exocarp layer is followed by a mesocarp with parenchyma cells in *A. koraiensis*, *A. reflexa*, and *A. narcissiflora* (Figs. 2b,d,j); however, this region is represented by a few layers of crushed cells in *A. raddeana* and *A. narcissiflora* subsp. *crinita* (Figs. 2f,h). The thickest endocarp was measured in *A. narcissiflora* (29.84±4.62 μm), followed by *A. raddeana* (25.49±5.25 μm). All the observed taxa have lignified (either palisade-like or sclereid-like) endocarp, regardless of the variation

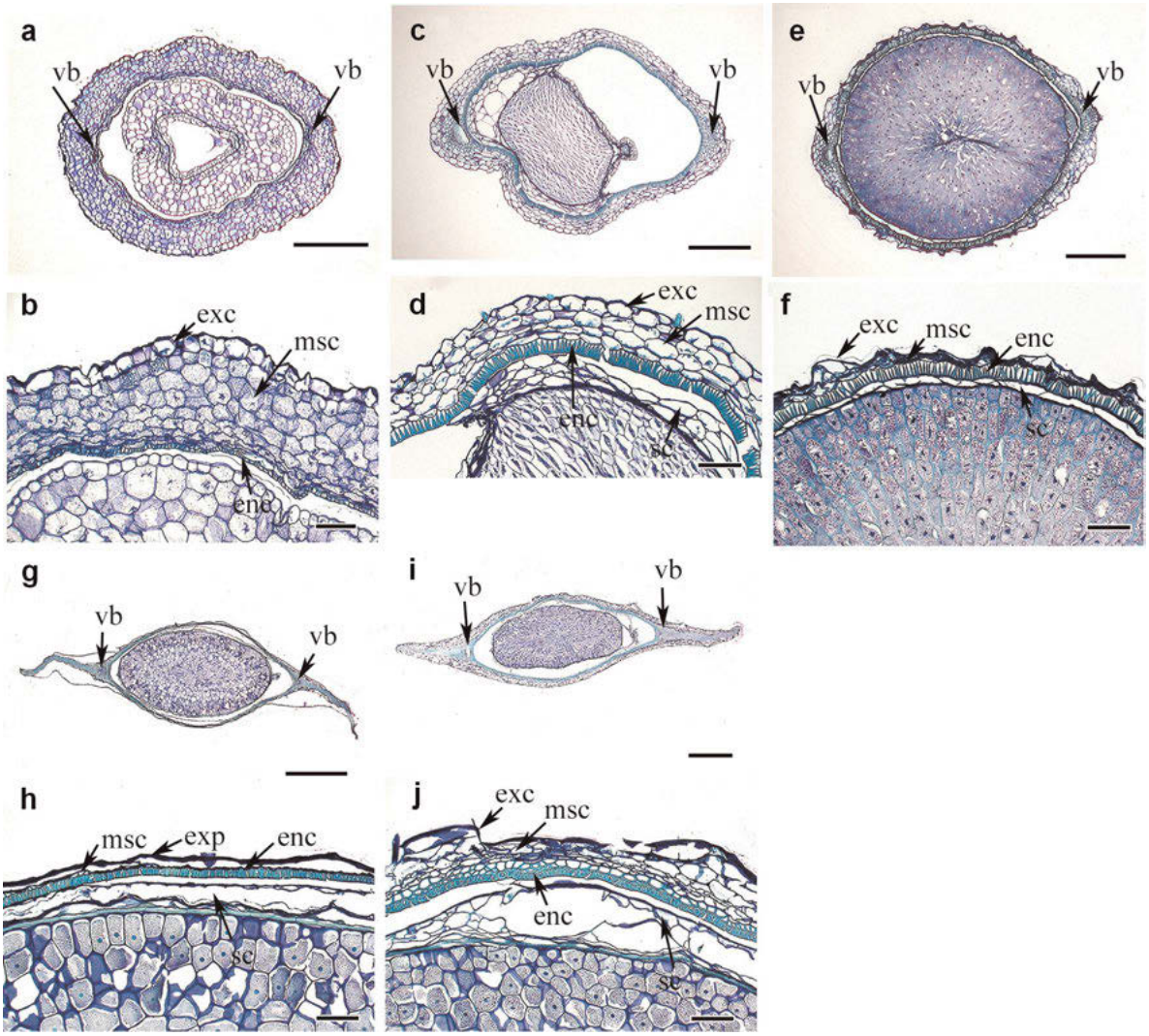

Fig. 2. Cross section of *Anemone* achenes (**a**,**b**) *A. koraiensis* (**c**,**d**) *A. reflexa* (**e**,**f**) *A. raddeana* (**g**,**h**) *A. narcissiflora* subsp. *crinita* (**i**,**j**) *A. narcissiflora*. Scale bars: **a**, **c**, **e** = 0.5 mm; **b**, **d**, **f**, **h**, **j** = 0.1 mm; **g**, **i** = 1 mm. Abbreviations: enc – endocarp, exc – exocarp, msc – mesocarp, sc – seed coat, vb – vascular bundle.

in their thickness (Figs. 2b,d,f,h,j,l). In all the species, the seed coat is feebly established, and either represented by a couple of parenchyma layers or containing degenerating cells.

HEPATICA MILL.

Three species of the genus *Hepatica* are included in this study. Achene morphology such as shape, size, color, and anatomy of the pericarp, endocarp, and seed coat displayed relatively similar qualitative features and quantitative measurements. The achenes are stalked, obovoid, pale yellow to brown or black in color, and pubescent (*Hepatica asiatica*, *H. insularis*) or glabrous (*H. maxima*) in the surface texture (Fig. 3; Table 2). *Hepatica maxima* has comparatively larger achenes (3.35±0.32 × 1.9±0.29mm) than *H. asiatica* (2.11±0.23 × 1.32±0.15mm) and *H. insularis* (2.43±0.19 × 1.41±0.11mm). The lateral wings are small in *Hepatica* and the persistent style develops into a long slender beak (Figs. 3a,b,c).

In the cross-section, *Hepatica* achenes are circular in outline (Figs. 4a,c,e). The cross-sectional diameter is equivalent to the size of an achene: the larger the achene, the bigger the endosperm diameter (Table 3). Likewise, the pericarp is thicker in *H. maxima* (274.38±27.83 μm) than in *H. asiatica* (99.04±8.35 μm) and *H. insularis* (126.85±13.57 μm) but the endocarp is relatively comparable in all species: thus, the ratio of the pericarp and endocarp thickness is relatively higher in *H. maxima* (Table 3). The exocarp is single-layered, cutinized with either tangentially elongated and rectangular (*H. asiatica*, *H. insularis*) or isodiametric (*H. maxima*) cells (Figs. 4b,d,f). The mesocarp is mostly crushed in *H. asiatica*, represented by two to three layers of parenchyma cells in *H. insularis* and three to five layers of elongated parenchyma cells with wavy cell walls in *H. maxima*. The endocarp is multi-layered, and the cells are highly lignified, but the seed coats in all the species are represented by two to three layers of degenerating cells (Figs. 4b,d,f).

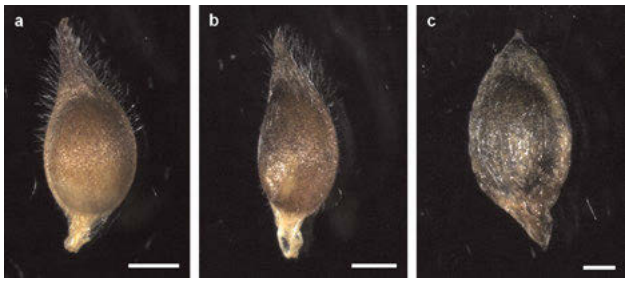

Fig. 3. Achenes of *Hepatica* under stereomicroscope (**a**) *H. asiatica* (**b**) *H. insularis* (**c**) *H. maxima*. Scale bars = 1 mm

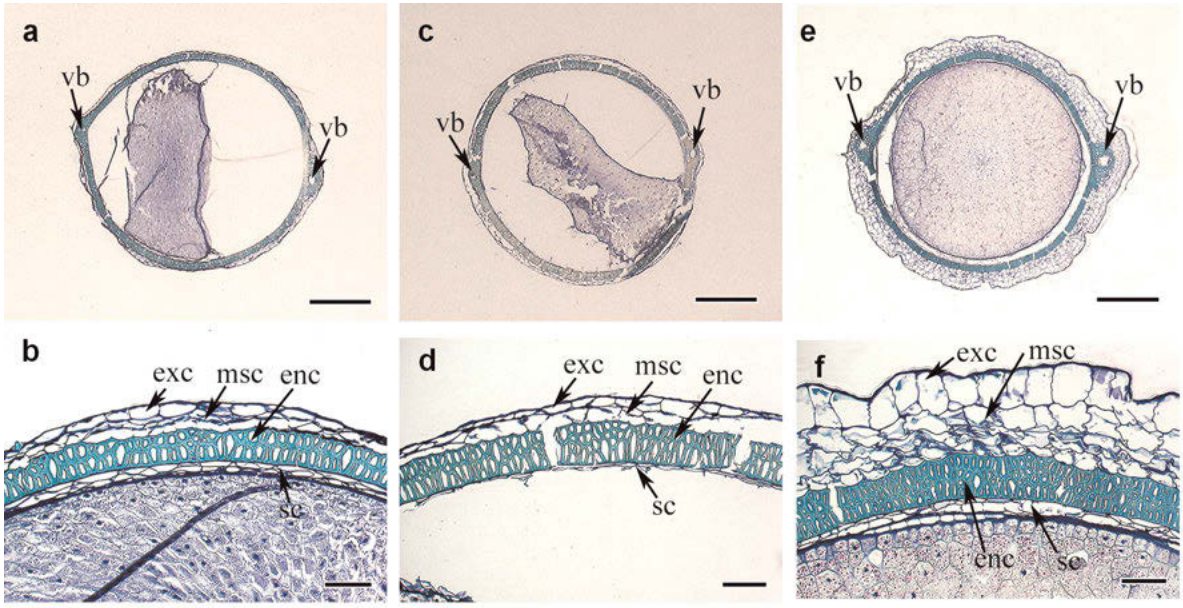

Fig. 4. Cross section of *Hepatica* achenes (**a**,**b**) *H. asiatica* (**c**,**d**) *H. insularis* (**e**,**f**) *H. maxima*. Scale bars: **a**,**c**,**d** = 0.75 mm; **b**,**d**,**f**, = 0.1 mm. Abbreviations: enc – endocarp, exc – exocarp, msc – mesocarp, sc – seed coat, vb – vascular bundle.

PULSATILLA MILL.

Four species of the genus *Pulsatilla* were observed. The results of the study indicate that *Pulsatilla* species exhibit very similar achene morphological and anatomical structures. The achenes are much longer than wider, elliptical in shape, brown to black in color, and profoundly have hirsute with white and shiny long hair (Fig. 5; Table 2). The largest measured achenes are in *P. tongkangensis* (4.07±0.24 × 1.17±0.51 mm) and the shortest measured achenes are in *P. dahurica* (2.7±0.3 × 0.87±0.08 mm). The lateral wings are narrow and the persistent style forms a long slender beak.

Fig. 5. Achenes of *Pulsatilla* under stereomicroscope (**a**) *P. dahurica* (**b**) *P. tongkangensis* (**c**) *P. cernua* (**d**) *P. koreana*. Scale bars = 1 mm

In the cross-section, the achenes are circular in outline (Figs. 6a,c,e,g). The cross-section diameter is comparable to the width of the achenes, as in *Anemone* and *Hepatica* species (Table 3). Corresponding with the achene size, the pericarp and the endocarp are thickest in *P. tongkangensis* (58.63 ±8.199 μm and 32.84±3.35 μm, respectively), however, the thinnest pericarp (32.11±5.78 μm) and endocarp (18.24±3.41 μm) are measured in *P. cernum*. The exocarp is mostly crushed or rarely present as fragmented cells in all four species (Figs. 6b,d,f,h). The mesocarp is represented by indistinguishable layers of degenerated cells, whereas the endotesta comprises a thick single layer of highly-lignified sclereid cells. The seed coat is also represented by degenerated cells, except in *P. tongkangensis* which has two- to three layers of elongated cells with thickened walls (Fig. 6d).

STATISTICAL ANALYSES

One-factor ANOVA was performed on nine quantitative achene traits and the differences between the species were found to be highly significant ($P<0.001$) (Table 3). Pearson's correlation coefficient also indicated a significant correlation between different achene traits. The differences in the thickness of the pericarp and endocarp are presented in boxplots (Figs. 7a,b). The relationships among the species through six quantitative and 11 qualitative features were revealed using cluster analysis (Fig. 8). The cluster analysis, based on the paired group (UPGMA) algorithm using Gower similarity index, revealed three groups. *Anemone narcissiflora* complex represented the basal group of the UPGMA tree, three *Hepatica* species remained at the top of the phenograms and the third group represented by two subgroups, in which *A. reflexa* and *A. koraiensis* separated first, and *A. raddeana* was grouped with four *Pulsatilla* species.

DISCUSSION

Molecular phylogenetic studies suggested, in a wider perception, that *Anemone* incorporated *Hepatica*, *Pulsatilla*, and *Knowltonia* (Hoot et al., 1994; Schuettpelz et al., 2002; Meyer et al., 2010). The group of these taxa also share some similar morphological characters, such as rosette basal leaves with a variety of perennating structures, an inflorescence with involucre leaves on the peduncle, petaloid sepals, and similar achene features. However, there have been differences in a few morphological features, such as close proximity to the flower and stalked achenes of *Hepatica*, an elongated plumose style in *Pulsatilla*, and berry-like fruits in *Knowltonia* and *Anemone* (Hoot et al., 1994). We compared the achene morphology and

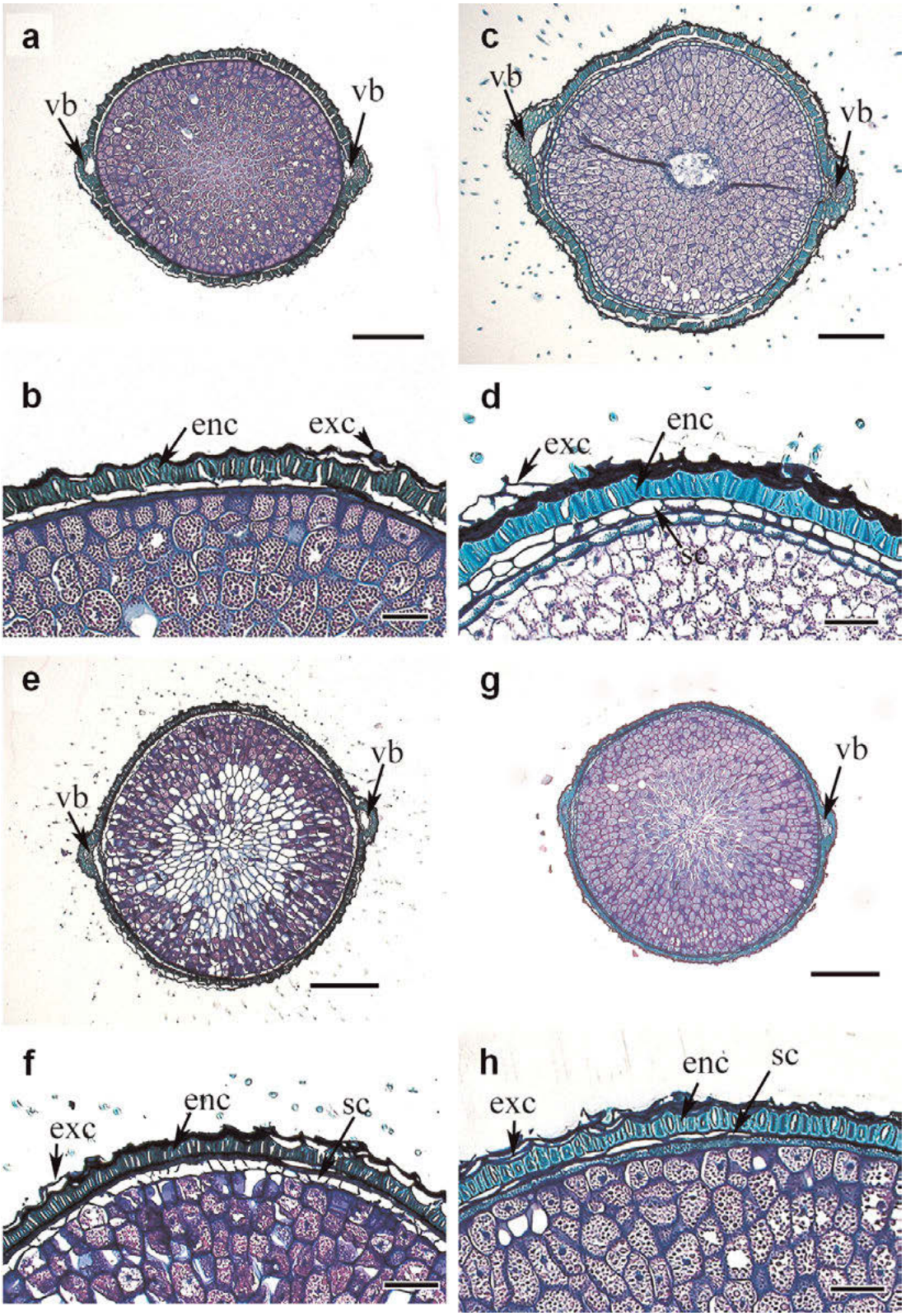

Fig. 6. Cross section of *Pulsatilla* achenes (**a**,**b**) *P. dahurica* (**c**,**d**) *P. tongkangensis* (**e**,**f**) *P. cernua* (**g**,**h**) *P. koreana*. Scale bars: **a**,**c**,**e**,**g** = 0.25 mm; **b**,**d**,**f**,**h** = 0.1 mm. Abbreviations: enc – endocarp, exc – exocarp, sc – seed coat, vb – vascular bundle.

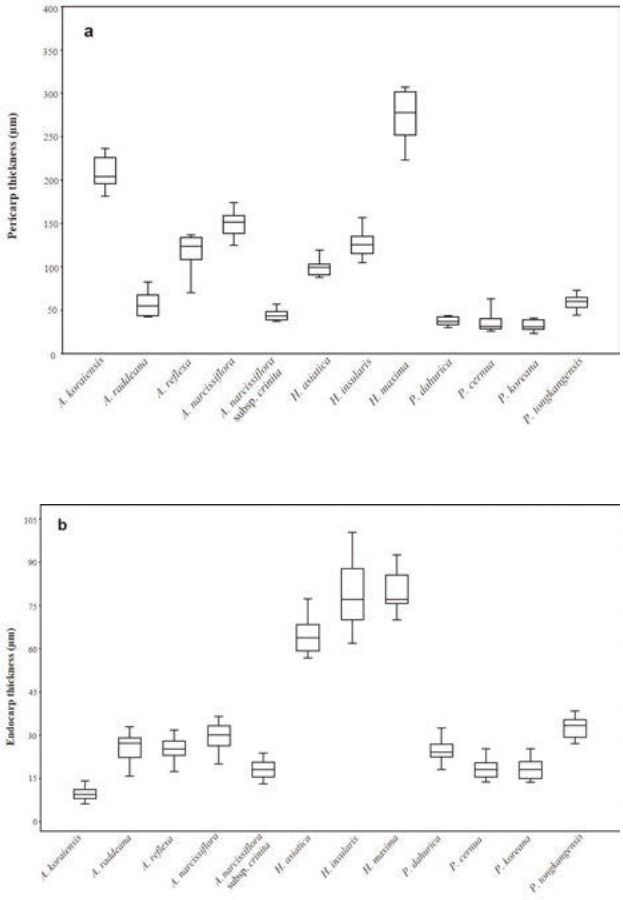

Fig. 7. Normal boxplot showing pericarp and endocarp thickness of achene (**a**) pericarp (**b**) endocarp.

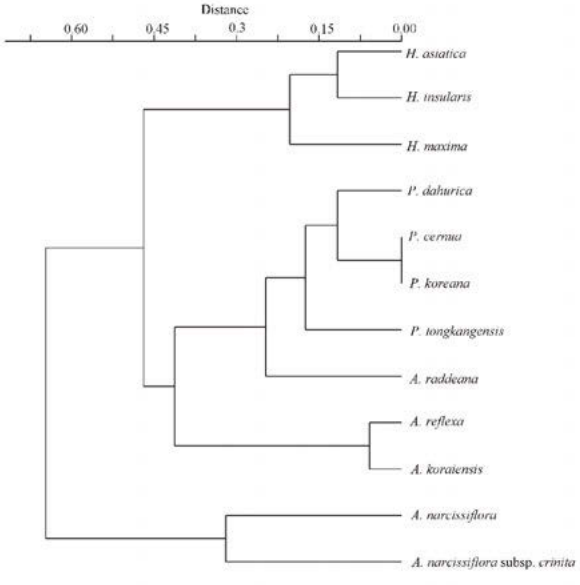

Fig. 8. UPGMA tree based on achene features of *Anemone*, *Hepatica*, and *Pulsatilla*.

anatomy of 12 taxa belonging to three genera, excluding *Knowltonia* from *Anemone* complex.

Exomorphological comparison revealed that the achenes of the studied taxa exhibited variations in the shape, size, color, surface, wings, and the remnant of the style. The ANOVA of the quantitative variables revealed significant differences in length and width of the achene among the taxa ($P<0.001$) (Table 3) and Pearson's correlation coefficients showed the positive relationship between the length and width ($r=0.934$, $P<0.01$) (Table 4). Among the qualitative features, the surface, wings, and style are highly variable within and between the genera. In previous studies, achene characters such as size, shape, and style have been proven to be useful for distinguishing the species in the genus *Anemone* (Saoud et al., 2007; Ziman et al., 2008, 2011; Maciejewska-Rutkowska and Antkowska, 2013) and the result of this study evidently corroborated it. In a recent study, Ghimire et al. (2020) reported that the achene size, permanent style, surface sculpture, shape in cross-section, and thickness of the exocarp and endocarp are valuable features, which can be used for species delimitation in *Clematis*. The five taxa of *Anemone* were clearly divided into two groups based on their shape, surface, and wings. *Anemone narcissiflora* and *A. narcissiflora* subsp. *crinita*, which belong to the sub-genus *Omalocarpus*, had obovoid and flattened achenes with a glabrous surface and wide wings, whereas *A. koraiensis*, *A. raddeana*, and *A. reflexa*, which belong to the sub-genus *Anemonanthea*, had elliptical achenes with a pubescent surface and narrow wings. However, the presence of a hooked style in all taxa of the *Anemone* differentiates the genus from *Hepatica* and *Pulsatilla* species, which had a slender style.

A similar result was obtained for achene anatomy. Achenes of *Hepatica* and *Pulsatilla* are circular in outline, whereas achenes of *Anemone* are oval to elliptical in cross-section. The quantitative variables indicated that the pericarp and endocarp thickness and the achene diameter in the cross--section of both planes vary significantly among the species ($P<0.001$). The pericarp thickness is positively correlated with the endocarp thickness ($r=0.498$; $P<0.01$) and the diameter in the cross--section of both planes ($r=0.487$; $P<0.01$ and $r=0.8$; $P<0.01$), whereas the endocarp thickness is negatively correlated with the achene length ($r=-0.318$; $P<0.01$) and width ($r=-0.167$; $P<0.05$). Among the qualitative features, the nature of the exocarp, mesocarp, and endocarp are important because the seed coat was poorly represented in all taxa.

TABLE 4. Pearson's correlation coefficients between different seed features in Anemoninae species. L/W = length and width ratio, P/E = pericarp and endocarp ratio, CSD1 = diameter parallel to cotyledon, CSD2 = diameter perpendicular to cotyledon, D1/D2 = CSD1 and CSD2 ratio.

	Length	**Width**	**L/W**	**Pericarp**	**Endocarp**	**P/E**	**CSD1**	**CSD2**	**D1/D2**
Length									
Width	**.934****								
L/W	**-.334****	**-.605****							
Pericarp	-.095	.062	**-.411****						
Endocarp	**-.318****	**-.167***	**-.308****	**.498****					
P/E	-.007	.042	**-.185****	**.510****	**-.317****				
CSD1	**.674****	**.838****	**-.820****	**.487****	.229	.098			
CSD2	.050	**.259***	**-.699****	**.800****	**.765****	.077	**.662****		
D1/D2	**.852****	**.875****	**-.492****	-.025	**-.333****	.063	**.766****	.042	

**Sig. at 0.01 level *Sig. at 0.05 level

In cross-section outline, *Anemone* achenes are oval or elliptical with narrow to wide extensions wherein the vascular bundles are present. The achene anatomy of *Anemone* was extensively studied in the past. Ziman et al. (2008) and Maciejewska-Rutkowska and Antkowska (2013) emphasized the taxonomic value of the pericarp in this genus. Before that, Chaudhary and Trifonova (1988) distinguished the epicarp, mesocarp, and endocarp of 11 species of *Anemone* and highlighted the significance of the number of cell layers and cell forms in individual pericarp layers. Even with a very limited sampling (five taxa), we found a similar result in this study. The exocarp is cutinized with rectangular to elongated cells in *A. koraiensis* and *A. reflexa*, but the cells are crushed in the other three taxa; however, a few fragmented exotestal cells were found in *A. narcissiflora*. A similar trend was observed for the mesocarp, except for the number of cell layers. However, the most characteristic feature in *Anemone* seems to be the nature of the endocarp, which clearly separated the two subgenera in our sampling. The endocarp is exclusively single-layered and the cells are highly lignified and palisade-like in *A. raddeana* and *A. reflexa*, which belong to the subgenus *Anemonanthea*. However, cells are highly lignified, sclereid and single-layered in *A. narcissiflora*, and two- to three-layered in *A. narcissiflora* subsp. *crinita*, which belongs to the subgenus *Omalocarpus*. The endocarp is a thin layer of thick-walled rectangular cells in *A. koraiensis*. According to Ghimire et al. (2020), the endocarp is the most consistent region in the *Clematis* species. Morphologically, *A. koraiensis* is closely related to *A. reflexa* and both species share similar vegetative forms, a white solitary flower with five sepals and a pubescent achene surface (Sung, 2017). The results of this study agree with those of Sung (2017) because the two species formed a distinct subclade on the *Pulsatilla-Anemone* clade in the UPGMA phenogram.

In *Hepatica*, the exotesta is well characterized by tangentially-elongated cells, as in *H. asiatica* and *H. insularis*, or by broadly rectangular cells, as in *H. maxima*. Following the exocarp, *H. maxima* also revealed three to five cell layers-thick mesocarp and a maximum of four-cell layered endocarp, however, *H. asiatica* and *H. insularis* exhibited one to two layered mesocarp and a maximum of three-layered endocarp. The result of this study supported the classification of Nakai (1952), who divided these three Korean species into two groups: *H. asiatica* and *H. insularis* with annual leaves and *H. maxima* with biennial leaves. In addition, Pfosser et al. (2011) found a genetically close relationship between *H. asiatica* and *H. insularis*. The qualitative achene features of four *Pulsatilla* species included in this study are astonishingly similar in every detail. It is not surprising because in a recent taxonomic study by Grey-Wilson (2014), all four investigated species belonged to the same section *Semicampanaria* of the subgenus *Pulsatilla*.

In conclusion, regardless of limited species samples, our results suggested that achene morphology and anatomy provide a useful taxonomic base for species delimitation in the studied genera. *Anemone* species showed a remarkable variation in

the nature of the endotesta that could contribute to the subgeneric classification of the genus. In addition, the pericarp structure, particularly the number of cell layers and cell forms in the exocarp and endocarp, could be used as an alternative source of species discrimination in *Anemome* and *Hepatica*.

AUTHORS' CONTRIBUTIONS

Conceptualization: Balkrishna Ghimire and Mi Jin Jeong; Experiments: Balkrishna Ghimire; Statistical analysis: Dabin Yum and Balkrishna Ghimire,; Manuscript writing: Balkrishna Ghimire; Review and editing: Dabin Yum, Jae Hyun Kim and Mi Jin Jeong; Funding acquisition: Jae Hyun Kim and Mi Jin Jeong.

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