NUMERICAL TAXONOMY AND SEED PROTEIN ANALYSIS OF *HYOSCYAMUS* SPECIES IN IRAN

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Abstract

Numerical taxonomy and seed storage protein analysis of *Hyoscyamus* species of Iran was carried out with the aim to illustrate species inter-relationship and to check the sub-generic taxonomic treatment proposed for the genus. Cluster analysis of morphological and protein data grouped the species in three separate clusters which supports the relationships of *H. niger* with *H. reticulatus* and *H. kurdicus* with *H. squarrosus* and *H. arachnoideus*. Discriminant analysis produced three separate groups with 100% correctness of predicted versus actual membership of the species studied, supporting the sub-generic treatment of the genus *Hyoscamus*.

Introduction

The genus *Hyoscyamus* L. belongs to the tribe *Hyoscyameae* Miers of *Solanaceae*. In Flora Iranica 18 *Hyoscyamus* species have been reported from Iran which have been divided into two subgenera of *Dendrotericon* and *Hyoscyamus* by Schönbeck-Temesy [20]. However, Khatamsaz [12] in her recent taxonomic study described *H. bornmulleri* as a new species for the country and considered some other species as synonymous, thereby reporting only 13 *Hyoscyamus* species from Iran. Moreover, she described the new subgenus *Parahyoscyamus* with two species of *H. malekianus* and *H. leptocalyx* which were considered to be the members of the subgenus *Dendrotericon* by Schönbeck-Temesy [20].

The earlier studies of the genus in Iran is restricted to a few chromosome number reports [1,8], anatomical and pollen grain studies [9,13]. Recently the authors reported the karological details of the 11 species from Iran showing the presence of two different basic chromosome numbers of x=14 and 17 in the genus *Hyoscyamus* [23].

The present article considers seed storage protein electrophoresis and numerical taxonomy of the genus trying to indicate the species relationships. Moreover, sub-generic taxonomic treatment of Khatamsaz [12] has been checked by using Discriminant analysis of morphological characters.

Keywords: *Hyoscyamus*; Cluster analysis; Discriminant analysis; Numerical taxonomy; SDS-PAGE; Seed storage proteins

Materials and Methods

Plant Materials

The species names, their localities and voucher numbers are given in Table 1. Voucher specimens of the species studied are deposited in the Central Herbarium of Iran (TARI). The present study was performed on the specimens available in the herbarium.

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Numerical Taxonomy

For numerical taxonomy analysis, seventy-two quantitative and qualitative morphological characters were studied according to the description provided by Khatamsaz [12] (Table 2).

Qualitative characters were coded as multistate characters [25,10,14] and the means of quantitative characters were used. Quantitative characters were measured on a minimum of 5 plants from each species except for *H. bornmulleri* with only one type of specimen available in the herbarium.

Protein Extraction and Electrophoresis

One hundred mg of each sample (25-50 dry seeds) were homogenized to obtain a fine meal. Proteins were extracted in a pre-cooled mortar and pestle over ice with a 0.39 M Tris phosphate buffer (pH 8.3). The resulting mixture was centrifuged at 15000 g for 10 min. The crude extracts were boiled for 5 min in 77 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulphate (SDS), 10% 2-mercaptoethanol and 3% glycerol [19]. Protein electrophoresis (SDS-PAGE) was carried out using 20 µg of protein in each lane. Vertical slab gels (1 mm thick) were electrophoresed at a constant current of 30 mA for 8 h. Coomassie Brilliant Blue G-250 was used overnight for gel staining followed by trichloro-acetic as a fixative.

Statistical Analysis

In order to group the species having morphological similarities, cluster analysis using single linkage (nearest neighbour), WARD (minimum variance spherical clusters) [10] and ordination of species on the first two principal component axes (PCA) was performed [15,22]. The Euclidean distance was used as a dissimilarity coefficient in cluster analysis of morphological data.

Variables were standardized (mean=0, variance=1) for numerical analysis [4,21].

In order to determine the most variable morphological characters among the species, a factor analysis based on principal components analysis (PCA) was performed. Varimax rotation was carried out after Kaiser normalization [16].

Taxonomic position of the species was checked against the proposed classification of Khatamsaz [12] by using discriminant analysis [14]. For each species, predicted group membership was estimated from canonical discriminant scores using Bayes rule [17] and checked against actual group membership, followed by

Table 1. Species studied, their codes, localities and voucher numbers (sub-genera treatment according to Khatamsaz (1998))

***Hyoscyamus* Species**	**Species code**	**Locality**	**Voucher number**
Subgenus *Dendrotrichon* Schönbeck-Temesy			
bornmulleri Khatamsaz	born	Shiraz-Bemo park	32707
insanus Stocks	insan	Ramhormoz, Baba-Ahmadi road	72964
tenuicaulis Schönbeck-Temesy	tenui	Bandar Abbas, Sirjan road	44782
Subgenus *Parahyoscyamus* Khatamsaz			
malekianus Parsa	malek	Balochestan-Taftan mountain	53092
leptocalyx Stapf.	lepto	Kermanshah, West Carand	16763
Subgenus *Hyoscyamus* L.			
niger L.	niger	Tehran, Karaj road	72893
reticulatus L.	neticula	Azarbayejan, Tabriz road	73143
squarrosus Griff.	squar	Kerman, Ravar	23080
kurdicus Bornm.	kurdic	Kordestan, Sanandaj	74014
arachnoideus Pojark.	arach	Hamedan, Galehbar mountain	74010
turcomanicus Pojark.	turco	Gorgan, Ramiyan	55672
pusillus L.	pusil	Tehran, Arak road	48095
senecionis var. *senecionis* Willd.	sen.sen	Yasoj, Dehdasht	72984
senecionis var. *bipinnatisectus* Boiss.	sen.bip	Khorasan, Birjand	72985

Table 2. Morphological characters and their coding

1 – Habit: Herbaceous=1, herbaceous with woody base=2
2 – Life cycle: Annual=1, biennial=2, perennial=3
3 – Rhizome: Absent=1, present=2
4 – Plant height (cm)
5 – Shape of stem: Angular=1, circular=2
6 – Stem from base: Single=1, multiple=2
7 – Stem diameter (mm), Stem branching: Simple=1, branched above the base=2
8 – Stem branching: Simple=1, branch above the base=2, branched from the base=3
9 – Stem habit: Strait=1, nodding=2, inclining=3, decumbent=4, erect or decumbent=5, caespitose=6
10 – Indumentum: Simple=1, simple and branched=2
11 – Gland: Presence=1, Absence=2
12 – Indumentum: Pubescent=1, tomentose=2, lanate=3, villous=4, cobwebby=5, villous-tomentose=6
13 – Type of leaf: Non succulent=1, succulent=2
14 – Shape of rosette leaves: Ovate=1, elliptic=2, lanceolate=3, ovate-lanceolate=4, ovate-angulate=5, reniform-angulate=6, triangular, rhomboid or semi-orbicular=7, triangular, rhomboid or lanceolate=8
15 – Length of rosette leaves (cm)
16 – Width of rosette leaves (cm)
17 – Rosette leaves apex: acute=1, obtuse or acuminate=2
18 – Rossete leaves base: Cuneate=1, broadly cuneate=2, cordate=3, truncate=4, cordate or cuneate or truncate=5
19 – Rosette leaves margin: dentate=1, angular=2, lobed=3, entire or undulate=4, sinuate or laciniate=5, double pinnatified=6
20 – Petiole of rosette leaves: Sessile=1, petiolate=2
21 – Cauline leaves shape: Ovate=1, triangular=2, lanceolate=3, obovate=4, ovate-angular=5, ovate-lanceolate=6, angular-reniform=7, triangular, rhomboid or lanceolate=8, triangular, rhomboid or subcircular=9
22 – Cauline leaves length (cm)
23 – Cauline leaves width (cm)
24 – Cauline leaves apex: acute=1, acuminate=2, obtuse=3, aristed=4, acuminate or obtuse=5, obtuse or aristed=6, obtuse or acure=7
25 – Cauline leaves base: cuneate=1, cordate=2, perforate=3, broadly cuneate=4, cordate or truncate=5, roundish, cuneate or truncate=6
26 – Cauline leaves margin: Lanceolate=1, dentate=2, lacerate=3, bipinnatipatite=4, entire or repand=5, entire angulate=6, entire or lobate=7, angulate to repand=8
27 – Length of inflorescence leaves (cm)
28 – Width of the inflorescence leaves (cm)
29 – Shape of inflorescence leaves: Lanceolate=1, ovate=2, triangular=3, triangular-reniform=4, obovate=5, lanceolate=6
30 – Inflorescence leaves length (cm)
31 – Inflorescence leaves width (cm)
32 – Apex of inflorescence leaves: Acute=1, acuminate=2, obtuse=3, aristed=4, acute or obtuse=5, acuminate or aristed=6
33 – Base of inflorescence leaves: Cuneate=1, cordate=2, broadly cuneate=3, perforate=4
34 – Margin of inflorescence leaves: Entire=1, dentate=2, entire to angulate=3, laciniate=4, entire or dentate=5, lacerate=6, double lacinate=7
35 – Petiole of inflorescence leaves: Present=1, absent=2
36 – Pedicel length (cm)
37 – Shape of pedicel: Erect=1, erect to contorted=2, erect to reflexed=3
38 – Shape of calyx: Funnel-Shaped=1, camprulate=2, clavate=3, campanulate to funnel-shaped=4, campanulate to clavate=5
39 – Calyx length (cm)
40 – Length of calyx teeth (cm)
41 – Shape of calyx teeth: Triangular=1, lanceolate=2, broadly triangular=3
42 – Apex of calyx teeth: Acute=1, cuspidate=2, obtuse=3, aristate=4, mucronate=5
43 – Calyx vein: Veined=1, weakly veined=2, reticulate=3, veinless=4, parallel veined=5
44 – Corolla length (cm)
45 – Corolla shape: Funnel-Shaped=1, campanulate=2, clavate=3
46 – Flower color: Yellow=1, white=2, purple=3, whitish violet=4, yellowish purple=5, yellow with purple veins=6
47 – Indumentum of corolla: Hairy=1, not hairy=2
48 – Carolla lobes length (cm)
49 – Shape of carolla lobes: Orbicular=1, broadly ovate=2, ovate=3
50 – Carolla diameter (cm)
51 – Shape of calyx length (cm)
52 – Length of fruiting calyx (cm)
53 – Length of fruiting calyx teeth (cm)
54 – Shape of fruiting calyx teeth: Triangular=1, lanceolate=2, broadly triangular=3

Table 2. Continued

55 – Apex of fruiting calyx length: Acute=1, obtuse=2, cuspidate=3, aristate=4, mucronate=5
56 – Form of fruiting calyx teeth: Erect to patent=1, patent to reflexed=2
57 – Fruiting calyx vein: Veined=1, reticulate=2, veinless=3, parallel-vveined=4
58 – Pedicel length at fruiting (cm)
59 – Pedicel shape at fruiting: Erect=1, contorted=2, erect to reflexed=3
60 – Capsule length (cm)
61 – Aperture length (cm)
62 – Aperture diameter (cm)
63 – Fruit shape: Ovate=1, ovate to oblong=2, orbiculate=3, little orbiculate=4, ellipsoid=5, ellipsoid to ovate=6
64 – Stamen length (cm)
65 – Size of stamens: Equal=1, unequal=2
66 – Type of stamen: Included=1, excluded=2
67 – Stamen situation: Base of stamens joining to corolla
68 – Style length (cm)
69 – Seed shape: Reniform=1, little reniform=2, lens to reniform=3
70 – Seed length (cm)
71 – Seed width (cm)
72 – Seed surface: Raguse=1, reticulate to glandular=2, glandular reticulate=3, punctate=4

grouping of the species based on the first two discriminant factors.

In order to group the species being similar in protein bands cluster analysis, single linkage and WARD methods were performed. For this purpose, RM (Relative Mobility) values of protein bands were estimated and proteins having similar RM were taken as similar [27]. Each protein band was taken as a qualitative character and coded as 1 (presence) versus 0 (absence) [2,3]. Jaccards' similarity index [25] was determined among the species followed by cluster analysis (Figs. 1-7).

In order to determine the most variable protein bands among the species, factor analysis based on principal components analysis (PCA) was performed [24].

The mental test was employed to indicate association between protein variation and morphological differences [26]. For this purpose dissimilarity matrices that were calculated between taxa for morphological and protein data were plotted against each other [18]. The average taxonomic distance coefficient [6] was used for morphological characters and for protein data, Jaccards' similarity coefficient was calculated and deducted from 1 [26]. Correlation between these two dissimilarity coefficients was finally determined. SPSS [17] and NTSYS [18] softwares were used statistical analysis.

Quantitative variation in proteins was determined by densiometry of bands using Corning 710 Flurimeter/Densitometer at wave length 520 nm.

Results and Discussion

Numerical Taxonomy

Morphological characters and their coding are presented in Table 2. It is suggested to perform different methods of clustering particularly single linkage and WARD. The common clusters of these methods may be considered as the true clusters [4,10]. Rohlf [18] suggested that in the determination of cophenetic correlation between clustering results and the original data, a high cophenetic value indicates distinctness of the clusters. Different clustering methods such as WARD and single linkage were employed on morphological and protein data, all producing the same results.

Phenogram of cluster analysis (single linkage) is presented in Figure 1. Three major clusters can also be recognized as being supported by ordination of the species based on a principal components analysis (Fig. 2). The first cluster is comprised of *H. niger, H. turcomanicus, H. arachnoideus, H. reticulatus, H. squarrosus, H. kurdicus, H. pusillus, H. senecionis* var. *senecionis* and *H. senecionis* var. *bippinatisectus* of the subgenus *Hyoscyamus*. The second cluster is comprised of *H. malekianus* and *H. leptocalyx* of subgenus *Parahyoscyamus* and the third cluster is comprised of *H. bornmulleri, H. tenuicaulis* and *H. insanus* of the subgenus *Dendrotrichon*. Fit of the clusters to the original data was confirmed by obtaining high cophenetic correlation ($r>0.80$).

In order to check sub-generic taxonomic treatment of Khatamsaz [12], a discriminant analysis was performed. The results supported her taxonomic treatment showing the presence of three separate groups (Fig. 3). 100% correctness of classification was obtained for predicted versus actual group membership, supporting the groups formed. Therefore, the separation of three subgenera as suggested by Khatamsaz [12], is supported by cluster and discriminant analysis of morphological data.

It is also interesting to mention that members of subgenera *Hyoscyamus* possess 2n=34 and 68 chromosome number (x=17) [23], while *H. malekianus* from subgenus *Parahyoscyamus* (*H. leptocalyx* could

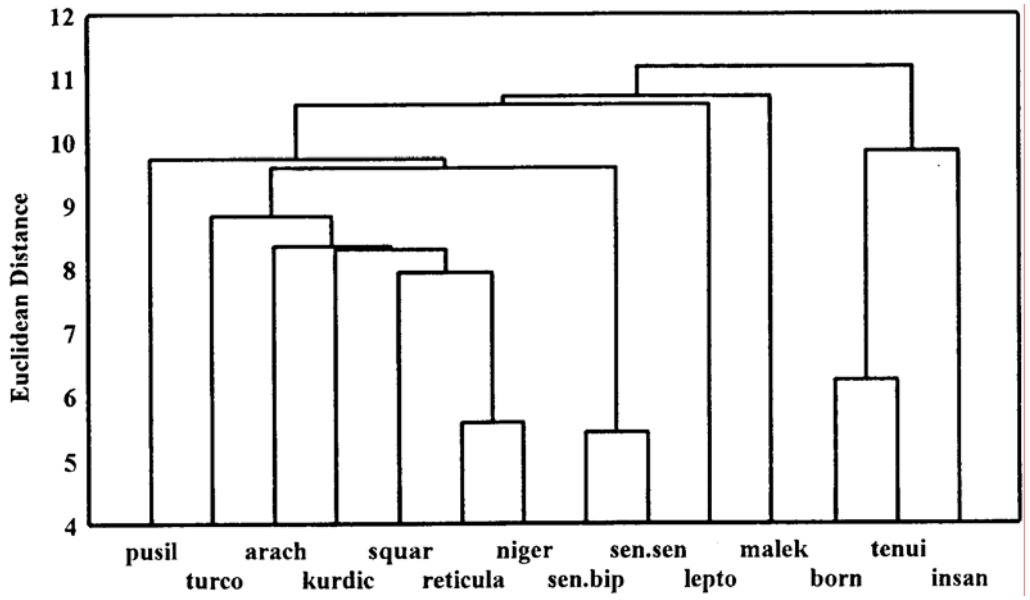


Figure 1. Cluster analysis (single linkage method) of morphological data (species codes as in Table 1).

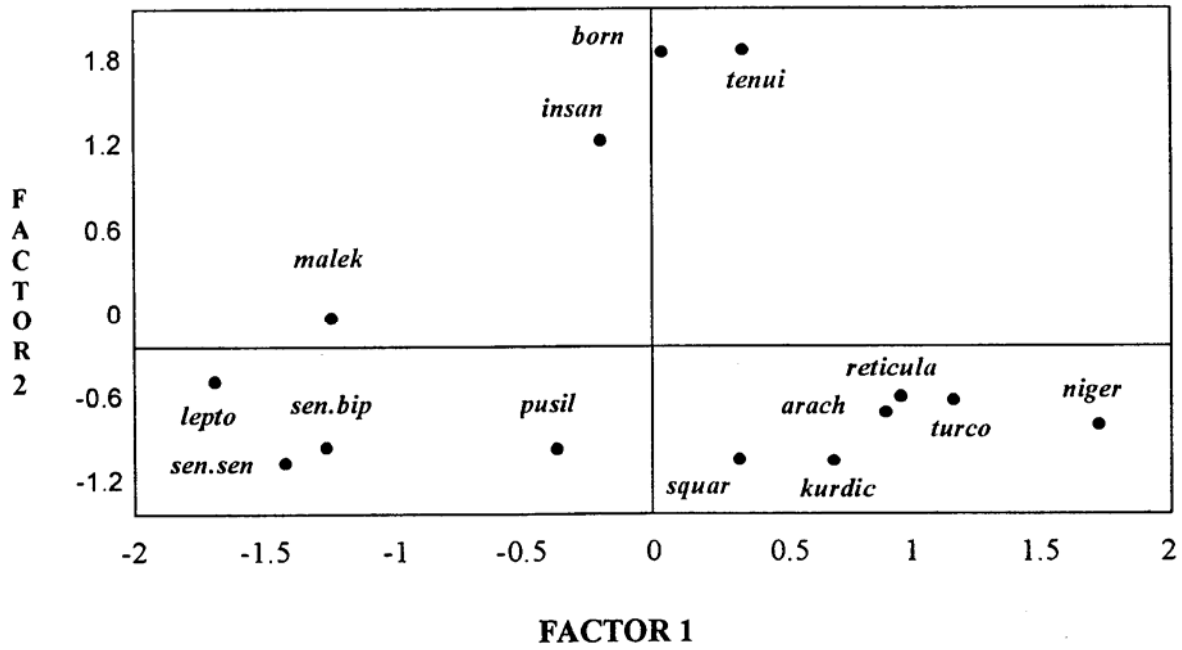


Figure 2. Ordination of morphological data on the first two axes of factor analysis (species codes as in Table 1).

not be worked) and members of subgenus *Dendrotrichon* possess 2n=28 (x=14). Therefore, with regard to the basic chromosome number (x), separation of *H. malekianus* from subgenus *Hyoscyamus* is also supported.

Factor analysis of morphological characters showed that the first six factors describe about 69% of total variance. The first component comprises about 21% of the total variance to which the base and petiole of inflorescence leaves are highly correlated. Habit of plant, shape of stem, indumentum, leaf type, apex of calyx teeth and stamen length are highly correlated with the second component (comprising 19% of total variance). Length, width, margin and petiole of the

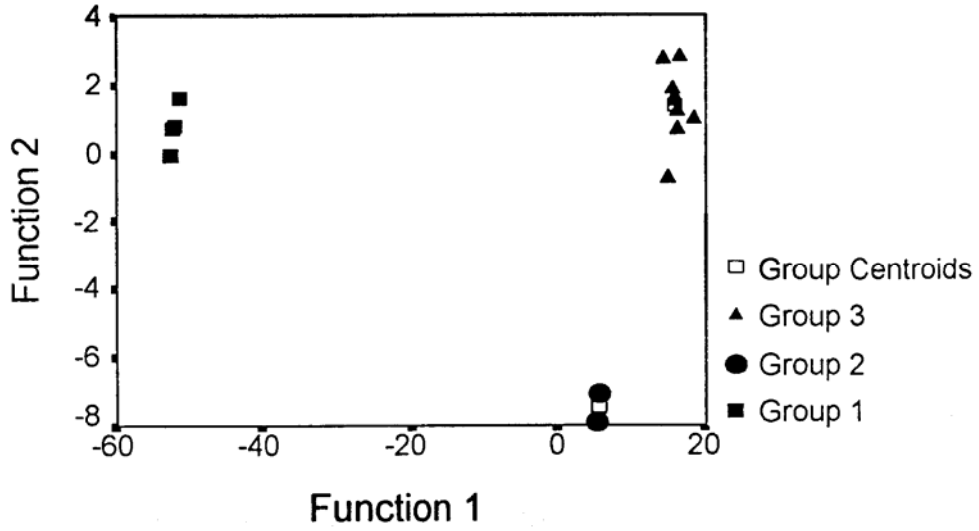


Figure 3. Discriminant analysis of morphological data. Group 1=*H. insanus, H. tenuicaulis, H. bornmulleri;* Group 2=*H. malekianus, H. leptocalyx;* Group 3=rest of the species.

rosette leaves, pedicel length and shape at fruiting, calyx vein, stamen length and fruit shape are highly correlated (>0.70) with the other factors. These characters are the most variable characters and may be used to differentiate among the *Hyoscyamus* species.

Protein Electrophoresis

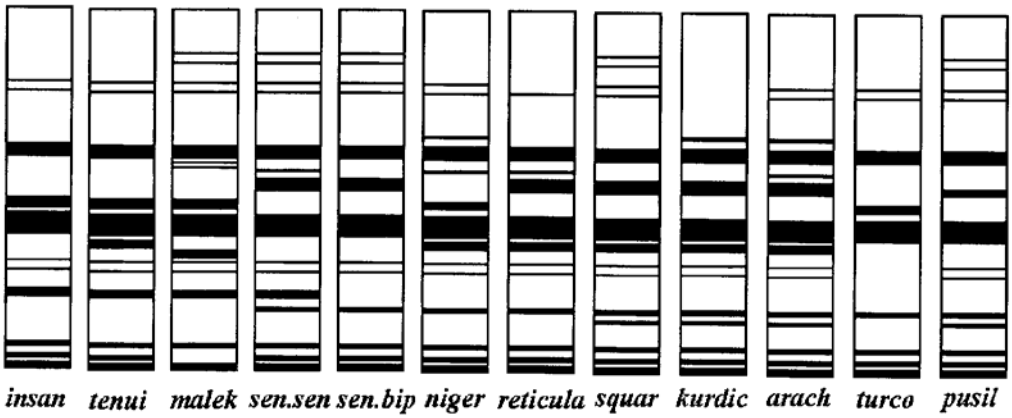
Protein bands obtained and their R. M values are presented in Table 3. In total, 21 bands were identified (Figs. 4 & 5) ranging from 9 in *H. turcomanicus* to 16 in *H. malekianus*. Bands 4, 6, 11, 19 and 21 are common in all the species and may be taken as the genus specific bands. Bands 7, 13 and 16 are specific for *H. malekianus* and can be taken as species specific.

The curves obtained from densitometry of protein bands are presented in Figure 6. The curves not only

Table 3. Distribution of protein bands in *Hyoscyamus* species, plus RM values

		Species											
Band	**RM**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	0.23	0	0	1	1	1	0	1	1	0	0	0	1
2	0.24	0	0	1	1	1	0	1	1	0	0	0	1
3	0.30	1	1	1	1	1	1	0	1	1	1	1	1
4	0.32	1	1	1	1	1	1	1	1	1	1	1	1
5	0.35	0	0	0	0	0	1	0	0	1	0	0	0
6	0.37	1	1	1	1	1	1	1	1	1	1	1	1
7	0.39	0	0	1	0	0	0	0	0	0	0	0	0
8	0.47	0	0	1	1	0	1	1	0	0	1	0	0
9	0.50	0	0	0	1	1	0	1	1	1	1	0	1
10	0.54	1	1	1	0	0	1	0	0	0	0	1	0
11	0.58	1	1	1	1	1	1	1	1	1	1	1	1
12	0.61	0	1	0	0	0	1	1	1	1	1	0	0
13	0.63	0	0	1	0	0	1	1	1	1	1	0	0
14	0.66	1	1	1	1	1	1	1	1	1	1	0	1
15	0.69	1	1	1	1	1	1	1	1	1	1	0	1
16	0.76	0	0	1	0	0	0	0	0	0	0	0	0
17	0.79	1	1	1	1	0	1	1	1	1	1	1	1
18	0.82	0	0	0	1	1	0	1	1	1	1	0	1
19	0.86	1	1	1	1	1	0	0	1	0	1	1	1
20	0.90	1	1	0	1	1	1	1	1	1	1	1	1
21	0.92	1	1	1	1	1	1	1	1	1	1	1	1

Species sequence as Figure 3



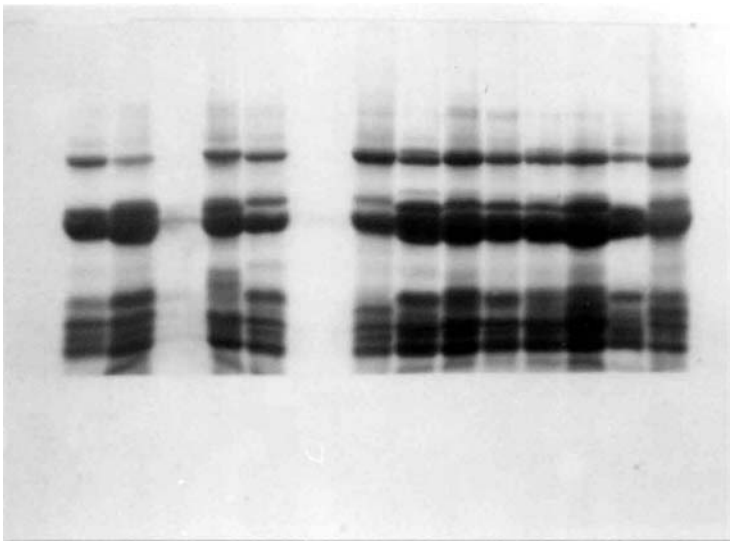


Figure 4 & 5. SDS-PAGE protein bands of species studied (species abbreviations as Table 1).

show the presence of common protein bands among the species but also indicate their quantitative differences. Such quantitative differences have been considered to represent different frequency of common genes among different populations/cultivars [7].

Jaccard similarity index determined a high correlation value (>0.60) among the species except for *H. malekianus* (<0.05). Cluster analysis of protein data is presented in Figure 7. Three main clusters can be recognized. *H. insanus* and *H. tenuicaulis* from subgenus *Dendrotrichon* from the first cluster. The second cluster is comprised of *H. turcomanicus, H. niger, H. reticulatus, H. kurdicus, H. arachnoideus, H. squarrosus, H. senecionis* var. *senecionis, H. pusillus* and *H. senecionis* var. *bipinnatisectus,* all from subgenus *Hyoscyamus*. *H. malekianus* from subgenus *Parahyoscyamus* stands in the third cluster. Therefore, the separation of three genera of *Hyoscyamus* is also supported by protein data.

Khatamsaz [12] considers *H. niger* close to *H. reticulatus* and that *H. arachnoideus* shows the relationship with *H. squarrosus* and *reticulatus*. Both morphological and protein cluster analysis supports such consideration. *H. pusillus* is considered close to *H. turcomanicus* which is not supported in the present study; it shows relationship with *H. senecionis* in both cluster analysis of morphological and protein data.

Factor analysis of protein data indicated that the first 4 factors comprise 80% of total variance. Bands number 2, 3, 7, 13-16, 18 and 20 which are highly correlated with these factors (>0.70) are the most variable bands among the species studied.

Seed storage proteins are coded by genes [5], therefore, variation in protein bands of *Hyoscyamus* species is indicative of genomic changes taken place during the species diversification.

The mental test performed did not show any association between changes in seed storage proteins

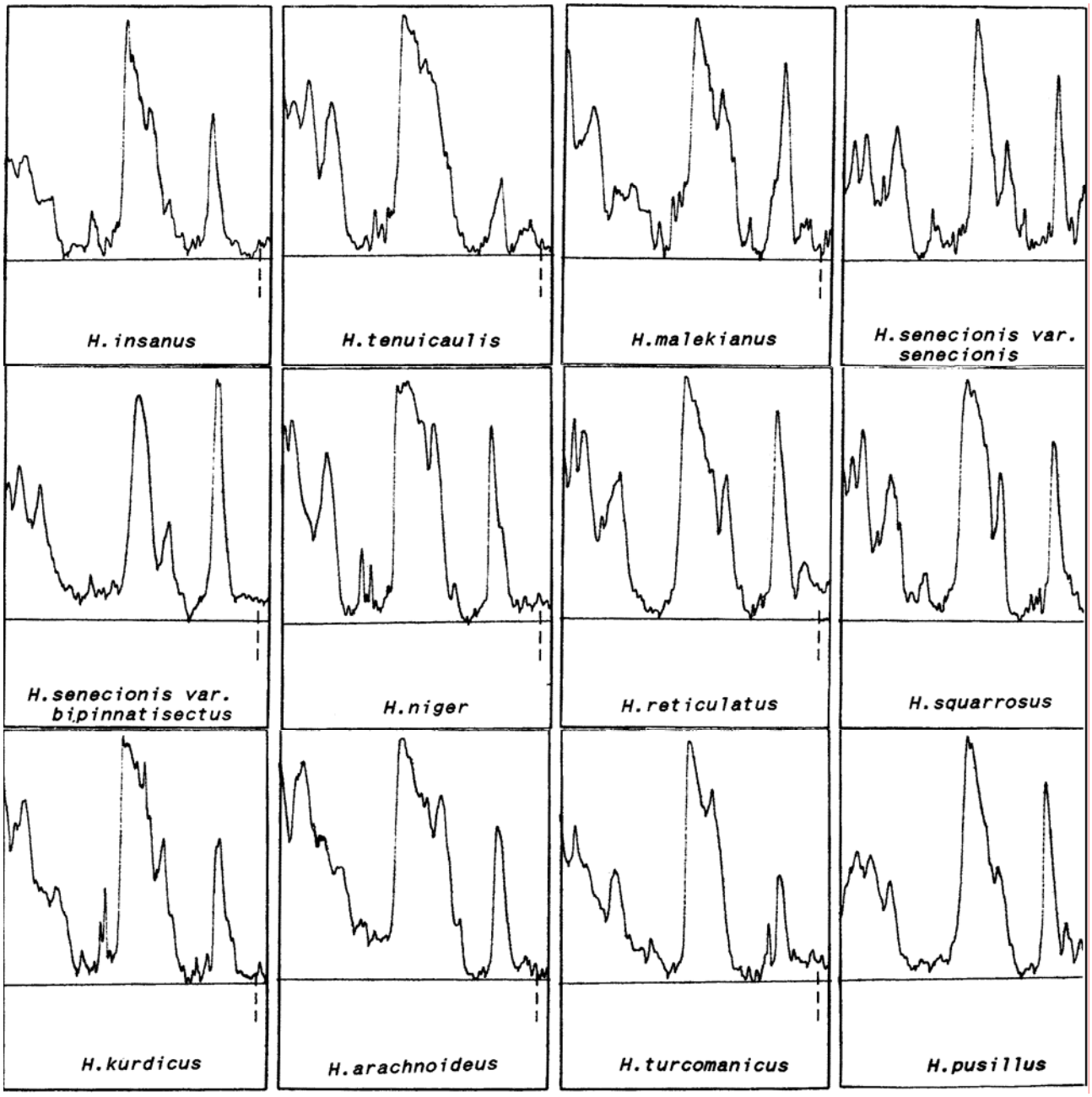


Figure 6. Curves obtained from densitometry of protein bands.

and morphological characters (r=-0.12, P=0.15). Therefore, changes that occurred in the genes coding seed storage proteins are not associated with genomic changes related to morphological characters, as it is supported by differences observed in some parts of clustering results obtained from morphological and protein data.

In short, the main findings of the present paper are:

1. Multivariate statistical analysis of morphological and protein data support the sub-generic treatment of the genus proposed by Khatamsaz [12].
2. Morphological analysis showed that characters like calyx vein, stamen length and fruit shape (formerly not used in the species differentiation) may be used in taxonomy of the genus.
3. *H. niger* is close to *H. reticulatus* while *H. arachnoideus* shows the relationship with *H. squarrosus* and *reticulatus*.

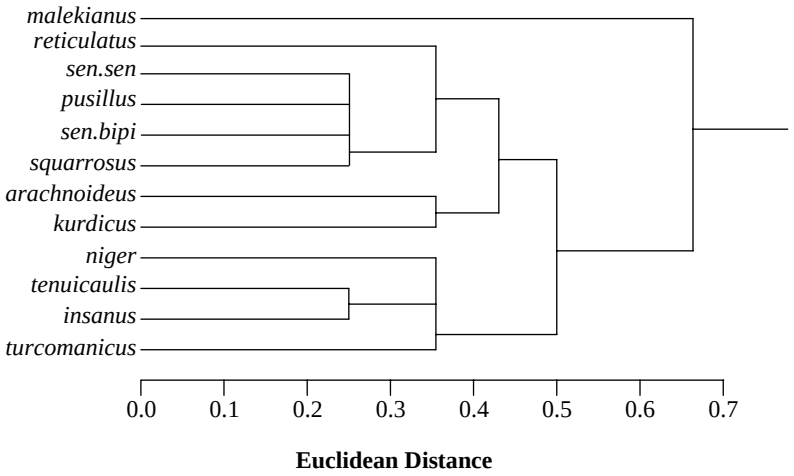


Figure 7. Cluster analysis (single linkage) of protein data.

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