A phylogenetic analysis of the wild *Tulipa* species (Liliaceae) of Kosovo based on plastid and nuclear DNA sequence

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**Abstract**

In Kosovo, the genus *Tulipa* is represented by eight taxa, most of which form a species complex surrounding *Tulipa scardica*. To investigate the phylogenetic relationship of these *Tulipa* species a Bayesian analysis was undertaken using the ITS nuclear marker and trnL-trnF, rbcL and psbA-trnH plastid markers. The resulting phylogenetic trees show that Kosovarian *Tulipa* species consistently group into two main clades, the subgenera *Eriostemones* and *Tulipa*. Furthermore, our analyses provide some evidence that the subspecies of *Tulipa sylvestris* are genetically distinguishable, however not significantly enough to support their recategorisation as species. In contrast, the markers provide some novel information to reassess the species concepts of the *T. scardica* complex. Our data provide support for the synonymisation of *Tulipa luanica* and *Tulipa kosovarica* under the species *Tulipa serbica*. Resolution and sampling limitations hinder any concrete conclusion about whether *Tulipa albanica* and *T. scardica* are true species, yet our data do provide some support that these are unique taxa and therefore should continue to be treated as such until further clarification. Overall, our work shows that genetic data will be important in determining species concepts in this genus, however, even with a molecular perspective pulling apart closely related taxa can be extremely challenging.

**KEYWORDS**

Balkans, barcoding, ITS, phylogenetics, species concepts I trnL-trnF, *Tulipa*

**1 | INTRODUCTION**

Species of the genus *Tulipa* L. (Liliaceae) have great economic, horticultural, and ecological value1 while also being culturally significant in many areas of the world.2 They are bulbous monocots characterized by a diverse range of variable vegetative and floral traits, which were traditionally used to define species concepts in this genus. Furthermore, the vegetative and floral traits often show a high degree of plasticity, sometimes, even within populations of a species.2-4 Due to this and the long horticultural history of tulips, creating a stable taxonomic framework for the genus has been extremely difficult, despite the existence of a large body of literature.3-6 and so classifications of *Tulipa* have been revised several times.7 The total number of extant *Tulipa* species varies between publications, although generally ranges from 40 to 150 species.5,8 In the World Checklist of Selected Plant Families,9 516 names are listed for *Tulipa*, but only 102 taxa have been accepted, while in the Plant List10 499 names are listed for *Tulipa* and 120 taxa have been accepted. According to the most complete evaluation of the genus to date,3 only 76 species are accepted, but since this work, a number of new species have been described.11-14

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number of *Tulipa* species native to the Balkan Peninsula is only a small proportion of the global diversity, varying from 15\(^{11}\) to 22\(^{9}\) species. In Kosovo, the genus *Tulipa* is represented by eight taxa (six species and two subspecies), belonging to the subgenera *Eriostemones* and *Tulipa*. In general, researchers working on these species have used different morphological traits to define the taxonomic relationship between them. The subgenus *Eriostemones*, is generally represented by *Tulipa sylvestris* and at the lower taxonomic level by two subspecies;\(^{16}\) *T. sylvestris* subsp. *sylvestris* only accepted by the World Checklist of Selected Plant Families\(^{7}\) and *Tulipa sylvestris* subsp. *australis* (Link) Pamp (accepted subsp.). While the subgenus *Tulipa* is represented by several species, *Tulipa gesneriana* L.\(^{16}\) is sometimes treated as a wild species, although it is not thought to grow in a truly wild state\(^2\) and is known as the oldest species in the group.\(^2\) Due to the similarities between these species, they have sometimes been treated as synonyms, and are often erroneously identified and misclassified.

Studies focused on defining species concepts within the *scardica* complex have primarily used morphological characteristics and geographical distributions. However, in addition, karyological analyses have been undertaken for *T. albanica*,\(^{21}\) and *T. luanica*,\(^{11}\) as well as measurements of nuclear genome size (DNA 2C-values) for *T. albanica*,\(^{21,22}\) *T. scardica*,\(^6\) *T. kosovarica*, and *T. luanica*.\(^{22}\) However, DNA content and cytogenetic analyses have not been undertaken for all species present in Kosovo and so understanding of species relationships is currently limited.

DNA/molecular markers have emerged in the last few decades as a powerful tool in plant systematics and have become an important, inexpensive, reliable technique for exploring phylogenetic relationships.\(^{23}\) Molecular phylogenetic analysis using sequences from nuclear ribosomal DNA (nrDNA) and chloroplast DNA (cpDNA) have previously been successfully used to determine relationships between species within the genus *Tulipa*. Thus, we decided to use *Tulipa* DNA sequences from the ITS region,\(^{2,7,24,25}\) *tml-trnF* region,\(^{26}\) *psbA-trnH* region, and *rbcL* region\(^{27}\) to undertake a phylogenetic analysis of Kosovarian tulip diversity. This work aimed to improve understanding of species concepts across the wild-growing *Tulipa* species of Kosovo, especially the *scardica* complex, with a view to inform tulip conservation, evolutionary understanding, and the broader taxonomic positioning of Kosovarian tulip species.

2 | RESULTS

The ITS sequences (ITS1, complete 5.8S rDNA gene, ITS2 and a small part of 26S rDNA gene) of *Tulipa* species in the dataset ranged from 616 to 655 bp. The in-group alignment included 66 ambiguous positions. Sixty-seven positions were potentially informative, 33 potentially informative indels, and 60.0% G + C content (Table 1). The sequence length of ITS1 ranged between 229 and 233 bp, 5.8S rDNA between 162 and 166 bp, ITS2 between 225 and 231 bp and 26S rDNA (partial) was consistently 26 bp. Tulip samples showed an average of 141 and 143 conserved sites for ITS1 and ITS2, respectively. The *tml-trnF* sequences of *Tulipa* species in the dataset ranged from 765 to 788 bp in length. The in-group alignment had 46 ambiguous positions. Analyzed sequences showed eight potentially informative characters, 16 potentially informative indels, and 31.2% G + C content (Table 1). The *tml-trnF* region was made up of *tml* 631 to 692 bp, *trnF* 57–64 bp and *igs* 25 bp for each sequence, respectively. The *rbcL* sequence length in the dataset ranged from 488 to 597 bp. In-group alignment includes three ambiguous positions. Analyzed sequences showed three potentially informative characters, five potentially informative indels, and 44.0% G + C content (Table 1). The *psbA-trnH* sequences in the dataset ranged from 488 to 597 bp in length. The in-group alignment had 35 ambiguous positions. Analyzed sequences showed 15 potentially informative characters, 93 potentially informative indels, and 32.6% G + C content (Table 2). The combined *ITS + tml-trnF + psbA-trnH + rbcL* sequences for species ranged from 2405 to 2469 bp in length. The alignment showed 134 ambiguous positions, 2272 conserved sites, 113 potentially informative characters, and 125 potentially informative indels, and an average 42.1% G + C content (Table 1).

2.1 | Phylogenetic analysis

In total, 106 sequences were used in the phylogenetic analysis. The phylogenetic trees for all datasets (separate ITS, *tml-trnF*, *psbA-trnH*, and *rbcL* trees as well as the combined *ITS + tml-trnF + psbA-trnH + rbcL* datasets) were generated through a Bayesian analyses. Resolution was relatively weak for all trees produced from single markers while the best resolution was obtained from the phylogenetic tree created using the combined *ITS + tml-trnF + psbA-trnH + rbcL* dataset.
The phylogenetic analysis based on 31 ITS sequences is shown in Figure 1. The generated tree shows that the Tulipa taxa are divided into two main clades with strong support (BPP = 1). The first clade includes specimens of the subgenus Eriostemones (T. sylvestris, including both subspecies), while the second clade includes members of the subgenus Tulipa (T. albanica, T. kosovarica, T. luanica, T. scardica, T. serbica, Tulipa ulophylla, Tulipa tschimganica, T. suaveolens, Tulipa jula, and T. gesneriana). In the first clade, the wild-collected specimens of T. sylvestris subsp. sylvestris (T21 and T23) are separated from the wild T. sylvestris subsp. australis (T22), while all wild-collected specimens are more closely related to each other than to the T. sylvestris subsp. sylvestris sequence obtained from GenBank (BPP = 1%). In the second clade, all species from the scardica complex form a single clade (T. albanica, T. kosovarica, T. luanica, T. scardica, and T. serbica), with specimens of T. ulophylla, T. x tschimganica, T. suaveolens, T. julia, and T. gesneriana all more distantly related. The species T. x tschimganica (section Spiranthera), T. ulophylla (section Tulipanum), and T. julia were all identifiable as separate taxonomic entities (BPP = >0.9), while T. suaveolens and T. gesneriana formed a strongly supported clade (BPP = 1) that was sister to the scardica complex, indicating that the sequences under the name T. gesneriana may in fact be T. suaveolens.

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| Potential species                  | Sequence ID                      | Collection locality | Country   | Longitude | Latitude | Altitude | ITS accession number | trnL-trnF accession number | rbcl accession number | psba-trnH accession number | Uni. of Prishtina herb. Acces. no. |
|-----------------------------------|----------------------------------|---------------------|-----------|-----------|----------|----------|---------------------|-----------------------------|------------------------|-------------------------------|-------------------------------|
| T. albanica                       | T._albanica_T1 (yellow flower)   | Surroj              | Albania   | 42° 2.744'N | 20° 20.037'E | 622       | MN336199            | MN446897                   | MZ147066               | MZ147043                     | 00000158                     |
| T. albanica                       | T._albanica_T2 (reddish maroon flower) | Surroj       | Albania   | 42° 2.744'N | 20° 20.037'E | 622       | MN336200            | MN446898                   | MZ147067               | MZ147044                     | 00000157                     |
| T. albanica                       | T._albanica_T3 (reddish maroon /yellow flower) | Surroj       | Albania   | 42° 2.744'N | 20° 20.037'E | 622       | MN336201            | MN446899                   | MZ147068               | MZ147045                     | 00000156                     |
| T. kosovarica                     | T._kosovarica_T4                 | Goriç              | Kosovo    | 42° 26.689'N | 20° 45.337'E | 659       | MN336202            | MN446900                   | MZ147069               | MZ147046                     | 00000155                     |
| T. kosovarica                     | T._kosovarica_T5                 | Goriç              | Kosovo    | 42° 26.689'N | 20° 45.337'E | 659       | MN336203            | MN446901                   | MZ147070               | MZ147047                     | 00000154                     |
| T. kosovarica                     | T._kosovarica_T6                 | Koznik             | Kosovo    | 42° 30.334'N | 20° 33.987'E | 425       | MN336204            | MN446902                   | MZ147071               | MZ147048                     | 00000153                     |
| T. kosovarica                     | T._kosovarica_T7                 | Koznik             | Kosovo    | 42° 30.334'N | 20° 33.987'E | 425       | MN336205            | MN446903                   | MZ147072               | MZ147049                     | 00000152                     |
| T. kosovarica                     | T._kosovarica_T8                 | Koznik             | Kosovo    | 42° 30.334'N | 20° 33.987'E | 425       | —                   | MN446904                   | MZ147073               | MZ147050                     | 00000151                     |
| T. species                        | T._species_T9                    | Krimi              | Kosov      | —          | —         | —        | MN336206            | —                           | MZ147074               | MZ147051                     | 00000150                     |
| T. luanaica                       | T._luanaica_T10                  | Pashtrik           | Kosovo    | 42° 14.966'N | 20° 30.399'E | 1041      | —                   | MN446905                   | MZ147075               | MZ147052                     | 00000149                     |
| T. luanaica                       | T._luanaica_T11                  | Pashtrik           | Kosovo    | 42° 14.966'N | 20° 30.399'E | 1041      | MN336207            | MN446906                   | MZ147076               | MZ147053                     | 00000146                     |
| T. luanaica                       | T._luanaica_T12                  | Pashtrik           | Kosovo    | 42° 14.966'N | 20° 30.399'E | 1041      | MN336208            | MN446907                   | MZ147077               | MZ147054                     | 00000147                     |
| T. luanaica                       | T._luanaica_T13                  | Qafé Prush         | Kosovo    | 42° 18.275'N | 20° 23.529'E | 580       | MN336209            | MN446908                   | MZ147078               | MZ147055                     | 00000148                     |
| T. luanaica                       | T._luanaica_T14                  | Qafé Prush         | Kosovo    | 42° 18.275'N | 20° 23.529'E | 580       | MN336210            | MN446909                   | MZ147079               | MZ147056                     | 00000145                     |
| T. scardica                       | T._scardica_T15                  | Krivenik           | Kosovo    | 42° 6.254'N  | 21° 14.958'E | 575       | MN336211            | MN446910                   | MZ147080               | MZ147057                     | 00000167                     |
| T. scardica                       | T._scardica_T16                  | Krivenik           | Kosovo    | 42° 6.254'N  | 21° 14.958'E | 575       | MN336212            | —                           | MZ147081               | MZ147058                     | 00000166                     |
| T. scardica                       | T._scardica_T17                  | Krivenik           | Kosovo    | 42° 6.254'N  | 21° 14.958'E | 575       | MN336213            | —                           | MZ147082               | MZ147059                     | 00000165                     |
| T. serbica                        | T._serbica_T18                   | Serboc             | Kosovo    | 42° 58.067'N | 20° 49.757'E | 596       | MN336214            | MN446911                   | MZ147083               | MZ147060                     | 00000164                     |
| T. serbica                        | T._serbica_T19                   | Serboc             | Kosovo    | 42° 58.067'N | 20° 49.757'E | 596       | MN336215            | MN446912                   | MZ147084               | MZ147061                     | 00000163                     |
| T. serbica                        | T._serbica_T20                   | Serboc             | Kosovo    | 42° 58.067'N | 20° 49.757'E | 596       | MN336216            | MN446913                   | MZ147085               | MZ147062                     | 00000162                     |
| T. sylvestris ssp. sylvestris      | T._sylvestris_ssp. _sylvestris_T21 | Goriç            | Kosovo    | 26° 7.474'N  | 20° 45.293'E | 665       | MN336217            | MN446914                   | MZ147086               | MZ147063                     | 00000161                     |
| T. sylvestris ssp. australis       | T._sylvestris_ssp. _australis_T22 | Devë              | Kosovo    | 42° 19.950'N | 20° 20.517'E | 700       | MN336218            | MN446915                   | MZ147087               | MZ147064                     | 00000160                     |
| T. sylvestris ssp. sylvestris      | T._sylvestris_ssp. _sylvestris_T23 | Devë              | Kosovo    | 42° 19.950'N | 20° 20.517'E | 700       | MN336219            | MN446916                   | MZ147088               | MZ147065                     | 00000159                     |
the members of the subgenus Eriostemones and that of the subgenus Tulipa albeit with very limited resolution. In the Eriostemones clade, the specimens of T. sylvestris subsp. sylvestris formed a clade separate from T. sylvestris subsp. australis (BPP 0.9). Within the Tulipa clade the Bayesian analysis provided extremely limited resolution (BPP < 0.5) to distinguish between taxa especially those of the scardica complex (T. albanica, T. kosovarica, T. liuica, T. scardica, and T. serbica). Nonetheless, there was some support that T. x tschimganica and T. gesneriana were genetically distinct from the specimens in the scardica complex (BPP = 0.95). Here, we also note that Erythronium japonicum appears more closely related to Tulipa specimens than to Amana specimens, which contradicts the expected relationship of these genera providing some evidence that this is not a taxonomically informative marker.

2.6 Combined ITS, trnL-trnF, psbA-trnH, and rbcl dataset

The phylogenetic tree obtained from the combined ITS, trnL-trnF, psbA-trnH, and rbcl sequences provided the most strongly supported tree structure for the specimens analyzed (Figure 5). The phylogenetic tree is divided into two main clades, the subgenus Eriostemones and the subgenus Tulipa with strong support for this separation (BPP = 1). Within the Eriostemones subgenus, the specimens of T. sylvestris subsp. sylvestris fall together with T. sylvestris subsp. australis sister to these (BPP = 1). Within the Tulipa clade, the analyzed taxa divided into three clear genetically distinct clades of the tree. Both T. albanica and T. scardica, appear as taxonomically distinct clades, although T. scardica is only represented by a single specimen (BPP = 1), while a group consisting of T. kosovarica, T. liuica, and T. serbica created a third separate clades of the tree, which was strongly supported (BPP = 1). Within this last grouping the species concepts are not monophyletic. We also note here that within the outgroup Amana edulis and A. erythronioides do not fall together as expected; however, all members of the outgroup do sit outside the Tulipa clade.

3 DISCUSSION

In this study, we use the genetic markers ITS, trnL-trnF, psbA-trnH, and rbcl to undertake a molecular phylogenetic analysis of Kosovarian tulip diversity. Our data highlight the informativeness and limitations of the ITS nuclear marker and plastid markers trnL-trnF, rbcl, and psbA-trnH in investigating evolutionary relationships between species of wild Tulipa. In general, we found that subgenera can be reliably separated by a range of single genetic markers; however, that separating more closely related species requires a combination of markers. Our most informative tree provides evidence that the scardica complex has been over split and specifically that T. liuica and T. kosovarica should be synonymised under T. serbica. While our data also provide some support for the existence of T. albanica and T. scardica as unique taxa, as well providing some evidence that the
subspecies of *T. sylvestris* can be distinguished genetically although should be maintained as a single species.

In general, phylogenetic trees generated using ITS sequence data had better resolution than those generated from single plastid markers, including the *trnL-trnF* marker which is in line with previous research. The *rbcl* tree was the least informative as it had extremely weak resolution across the analyzed taxa, which supports previous reports of the marker performing poorly. The *psbA-trnH* marker provided somewhat better resolution than *rbcl*, but still lacked enough informative sites to separate the *scardica* complex and also unexpectedly placed an *Amana* specimen within the *Tulipa* clade showing it is not necessarily a reliable genetic marker. Our phylogenetic analyses also showed that the unidentified *Tulipa* species (sample T9, Table 2) sequenced from herbarium material at the Herbarium of the University Prishtina, falls into the *scardica* complex, but we lack the resolution to identify it as an existing or new species. It is, therefore, clear from our work and previous research that single genetic markers can only provide reliable resolution at the subgenera level.

The use of sections within the genus *Tulipa* was actively discouraged until further in-depth genetic studies could be undertaken. Yet, we wanted to briefly explore how our ITS tree fits into the taxonomic framework developed by Zonneveld. The phylogenetic tree based on the ITS marker we generated had monophyletic groups that represented the *Eriostemones* section *Sylvestres* and the *Tulipa* sections *Spiranthera* and *Tulipa*. Yet, our tree shows that the section *Tulipanum* in the *Tulipa* subgenera does not form a monophyletic group, with the specimen of *T. julia* shown to be more closely related to the species of the section *Tulipa* than to *T. ulophylla* of the same section. There are significant limitations in our assessment of sections of the genus *Tulipa* both in terms of the genetic marker used as well as in the extremely poor species representation. We therefore do not make any conclusive statements about the use of sections in the genus *Tulipa* but do note that these may not all hold as more genetic data become available.

Unsurprisingly, our most informative tree was generated using the combined dataset that included all the markers (ITS, *trnL-trnF*, *psbA-trnH*, and *rbcl*). This, like the single marker trees, separated the *Tulipa* taxa into two main clades, representing the subgenus *Eriostemones* and the subgenus *Tulipa*, which are clearly stable monophyletic taxonomic groupings. Among the newly sequenced species of the *Eriostemones* clade, there was some distinguishable difference between *T. sylvestris* subsp. *sylvestris* and *T. sylvestris* subsp. *sylvestris* from Kosovo. Our work therefore suggests that these subspecies should continue to be treated as separate taxa; however, within our work, we did not incorporate enough specimens or have the resolution to classify these as unique species. These subspecies are known to have different chromosome numbers, with *T. sylvestris* subsp. *sylvestris* a diploid form of *T. sylvestris*, and *T. sylvestris* subsp.
sylvestris encompassing triploid or tetraploid forms of T. sylvestris. Yet, the native range of these subspecies remains unclear, and many morphologically intermediate forms are known to occur in the wild. Further cytotaxonomic studies will therefore be needed to investigate the chromosome numbers of the specimens located in Kosovo to confirm their taxonomic identity, while extensive in-depth molecular work will be needed to unentangle this widespread, complicated taxon. In the subgenus Tulipa, the grouping together of the species T. scardica, T. serbica, T. albanica, T. kosovarica, and T. luanica into a clade provides strong evidence of a close relationship between these taxa, confirming the existence of the scardica complex. Our combined tree highlighted the genetic distinctness of T. albanica and T. scardica from the other species in this complex, while leaving the other three taxa in a clade where none were monophyletic. This provides evidence for the oversplitting of this complex and the need to synonymize some of the taxa under one species name, specifically T. luanica and T. kosovarica under T. serbica.

The scardica complex remains a controversial group of species due to the many morphological similarities between these taxa. There has been significant confusion around species concepts, including in the use of the name T. gesneriana. In some instances, T. scardica has been synonymized under the name T. gesneriana, however, T. gesneriana is likely not a true species. This taxonomic confusion is highlighted again in the varied acceptance of T. gesneriana as a species across different classification bodies; it is not accepted by Flora Europea, but is by the World Checklist of Selected Plant Families. Today, there are five species recognized as part of this complex.

T. scardica was the first species described from this complex, and individuals of this species show significant variation in several morphological characters, such as leaf form, flower color, length of filaments, and anthers in different distribution areas. Tulipa serbica, the second species named in this complex was described from Mt Rogozna and was originally thought to be a population of T. scardica, before being described as a new species. Both species are thought to be closely related with T. serbica only morphologically differing from T. scardica in its paler, unspotted perianth segments, pale (not blackish) staminal filaments, dull violet (not yellowish), and acute anthers.

Tulipa albanica was recorded as a new species in Northeast Albania in 2010; it has recently been found growing in Kosovo as well. It shows significant variation in several morphological characters from the other species in the group; it has a unique combination of yellow perianth bases without black blotches, yellow filaments, and violet-purple pollen. The plant’s campanulate flowers exist in two color forms, yellow to golden-yellow or carmine-scarlet turning deep reddish maroon, with a dominance of the golden-yellow flowers. Some
FIGURE 3  Phylogenetic trees based on psbA-trnH sequences, including posterior probabilities (BPPs) (>0.5) provided above each node.

FIGURE 4  Phylogenetic trees based on rbcL sequences, including posterior probabilities (BPPs) (>0.5) provided above each node.
individuals have an intermediate color of yellow to reddish maroon. Yet, *T. albanica* also shares many morphological similarities with *T. scardica*, *T. kosovarica*, and *T. lyansica*. *T. kosovarica* collected for the first time along the Mrasori river (Mirusha region) at the foot of Mt Kozniku in 2010 was originally thought to be a population of *T. scardica* 
,21 but in 2012, the material was revisited and described as a new species.20 Later, this species was recorded from several other locations such as Guriç, Llapushnik, Qafë Prush and Devë.16 *T. kosovarica* differs from *T. scardica* due to its white or whitish perianth base that is sometimes masked by obtrullate patches of maroon and violet, while *T. albanica* differs from this species by having yellow perianth bases without black blotches.20

*T. lyansica* is the most recent species described as a member of the *T. scardica* complex11 that shares many morphological characters with *T. albanica*, *T. kosovarica*, and *T. serbica*. However, *T. lyansica* also differs in several characters, including that it exclusively grows on limestone substrate rather than the serpentine substrate which other species grow on.

Across the literature, flower color has been one of the main characters used to discriminate the species of the *scardica* complex, but there is considerable variation in flower color within species.5,6,11,17,20,21 For example, flower color from within populations of *T. albanica* is reported to vary from yellow/golden-yellow to carmine-scarlet turning deep reddish maroon,21 with a range of intermediate colors. Furthermore, the flower color within species may differ in two aspects, first the blotch and the blotch margins may show differences in size and color intensity and second, within some species, anthocyanidins are lacking in certain accessions resulting in yellow or very light colors.35 Experiments are based on selection of accessions obtained from natural provenances, as well as mutation experiments with radiation showed that blotch margin and flower color can easily be influenced.35 Flower color is therefore not regarded as a suitable trait from which to make taxonomic decisions.2

Apart from flower morphological features, the characteristics of the bulb tunic have often been used to differentiate between *Tulipa* species and has generally been found to be a reliable character.36 Our samples of *T. sylvestris* subsp. *sylvestris* and *T. sylvestris* subsp. *australis* both had brownish black tunics, with straight hairs in the inner part of the tunic, located only around the root and on the throat of the bulb. Furthermore, the type and distribution of the trichomes in the tunic of the bulbs of *T. albanica*, *T. kosovarica*, *T. lyansica*, and *T. scardica* were also analyzed, here, the trichomes in the form of the straight hairs were located in the inner part of the tunic, densely covering all parts of the tunic. No differences were recorded in the type and distribution of trichomes in the tunics of the bulbs of *T. albanica*, *T. kosovarica*, *T. lyansica*, and *T. scardica*. Species of the *scardica* complex have also been investigated through genome size analyses, providing 2C values for most taxa. Considerable variation has been reported in the 2C value of *T. albanica* with both 54.15 pg21 and 43.86 pg22 being reported from separate experiments. *T. kosovarica*, *T. lyansica*, and *T. scardica* are recorded as having 45.71 pg, 47.49 pg, and 69 pg 2C values, respectively.5,22 The incongruent results for *T. albanica* reported (in references 20 and 21) were attributed to the origin of the plant material22: leaves collected from wild populations in bloom, vs adult leaves germinated from seeds collected from natural populations. This explanation seems somewhat unconvincing and makes it difficult to base any taxonomic decisions on 2C values for any of these species.
especially given that differences in genome sizes within species could be correlated with differences in habitat, plant phenotype, or caused by technical artifacts. In addition, the DNA content of T. serbica has not been measured so this cannot be linked to other species in the scardica complex. Overall, this means that our DNA sequence data are likely the best assessment of this species complex to date and should be used as a guide on how to classify these taxa into species over and above current cytogenetic data.

4 | MATERIALS AND METHODS

4.1 | Plant material

Eight taxa (six species and two subspecies) of the genus Tulipa were collected from wild populations between the months of April and May across 2017, 2018, and 2019. All Tulipa species were collected in Kosovo, except T. albanica, which was collected in Albania (Figure 6). One unidentified plant specimen of Tulipa sp. (sample T9, Table 2) was obtained from material provided by the Herbarium of the University Prishtina. T. kosovarica (locations Goriç and Koznik) and T. iuanica (locations Pashtrik and Qafë Prush) were collected from two different localities. Plant specimens were collected, and part of the young leaves was dried in silica gel for DNA extraction. The voucher specimens were deposited at the Herbarium of the University Prishtina, Kosovo and the Emory University Herbarium, Atlanta, USA. Detailed sample information is given in Table 2.

4.1.1 | DNA extraction, polymerase chain reaction (PCR), and sequencing

Genomic DNA was extracted from silica gel-dried material or herbarium specimens using the DNeasy Plant Mini Kit (Qiagen Hilden, Germany) according to the manufacturer’s instructions. The DNA quality was checked using agarose gel electrophoresis with 1.0% agarose gels containing 0.4 x PeqGreen (VWR, Erlangen, Germany) for 40 minutes at 120 V, which was documented using microDOC system with UV transilluminator (Cleaver Scientific LTD, Rugby, Warwickshire, UK) using 312 nm wavelength.

Extracted DNA was 1:50 diluted with deionized water and then used for PCR. The nuclear internal transcribed spacer region (ITS) and the chloroplast trnL-trnF, psbA-trnH, and rbcL markers were amplified and then sequenced from 23 samples of six species and two subspecies. For a 15-μL PCR reaction, 1 μL of diluted genomic DNA (equivalent to approximately 1-50 ng) was added to 14 μL master mix containing 1 x PCR buffer B, 2.5 mM MgCl2, 130 μM dNTP mix, 0.6 U Taq HOT FIREPol DNA polymerase (all reagents from Solis Bio-dyne, Tartu, Estonia) and 300 nM forward (ITS5 [5’-GGAAGGAGA AGTCGTAACAAGG-3’];40 or c [5’-CGAAATCGGTAGACGCTACG-3’];41 or rbcLaF- ATGTCACCACAAACAGAGACTAAAGC or psbA3f- GTTATGATGAACCTAATGCTC) and reverse primers (ITS4 [(5’-TCCTTCCGCTATTGATATGC-3’];42 or f [5’-ATTTGAACTGGTACA G-3’];41 or rbcL_ajf634R- GAAACGGTCTCTCCAACGCAT or trnHf- CGCGCATGGTGAGGATTTCC) (Sigma Aldrich, Taukirchen, Germany). The PCRs were performed in a MIC qPCR

FIGURE 6  Distribution of Tulipa sp. in Kosovo and their flower color variability
cyclers (Biomolecular Systems, Upper Coomera, Australia). PCR amplifications were performed with an initial denaturation step at 95 °C for 14:30 minutes, followed by 40 cycles at 95/58/72°C for 30/30/90 seconds, and a final elongation step of 7 minutes at 72°C. The amplified PCR fragments (2 μL of PCR products) were checked using electrophoresis in 1% agarose gels (low melting point agarose, Sigma Aldrich, Taufkirchen, Germany), using similar conditions as described above for genomic DNA.

Exonuclease I from Escherichia coli 20 U/μL (EXO I) and Thermosensitive Alkaline Phosphatase 1 U/μL (FastAP) (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) were premixed in the ratio 1:4 and stored in the freezer. 13 μL PCR products were mixed with 1.3 μL EXO I and FastAP mixture and incubated at 37°C for 15 minutes and 85°C for 15 minutes. Purified PCR products were diluted with distilled water and admixed with sequencing primers according to the requirements of the sequencing company. Sequencing was performed by Microsynth Austria (Vienna, Austria) using Applied Biosystems 3730 × 1 96 capillary DNA analyzer (Thermo Fisher Scientific). Every sequence was manually edited with CHROMAS vers. 2.6.6 (Technelysium, South Brisbane, Australia) and aligned with MEGA X software. Edited sequences were subjected to BLAST searches for preliminary analysis.

### 4.2 | Phylogenetic analyses

In total, 106 sequences obtained from 14 taxa were analyzed, 87 of them were newly generated sequences generated from eight Tulipa taxa (six species and two subspecies) collected from wild populations in Kosovo and 19 sequences were obtained from GenBank (Table 2). The ITS sequences for T. ulophylla (HF952978), T. tschimganica (HF952976), T. sylvestris subsp. sylvestris (HF952974), T. suaveolens (MK334468), T. julia (HF952964), T. gesneriana (MK335217, MK335224), the trnL-trnF sequences for T. ulophylla (HF953003), T. tschimganica (HF953001), T. sylvestris subsp. sylvestris (HF952999), T. suaveolens (HF952998), T. julia (HF952989), for rbcL T. gesneriana (KP711981), T. tschimganica (KM085539), and T. sylvestris subsp. Slyvestris (KM085538), were obtained from GenBank. The trees were rooted using A. edulis (obtained from GenBank: ITS MN173164, trnL-trnF HF953006, rbcL KC796897, and psba-trnH NC034707), Amana erythronioides (obtained from GenBank: ITS HF952982, trnL-trnF HF953007, rbcL NC03463, and psba-trnH EU939293) and E. japonicum (obtained from GenBank: ITS EU912083, trnL-trnF HF953009, rbcL D28156 and psba-trnH EU939295) as an outgroup.

ITS, trnL-trnF, psba-trnH, and rbcL sequences of most of the taxa were amplified and then sequenced from three specimens for each species, while the T. kosovarica (locality Goric) and T. luaonica (locality Qafë Prush) were amplified and sequenced successfully from two specimens per species. Due to the amplification failure of some specimens (ITS T8 and T10; trnL-trnF T9, T16, and T18), some species were represented by only one or two sequences.

The sequences were aligned using MEGA X software. For ITS analyses, in total 31 sequences were aligned to determine sequence statistics, 21 of them were newly generated, and 10 were obtained from GenBank, for trnL-trnF statistical analyses included 28 sequences (20 newly generated and eight obtained from GenBank) (Table 1). For rbcL analyses of 29 sequences were used, of them 23 were newly generated and six of them were obtained from Genebank, while for psbA-trnH 26 sequences were used for analyses of them 23 newly generated and three obtained from gene bank (outgroup species). Bayesian analyses were conducted through a Markov Chain Monte Carlo (MCMC) approach using BEAST v1.10.4 with the help of BEAGLE v3.1.0 library. The input files for BEAST were prepared in the corresponding BEAUTi program and maximum clade credibility trees generated and annotated in TreeAnnotator. The MCMC was run for 10 000 000 generations, with resulting phylogenetic trees sampled every 1000. A burn in period of 1 000 000 was used. All trees were visualized using Figtree (V.1.4.4) and Mega X software.

### 5 | CONCLUSIONS

Our phylogenetic analyses show that Kosovar tulips can easily be distinguished as either in the subgenera Eriostemones or Tulipa. Yet, within these subgenera, we found limited resolution to determine clear species relationships using the markers we selected. Nonetheless, we note that there was some genetic distinguishability between the subspecies of Tulipa sylvestris (australis and sylvestris) and that these should therefore continue to be classified as different subspecies but our work does not suggest that they should be raised to species level. In contrast, our data suggest that within the Tulipa subgenus, there has been over splitting of species within the sardica complex. With our novel genetic perspective, we suggest that T. luaonica and T. kosovarica can be synonymised under T. serbica, while both T. albanica and T. sardica were genetically distinct enough to continue to be treated as species. Further analyses with more extensive sampling and additional genetic markers will be necessary for a better understanding of the natural variability within the taxa of the sardica complex, but for now our study provides the most comprehensive genetic understanding of the complex diversity of tulips growing in and around Kosovo. This understanding will not only be crucial for taxonomic stability and future research, but also for identifying conservation priorities, especially given that threats to wild tulips are likely to increase in the near future.

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**CONFLICT OF INTEREST**

We confirm that there are no known conflicts of interest associated with this publication, the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order and contributions of authors listed in the manuscript has been approved by all of us.
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