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Research Article

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Posted Date: January 10th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1160711/v1>

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Molecular (ISSR, cp DNA, ITS) and Morphological study of the genus
Tragopogon L. (Asteraceae)

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25 Abstract

26

27 *Tragopogon* L. (Cichorioideae, Lactuceae, Scorzonerinae) is an Old World genus with 150 species, Rechinger in
28 Flora Iranica divided this genus in 13 section and 37 species that 26 species of them are exist in Iran. Safavi et al.
29 divided it into 26 species without sections in flora Iran. Despite the anatomical and molecular studies done around the
30 world, the exact classification of this genus is not clear due to the high number of secret species, hybridization,
31 polyploidy and rapid diversification. The morphology studies of 32 species and Molecular studies (ISSR, ITS, cp
32 DNA) of 22 species of the genus *Tragopogon* was investigated. The purpose of these studies are classification and
33 determination of interspecific relationship in this genus. Sections of *Rubriflori*, *Sosnowskya*, *Chromopappus*, *Majores*,
34 *Angustissimi*, *Krascheninnikovia* in flora of Iranica are confirmed on the basis of morphometry and molecular data.
35 Section of *Profundisulcati* in flora Iranica is confirmed on the base of morphometry data. The Species of *T. jesdianus*,
36 *T. porphyrocephalus*, *T. rezaiyensis* and *T. Stroterocarpus* in the flora of Iranica are not classified in any section which
37 we classified in the Rubriflori section, Cp DNA dendrogram are not useful for classification in this genus and
38 Chloroplast sequences are very similar among *Tragopogon* species, Therefore, the use of cp DNA markers in the
39 classification of this genus is not recommended.

40 **Keywords:** *Tragopogon*, Morphometry, ISSR, ITS, cp DNA.

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51 **Introduction:**

52 *Tragopogon* L. is an Old World genus of approximately 150 species that occurs across Eurasia from the Atlantic to
53 the Pacific Ocean, with a center of distribution in the Mediterranean region, the Middle East and Eastern Europe
54 (Mavrodiev et al. 2005; 2008a-c). The monophyly of the genus was strongly supported in a recent phylogenetic
55 analysis of Scorzonerinae based on internal transcribed spacer (ITS) sequence data (Mavrodiev et al. 2004).
56 *Tragopogon* includes biennial and perennial herbs with linear or linear-lanceolate leaves; solitary, simple, or sparingly
57 branched stems; one or only a few capitulum and receptacles without scales. The achenes of *Tragopogon* are usually
58 fusiform, with five to 10 more or less distinct ribs and a beak of varying length. The involucre bracts are always in
59 one row, ligulate flowers are yellow or purplish, and the pappus is in one row of mostly plumose hairs (Richardson
60 1976). It is almost impossible to identify the species without adequate knowledge of mature achene and ligule color.
61 *Tragopogon* is taxonomically difficult due to its high degree of species morphological variability, which in turn arises
62 due to frequent occurrence of inter-specific hybridization and different ploidy levels (Mavrodiev et al. 2008; Bell et
63 al. 2012). Occurrence of interspecific hybridization reported in the literature (Ownbey 1950; Ownbey & McCollum
64 1953; Pires et al. 2004; Buggs et al. 2008; Mavrodiev et al. 2008) or facultative apomixis recently found in *T. dubius*
65 (Kashin et al. 2007) Relationships within *Tragopogon* are poorly understood. Many species of *Tragopogon* have not
66 been placed in a section; most of these are narrow endemics that have been recognized and named but not treated
67 taxonomically furthermore, the genus has never been the subject of a comprehensive monograph. Regional floras have
68 provided treatments for species only in those geographic areas. (Mavrodiev et al. 2005). (Blanca and De la guardia,
69 1997) Schishkin (1961) In Komarov Flora Russia divided it in 17 section and 79 species that 10 species of them are
70 share with flora Iran. Rechinger (1977) according to schishkin in flora Iranica divided this genus in 13 section and 37
71 species that 26 species (11 species endemic) of them are exist in Iran (Rechinger 1977). Safavi et al. (2014) divided
72 it into 26 species without sections in flora Iran.

73 In an initial effort to resolve relationships within *Tragopogon*, we conducted a phylogenetic analysis of *Tragopogon*
74 species in Iran, using ISSR (ISSRs: Inter simple sequenced repeats), ITS and cp DNA (non-coding region of
75 chloroplast DNA) sequence data.

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78 **Materials and methods:**

79 **Plant material**

80 *Tragopogon* species were collected from natural habitats in Iran in 2015-2018. The voucher specimens are deposited
81 in the Shahid Beheshti University herbarium (HSBU), herbarium of Research Institute of Forests and Rangeland
82 (TARI) and herbarium of Museum of Natural History Vienna (W). (Table 1)

83 **Morphological studies**

84 26 species of this genus belonging to the flora of Iran and 6 species belonging to the flora of Iranica were studied.
85 Morphological characters studied were: Flower color, Anther tube color, Collar type, Investigation of branching in
86 plant, presence of indumentums in plant, indumentums of involucre bracts, Number of flowers per pile, status of
87 involucre bracts and ligule, Ratio length of bract to papus, shape of base leaves, Presence or absence of sheath in leaf,
88 condition of tips of stem leaves, Leaf margin type, Leaf type, Condition of middle vein thickness, Central achene
89 surface, Marginal achene surface, papus status, condition of papus and achene, papus type, Condition of beak to
90 achene, beak type, condition of tip of beak, Surface on the beak, diameter of inflorescence, length of involucre, Width
91 of involucre, length of bract, width of bract, number of involucre bracts, length of achene, width of achene, length of
92 papus, length of beak, Number of vine or wings on the achene, length of the base leaves, width of the base leaves,
93 Stem leaf length, stem leaf width, Number of basal leaf veins, ratio of length to width of the involucre, ratio of length
94 to width of the bract, ratio of length to width of the achene, ratio of length to width of the papus, ratio of length to
95 width of the beak, ratio of length to width of the base leaf, ratio of length to width of the stem leaf.

96 **DNA extraction and molecular assays**

97 Fresh leaves were collected from randomly selected plants and dried in silica gel powder. Genomic DNA was extracted
98 using a CTAB activated charcoal protocol (Sheidai et al. 2013). The quality of extracted DNA was examined by
99 electrophoresis on 0.8% agarose gels. four ISSR primers custom synthesized by UBC (the University of British
100 Columbia) were used: UBC807, UBC810, UBC 834, (AGC) 5GA. PCR reactions were performed in a 25µl volume
101 containing at final concentrations 10 mM Tris-HCl buffer at pH 8, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each
102 dNTP (Bioron, Germany), 0.2 µM of a single primer, 20 ng genomic DNA and 3 U of Taq DNA polymerase (Bioron,
103 Germany). The DNA amplification reactions were performed in a thermocycler (Germany) with the following

104 program: 5 min initial denaturation step at 94°C, 45s at 94°C; 1min at 50°C and 1:30 min at 72°C. The reaction was
105 completed with a 7 min extension step at 72°C. The amplification products were visualized by electrophoresis on 2%
106 agarose gels, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular
107 size ladder (Fermentas, Germany).

108 The ITS5 region was amplified with 0.2 µM primer ITS5 (5'-GGA AGT AAA AGTCGT AAC AAG G-3'; Bioron,
109 Germany), and primer ITS4 (5'-TCC TCC GCT TATTGA TAT GC -3') (White et al. 1990). PCR reactions were
110 performed in a 25µl volume containing at final concentration 10 mM Tris-HCl buffer at pH 8, 50 mM KCl, 1.5 mM
111 MgCl₂, 0.2 mM of each dNTP (Bioron, Germany), 20 ng genomic DNA and 3 U of Taq DNA polymerase (Bioron,
112 Germany). The following thermocycler parameters were used: 95°C for 2 min, followed by 33 cycles at 95°C for 30s,
113 56°C for 60s, and 72°C for 2 min, followed by one final extension step at 72°C for 7 min.

114 The plastid intergenic spacer psbA-trnH(GUG) was amplified and sequenced with universal primers following the
115 methodology of Shaw et al. (2005) and Timme et al. (2007). The psbA-trnHGUG forward primer was trnHGUG (5'-
116 CGC GCA TGG TGG ATT CAC AAT CC-3') and, the reverse primer was psbA (5'- GTT ATG CAT GAA CGT
117 AAT GCT C-3') (Table 2). Each 20 µl PCR reaction contained 10 µl of 2x PCR buffer, 0.5 mM of each primer, 200
118 mM of each dNTP, 1 Unit of Taq DNA polymerase (Bioron, Germany), and 1 µl of template genomic DNA at 20 ng
119 µl-1. The amplifications were performed in a Techne thermocycler (Germany) with the following program: 2 min
120 initial denaturation step 94°C, 1 min at 94°C; 1 min at 58°C and 1 min at 72°C. The reaction was completed by a final
121 extension step of 6 min at 72°C.

122 The PCR products were electrophoresed on 2.5% agarose gels and visualized through GelRed™ Nucleic Acid Gel
123 Staining. Fragment size was estimated using a 100 bp size ladder (Thermo- Fisher Scientific, Waltham, MA USA).

124 **Data analysis**

125 **Morphological analyses**

126 Grouping of the species was obtained by using UPGMA (Unweighted Paired Group Method with Arithmetic mean)
127 and Ward (Minimum spherical cluster method) as well as PCoA (Principal Coordinate Analysis) (Podani, 2000).
128 Morphological characters were first standardized (Mean = 0, Variance = 1) and used to establish Euclidean distances
129 among pairs of taxa (Podani, 2000; Sheidai et al., 2014). The obtained distances were used for clustering.

130 Morphological difference of the studied species was investigated by ANOVA (Analysis of Variance) and CVA
131 (Canonical Variance Analysis). PCA (Principal Components Analysis) was performed to identify the most variable
132 morphological characters (Podani, 2000). Morphometric analyses were performed by PAST ver. 2.17 (Hammer et
133 al., 2012).

134 **ISSR analysis**

135 Significances of genetic differences among the studied species was determined by AMOVA (Analysis of Molecular
136 Variance) with 1000 permutations for dominant molecular markers as implemented in GenAlex v.6.4 (Peakall and
137 Smouse 2006). Nei's genetic distances were determined among the studied species and used for clustering. For
138 grouping of the plant specimens, Neighbor Joining (NJ) clustering and PCoA were used (Podani 2000). GenAlex 6.4,
139 and PAST v.2.17 (Hammer et al. 2012), programs were used for these analyses. The genetic structure of species was
140 studied by STRUCTURE (Pritchard et al. 2000), for dominant markers (Falush et al. 2007), using the admixture
141 model. The Markov chain Monte Carlo simulation was run 20 times for each value of K for 106 iterations after a burn-
142 in period of 105. All other parameters were set at their default values.

143 **Cp-DNA and nuclear gene sequence analyses**

144 The intergenic chloroplast spacer psbA-trnHGUG as well as the ITS region nuclear sequences obtained for all the
145 studied species. The sequences obtained were aligned by MUSCLE program implemented in MEGA ver. 5 (Tamura
146 et al. 2011) and used to study the species relationship by different phylogenetic reconstruction methods like: Neighbor
147 Joining, UPGMA clustering (Unweighted paired group using average), and maximum parsimony and maximum
148 likelihood using MEGA v. 5 (Tamura et al. 2011) The proper model for sequence evolution was determined by the
149 same option provided in MEGA program.

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155 Table 1. *Tragopogon* species studied, their locality information, code and voucher number

	<i>Species</i>	<i>code</i>	<i>Locality</i>	<i>Voucher number</i>
1	<i>T. capitatus</i>	Cap	Iran: East Mazandaran	W-2000/06412
2	<i>T. vaginatus</i>	Vag1	Iran: West Azerbaijan: Sardasht- Piranshahr(IS)	TARI-7748
2	<i>T. vaginatus</i>	Vag2	Iran: West Azerbaijan: Ardalan village (IS)	TARI-65501
2	<i>T. vaginatus</i>	Vag3	Iran: Khalkhal to Ardebil	TARI-86534
2	<i>T. vaginatus</i>	Vag4	Iran: East Azerbaijan: Sahand (IS)	TARI-30669
2	<i>T. vaginatus</i>	Vag5	Iran: Kurdistan: Karabad (IS)	TARI-644
2	<i>T. vaginatus</i>	Vag6	Iraq: Sulaimaniya, inte Sulaimaniya et Dokan	W-1991/4989
2	<i>T. vaginatus</i>	Vag7	Iraq: Sulaimaniya, inter Kirkuk to ward Sulaimaniya	W- 1991/19529
3	<i>T. afghanicus</i>	Afg1	Afghanistan: qala salcan	W- 1956/3032
3	<i>T. afghanicus</i>	Afg2	afghanistan: Khurak	W-1956/3122
4	<i>T. paradoxus</i>	Par1	Afghanistan: N Darai suf, between qala sarkari and kupruk	W-1987/4287
4	<i>T. paradoxus</i>	Par2	Afghanistan: nordl. Pul e khomrie	W-1965/15880
5	<i>T. kemulariae</i>	Kem1	Iran: West Azerbaijan: Khoy, Qoture (IS)	TARI-84101
5	<i>T. kemulariae</i>	Kem2	Iran: Hamadan: Fakhr abad (IS)	TARI-4254
5	<i>T. kemulariae</i>	Kem3	Iran: Kurdistan: 20 km toward Hamadan (IS)	TARI-84907
5	<i>T. kemulariae</i>	Kem4	Iran: Weast Azerbaijan: Khoy, Grees village (IS)	TARI-84132
5	<i>T. kemulariae</i>	Kem5	Iran: Ardebil: Arasbaran (IS)	TARI-24712
5	<i>T. kemulariae</i>	Kem6	Iran: Ardebil: Arasbaran (IS)	TARI-24651
6	<i>T. sosnowsky</i>	Sos	Armenia,Sevan	W-2004/2395
7	<i>T. vvedenskyi</i>	Vve1	Afghanistan: Farakulum	W-1980/3311

7	<i>T. vvedenskyi</i>	Vv2	Iran: Tehran: Haraz valley	TARI-85712
7	<i>T. vvedenskyi</i>	Vv3	Iran: West Azerbaijan: Silvana to Salmas road (IS)	TARI-85193
7	<i>T. vvedenskyi</i>	Vv4	Iran: Semnan	TARI-40684
7	<i>T. vvedenskyi</i>	Vv5	Iran: Isfahan: Golestan koh (IS)	TARI-83087
7	<i>T. vvedenskyi</i>	Vv6	Iran: Gorgan: Almehr	TARI-12618
7	<i>T. vvedenskyi</i>	Vv7	Iran: Gorgan: Almehr	TARI-12619
7	<i>T. vvedenskyi</i>	Vv8	Iran: Mazandaran: Plur to Rineh (4 IS)(cp)(ITS)	HSBU-2018800
8	<i>T. longirostris</i>	Long1	Iran: Tehran: solghan valley	TARI-32617
8	<i>T. longirostris</i>	Lon2	Iran: Khorasan: Tandureh,Chehel mehr	TARI-50839
8	<i>T. longirostris</i>	Long3	Iran: Chahar mahale bakhtiari:Shalam zar,Agha saner	TARI-54586
8	<i>T. longirostris</i>	Long4	Iran: West Azerbaijan: Urmieh lake,Islamic island	TARI-86916
8	<i>T. longirostris</i>	Long5	Iran: Kermanshah	TARI-60769
8	<i>T. longirostris</i>	Long6	Iran: Kurdistan: Baneh to Marivan	TARI-29317
8	<i>T. longirostris</i>	Long7	Iran: Gorgan: Almehr	TARI-12620
8	<i>T. longirostris</i>	Long8	Iran: Fars:Nor abad	TARI-45696
8	<i>T. longirostris</i>	Long9	Iran: Alborz: Karaj- Chalus (IS)	HSBU-2018801
8	<i>T. longirostris</i>	Long1 0	Iran: Kurdistan: Baneh	HSBU-2018802
8	<i>T. longirostris</i>	Long1 1	Iran: Kurdistan: Saqez, Zagheh (2 IS)	HSBU-2018803
8	<i>T. longirostris</i>	Long1 2	Syria *	W-1980/296324
8	<i>T. longirostris</i>	Long1 3	Iraq: Sulaimaniya, inter Kirkuk to ward Sulaimaniya	W-1980/19526
8	<i>T. longirostris</i>	Long1 4	E- Afghanistan: Kabul, Band-I Kharghak	W-1980/619
9	<i>T. badakhshanicus</i>	Bad	Afghanistan: Wakhan, Ab-Gaj	W-1965/18843

10	<i>T. gracilis</i>	Grac1	Afghanistan: Paktya, Kotgay, Mandaher wald	W-1980/1725
10	<i>T. gracilis</i>	Grac2	East Afghanistan: Khost, chakmanni	W-1980/777
11	<i>T. gongylorrhizus</i>	Gon1	Iran: West Azerbaijan: Urmieh lake, Spireh island	TARI-15052
11	<i>T. gongylorrhizus</i>	Gon2	Iran: Gorgan #	TARI-15053
11	<i>T. gongylorrhizus</i>	Gon3	Iran: Gorgan: Golestan Park (IS)	W-1980/5141
11	<i>T. gongylorrhizus</i>	Gon4	Iran: Gorgan	TARI-5123
12	<i>T. buphthalmoides subsp. buphthalmoides</i>	Bupb1	Iraq: Suleimaniya to Ghala Daze	W-1980/19531
12	<i>T. buphthalmoides subsp. buphthalmoides</i>	Bupb2	Iran: Tehran: Dehnaar(IS)	HSBU -4006
12	<i>T. buphthalmoides subsp. buphthalmoides</i>	Bupb3	Iran: West Azerbaijan: Sardasht, Gerzhal(IS)	HSBU -4007
12	<i>T. buphthalmoides subsp. buphthalmoides</i>	Bupb4	Iran: West Azerbaijan: Jaldian	HSBU -4008
12	<i>T. buphthalmoides subsp. buphthalmoides</i>	Bupb5	Iran: Mazandaran: Siah bisheh(IS)	HSBU -4009
12	<i>T. buphthalmoides subsp. buphthalmoides</i>	Bupb6	Iran: Mazandaran: Rineh	HSBU -4010
12	<i>T. buphthalmoides subsp. buphthalmoides</i>	Bupb7	Iran: Alborez: Gach sar (IS)	HSBU -4011
12	<i>T. buphthalmoides subsp. buphthalmoides</i>	Bupb8	Iran: Kurdistan: Kani bard	HSBU -4012
12	<i>T. buphthalmoides subsp. buphthalmoides</i>	Bupb9	Iran: Isfahan: Naein (IS)	HSBU -4013
12	<i>T. buphthalmoides subsp. buphthalmoides</i>	Bupb10	Iran: Semnan	HSBU -4014
12	<i>T. buphthalmoides subsp. Latifolius</i>	Buphb11	Iran: Kurdistan: Saqez	HSBU -4015

12	<i>T. bupthalmoides</i> <i>subsp. Latifolius</i>	Bupl1	Iran: Kurdistan: Boein (IS)	HSBU -4016
12	<i>T. bupthalmoides</i> <i>subsp. Latifolius</i>	Bupl2	Iran: Mazandaran: Rineh (IS)	HSBU -4017
12	<i>T. bupthalmoides</i> <i>subsp. Latifolius</i>	Bupl3	Iran: Alborez: Gachsar (IS)	HSBU -4018
12	<i>T. bupthalmoides</i> <i>subsp. Latifolius</i>	Bupl4	Iran: Tehran: Dehnar (IS)	HSBU -4019
12	<i>T. bupthalmoides</i> <i>subsp. Latifolius</i>	Bupl5	Iran: West Azerbaijan: Mosalan	HSBU-4020
12	<i>T. bupthalmoides</i> <i>subsp. Latifolius</i>	Bupl6	Iran: West Azerbaijan: Sardasht, Gerzhal (IS)(ITS)	HSBU-4021
13	<i>T. rechingeri</i>	Rec1	Iran:(Hamadan: Agh blagh (IS)	TARI-3392
13	<i>T. rechingeri</i>	Rec2	Iran: Hamadan: 20 km Asad abad to Akhtachi (IS) village	TARI-3443
13	<i>T. rechingeri</i>	Rec3	Iran: Hamadan: 20 km Asad abad to Akhtachi village(IS)	TARI-3444
13	<i>T. rechingeri</i>	Rec4	Iran: Hamadan:8 km East Ganj nameh (IS)	TARI-36848
13	<i>T. rechingeri</i>	Rec5	Iraq: Kurdistan: Erbil, Haji omran *	W-1980/5240
14	<i>T. bornmuelleri</i> <i>var. bornmuelleri</i>	Borb1	Iran: Kurdistan: 36 km Sanandaj to Kamiaran, (2IS) Noshor Valley	TARI-60592
14	<i>T. bornmuelleri</i> <i>var. bornmuelleri</i>	Borb2	Iran: Kurdistan: Maran village (2IS)	TARI-60418
14	<i>T. bornmuelleri</i> <i>var. bornmuelleri</i>	Borb3	Iran: Kurdistan: Saqez to Baneh (2 IS)	TARI-2951

14	<i>T. bornmuelleri</i> <i>var. bornmuelleri</i>	Borb4	Iran: Kurdistan: Ravandoz	W-1986/1645
14	<i>T. bornmuelleri</i> <i>var.</i> <i>bornmuellerVai</i>	Borb5	Iran: Kurdistan: Riwandous in m. Sakrisakran	W-1966/5248
14	<i>T. bornmuelleri</i> <i>var. bornmuelleri</i>	Borb6	Iraq: Kurdistan:Erbil	W-1980/11190
14	<i>T. bornmuelleri</i> <i>var. latifolius</i>	Bor11	Iran: Kermanshah to Kamiaran (IS)(ITS)	TARI-87735
14	<i>T. bornmuelleri</i> <i>var. latifolius</i>	Bor12	Iran: Kermanshah:Mansure agha village, Shahoo mountain	TARI-89153
15	<i>T. acanthocarpus</i>	Aca1	Iran: Mazandaran: Rineh, Abgarm	TARI-5486
15	<i>T. acanthocarpus</i>	Aca2	Iran: Zanjan: 10 km Mah neshan, Ghezal ozan river	TARI-61228
15	<i>T. acanthocarpus</i>	Aca3	Iran: East Azerbaijan: Marand to Jolfa, Zenoz, 22 km Zenoz to Kohe Kamar	TARI-84034
15	<i>T. acanthocarpus</i>	Aca4	Iran: West Azerbaijan: Urmieh University (IS)	TARI-17743
15	<i>T. acanthocarpus</i>	Aca5	Iran: Alborz: Karaj, Research center to Alborz	TARI-1340
15	<i>T. acanthocarpus</i>	Aca6	Iran: Hamadan: Absard (IS)	TARI-5464
15	<i>T. acanthocarpus</i>	Aca7	Iran: Markazi: Arak, Miran khaneh, Sefid khani (IS)	TARI-47745
15	<i>T. acanthocarpus</i>	Aca8	Iran: Mazandaran: Dashte Nazir (IS)	HSBU-2018804
15	<i>T. acanthocarpus</i>	Aca9	Iran: Kurdistan: Salavat abad village (IS)	HSBU-2018805
15	<i>T. acanthocarpus</i>	Aca10	Iran: Tehran: Firuz koh (IS)	HSBU-2018806
16	<i>T. graminifolius</i>	Gra1	Iran: Tehran: Chitgar (IS)	HSBU-14466
16	<i>T. graminifolius</i>	Gra2	Iran: North Khorasan:Ziarat, Shirvan (IS)	HSBU-14466
16	<i>T. graminifolius</i>	Gra3	Iran: East Azarbaijan: Ilkhchi (IS)	HSBU-14469
16	<i>T. graminifolius</i>	Gra4	Iran: West Azarbaijan: Orumieh	HSBU-14470
16	<i>T. graminifolius</i>	Gra5	Iran: Markazi: Arak, Gavkhaneh(IS)	HSBU-14471
16	<i>T. graminifolius</i>	Gra6	Iran: Isfahan: Semirom (IS)	HSBU-14472

17	<i>T. reticulatus</i>	Ret1	Iran: West Azarbaijan: Orumieh lake, Kabodan island	TARI-24916
17	<i>T. reticulatus</i>	Ret2	Iran: Khozestan: Deh dez (IS)	TARI-63121
17	<i>T. reticulatus</i>	Ret3	Iran: West Azarbaijan: Ghasemloo(IS)	TARI-4184
17	<i>T. reticulatus</i>	Ret4	Iran: Hamedan: Abas abad(IS)	TARI-4252
17	<i>T. reticulatus</i>	Ret5	Iran: kurdistan:baneh,boeen(IS)	TARI-2081
17	<i>T. reticulatus</i>	Ret6	Iran: Mazandaran:Polor,Haraz Road	TARI-4461
18	<i>T. kotschy</i>	Kot1	#	W-1986/32097
18	<i>T. kotschy</i>	Kot2	Iran: Mazandaran:91 Km Karaj to Chalous(IS)	TARI-5430
18	<i>T. kotschy</i>	Kot3	Iran: East Azerbaijan :Kandovan(IS) (cp)	TARI-4504
18	<i>T. kotschy</i>	Kot4	Iran: Tehran:gajereh(IS)	TARI-24193
18	<i>T. kotschy</i>	Kot5	Iran: Tehran:Gajereh(IS)	TARI-5473
18	<i>T. kotschy</i>	Kot6	Iran: Mazandaran:8 km to polor	TARI-86047
18	<i>T. kotschy</i>	Kot7	Iran: Gorgan:Aalmeh	TARI-12619
18	<i>T. kotschy</i>	Kot8	Iran: Lorestan:Khoram Abad(IS)	HSBU-2018813
19	<i>T. marginatus</i>	Mar1	Iran: West Azarbayejan:20 Km to Seyah Rod	TARI-86729
19	<i>T. marginatus</i>	Mar2	Iran: West Azarbayjan :Arasbaran,Topkhaneh	TARI-81373
19	<i>T. marginatus</i>	Mar3	Iran: Karaj:Kondar(IS)	TARI-4374
19	<i>T. marginatus</i>	Mar4	Iran: Yazd:Tarzjan Mountain,South Eastern Shirkoh	TARI-1452
19	<i>T. marginatus</i>	Mar5	Iran: West Azarbayjan: Uromieh,Sero Road(IS)	TARI-7708
19	<i>T. marginatus</i>	Mar6	Iran: Ardabil:Arasbaran(IS)	TARI-20954
19	<i>T. marginatus</i>	Mar7	Iran: West Azarbayjan:Sahand Mountain(IS)	TARI-99404
19	<i>T. marginatus</i>	Mar8	Iran: Gilan:Damash to Jirandeh	TARI-13515
20	<i>T. maturatus</i>	Mat	Iran: Golestan:Golestan jungle,Tangeh Rah	W-1976/10148
21	<i>T. coloratus</i>	Colo1	Iran: Gorgan	TARI-296322

21	<i>T. coloratus</i>	Colo2	Iran: Gorgan:18 Km from Maraveh Tapeh to Ash Khaneh	TARI-55598
21	<i>T. coloratus</i>	Colo3	Iran: Gorgan:43 Km Rodbar to Gilan	TARI-60180
21	<i>T. coloratus</i>	Colo4	Iran: Azarbayjan:30 Km Southern Khalkhal(IS)	TARI-36251
21	<i>T. coloratus</i>	Colo5	Iran: Zanjan:25 Km from Gilvan to Zanjan	TARI-60280
21	<i>T. coloratus</i>	Colo6	Iran: Tehran:Sorkkeh Hesar(IS)	TARI-5476
21	<i>T. coloratus</i>	Colo7	Iran: Tehran:Hesarak	TARI-5481
21	<i>T. coloratus</i>	Colo8	Iran: Arak:Miran house,Kooh Sefid Khani(IS)	TARI-47748
21	<i>T. coloratus</i>	Colo9	Iran: Kermanshah:Kamyaran,Varmanjeh,Padegan (IS) Shahid Rajaei	TARI-87737
21	<i>T. coloratus</i>	Colo10	Iran: Kermanshah:Tagh Bostan,Tangeh Konesht(IS)	TARI-87738
21	<i>T. coloratus</i>	Colo11	Iran: Ardabil:Arasbaran protect region,Shib (IS) Jonobi Jangal Tahghighati	TARI-81348
22	<i>T. pterocarpus</i>	Pte1	Iran: Chahar Mahal Bakhtyari:Shahr Kord,Shams Abad	TARI-96641
22	<i>T. pterocarpus</i>	Pte2	Arak:Arak to Khomeyn,Koh Vercheh to Istgah Macrowave Iran:	TARI-48044
22	<i>T. pterocarpus</i>	Pte3	Iran: Ghazvin:Almot Region,Balaye Rostaye Moalem Kalateh	TARI-50992
22	<i>T. pterocarpus</i>	Pte4	Iran: Alborz:40 Km Karaj Chalos,Seyeda Chal	TARI-5447
22	<i>T. pterocarpus</i>	Pte5	Iran: Ardabil:8 Km north Khalkhal,Kelar Abad Road	TARI-34091
22	<i>T. pterocarpus</i>	Pte6	Iran: Isfahan:Ghamishlo protect region,Khersak(IS)	TARI-1074
22	<i>T. pterocarpus</i>	Pte7	Iran: Kurdistan:36km Sanandaj to Kamyaran, (IS) noshur valley	TARI-60624
22	<i>T. pterocarpus</i>	Pte8	Iran: West Azarbaijan: Khoy, Razy valley(IS)	TARI-1392
22	<i>T. pterocarpus</i>	Pte9	Iran: Semnan: Abr jungle (IS)	HSBU-2018814
22	<i>T. pterocarpus</i>	Pte10	Iran: Tehran: Chalooos road, Moroud (IS)	HSBU-2018815
23	<i>T. collinus</i>	Coli1	Iran: Gilan: between Gazvin and Rudbar	TARI-27662

23	<i>T. collinus</i>	Coli2	Iran: Khorasan: Birjand, Western part, Asfaraz village	TARI-83349
23	<i>T. collinus</i>	Coli3	Iran: Khorasan: 42 km Birjand	TARI-84745
23	<i>T. collinus</i>	Coli4	Iran: Khorasan: Birjand, around Sar bishe, (IS) Salmabad	TARI-83340
23	<i>T. collinus</i>	Coli4	Iran: Tehran: Saveh, Rud shor (IS)	TARI-9632
23	<i>T. collinus</i>	Coli5	Iran: Tehran: Saveh, Rud shor(IS)	TARI-9633
23	<i>T. collinus</i>	Coli6	Iran: Isfahan: Semirom, Abshar (IS)	TARI-88600
23	<i>T. collinus</i>	Coli7	Iran: Isfahan: Semirom, Abshar(IS)	TARI-88601
24	<i>T. caricifolius</i>	Car1	Iran: Tehran: Homand, Absard	TARI-5465
24	<i>T. caricifolius</i>	Car2	Iran: Mazandaran: Chalus, Dashte Nazir(IS)	TARI-1377
24	<i>T. caricifolius</i>	Car3	Iran: Karaj: 12 km northwest Karaj	TARI-5470
24	<i>T. caricifolius</i>	Car4	Iran: East Azarbaijan: 20 km Tabriz to Tehran	TARI-28013
24	<i>T. caricifolius</i>	Car5	Iran: Yazd: 50 km east Bafegh, Hamsuk village(IS)	TARI-56083
24	<i>T. caricifolius</i>	Car6	Iran: Bandar Abas: Geno mountain	TARI-16083
24	<i>T. caricifolius</i>	Car7	Iran: Kerman: 50 kmWest of Khajeh Mountain (IS)	TARI-56206
24	<i>T. caricifolius</i>	Car8	Iran: Baluchestan: Taftan mountain, Tamndan (IS) region	TARI-53202
24	<i>T. caricifolius</i>	Car9	Iran: Tehran: Darband sar(IS)	TARI-49230
25	<i>T. bakhtiaricus</i>	Bak1	Iran: Chahar mahale Bakhtiari: Brojen, Research Institute of Forests and Rangelands (IS)(cp)	TARI-54377
25	<i>T. bakhtiaricus</i>	Bak2	Iran: Chahar rmahale Bakhtiari: Brojen, Bar aftar (IS) mountain	TARI-54767
25	<i>T. bakhtiaricus</i>	Bak3	Iran: Fars: Kharman mountain(IS)	TARI-46922
25	<i>T. bakhtiaricus</i>	Bak4	Iran: Chahar mahale Bakhtiari:Vardandjan to (IS) Kakolak	TARI-54126
25	<i>T. bakhtiaricus</i>	Bak5	Iran: Gilan: Loshan to Amar loo(IS)	TARI-5445
26	<i>T. gaudanicus</i>	Gau1	Iran: Fars: Kazeron, ketel (pir zan)(IS)	TARI-9145

26	<i>T. gaudanicus</i>	Gau2	Iran: North Khorasan: Birjand, Kharashad(IS)	TARI-12137
26	<i>T. gaudanicus</i>	Gau3	Iran: Kurdistan: around Salavat abad river, East (IS) Sanandaj	TARI-297
26	<i>T. gaudanicus</i>	Gau4	Iran: Khorasan: Hezar masjed mountain(IS)	TARI-4842
26	<i>T. gaudanicus</i>	Gau5	Afghanistan: Abe chist	W-1980/3031
27	<i>T. montanus</i>	Mon1	Iran: Tehran: Firoz koh, Veresk bridge (IS)	TARI-1342
27	<i>T. montanus</i>	Mon2	Iran: Golestan: Gorgan,Golestan jungle park, near (IS) Bojnord	TARI-11056
27	<i>T. montanus</i>	Mon3	Iran: Golestan: Gorgan,Golestan jungle park, near (IS) Bojnord	TARI-11057
27	<i>T. montanus</i>	Mon4	Iran: Golestan: Gorgan,Golestan jungle park, near Bojnord	TARI-11058
27	<i>T. montanus</i>	Mon5	Iran: Golestan: Almeh jungle (IS)	TARI-4284
27	<i>T. montanus</i>	Mon6	Iran: Golestan: Almeh jungle (IS)	TARI-4285
27	<i>T. montanus</i>	Mon7	Iran: Golestan: Almeh jungle(IS)	TARI-4286
27	<i>T. montanus</i>	Mon8	Afghanistan: 65 km north west Harat	W-1980/4276
28	<i>T. erostris</i>	Ero	Iran: Kurdistan: Sanandaj to Kamyaran, Avalan mountain	TARI-9439
29	<i>T. jesdianus</i>	Jez1	Iran: Yazd: Taft to Nier, Sakhoid neck (IS)	TARI-77549
29	<i>T. jesdianus</i>	Jez2	Iran: Semnan: Shahrood, Shish mountain(IS)	TARI-28673
29	<i>T. jesdianus</i>	Jez3	Iran: Yazd: Nodoushan, between Sadr abad and Hemat abad	TARI- 77777
29	<i>T. jesdianus</i>	Jez4	Iran: Bandar abas: Geno mountain(IS)	TARI-39783
29	<i>T. jesdianus</i>	Jez5	Iran: Semnan: Turan protect region, western Shotor koh	TARI-28457
29	<i>T. jesdianus</i>	Jez6	Iran: Semnan: Turan protect region, 4 km to Delbar	TARI-28953
29	<i>T. jesdianus</i>	Jez7	Iran: Semnan: Turan protect region, 4 km to Delbar	TARI-28954
29	<i>T. jesdianus</i>	Jez8	Iran: Semnan: Turan protect region, southern part (IS) Kalateh mountain	TARI-28803

29	<i>T. jesdianus</i>	Jez9	Iran: Semnan: 30 km northern west Shahrud, (IS) between Tash and Chahar bagh	TARI-21160
30	<i>T. porphyrocephalus</i>	Pro1	Iran: West Azarbaijan: Rezaeieh lake	W-1964/4029
30	<i>T. porphyrocephalus</i>	Pro2	Iran: Chahar mahale Bakhtiari: Shahre kord, (IS) Harchegan	TARI-62060
30	<i>T. porphyrocephalus</i>	Pro3	Iran: Kurdistan: 40 km north Kamyaran to (IS) Sanandaj, Faghieh Soleiman	TARI-370
31	<i>T. rezaiyensis</i>	Rez1	Iran: Khorasan: Torbate Heidarieh to Mashhad, 5 km Kameh Sofla	TARI-84842
31	<i>T. rezaiyensis</i>	Rez2	Iran: Tehran: Gilavand	HSBU-2018816
31	<i>T. rezaiyensis</i>	Rez3	Iran: Tehran: Shahid Beheshti university	HSBU-2018817
31	<i>T. rezaiyensis</i>	Rez4	Iran: Tehran: Abali	HSBU-2018818
31	<i>T. rezaiyensis</i>	Rez5	Iran: West Azarbaijan: Rabus valley to Rezaeieh	W-2000/5182
32	<i>T. stroterocarpus</i>	Str	Iran: West Azarbaijan: Sardasht	W-1980/5183

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158 Results

159 Morphometry

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161 UPGMA, NJ dendrogram, Ward and PCA plot and Parsimony produced similar results therefore, only UPGMA

162 dendrogram is presented here (Fig 1). species delimitation between the studied species are determined from each other,

163 *T. capitatus*, *T. vaginatus*, *T. afghanicus*, *T. paradoxus* located in one cluster Which *T. capitatus*, *T. afghanicus* and

164 *T. paradoxus* belong to the flora of Iranica and do not exist in Iran. Populations belonging to *T. kemulariae* together,

165 populations belonging to *T. longirostris* together and populations belong to *T. gngylorhizus* to gether have formed

166 cluster.

167 *T. badachschanicus* and *T. gracilis* species and *T. sosnovsky* and *T. wedenskyi* species show the closest relationship
168 with each other. The species *T. badachschanicus*, *T. gracilis* and *T. sosnovsky* do not exist in Iran and belong to the
169 flora of Iranica.

170 *T. kotschy*, *T. marginatus* and one population of *T. reticulatus* together in a cluster and populations of *T. collinus*, *T.*
171 *caricifolius*, *T. bakhtiaricus*, *T. gaudanicus* and one population of *T. montanus* are located in the same cluster. The
172 populations of *T. buphthalmoides*, *T. bornmuelleri*, *T. rechingeri*, *T. acanthocarpus*, *T. graminifolius* with five
173 populations of *T. reticulatus* are in the same cluster. The populations of *T. pterocarpus* and *T. coloratus* are located in
174 two separated clusters and are located close to each other.

175 PCA analysis of morphological characters revealed that the first 3 PCA components comprise about 76% of total
176 variability. Diameter of inflorescence, flower color, number of bract and length of beak showed the highest level of
177 correlation with the first PCA component (40), while character length of papus, length of achene, status of involucre
178 bracts and ligule were highly correlated with the second PCA component (12.81). Therefore, these are the most
179 variable morphological characters among the studied species.

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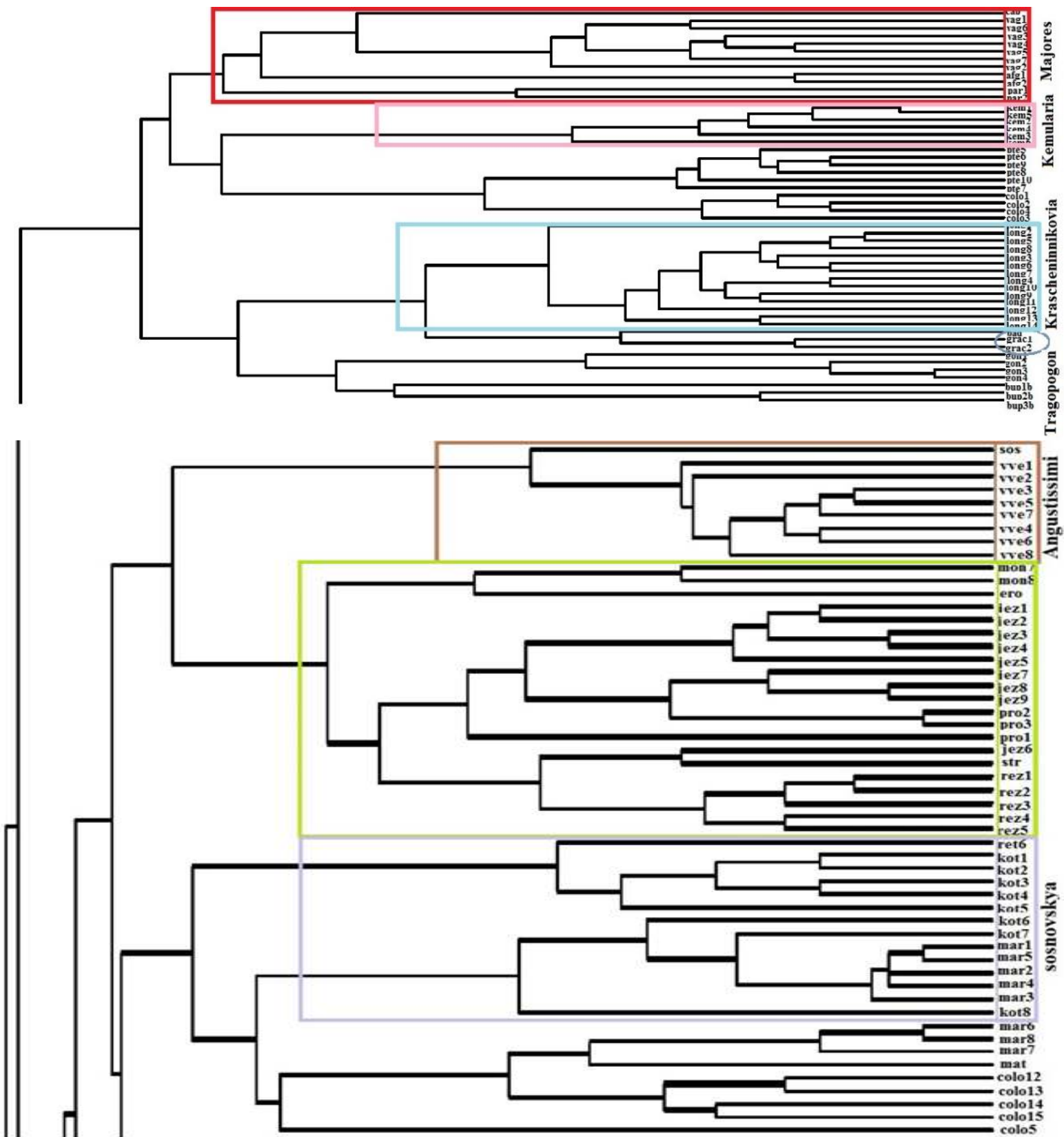
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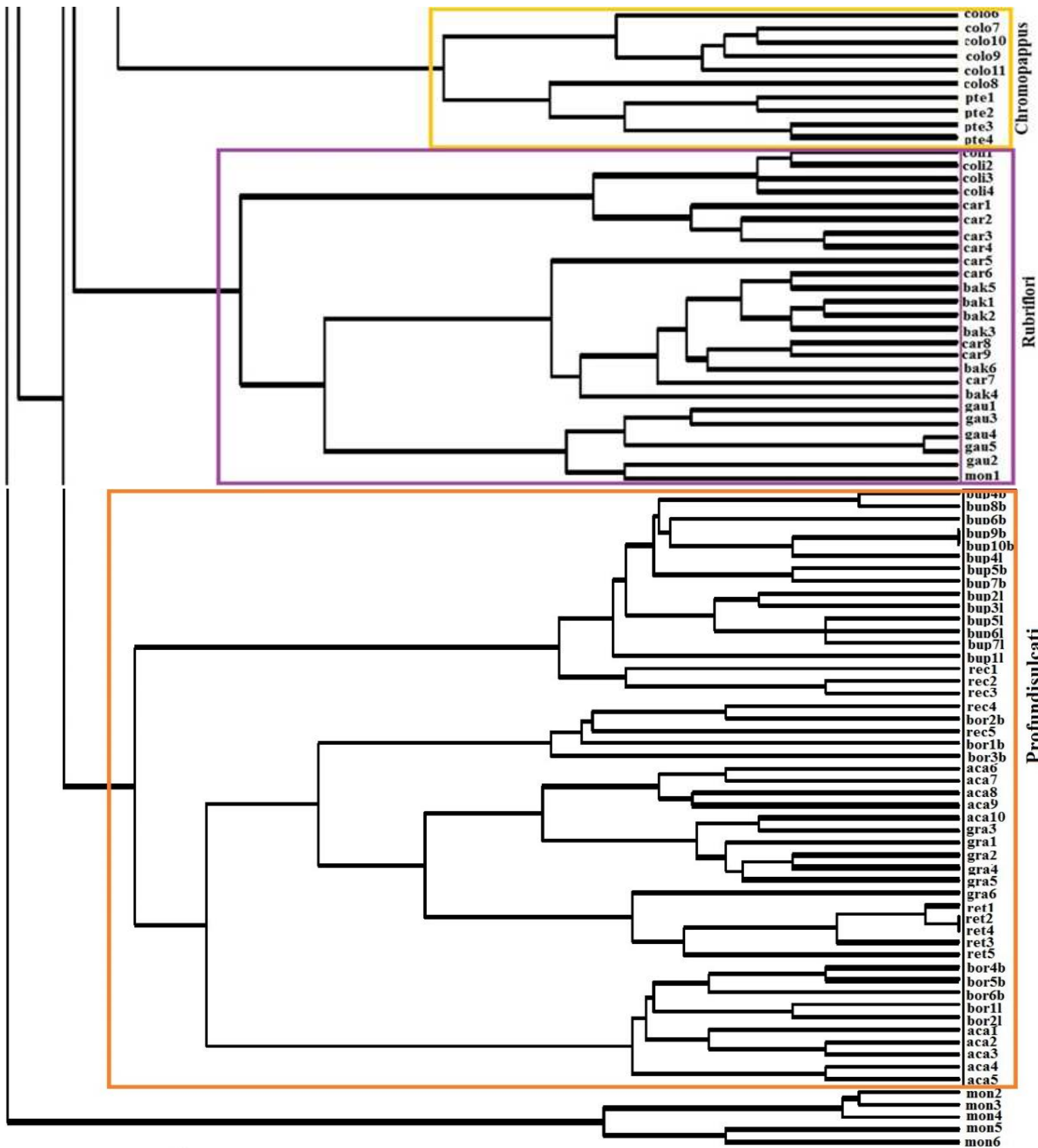
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194 Fig 1. UPGMA dendrogram of morphological characters among *Tragopogon* species (Species code are according to
195 Table 1).

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197 **ISSR markers**

198 This study was performed on 110 individuals belonging to 102 populations of 22 species of this genus. We obtained
199 153 reproducible ISSR bands from almost all ISSR primers used. These bands formed our initial data matrix. AMOVA
200 test revealed the presence of significant molecular difference among the studied populations ($P = 0.01$). It also revealed
201 that 60% of total variance occurred due within species genetic variability, while 40% was due to among species genetic
202 difference. UPGMA, NJ dendrogram and PCOA, PCA, PCO plot produced similar results. Therefore, only NJ
203 dendrogram is presented here (Fig. 2). Almost populations of each species were located close to each other. This
204 indicates that the ISSR molecular marker can be used to determine the species delimitation of *Tragopogon*. ISSR
205 determine delimitation of species and show relationship between species in this genus. ISSR can determine
206 delimitation of species and relationship between species in this genus. Two populations of *T. gaudanicus* with species
207 of *T. jesdianus*, *T. porphyrocephalus*, *T. bakhtiaricus*, *T. montanus* and *T. caricifolius* are in one main cluster.
208 Populations of *T. vvedenskyi*, *T. vaginatus*, *T. graminifolius* are located in separated cluster. Species of *T. kotschy* and
209 *T. marginatus* are showed close relationship. Populations of *T. longirostris* are located in one cluster and are closely
210 related to *T. gongylorrhizus*. A number of *T. buphthalmoides* populations and *T. bornmuelleri* populations are show
211 close relationship. All populations of *T. kemulariae* were placed in a cluster. The Evanno test produced delta $k = 7$ as
212 the best number of genetic groups. The STRUCTURE plot based on $k = 7$ (Fig. 3) identified seven genetic groups/gene
213 pools. The genetic affinity revealed by STRUCTURE analysis was almost in agreement with the NJ tree result.

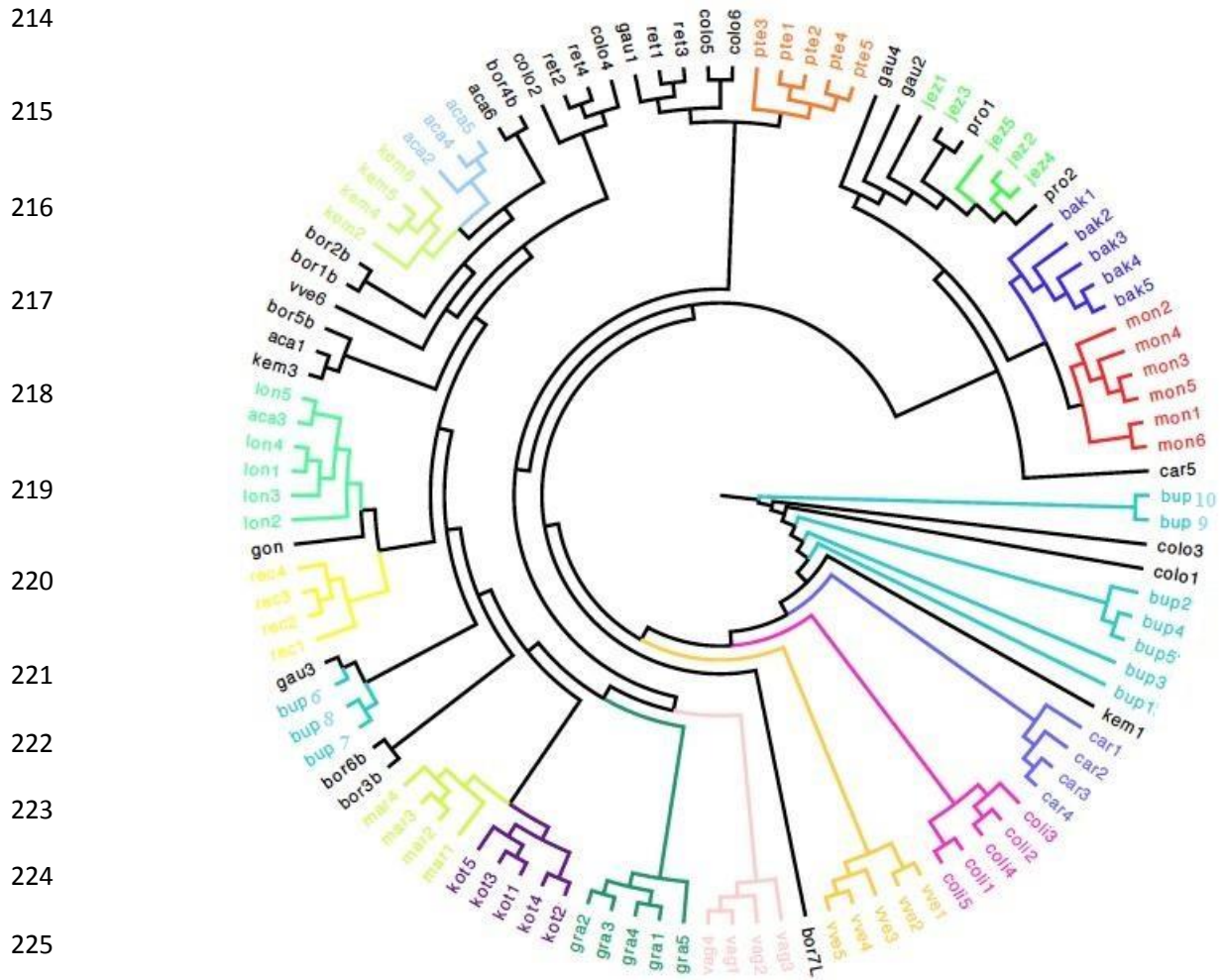
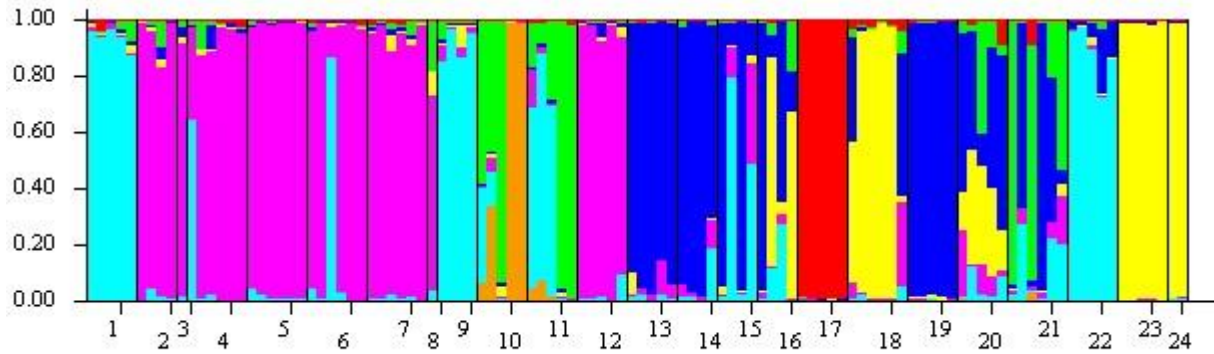


Fig 2. UPGMA tree of *Tragopogon* species based on ISSR data (Species code are according to Table 1).



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Fig 3. STRUCTURE plot of *Tragopogon* species based on ISSR

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1: *T. graminifolius*, 2: *vaginatus*, 3: *T. gongylorrhizus*, 4: *T. vvedenskyi*, 5: *T. kemulariae*, 6: *T. acanthocarpus*, 7: *T. bornmuelleri*
 241 var. *bornmuelleri*, 8: *T. bornmuelleri* var. *latifolius*, 9: *T. rechingeri*, 10: *T. bupthalmoides* var. *bupthalmoides*, 11: *T.*
 242 *bupthalmoides* var. *latifolius*, 12: *T. longirostris*, 13: *T. kotschy*, 14: *T. marginatus*, 15: *T. reticulatus*, 16: *gaudanicus*, 17: *T.*
 243 *bakhtiaricus*, 18: *T. montanus*, 19: *T. collinus*, 20: *T. caricifolius*, 21: *T. coloratus*, 22: *T. pterocarpus*, 23: *T. jesdianus*, 24: *T.*
 244 *porphyrocephalus*.

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246 ITS and Cp-DNA sequences

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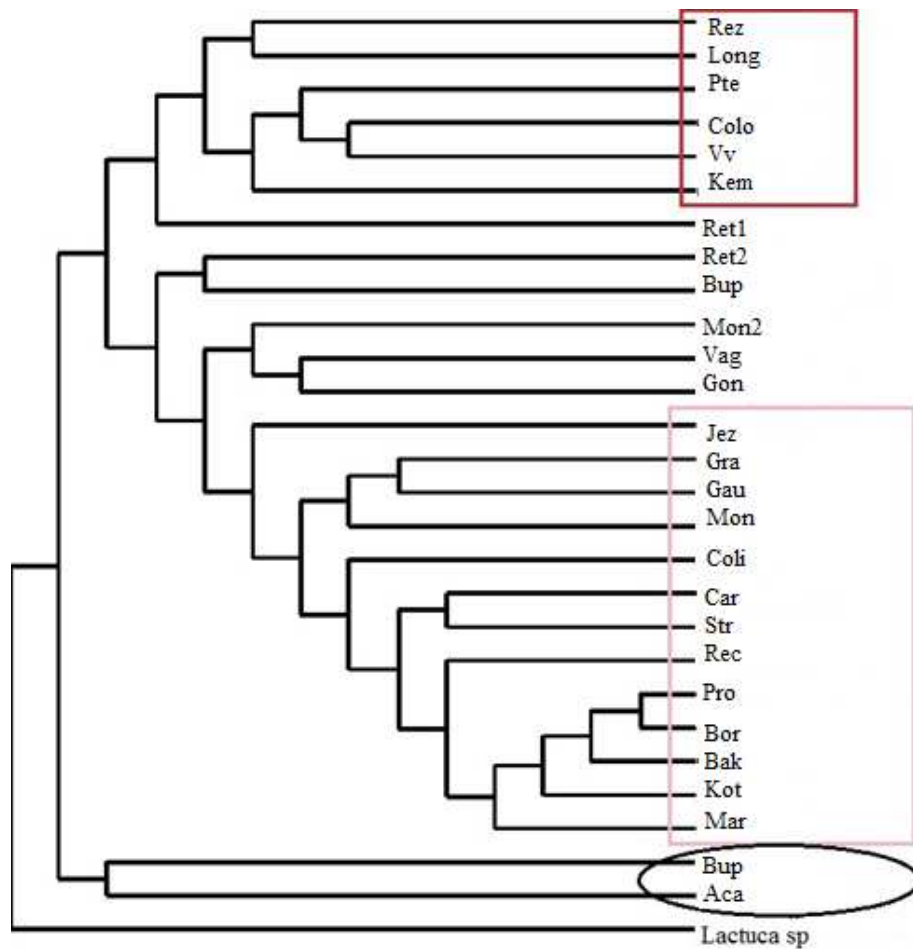
NJ, Maximum parsimony and maximum likelihood trees produced similar results and therefore ML tree is presented
 248 and discussed (Fig 4). The ML tree based on ITS has two main clusters, *T. bornmuelleri* var. *latifolius* is separated
 249 from the rest of the species and second cluster is divided into two sub-clusters, One of the sub-clusters includes *T.*
 250 *bupthalmoides* and *T. acanthocarpus*. The rest of the species are arranged in sub clusters. *T. gaudanicus*, *T. montanus*,
 251 *T. collinus*, *T. caricifolius*, *T. jesdianus*, *T. stroterocarpus*, *T. porphyrocephalus*, *T. bakhtiaricus*, *T. graminifolius*, *T.*
 252 *rechingeri*, *T. bornmuelleri*, *T. kotschy* and *T. marginatus* are in one sub-cluster and *T. rezaiyensis*, *T. longirostris*, *T.*
 253 *pterocarpus*, *T. coloratus*, *T. vvedenskyi*, *T. kemulariae* are in one sub-cluster. The tree based on cp DNA data showed
 254 poor clustering (Fig 5). *T. marginatus*, *T. colinus* and *T. rechingeri* are arranged in one cluster. Cp DNA data are not
 255 suitable for classifying and examining relationship between species in this genus, The results based on ITS sequences
 256 are more efficient than the results based on cp DNA for species delimitation and relationship between species.

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264 Fig 4. ML tree based on ITS sequence data in *Tragopogon* genus (*Lactuca sp.*: out group, Species code are according
265 to Table 1).

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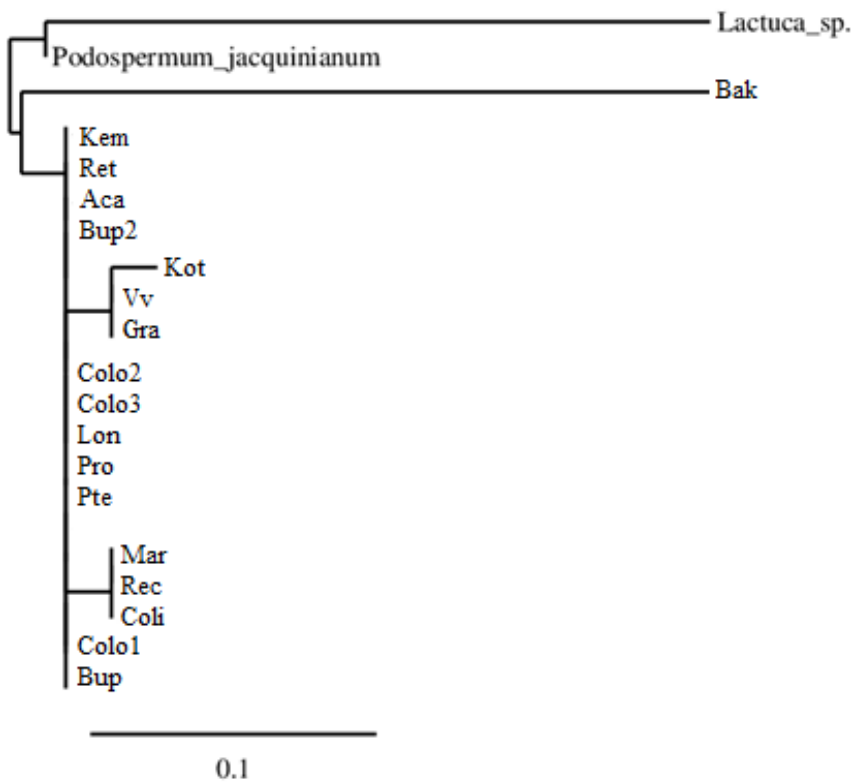
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288 Fig 5. ML tree based on cp DNA data in *Tragopogon* genus (Lactuca sp: out group, Species code are according to
289 Table 1).

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292 **DISCUSSION**

293 Based on the morphological studies, the species related to each section were placed close to each other and the border
294 between the sections is almost clear. Species of section Majores (*T. capitatus*, *T. vaginatus*, *T. afghanicus*, *T.*
295 *paradoxus*) section of Kemulariae (*T. kemulariae*) section of Krascheninnikovia (*T. longirostris*) section of
296 Tuberosi(*T. gongylorrhizus*)section of *Tragopogon* (*T. gracilis*, *T. badachschanicus*) section of Angustissimi((*T.*
297 *sosnovsky* , *T. vvedenskyi*) are clustered based on morphometric data and correspond to the sections of Iranica flora .
298 Marodive et al. 2005 have proven that *Tragopogon* and *Majores* sections are monophylic based on ITS-ETS data.
299 Sections include Kemulariae (*T. kemulariae*), Krascheninnikovia (*T. longirostris*) Tuberosi (*T. gongylorrhizus*)

300 confirmed based on ISSR data. Majores, Angustissimi, Krascheninnikovia and Tuberosi sections have been confirmed
301 based on morphometric and ISSR studies, which are consistent with studies by Azizi et al. 2021 and Iranica flora.

302 Species of *T. kotschy*, *T. marginatus*, *T. reticulatus* and *T. maturatus* are introduced according to the flora of Iranica
303 in Sosnowskya section, which in the present morphometric studies, except for *T. maturatus*, are arranged in a cluster.
304 Based on morphometric data of *T. collinus*, *T. caricifolius*, *T. bakhtiaricus*, *T. gaudanicus* and *T. montanus* are in the
305 same cluster and correspond to section Rubriflori in the flora of Iranica.

306 *T. buphthalmoides*, *T. bornmuelleri*, *T. rechingeri*, *T. acanthocarpus*, *T. graminifolius*, *T. reticulatus* are based on
307 morphometric data in a cluster, Species of *T. buphthalmoides*, *T. bornmuelleri*, *T. rechingeri*, *T. acanthocarpus*, are
308 classified in the profundisulcate section based on flora of Iranica. *T. graminifolius* is classified in the Brevirostres
309 section and *T. reticulatus* is classified in the Sosnowskya section in the flora of Iranica. Based on morphometric data,
310 two species *T. graminifolius* and *T. reticulatus* are in section profundisulcati. Subsequently, based on studies of seed
311 morphology and morphology by Sukhorukov and Nilova (2015) and molecular studies of ITS, ETS by Mavrodiev et
312 al. (2005) has also been confirmed. Two species of *T. petrocarpus* and *T. coloratus* are located in two separate clusters
313 close to each other based on morphometric data and ISSR data. According to the flora of Iranica, species of *T.*
314 *coloratus* and *T. petrocarpus* have many similarities to each other and are located in the section of Chromopapus
315 Pollen studies performed by Azizi et al. (2021) also confirm this section, therefore We confirm the chromopapus
316 section.

317 Rubriflori sections based on morphological, ISSR, ITS data confirms the flora of Iranica and pollen studies by Azizi
318 et al. (2021). Classification of Iranian endemic species such as *T. jesdianus*, *T. erostris*, *T. porphyrocephalus*, *T.*
319 *rezaiyensis*, *T. stroterocarpus* in the flora of Iranica is unknown, Marodive 2012 Based on 7 nuclear loci (Adh, GapC,
320 LFY, AP3, PI, ITS, ETS) studies species of *T. rezaiyensis* in B clade, species of *T. jesdianus* in C clade, species of *T.*
321 *stroterocarpus* in D clade, species of *T. porphyrocephalus* in F clade classified. Based on our morphometric studies,
322 these species belong to the Rubriflori section, ISSR data also proves that two species of *T. porphyrocephalus* and *T.*
323 *jesdianus* belong to the Rubriflori section. Rubriflori section introduced in Iranica flora has been proved by
324 morphometric and ISSR data. The species *T. porphyrocephalus*, *T. stroterocarpus* and *T. jesdianus* belong to Rubriflori
325 section based on ITS data results which is consistent with the results of pollen data by Azizi et al. (2021).

326 According to Marodive et al. (2005) studies, *T. jesdianus* species were classified in Collini (Rubriflori) section, which
327 this section has *T. montanus*, *T. bornmuelleri*, *T. collinus*, *T. jesdianus*, *T. marginatus*.

328 Populations of *T. vaginatus* species in one cluster as well as populations of *T. vvedenskyi* species in one cluster and
329 populations of *T. graminifolius* species in one cluster are arranged, Each of these species, according to the flora of
330 Iranica, are placed in separate sections under the names of Majores, Angustissimi, Brevirostris, respectively The ISSR
331 data results confirm these three sections.

332 According to the ISSR data, populations of *T. longirostris* are located in a cluster and confirm the Krascheninnikovi
333 section of the flora of Iranica.

334 According to ITS data, *T. rezaiyensis*, *T. longirostris*, *T. ptrocarpus*, *T. coloratus*, *T. vvedenskyi*, *T. kemulariae* are
335 related to each other Which is almost consistent with clade B by Marodive et al. 2012 studies based on 7 nuclear genes
336 (Adh, GapC, LFY, AP3, PI, ITS, ETS).

337 According to ITS data, *T. bupthalmoides* and *T. acanthocarpus* species are located in a cluster that confirms the
338 Profundisulcati section of Iranica flora.

339 Cp DNA dendrogram are not useful for classification in this genus and Chloroplast sequences are very similar among
340 *Tragopogon* species, Therefore, the use of cp DNA markers in the classification of this genus is not recommended.

341 **Compliance with ethical standards**

342 **Conflict of interest** The authors declare that they have no conflict of interest.

343

344 **References**

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