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RESEARCH ARTICLE

Molecular phylogenetics and morphological diversification in the genus *Gagea* (Liliaceae)

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Abstract

The genus *Gagea* exhibits high morphological diversity and complex taxonomy. In this study, we analyzed the phylogenetic relationships and morphological differentiation within *Gagea* using *ITS* sequences from 32 species across 9 sections. Bayesian analysis revealed 3 main clades, partially congruent with morphological traits. Our results highlight both the value of molecular data for species delimitation and the need for taxonomic revision due to cases of morphological convergence and incongruence. This study contributes to a deeper understanding of evolutionary patterns in *Gagea*.

Keywords: *Gagea*; *ITS* sequences; molecular phylogeny; morphological traits; section classification; species delimitation

Introduction

The genus *Gagea* Salisb. (Liliaceae Juss.) comprises approximately 250-300 species (1-3), many of which have been described during the last decade (4-9). Members of this genus exhibit substantial morphological diversity in their underground structures, including bulb configuration, as well as variation in the number of basal leaves, floral morphology and seed structure. Levichev (1999) proposed a classification of *Gagea* underground organs based on the number of bulbs and characteristics of the root system. Later, in a comparative study of morphological and physiological traits in the genera *Lloydia* Salisb. ex Rchb., *Gagea* Salisb. and *Kharkevichia* Levichev (Liliaceae), Levichev (2013) emphasized the diagnostic importance of features such as the cross-sectional shape of the scape, basal and bract leaves. Additional taxonomically relevant traits include inflorescence structure, tepal morphology, anther architecture and seed characteristics.

The genus *Gagea* has been the subject of numerous molecular and taxonomic studies aimed at elucidating its evolutionary relationships and classification (9-11). One of the earliest molecular phylogenetic studies analyzed 7 species of *Gagea* from Germany using chloroplast and nuclear DNA sequences, identifying 2 subspecies within *G. bohemica* and providing evidence that *G. pomeranica* may have a hybrid origin (11). The study presented here also considered the debated generic status of *Lloydia*, which has long been controversial and in recent years increasingly treated as part of *Gagea* (10, 11): *Gagea* and *Lloydia Salisb*. ex Rchb. A phylogenetic analysis in 2008 using both molecular and morphological data to explore relationships within and between these 2 genera was conducted (8). The primary focus was on *Gagea* and 58 species were included in their analysis. These new data led to the revision of previous classification systems, including Pascher's subdivision into 2 subgenera.

Subsequent broader analyses, which included *Lloydia*, confirmed the non-monophyly of traditionally defined groups, highlighting the need for taxonomic revision (11). Further studies concentrated on specific species complexes and sections. Research on the *G. reticulata* complex revealed the presence of multiple copies of nuclear genes and complex relationships suggesting introgressive hybridization or retention of ancestral polymorphism (12). Analysis of section Didymobulbos identified a Mediterranean group undergoing reticulate evolution, which played a key role in diversification and supported the description of new taxa (13). This work also emphasized the complexity of species boundaries within the *G. lutea* complex, with 1 German population identified as a likely hybrid (14), supporting the notion of frequent reticulate evolution within the genus (15, 16).

More geographically focused studies provided insight into regional diversification (17). Investigations in northwestern China revealed new species and underscored the role of hybridization in the diversification of section Minimae (18).

Analysis of populations in Kazakhstan found evidence of ongoing hybridization and identified a new high-mountain species (8).

Among the most comprehensive studies combined the molecular phylogeny with spatial distribution analyses and concluded that *Gagea* originated in southwestern Asia and diversified across the Irano-Turanian region during the last 3 million years (18).

Despite the valuable contributions of previous phylogenetic studies to our understanding of this taxonomically complex genus, relationships within and among its sections remain partly unresolved. Amplification was carried out using primers *ITS-1p* and *ITS-2* to amplify both *ITS1* and *ITS2* regions of the *nrITS*. The complete *ITS* region (*ITS1–5.8S–ITS2*) was not sequenced; instead, we focused on ITS1 and ITS2, which are widely used in phylogenetic studies of *Gagea*.

Materials and Methods

Intragenomic polymorphism was investigated using locus-specific NGS sequencing on the Illumina MiSeq platform. DNA was extracted from both freshly collected leaf material obtained during field expeditions and herbarium specimens using the Qiagen DNeasy Plant Mini Kit (Qiagen, Germany), according to the manufacturer's instructions with slight modifications. Library preparation was carried out using total genomic DNA (19) and primers *ITS-1p* (20) and *ITS-2* (21, 22). These primers were selected because they have been successfully applied in previous phylogenetic studies of *Gagea* and related genera, ensuring comparability of our results with earlier works. Amplification was conducted using a BioRad T-100 thermal cycler with the Plant PCR Master Mix (Thermo Scientific) under the following cycling conditions: initial denaturation at 98 °C for 5 min; 30 cycles of 98 ° C for 5 s, 56 °C for 5 s and 72 °C for 15 s; final extension at 72 °C for 1 min; storage at 4 °C.

Raw reads were processed using FastQC (Babraham Bioinformatics), Trimmomatic (23), Fastq-join (23) and Vsearch (24). Quality-filtered reads were aligned using MEGA7 (25) and taxonomic identity was verified via BLAST searches against the NCBI GenBank database.

A total of 32 *Gagea* species from 9 sections and 2 *Tulipa* species (outgroups) were included in the analysis. Of these, 20 *ITS* sequences (26) were retrieved from GenBank, while 12 sequences were newly generated in this study. The resulting *ITS* sequences ranged from 590 to 645 bp in length. Plant materials were collected from different regions of Uzbekistan, representing the main distribution areas of the genus within the country and were selected to represent 9 sections of *Gagea*, ensuring broad taxonomic coverage. Only specimens with voucher numbers deposited in TASH and LE were included. In addition, only high-quality, non-chimeric *ITS* sequences were retrieved from GenBank for phylogenetic analyses. Sequence alignment was performed with ClustalW in MEGA X.

The best-fit substitution model was determined using jModelTest v2.1.10, with the Akaike Information Criterion (AIC) identifying the GTR+I+G model as optimal. Phylogenetic analyses were performed using 2 approaches: Maximum Likelihood (ML), conducted using MrBayes v3.2.7a (25), implemented in IQ-TREE v2.1.2 (27), with nodal support estimated from 1000 ultrafast bootstrap replicates; and Bayesian Inference (BI). Two independent MCMC chains were run for 2 million generations, sampling every 1000 generations, with the first 25 % discarded as burn-in. Posterior probability (PP) values were calculated and mapped on the consensus tree. The final phylogenetic tree was visualized using FigTree v1.4.4 (28).

Voucher specimens are listed in Table 1, including herbarium accessions from TASH and LE.

To integrate morphological traits with phylogenetic relationships, a combined heatmap analysis was performed in R. Morphological traits were coded as binary and multistate characters, clustered using Euclidean distance and then mapped onto the phylogenetic trees generated by IQ-TREE. Heatmap visualizations were produced using the R packages ggtree, pheatmap and ggplot2.

Result and Discussions

The phylogenetic reconstruction using BI and ML methods based on the nrITS region (ITS1–5.8S–ITS2) for 32 accessions of *Gagea* (including 2 Tulipa species as outgroups) revealed 3 well-supported major clades (Fig. 1). The molecular tree topology generated by both BI and ML trees were reconstructed and the ML topology was largely congruent and confirmed by 1000 ultrafast bootstrap replicates and posterior probability values, thus ensuring the robustness of the phylogenetic relationships inferred.

The first clade (Clade I) comprised species traditionally placed in sections *Plecostigma*, *Graminifolia* and *Platyspermum*, such as *G. pseudoreticulata*, *G. afghanica*, *G. hissarica* and *G. vvedenskyi*. Notably, *G. nabievii*, despite being morphologically classified within *Graminifolia*, was found to group with species from *Plecostigma*, Such incongruence between morphology and molecular data may result from incomplete lineage sorting, hybridization, or parallel evolution, which are frequent in *Gagea*. This clade exhibited high support (PP = 0.92; BS = 98) in both BI and ML analyses and reflects a coherent genetic lineage.

Clade II included taxa from the sections *Graminifolia* (*G. sogdiana*, *G. pakistanica*), *Platyspermum* (*G. kamelinii*, *G. wallichii*, *G. setifolia*, *G. divaricata*) and *Incrustata* (*G. circumflexa*). This group demonstrated strong congruence between molecular and morphological data, which reinforces the naturalness of sectional boundaries among these taxes. The consistency across data types supports previous infrageneric classifications was proposed (29 - 33). It is worth noting that within Clade II, *G. kamelinii*, *G. wallichii*, *G. setifolia*, *G. vegeta* and *G. divaricata* were recovered as a single cluster with full bootstrap support (100 %). This pattern most likely reflects the limited resolution of the ITS region in distinguishing recently diverged or morphologically similar taxa, a phenomenon also documented in other monocot lineages.

Clade III was the most taxonomically diverse and included species from *Stipitatae*, *Minimoides*, *Fistulosae*, *Davlianidze* and *Dschungaricae*, such as *G. stipitata*, *G. reinhardii*, *G. capusii*, *G. turkestanica* and *G. filiformis*. Despite exhibiting notable morphological divergence, especially in perianth size, bulb morphology and growth form, these taxa clustered into a single well-supported clade, suggesting a shared evolutionary

Table 1. Twelve species representing 3 subgenera were selected for sequencing from herbarium collections of TASH and LE

Sl. No	Subgenus	Species name	Collection site	Collection date	Herbarium accession	GenBank accession	Reference
1	*Minimoides*	*Gagea* × *absurda* Levichev	Fergana Region, Kokand, Baulna, Bish-Kunysh Pass	01.06.1913	DNA214/21	PV932931	Present study
3	*Gagea*	*G. nabievii* Levichev	Fergana Region, Khodzhrabat Pass	20.07.1959	DNA222/21	PV819495.1	Present study
4	*Gagea*	*G. pseudoreticulata* Vved.	Bukhara, sandy hills near the fortress of Kushka	22.03.1913	DNA219/21	PV932939	Present study
6	*Gagea*	*G. hissarica* Lipsky	Pamir-Alai, Alai Range, Taldyk Pass, near Syny	14.07.1959	DNA215/21	PV932932	Present study
7	*Gagea*	*G. tulipaeformis* Levichev ex M.Pop.	Kashkadarya Region, Gissar Range, Mount Maidanak, Langar River basin.	03.05.2017	544	PV932935	Present study
8	*Gagea*	*G. kamelinii* Levichev	Western Pamir-Alai. Zarafshan ridge. Sarykul village	15.03.2019	41003	PV932933	Present study
10	*Gagea*	*G. sogdiana* M.Pop.	Zeravshan ridge near the village of Saigus	12.04.2019	410	PV932938	Present study
11	*Gagea*	*G. pakistanica* Levichev et Ali	Surkhandarya Region, Baysun	27.02.2019	4724	PV932936	Present study
12	*Gagea*	*G. wallichii* Levichev et Ali	Surkhandarya Region, southern slope of the Susyztau Range, Panjob ravine, narrow rocky gorge above the village of Panjob	20.03.2019	41002	PV932940	Present study
14	*Minimoides*	*G. reinhardii* Levichev	Baysun, Omonkhona, Zavboshi	12.06.2019	G22	PV932934	Present study
17	*Gagea*	*G. calyptrifolia* Levichev	Alai Range, western part, vicinity of Vuadil settlement	20.03.1965	647	PV932937	Present study
18	*Bulbiferae*	*G. vvedenskyii*	Upper reaches of Bashkyzylsay, Buzbash Saddle	16.06.1978	DNA220/21	PX244199	Present study
19	*Bulbiferae*	*G. afghanica* A. Terracc.				AM087953	(8)
20	*Incrustata*	*G. circumplexa* Vved.				AM265529	(36)
21	*Gagea*	*G. divaricata* Regel				LN874797	(37)
22	*Gagea*	*G. setifolia* Baker				EU912068	(14)
23	*Gagea*	*G. vegeta* Vved.				EU912076	(14)
24	*Gagea*	*G. graminifolia* Vved.				FR689769	(18)
25	*Minimoides*	*G. gageoides* (Zucc.) Vved.				KU232874	Coskun et al. (Unpublished)
26	*Minimoides*	*G. chomutovae* (Pascher) Pascher				KU232868	Coskun *et al.* (Unpublished)
27	*Minimoides*	*G. capillifolia* Vved.				AM087951	(8)
28	*Minimoides*	*G. dschungarica* Regel				AM087952	(36)
29	*Minimoides*	*G. stipitata* Merckl. ex Bunge				AM409336	(36)
30	*Minimoides*	*G. ova* Stapf				AM287277	(8)
31	*Minimoides*	*G. filiformis* (Ledeb.) Kar. et Kir.				MT923860	Unpublished
32	*Minimoides*	*G. davlianidzeae* Levichev				FR689759	(18)
33	*Minimoides*	*G. pseudominutiflora*				AM493957	(8)
34	*Didymobolbos*	*G. tenera* Pascher				AM422460	(8)
35	*Gagea*	*G. capusii* A.Terracc.				AM422455	(8)
36	*Gagea*	*G. turkestanica* Pascher				LN874868	(37)

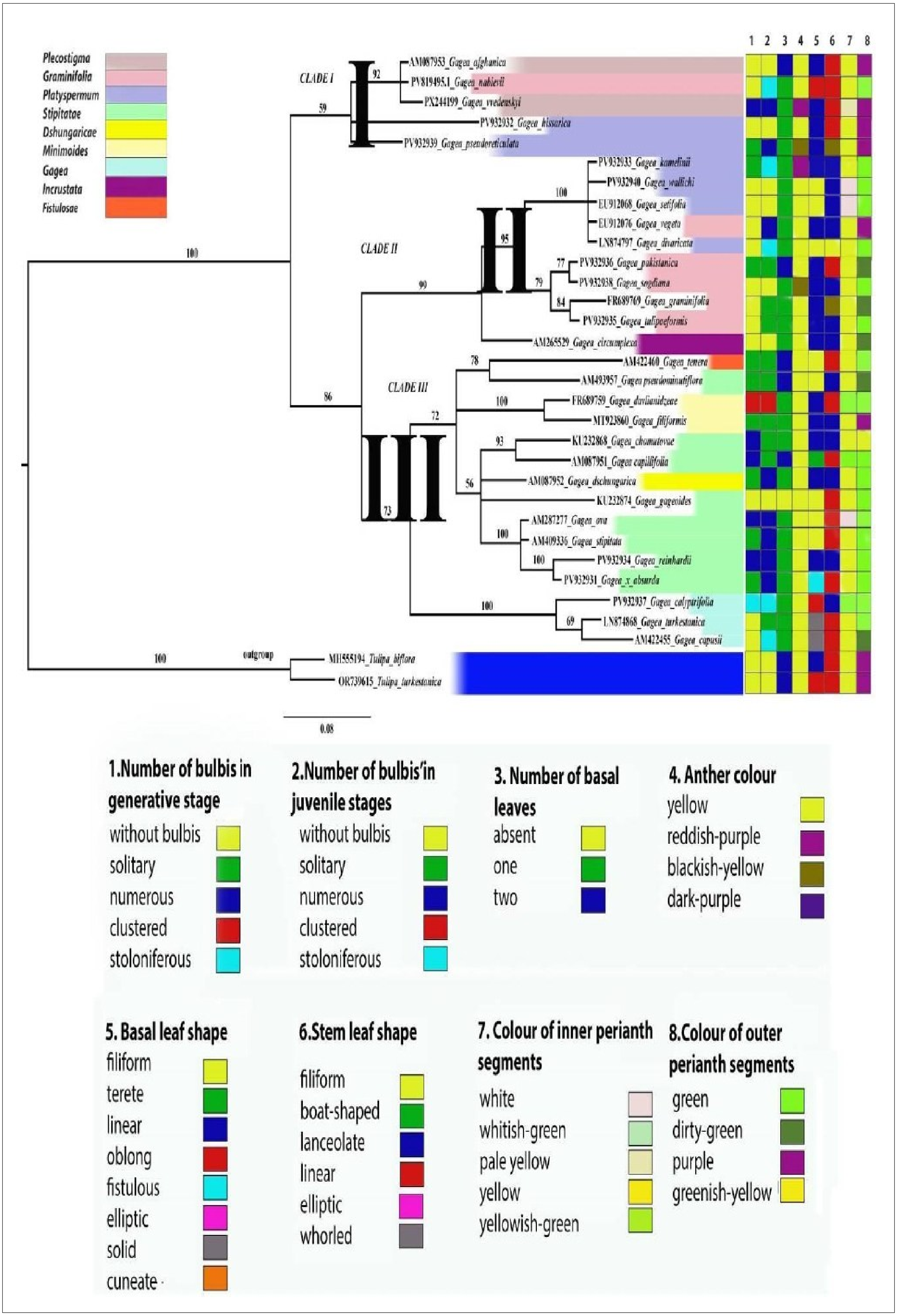

Fig. 1. Bayesian phylogenetic tree (GTR+G model) of 32 *Gagea* species based on ITS sequences. Three main clades (Clade I–III) are indicated, with posterior probabilities and bootstrap support values shown at the nodes.

origin possibly shaped by ecological diversification (32, 33).

To explore the correspondence between morphological characters and phylogenetic groupings, a character matrix including 8 morphological traits was mapped alongside the molecular tree (Fig. 1). These included bulb number, basal and stem leaf number and shape and perianth coloration. The matrix revealed a general congruence between morphological traits and genetic groupings, although exceptions such as *G. nabievii* and *G. liotardii* suggest morphological plasticity or misinterpretation of phenotypic traits.

Photographic documentation of representative species (Fig. 2 & 3) further confirmed diagnostic morphological traits such as tepal shape and color, bulb clustering and basal leaf morphology. These visual records were instrumental in verifying character scoring and validating morpho-molecular congruence.

Taken together, the integration of ITS molecular data with detailed morphological and geographical evidence provides a refined framework for understanding species boundaries, evolutionary trends and taxonomic structure within the genus *Gagea*. Although some discordances remain, especially among morphologically intermediate or variable taxa, the phylogenetic signal recovered from nuclear ITS sequences have proven effective for delimiting clades and informing sectional revisions.

Future work involving genome-wide datasets (e.g., plastome phylogenies or RADseq) and expanded taxon sampling will be essential for resolving complex relationships in problematic groups such as *Graminifolia* and *Stipitatae* and for testing hypotheses of hybridization and reticulate evolution suggested by earlier authors (34, 35).

Conclusion

This study provides new insights into the evolutionary relationships within the genus *Gagea* using an integrative approach that combines molecular (*ITS1* and *ITS2*) and morphological data. The recovered phylogenetic tree revealed 3 major clades that partially align with traditional sectional classifications.

The first clade demonstrated discordance between morphology and molecular data in the placement of *G. nabievii*, suggesting convergent traits or misclassification. The second clade showed a high degree of congruence, particularly among *Graminifolia*, *Platyspermum* and *Incrustata*, validating the naturalness of these groupings. The third clade, the most taxonomically diverse, included species from several morphologically distinct sections, yet formed a coherent genetic cluster.

Overall, the integration of molecular and morphological

Fig. 2. Photographs of selected *Gagea* species: 1- *G. sogdiana*. 2- *G. filiformis*. 3- *G. ova*. 4- *G. reinhardii*. 5- *G. chomutowae*. 6- *G. tenera*. 7- *G. vegeta*. 8- *G. liotardii*. 9- *G. taschkentica*.

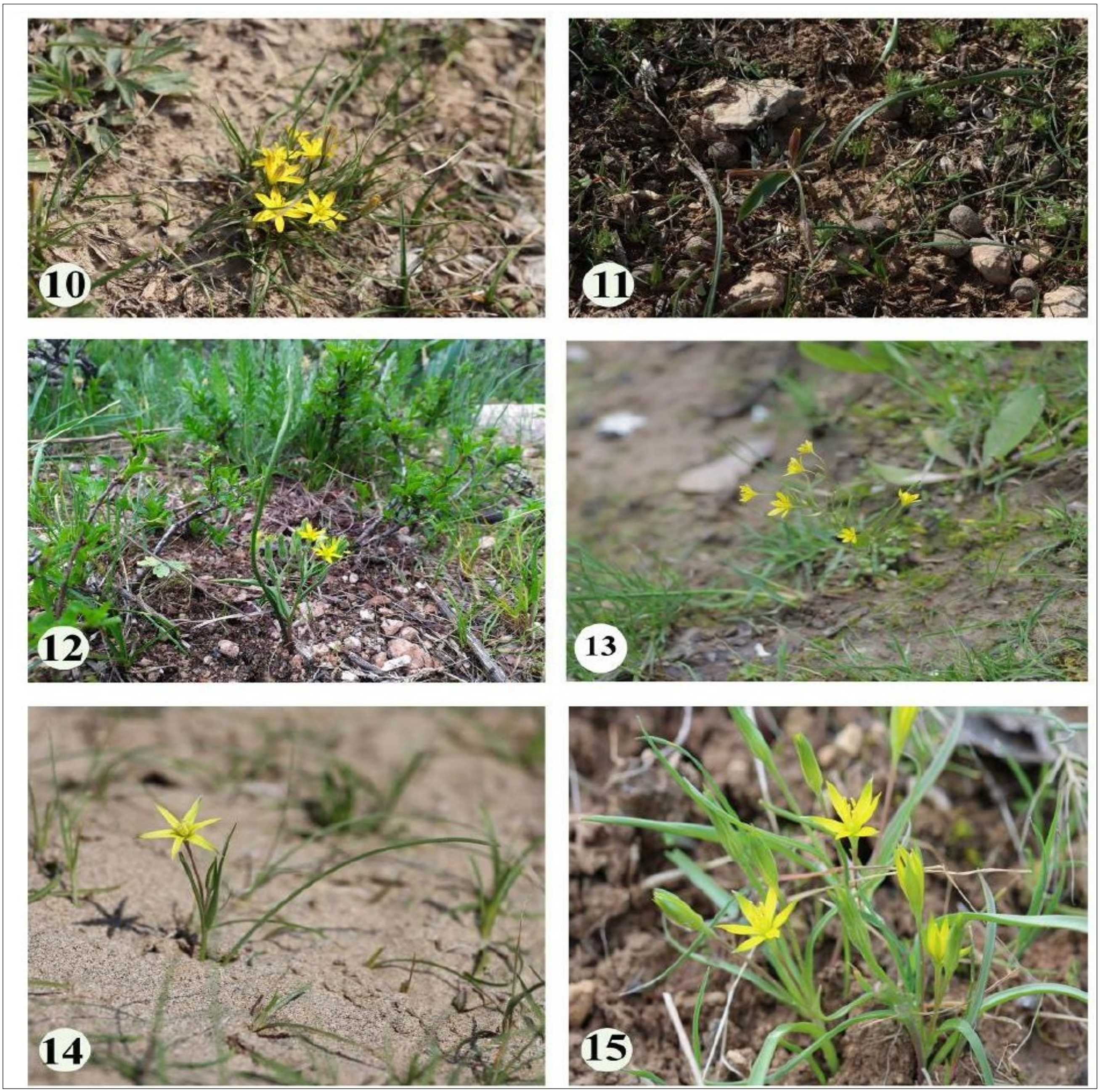

Fig. 3. Photographs of selected *Gagea* species: 10- *G. olgae*. 11- *G. turkestanica*. 12- *G. popovii*. 13- *G. gageoides.* 14- *G. divaricata*. 15- *G. graminifolia*.

datasets proved effective for resolving complex taxonomic relationships in *Gagea*. While some discrepancies remain, the results support the reevaluation of certain sectional boundaries and offer a framework for future taxonomic revisions in the genus.

The spatial distribution maps (Fig. 4 & 5) further substantiate the phylogenetic clades identified in this study. For example, species clustered in Clade 1, such as *G. afghanica* and *G. pseudoreticulata*, are primarily found in the southern arid mountainous regions, while Clade 2 species (*G. sogdiana*, *G. setifolia*) are more broadly distributed in the central and eastern regions. These patterns suggest that geographic isolation and ecological heterogeneity may have played significant roles in driving lineage diversification in *Gagea* within Uzbekistan.

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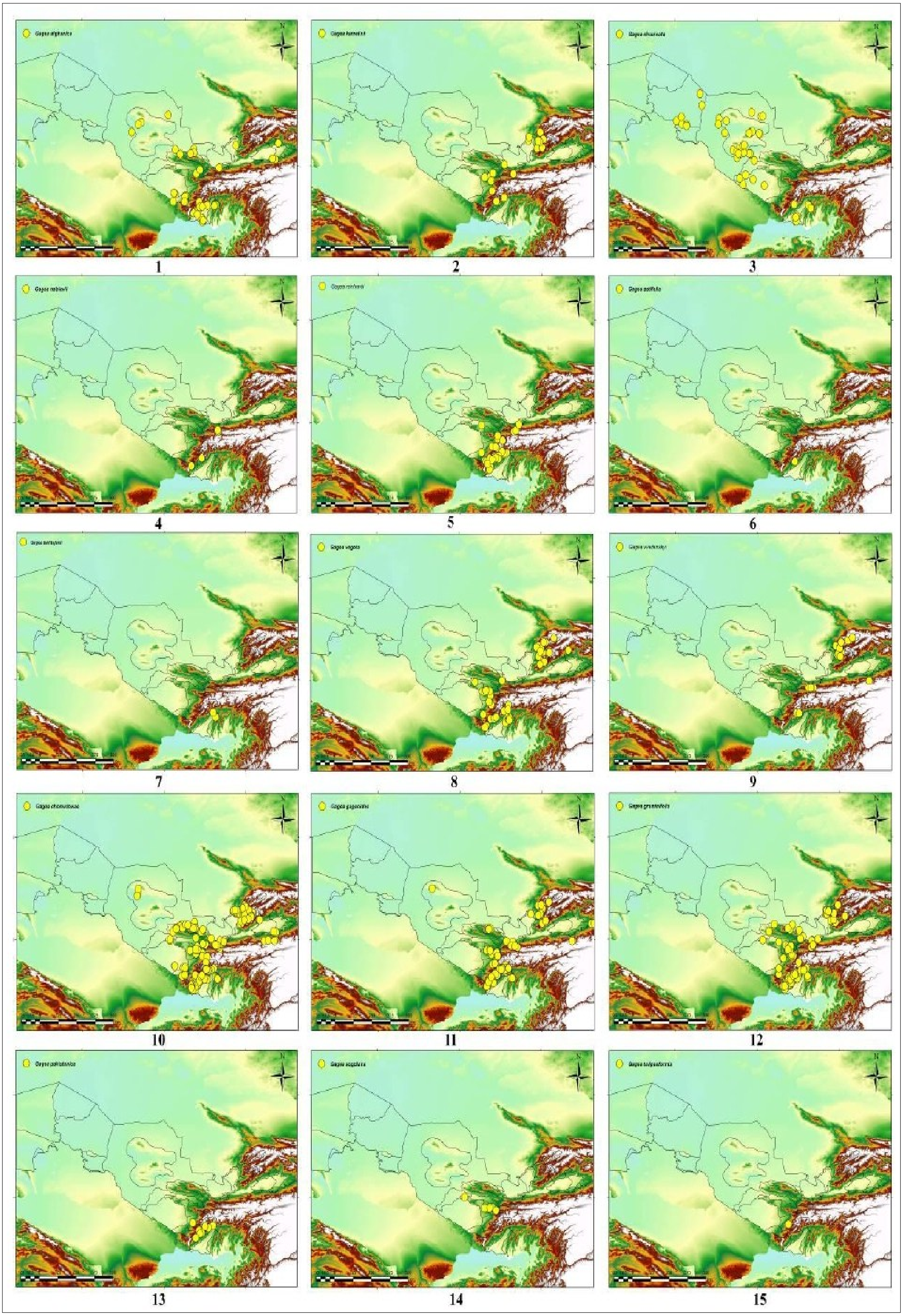
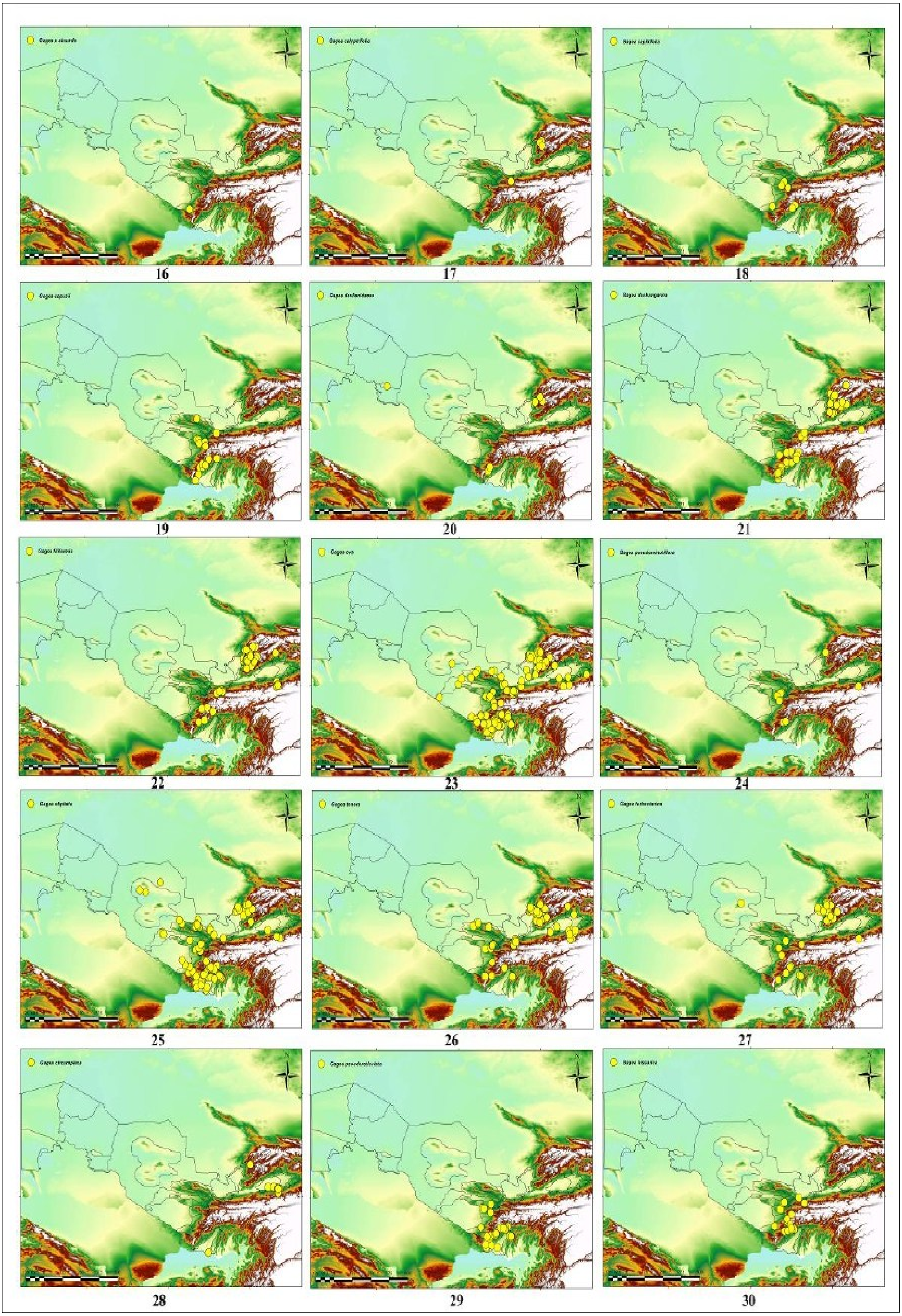


Fig. 4. Distribution maps of *Gagea* species in Uzbekistan. 1- *G. afghanica;* 2- *G. kamelinii;* 3- *G. divaricata;* 4- *G. nabievii;* 5- *G. reinhardii;* 6- *G. setifolia*; 7- *G. takhtajanii;* 8- *G. vegeta;* 9- *G. vvedenskyi;* 10- *G. chomutowae;* 11- *G. gageoides;* 12- *G. graminifolia;* 13- *G. pakistanica;* 14- *G. sogdiana;* 15- *G. tulipaeformis.*


16 17 18

19 20 21

22 23 24

25 26 27

28 29 30

Fig. 5. Distribution maps of *Gagea* species in Uzbekistan. 16- *Gagea x absurda;* 17- *G. calyptrifolia;* 18- *G. capillifolia;* 19- *G. capusii;* 20- *G. davlianidzeae;* 21- *G. dschungarica;* 22- *G. filiformis;* 23- *G. ova;* 24- *G. pseudominutiflora;* 25- *G. stipitata;* 26- *G. tenera;* 27- *G. turkestanica;* 28- *G. circumplexa;* 29- *G. pseudoreticulata;* 30- *G. hissarica.*

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Authors' contributions

GK contributed to the introduction, conducted field expeditions and participated in writing the results and discussions. IL contributed to the conclusions section and co-wrote the results and discussion. AR and AG were responsible for molecular analyses and preparing the materials and methods section. DJ contributed to the introduction and took part in data processing and analysis. BK and IE were involved in phylogenetic tree construction and interpretation. AN contributed to the introduction and co-authored the materials and methods section. ZY co-wrote the results and discussion section. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest.

Ethical issues: None

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