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COMPARATIVE STUDY OF SIX ISOENZYME SYSTEMS IN SOME SPECIES OF *HYOSCYAMUS* L., FROM IRAN

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Abstract

The electrophoretic patterns of 6 enzyme systems viz., lactate dehydrogenase (LDH), malate dehydrogenase (MDH), esterase (EST), superoxide dismutase (SOD), peroxidase (PRX) and polyphenoloxidase (PPO) obtained from 15 seed samples (OTUs) belonging to 7 species of *Hyoscyamus* L., from Iran were compared in order to clarify taxonomic relationships within the genus. Results show that the species. *H. tenuicaulis* is completely distinct from the other species. Among the others *H. arachnoideus* and *H. niger* are very heterogenous. *H. kurdicus* shows a close relationship to *H. niger* and *H. turcomanicus* is very close to *H. arachnoideus*.

Introduction

Hyoscyamus L., is a genus of Solanaceae family. In Flora Iranica, 18 species of *Hyoscyamus* have been reported from Iran (Schönbeck–Temesy, 1972). Then some of these species were considered synonymous and a new species, *H. bornmulleri* was added in Flora of Iran (Khatamsaz, 1998). Therefore she has only reported 13 species of *Hyoscyamus* from Iran.

The genus is distributed in both old (Komarov, 1955; Hawkes, 1972; Baytop, 1978 & Nasir, 1985) and new worlds (www.funet.fi/pub/sci/bio/life/plants). Some species have extensive distributation like *H. niger* that is cosmopolitan. It seems that Iran with three endemic species viz., *H. tenuicaulis*, *H. malekianus* and *H. bornmulleri*, is an important diversification center of the genus.

This genus also have been divided into two subgenera of *Dendrotericon* and *Hyoscyamus* (Schönbeck–Temesy, 1970). Recently the new subgenus *Parahyoscyamus* with two species of *H. malekianus* and *H. leptocalyx* has been reported (Khatamsaz, 1998). In previous study (Schönbeck–Temesy, 1972), these two species were included in subgenus *Dendrotericon*.

On the other hand, particular solanaceous genera, such as *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus* and *Scopolia* are characterized by the presence of the pharmacologically important, anticholinergic alkaloids hyoscyamine and scopolamine (Hibi *et al*., 1992). The extracts of the plants and the isolated pure alkaloids atropine and scopolamine have been used for medicinal purposes for many centuries (Sauerwein *et al*., 1993).

The identification of the species of the genus is very complex, difficult and often confused (Khatamsaz & Zangirian, 1998). Scanning electron microscopy of pollen grains (Khatamsaz & Zangirian, 1998) have also been described. Karyological studies have also been made on 11 species of this genus collected from Iran and have shown two different basic chromosome number that is x=14 and x=17 (Sheidai *et al.,* 1999). Interrelationship of these species have been studied through seed storage protein electrophoresis and numerical taxonomy (Sheidai *et al.,* 2000).

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The ability to separate electrophoretically multiple molecular forms of various enzyme extracts of plant materials has proved a useful tool to identify phylogenetic relationships (Torres *et al.*, 1979). Isozymes have also been used to identify different species and varieties of crop plants like potato varieties (Nieto *et al.*, 1990), garlic clones (Pooler &Simon, 1993), *Allium* species (Abdul Wahab, 1994), hybrids of rice (Farooq *et al.*, 1996), populations of *Lythrum salicaria* (Strefeler *et al.*, 1996), crop and wild species of *Amaranthus* (Chan & Sun, 1997), cotton varieties (Farooq *et al.*, 1999) and some perennial plants such as *Musa acuminata* (Jarret & Litz, 1986), peach (Arulsekar *et al.*, 1986), *Pyrola japonica* (Huh *et al.*, 1998) and species of the genus *Pinus* (González-Andrés *et al* ., 1999).

In this research we have tried to describe 6 isoenzyme systems in some species of *Hyoscyamus* collected locally. The objective was to study the usefulness of such methods for separating the species and grouping within the genus.

Materials and Methods

Plant materials: Seven species of the genus *Hyoscyamus* were used in the present study. Details of the materials along with seed sources, date of collection and distribution are listed in Table 1.Voucher specimens are deposited either in the central herbarium of Iran (TARI) or Tehran University Herbarium (TUH).

Enzyme extraction: 1g of seeds were homogenized in extraction buffer [0.05M tris–HCl pH: 8.3 (1.5 w/v)] using a mortar and pestle pre–cooled with ice. The homogenized meal was centrifuged at 15000 rpm for 30 min., at 4°C. The supernatant (enzyme extract) was filtered through cheese–cloth. This extract was either immediately used for electrophoresis or stored at -20°C.

Enzyme electrophoresis: Polyacrylamide gel electrophoresis (PAGE) was performed in a vertical system, thermostated at 4–6°C by a circulator cooling bath. Enzyme samples were loaded on to 21 x 22.8 cm plates with 4 mm thickness, which can accommodate 17 samples. Reservoir buffer was cold tris–glycine (pH 8.3) while bromophenol blue was used as marker front– dye. Gels were electrophoresed at a constant current of 5 mA per gel during 24 h. For all the enzymes, 90µl extract per well was loaded except for enzyme esterase only 40 µl extract was loaded.

Enzyme staining: Isozyme esterase (EST); (E.C.3.1.1.1) was stained according to the procedure described by Desborough *et al*., (1967). Peroxidase (PRX); (E.C.1.11.1.7) and polyphenoloxidase (PPO); (E.C.1.10.3.2) were stained according to Vanloon (1971). Lactate dehydrogenase (LDH); (E.C.1.1.1.27), malate dehydrogenase (MDH); (E.C.1.1.1.37), and superoxide dismutase (SOD); (E.C. 1.15.1.1) were stained according to Wendel & Weeden (1990).

Numerical analysis: The bands produced by each sample were counted individually and the relative mobility (Rm) was determined. The presence or absence of each band was treated as a binary character (coded 1 and 0, respectively). The allozyme and the specific locus was designated by numerical numbers superscripting the enzyme and the locus number e.g., EST–2^3 means allele no. 3 at EST locus–2 and PRX–1^2 means allele no.2 at PRX locus–1. The Jaccard's similarity index was calculated for the OTUs using the above-mentioned matrix. Data were analysed using the SAHN (Sequential Agglomorative Hierarchial and Nested) option of NTSYS–PC (Version 1.60) system of Rohlf (1990).

Table 1. Plant materials.

No.	Species	Seed source	Date of collection	Distribution	Code
1.	*Hyoscyamus arachnoideus* 1	Azarbayejan	JUN.1999	Iran, Iraq.	15
2.	*Hyoscyamus arachnoideus* 2	Tehran	JUL.1996	Iran, Iraq.	11
3.	*Hyoscyamus arachnoideus* 3	Zanjan	JUN.1999	Iran, Iraq.	10
4.	*Hyoscyamus arachnoideus* 4	Ardebill	JUN.1999	Iran, Iraq.	8
5.	*Hyoscyamus arachnoideus* 5	Mazandaran	JUL.1996	Iran, Iraq.	1
6.	*Hyoscyamus niger* 1	Tehran	JUL.1996	Iran.Iraq,middle Asia,north, Africa, Europe,Turkey, Russia,Caucasia,Ciberia, Afghanistan, Pakistan	12
7.	*Hyoscyamus niger* 2	Azarbayejan	JUN.1999	Iran, Iraq, middle Asia, north Africa, Europe, Turkey, Russia, Caucasia, Ciberia, Afghanistan, Pakistan	9
8.	*Hyoscyamus niger* 3	Mazandaran	JUL.1999	Iran, Iraq, middle Asia, north Africa,Europe, Turkey, Russia, Caucasia, Ciberia, Afghanistan, Pakistan	5
9.	*Hyoscyamus reticulatus* 1	Khorasan	JUL.1998	Iran, Iraq, Europe, Turkey, Syria, Egypt	4
10.	*Hyoscyamus reticulatus* 2	Tehran	JUL.1996	Iran, Iraq, Europe,Turkey, Syria, Egypt	3
11.	*Hyoscyamus squarrosus* 1	Mazandaran	JUL.1999	Iran, Afghanistan, Pakistan, middle Asia	7
12.	*Hyoscyamus squarrosus* 2	Tehran	JUL.1996	Iran, Afghanistan, Pakistan, middle Asia	2
13.	*Hyoscyamus kurdicus*	Kordestan	AUG.1996	Iran, Iraq, Syria	14
14.	*Hyoscyamus tenuicaulis*	Lorestan	AUG.1996	Iran: Endemic	13
15.	*Hyoscyamus turcomanicus*	Golestan	JUL.1996	Iran, Turcomanestan	6

Dendrogram was constructed by the UPGMA (Unweighted Paired Group Method Average) of Sneath & Sokal, (1973).

Results and Discussion

Electrophoretic profiles of different isozymes are shown in Table 2. Polyphenoloxidase (PPO), appeared as a mixture of monomeric and dimeric enzyme coded by four loci. PPO–1 and PPO–2 loci appeared as typical of a monomeric enzyme with two alleles. PPO–1^1 expressed in sample 7 and PPO–1^2 only expressed in sample 5. PPO–2 locus expressed in samples 8 and 15 with two alleles and in sample 12 with only one. Locus–3 expressed in samples 3, 8, 9, 10, 12, 13, 14 and 15 with three, in sample 5 and 6 with two alleles and in sample 4 with one allele. Samples 1, 2 and 11 showed no PPO activity.

Peroxidase (PRX), are expressed as 6 different alleles at three loci. Three zones of PRX activity were present on acrylamide gels. It appeared to be coded by three loci in different species of *Hyoscyamus.* The PRX–1 locus appeared as monomeric and expressed only in sample 7. Locus–2 and locus–3 exhibiting three alleles per locus. They appeared as dimeric. Therefore, this enzyme possesed a mixture of monomeric and dimeric activity as shown in previous study (Brewbaker *et al.,* 1985). PRX–2 occured in sample 5 with two and in samples 8, 14 and 15 with three alleles. PRX–3 occured in sample 12 with one, in sample 4, 6 and 10 with two and in sample 7 with three alleles. Samples 1, 2, 3, 9, 11 and 13 showed no PRX activity.

Esterase (EST), should prove useful as a diagnostic tool for cultivar identification in view of its extensive polymorphism for this enzyme (Jarret & Litz, 1986). The extension of this isoenzyme is usually considered unstable and highly dependent on environmental conditions when analyzed on leaves and other vegetative organs. However, when esterases are studied in seeds, they are considered valid for characterization as they are less likely to be affected by environmental conditions (González–Andrés *et al.,* 1999). Isozyme esterase exhibited one of the most complicated zymograms. It appeared as a mixture of monomeric and dimeric enzyme as it was shown in previous studies (Tanksley & Rick, 1980; Wehling & Schmidt–Stohn, 1984). On the other hand, one of the earliest studies on esterase isozymes from *Solanum* tubers assumed that three alleles Ea, Eb and Ec each control the production of a different monomer which combine to give 15 esterase isozymes (Desborough *et al.,* 1967). In this study, it appeared to be controlled by 7 loci. Locus–1, locus–3, locus–4 and locus–6 had two alleles. Locus–2 had three alleles. Locus–5 and locus–7 had only one allele. All of the alleles did not express simultaneously in one sample and they showed different isozyme patterns.

Superoxide dismutase (SOD) revealed 10 isozymes. It appeared as a mixture of monomeric and dimeric enzyme. In previous studies in rye, wild grasses of the tribe *triticeae* (Jaaska & Jaaska, 1982) and in maize (Baum & Scandalios, 1981) it was known as a dimeric or tetrameric isozyme. *Vigna* species (Pasquet & Vanderborght, 1999) showed only one isozyme. In the present study it appeared to be coded by 10 loci. SOD–1 had one allele and expressed in samples 11, 12, 13, 14 and 15. SOD–2, SOD–4, SOD–9 and SOD–10 each contained two alleles, locus–3 and locus–7 are monomorphic and comprised of one allele that occured in all samples. SOD–5 had one allele and only expressed in sample 13 (i.e., *H. tenuicaulis*). SOD–8 also had one allele that expressed in sample 12 (i.e., *H. niger* 1) and finally locus 6 had three alleles. This isozyme showed to be dimeric and had different banding patterns between different samples.

Table 2. Presence/absence data matrix of the isoenzymatic study.

Band	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
EST															
1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	1	0	0	0	1	0	1	1
3	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
4	1	0	0	1	0	1	0	1	0	0	1	0	0	1	1
5	1	0	1	1	0	1	0	0	0	1	1	1	0	1	1
6	0	0	1	1	0	0	0	1	1	1	1	1	0	0	0
7	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0
9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
10	1	1	1	1	0	1	0	0	1	1	1	1	0	0	0
11	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
12	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
13	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
LDH															
1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1
2	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1
3	1	1	1	1	0	1	1	0	1	1	1	1	0	1	0
4	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0
5	0	0	1	1	0	1	0	0	1	1	0	0	0	0	1
MDH															
1	1	1	0	1	1	1	1	1	1	1	1	0	0	1	1
2	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1
3	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1
4	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1
PRX															
1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
2	0	0	0	1	0	1	1	0	0	1	0	1	0	0	0
3	0	0	0	1	1	1	1	1	0	1	0	0	0	1	1

Band	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
4	0	0	0	0	1	0	0	1	0	0	0	0	0	1	1
5	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1
6	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
PPO															
1	0	0	0	1	0	1	1	0	1	1	0	0	0	0	0
2	0	0	1	1	0	1	1	1	1	1	0	1	1	1	1
3	0	0	1	0	1	1	0	1	1	1	0	1	1	1	1
4	0	0	1	0	1	0	0	1	1	1	0	1	1	1	1
5	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1
6	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
7	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
8	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
SOD															
1	0	0	0	0	0	0	1	1	1	1	0	0	0	1	1
2	0	1	1	1	0	0	1	1	1	1	0	0	0	1	1
3	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
4	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0
5	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
7	0	0	0	0	0	1	1	0	1	1	0	0	0	1	1
8	0	0	0	0	0	1	1	1	1	1	0	0	0	0	1
9	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
11	0	1	0	0	0	0	0	1	1	0	0	0	0	0	1
12	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1
13	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
14	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
16	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1

Malate dehydrogenase (MDH), has been studied in many plants including *Musa acuminata* Colla (Jarret & Litz, 1986), *Festuca* (Aiken *et al.*, 1993) and it has been reported to be a dimeric enzyme. Eight bands were observed in two zones of activity in individuals assayed for MDH in *Lythrum salicaria* (Strefeler *et al.*, 1996). In *Vigna luteola* and *Vigna marina* (Sonnante *et al.*, 1997), three putative loci were reported for this enzyme. In the present study, four bands and two zones of activity were resolved in different samples. It appeared to be coded by two genes at MDH–1 and MDH–2 loci. Both loci appeared as monomeric. MDH–2 had no activity in samples 12 and 13 (i.e., *H. niger*1 & *H. tenuicaulis*).

Isozyme Lactate dehydrogenase (LDH), appeared as a monomeric enzyme coded by three loci. LDH–1 and LDH–3 exhibited two alleles while LDH–2 was exhibiting one allele in different samples. LDH–1 and LDH–3 showed monomeric and monomorphic pattern. Sample 13 (*H. tenuicaulis*) showed no LDH activity.

There are 52 bands (29 loci) that can result from combinations of these six enzyme systems, five bands of them (10.3%) are found in all taxa and were removed in data analysis. The zymograms and profiles of these enzymes are shown in Fig. 1.

It may be mentioned that quantitative variation, as differences in intensity and thickness of bands, is not taken into consideration when coding for the numerical analysis, and only the presence/absence of each band using electrophoresis was used to produce a classification (Fig. 2).

In the obtained dendrogram, there are two main branches within the genus *Hyoscyamus*. The first branch separates *H. tenuicaulis* from the other species and it has the lowest similarity with the rest of the species that is in accord with the morphological studies (Khatamsaz, 1998) and other numerical taxonomic works (Sheidai *et al.*, 2000). The second group contains other species, among which *H. arachnoideus* is the most heterogeneous species. The five different samples of this species have different positions. *H. niger* is also a very heterogenous species (Fig. 2). *H. arachnoideus* 2 and *H. arachnoideus* 5 showed the highest similarity, with values around 0.65. *H. turcomanicus* is closely related to *H. reticulatus* and this is in consistence with numerical taxonomic works (Sheidai *et al.*, 2000). *H. kurdicus* shows close relationship to *H. arachnoideus.* The close relationship between *H. kurdicus* and *H.arachnoideus* that is shown here is supported by earlier electrophoretic and numerical taxonomic works (Sheidai *et al.*, 2000). *H. squarrosus* 1 and *H. squarrosus* 2 have a similarity level more than 0.35.

Anyway, none of the methods which were used to determine relationships between the species of *Hyoscyamus* are not useful enough (e.g., Khatamsaz 1998; Sheidai *et al.*, 1999; Sheidai *et al.*, 2000) and more studies are needed to this goal. Using such characters in the study of enzyme variation in this genus may represent that it is not a long time that these species derived from a maternal species.

This study showed intraspecies variation of isoenzymatic banding pattern. It suggests that we should primarily investigate genetic variation within and between different populations and then interspecies genetic distance. The isolation of species *H. tenuicaulis* showed that it is a long time that it has derived from the other species.

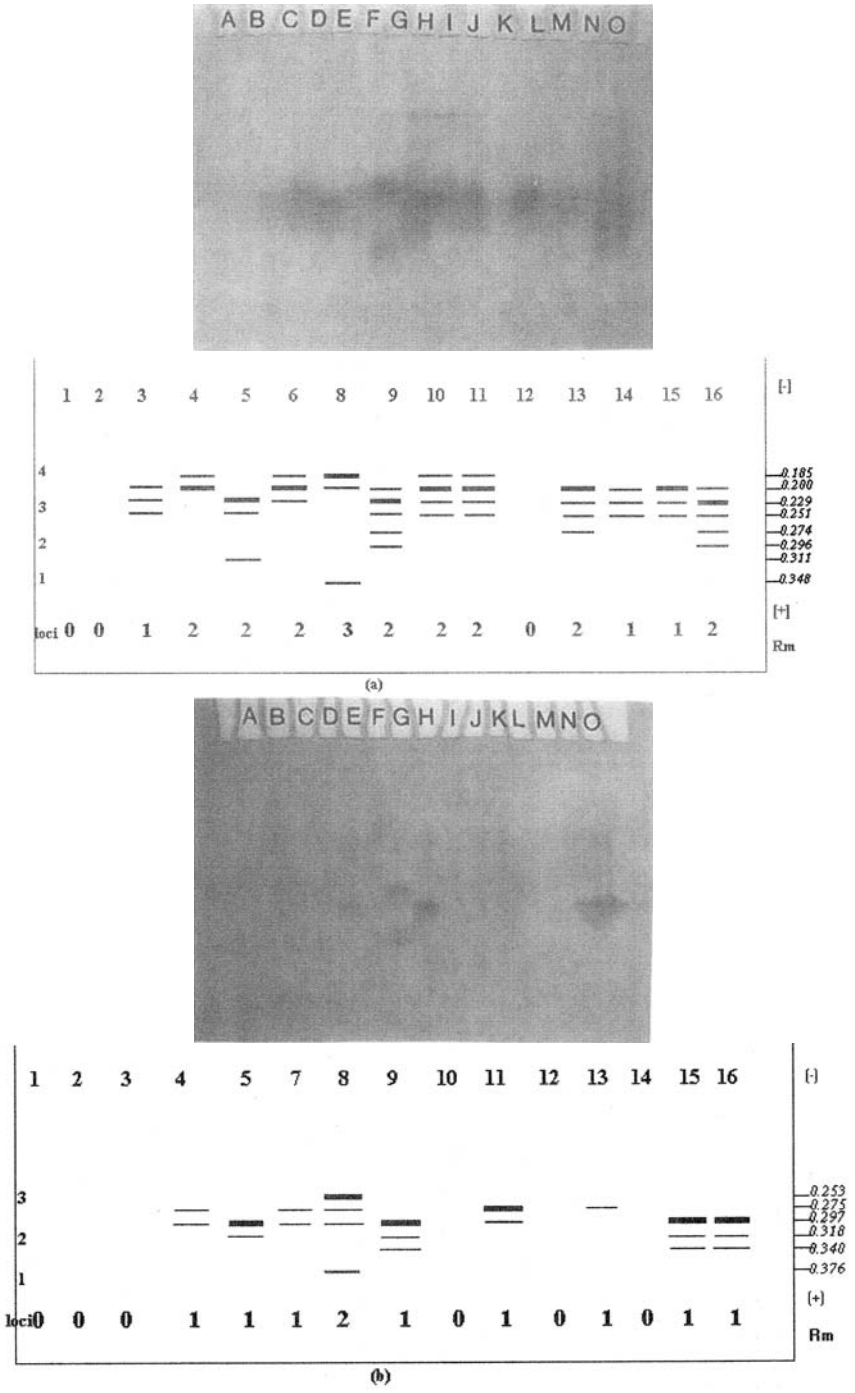

Fig. 1. Zymograms and profiles showing isozyme bands in seeds. (a) PPO, (b).

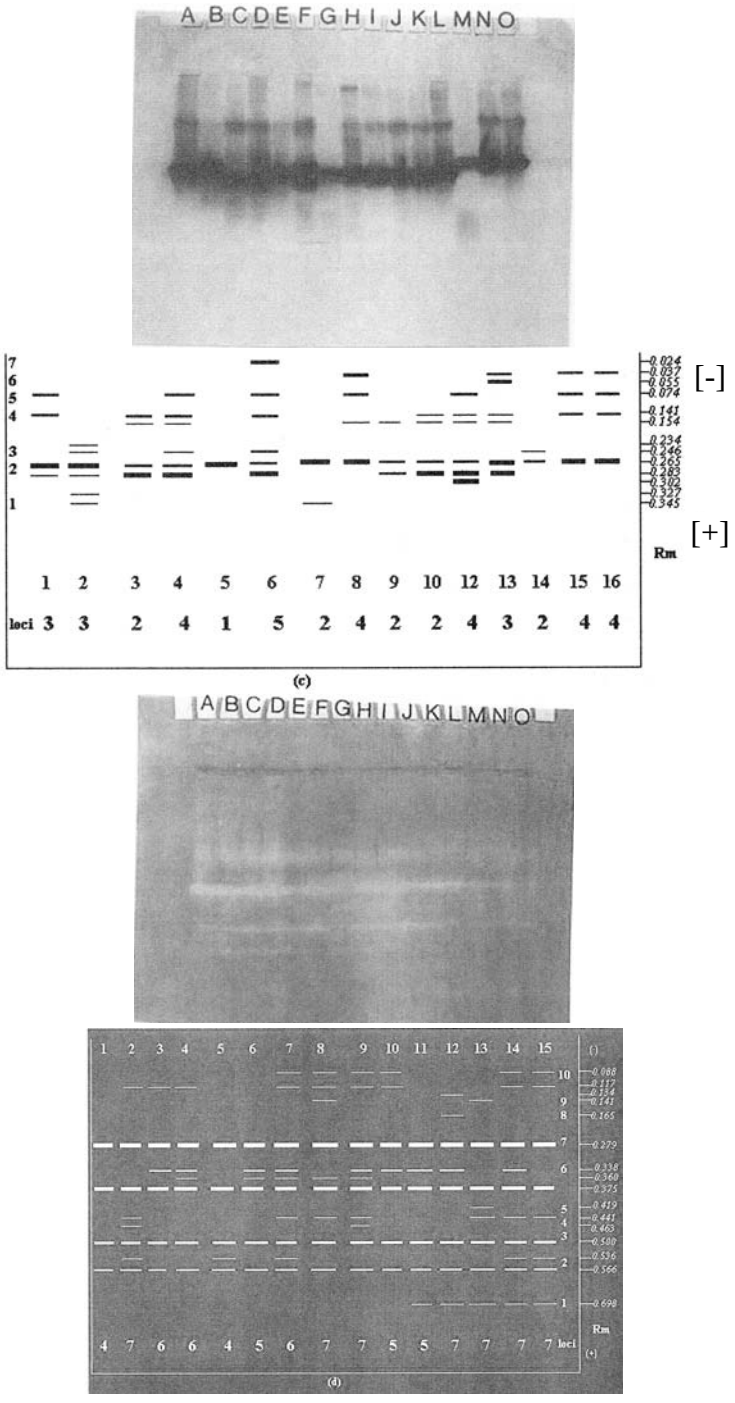

Fig. 1. Zymograms and profiles showing isozyme bands in seeds. (c) EST, (d) SOD.

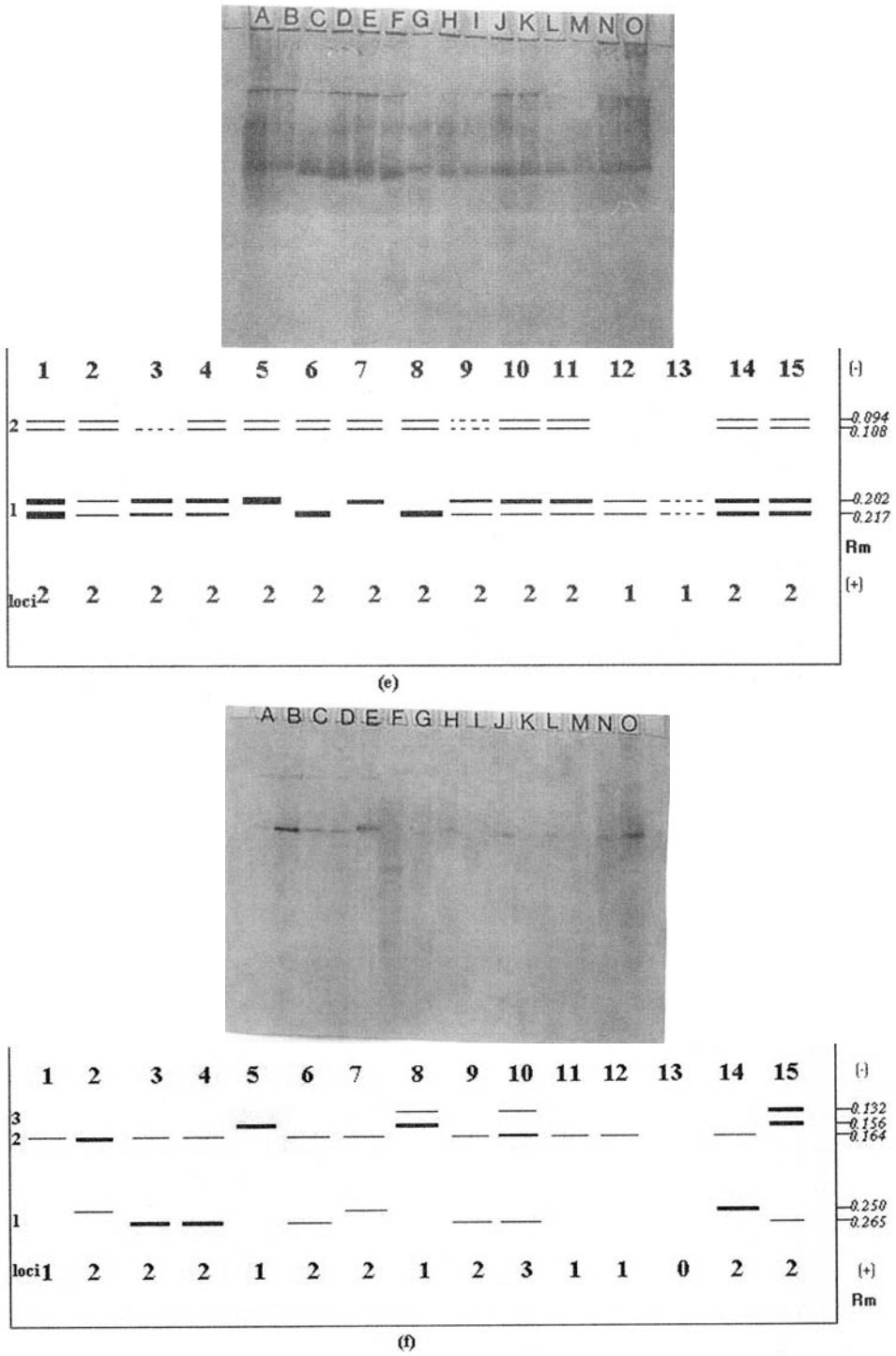

Fig. 1. Zymograms and profiles showing isozyme bands in seeds. (e) MDH, (f) LDH.

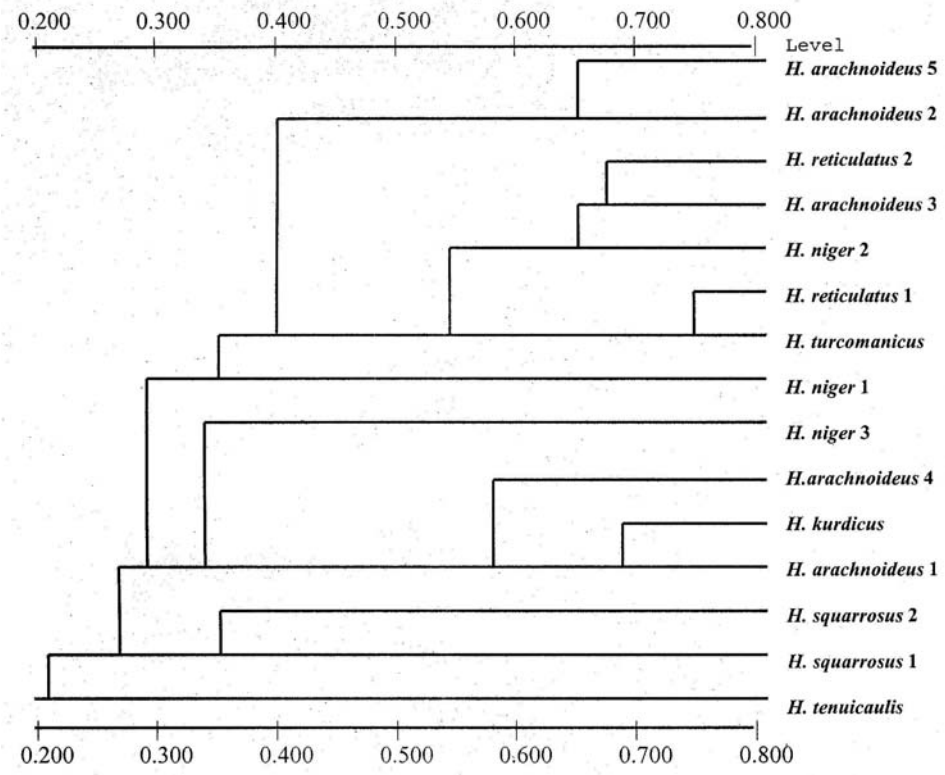

Fig. 2. Dendrogram illustrating the relationships of the investigated OTUs of *Hyoscyamus* based on isozyme data.

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