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Electrophoretic study of seed storage proteins in the genus *Hypericum* L. in North of Iran

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Abstract. In this research we studied the electrophoretic of seed storage proteins in the genus *Hypericum* L. from Iran. The plant samples were collected from various phytogeographical regions of Iran to study the seed storage proteins. The study was performed to determine the boundary among different species of genus *Hypericum* using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). All samples belong to three species of *H. perforatum*, *H. tetrapterum* and *H. androsaemum*. A total of 22 protein bands were observed in the studied species. The results show that *H. perforatum, H. tetrapterum* are closely related based on seed storage proteins. A closely relationship and high protein similarity (J=0.66) were found between *H. perforatum, H. tetrapterum*. Electophoretic results compared with earlier molecular and morphological studies. The highest number of bands was observed in Kordkoy1 population (Pop12) and Gardane heyran population (Pop20) of *H. perforatum* and the lowest in Gorgan/ Naharkhoran population (Pop 25) of *H. androsaemum.* Our results showed the species of *Hypericum* were placed intermixed. The aim of this study to delimit species in the genus *Hypericum* and used these seeds storage protein for the correct identification.

Keywords. *Hypericum*, North of Iran, Species relationships, SDS-PAGE.

INTRODUCTION

The genus *Hypericum* (Guttiferae, Hypericoideae) is perennial, belonging to the Hypericaceae family, having 484 species in forms of trees, shrubs, and herbs, distributed in 36 taxonomic sections (Crockett and Robson 2011). The species of the family are distributed worldwide in the temperate zones but are absent in extreme environmental conditions such as deserts and poles. Iranian species of this genus grow mainly in north, northwest and center of Iran and form floristic elements of Hyrcanian mountainous areas, Irano-Turanian, Mediterranean and Zagros elements. They generally prefer steep slopes of rocky and calcareous cliffs and margin of mountainous forests (Robson 1968; Azadi 1999). Robson (1968) introduced 21 species in the

area covered by Flora Iranica. Robson (1977) and Assadi (1984) reported *H. fursei* N. Robson and *H. dogonbadanicum* Assadi as two endemics of North and South West of Iran. In Flora of Iran, Azadi (1999) identified 19 species, 4 subspecies arranged in 5 sections (comprising *Campylosporus* (Spach) R. Keller, *Hypericum*, *Hirtella* Stef., *Taeniocarpum* Jaub. & Spach. and *Drosanthe* (Spach) Endl.), and two doubtful species including *H. heterophyllum* Vent. and *H. olivieri* (Spach) Boiss.

Hypericum species are generally known locally in Iran with the names "Hofariqun" which Ebn Sina (or Bo Ali Sina) called it (Rechinger, 1986). Plants of the genus *Hypericum* have traditionally been used as medicinal plants in various parts of the world. *Hypericum perforatum* L. is the source to one of the most manufactured and used herbal preparations in recent years, especially as a mild antidepressant, and thus is the most studied *Hypericum* species (Mozaffarian, 1998). According to Brutovska´ *et al*. (2000), *H. perforatum* is probably originated from autopolyploidization of an ancestor closely related to diploid *H. maculatum*. The chemical composition of *H. perforatum* oil has been the subject of many researcher in recent past (Cakir *et al*. 1997; Baser *et al*. 2002; Osinska 2002; Schwob *et al*. 2002; Mockute *et al*. 2003; Smelcerovic *et al*. 2004). The methanolic extract from the aerial parts of *Hypericum* plants typically contain hypericins, hyperforins and phenolic compounds (Osinska 2002).

Proteins and enzymes, characterized as primary gene products, are important parameters in biochemical taxonomy. Storage proteins separated by electrophoretic methods are thought to undergo the process of evolution with relative slowness due to their "non-essential nature" (Margoliash and Fitch 1968), while enzymes are thought to be extremely sensitive to selection pressures in evolution and thus to survival of the organism (McDaniel 1970). Analysis of proteins and isozymes is a tool for supplementing the evidence obtained by comparative morphology, breeding experiments and cytological analysis. Seed protein electrophoresis for the study of phylogenetic relationship in *Capsicum* L. was performed by Panda *et al*. (1986).

Although phenotypic traits are important for diversity studies, they need to be supported by molecular markers to give robust genetic diversity estimates (Esfandani-Bozchaloyi *et al*. 2018a, 2018b, 2018c, 2018d). Genetic diversity studies in *Capsicum* using morphological, cytological and biochemical marker systems (Kaur and Kapoor 2001; Gopinath *et al*. 2006) are also conducted. The data on agronomic, morphological and physiological plant traits are generally used to estimate the magnitude of genetic diversity present in the germplasm. However, such data may not provide an accurate indication of genetic diversity because of environmental influences upon the expression of observed traits and also the time consuming and laborious field evaluation procedures. The introduction of biochemical techniques like Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), isozyme markers has been particularly helpful in deducing systematic relationships between groups where morphological and cytological data were not corollary. SDS-PAGE is an economical, simple and extensively used technique for describing the seed protein diversity of crop germplasm (Fufa *et al*. 2005; Iqbal *et al*. 2005). Furthermore, seed proteins, used as genetic markers convey greater precision to measures of genetic diversity because they are the primary products of structural genes (Srivalli *et al*. 1999). Seed protein electrophoresis for the study of phylogenetic relationship in *Capsicum annuum* was performed by Panda *et al*. (1986) and of diploids and tetraploid hybrids of *Capsicum* was initiated by Srivalli *et al*. (1999). There is no report of SDS-PAGE in *Hypericum* species in Iran.

The present study was conducted on the genetic diversity of *Hypericum* genotypes from different locations which will be useful for breeding programmes and also for conservation of germplasm. To use genetic resources adequately, it is necessary to understand the extent and pattern of genetic diversity. Therefore, an attempt with the present investigation was undertaken to evaluate the extent of variability existing in 29 geographical populations belonging to three species of *Hypericum* of North region of Iran through seed protein analysis to provide a scientific basis for future selection and crop improvement program. The objective of this study was to assess the level of seed electrophoretic patterns of *Hypericum* taxa in Iran and used it for the correct taxonomy of the genus.

MATERIAL AND METHODS

Plant Material

Extensive field visits and collections were undertaken during 2016-2017 throughout the north of Iran. In present study 63 plant samples from 29 geographical populations belonging to three species of *Hypericum* in Iran were collected from field: *H. perforatum* L., *H. tetrapterum* Fries. and *H. androsaemum* L. Different references were used for the correct identification of species (Rechinger, 1999 , Azadi, 1999). The details of the voucher specimens and their localities are given in Table 1 and Fig 1. All materials were examined with a stereomicroscope (NIKON-SMZ1) and all voucher specimens

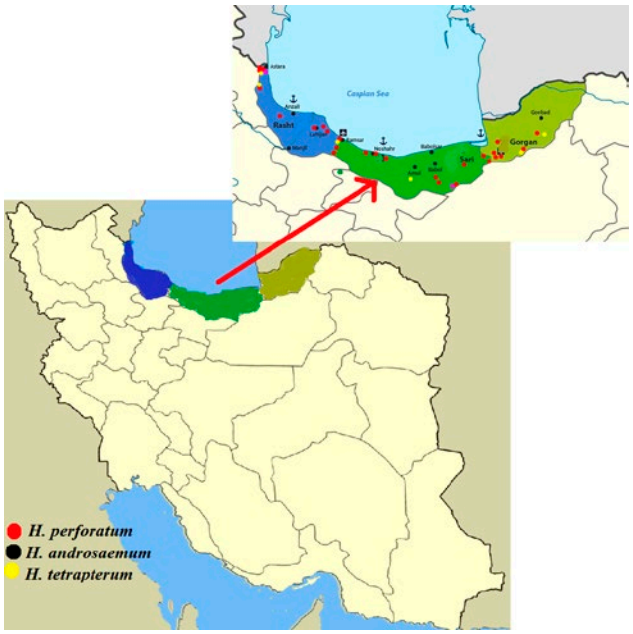

Figure 1. Distribution map of *Hypericum* populations studied.

are deposited at the University of Mazandaran Herbarium (HUMZ).

Protein extraction & Electrophoresis

An amount of 0.1 g of mature seeds were selected from each population and crushed in liquid nitrogen at low temperature. After obtaining a fine powder, proteins were extracted under cool conditions with 3 ml of Tris-Glycin buffer (pH 8). The resulting samples were centrifuged twice for 5 min at 11000 g. The protein electrophoresis was based on Laemmlis procedure (1970), using a discontinuous vertical slab gel. The separating gel comprises 12 ml of 30 % acrylamid

stock solution, 2.5 ml Tris-HCl 1.5 M (pH 8.8), 100 µl SDS 10 %, 2.3 ml water, 7 µl TEMED, and 60 µl APS (Ammonium per Sulfate). After polymerization of the separating gel, the stacking gel with 530 µl of 30 % acrylamide stock solution, l ml Tris 0.5 M (pH 6.8), 40 ml SDS 10 %, 2.37 ml water, 5µl TEMED, and 40 µl APS was polymerized on the separating gel.

The electrophoresis was carried out at a constant voltage of 100V for 7-15h. Gels were stained in Coomasie Brilliant Blue for 1-2 h and overnight distained with acetic acid and methanol (Laemmli 1970).

We used Jaccard similarity coefficient. Standard proteins (b-galactosisase, Ovalbumin, Lactate dehydrogenase, lactoglobulin-b, Lysozyme and Bovine serum albumin) were used to evaluate the molecular weight of the unknown proteins. The protein density was determined by Bradford Protocol(Bradford, 1976).

Protein banding profile analysis

Number and location of each protein band were identified and their RF (relative factor) and molecular weight were estimated. In statistical analysis, each protein band was considered as a qualitative character and coded as 1 (presence) versus 0 (absence). For grouping of the plant specimens,Ward (minimum spherical characters) were used (Podani 2000). PCA (principal components analysis) biplot was used to identify the most variable characters among the studied populations (Podani 2000). PAST version 2.17 (Hammer *et al.* 2012)

RESULTS

A total of 22 protein bands were observed for these taxa. (Fig. 2 and Table 2). All studied taxa had bands 76.12 KD and 45 KD except for Gorgan/Naharkhoran population (Pop 25) of *H. androsaemum*. The highest number of bands was observed in Kordkoy1 population (Pop12) and Gardane heyran population (Pop20) of *H. perforatum* and the lowest in Gorgan/Naharkhoran population (Pop 25) of *H. androsaemum* (Fig. 2 and Table 2).

In order to find out the most variable protein bands in the studied taxa, a Principal Component Analysis was implemented. Primitive analysis showed that three factors were responsible for 62.37 % of total studied variation in the taxa. In the first factor, with almost 37.81 % of the total variation, bands 6.12, 9.87, 34.87, 51.12 KD had the highest correlation. In the second factor, with about 14.63 % of the observed variation, bands 27.37, 30.19, 76.77 and 81.67 KD had the highest positive correlation. In the third factor, with 9.92 % of the total variation, bands 21.54, 78.14, 93.16 KD had the highest correlation.

Both clustering and PCA analyses of the *Hypericum* species studied produced similar groupings and therefore only WARD clustering characters are presented here (Fig. 3). Two major clusters were formed in WARD clustering (Fig.3). WARD clustering, of the studied populations did not entirely delimit the studied species and revealed that plants in these species are intermixed. In WARD dendrogram, a higher degree of intermixture occurred between *H. perforatum*, *H. tetrapterum* and *H. androsaemum*. Also WARD dendrogram revealed that although population of the species *H. perforatum* is more distinct than the other two species, but it showed a high degree of intraspecific genetic variability as they are positioned in different places of the dendrogram.

Table 1. Voucher details of *Hypericum* species examined in this study from Iran.

Population No.	Species	Population cod	Locality / Voucher number
1	*H. perforatum* L.	Hp1	Mazandaran , Ramsar, 1723 HUMZ
2	*H. perforatum* L.	Hp2	Mazandaran , Ramsar/Javaherde1,1724 HUMZ
3	*H. perforatum* L.	Hp3	Mazandaran , Ramsar/Javaherde2/daryache ghoo,1725 HUMZ
4	*H. perforatum* L.	Hp4	Mazandaran , Savadkoh/Alasht,1726 HUMZ
5	*H. perforatum* L.	Hp5	Mazandaran , Babolkenar1,1727 HUMZ
6	*H. perforatum* L.	Hp 6	Mazandaran , Babolkenar2,1728 HUMZ
7	*H. perforatum* L.	Hp 7	Mazandaran , Galogah1,1729 HUMZ
8	*H. perforatum* L.	Hp 8	Mazandaran , Galogah2,1730 HUMZ
9	*H. perforatum* L.	Hp9	Mazandaran , Aliabad katool,1731 HUMZ
10	*H. perforatum* L.	Hp10	Golestan , Gorgan,1732 HUMZ
11	*H. perforatum* L.	Hp 11	Golestan , Ziarat,1733 HUMZ
12	*H. perforatum* L.	Hp 12	Mazandaran ,Kordkoy1,1734 HUMZ
13	*H. perforatum* L.	Hp13	Mazandaran , Kordkoy2,1736 HUMZ
14	*H. perforatum* L.	Hp14	Mazandaran , Kelachay,1737 HUMZ
15	*H. perforatum* L.	Hp15	Guilan , Langrood,1738 HUMZ
16	*H. perforatum* L.	Hp16	Guilan , Lahijan/Bam Lahijan,1739 HUMZ
17	*H. perforatum* L.	Hp17	Guilan , Somesara,1740 HUMZ
18	*H. perforatum* L.	Hp18	Guilan , Asalem,1741 HUMZ
19	*H. perforatum* L.	Hp19	Guilan , Heyran,1742 HUMZ
20	*H. perforatum* L.	Hp20	Guilan , Gardane heyran,1743 HUMZ
21	*H. perforatum* L.	Hp21	Mazandaran , Nowshahr/Sisangan,1744 HUMZ
22	*H. perforatum* L.	Ht22	Guilan , Astara,1745 HUMZ
23	*H. tetrapterum* Fries.	Ht23	Mazandaran , Savadkoh/Alasht,1746 HUMZ
24	*H. tetrapterum* Fries.	Ha24	Guilan , Asalem,1747 HUMZ
25	*H. androsaemum* L.	Ha25	Golestan , Gorgan/Naharkhoran,1748 HUMZ
26	*H .androsaemum* L.	Ha26	Guilan , Astara,1759 HUMZ
27	*H. androsaemum* L.	Ha27	Mazandaran, Ramsar/Bam Ramsar,1750 HUMZ
28	*H. androsaemum* L.	Ha28	Mazandaran , Aliabad Katool/ Kabodval,1751 HUMZ
29	*H. androsaemum* L.	Ha29	Mazandaran , Amol/Sangchal,1752 HUMZ

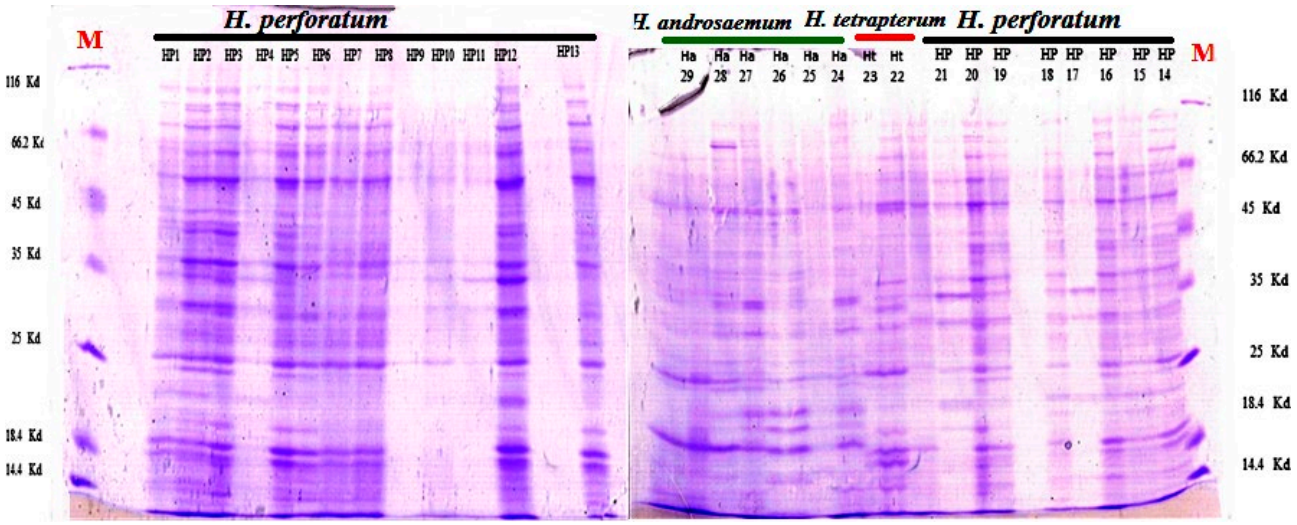

Figure 2. SDS-PAGE electrophoresis profiles of the studied population of *Hypericum*. Note: Populations abbreviations are according to Table 1.

Table 2. Band number and molecular weight for each studied population of *Hypericum*. (1- band is present in the seed sample, 0- band is absent in the seed sample).

Band No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
MW(kd)	99	95	94	92	85	82	76	68	65	62	59	55	51	45	40	37	34	27	22	18	12	8
Pop																						
1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1
2	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
3	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
4	1	0	0	0	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0	1	1	1
5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
7	0	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	0	0	1	1	1
8	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	0	0	1	1	1
9	0	1	1	1	1	1	1	0	0	0	0	1	1	1	0	1	1	0	0	1	1	1
10	0	0	0	0	1	1	1	0	0	0	0	1	1	1	0	0	1	0	0	1	0	0
11	1	1	1	0	1	1	1	0	0	0	0	1	1	1	0	0	1	0	0	1	0	0
12	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
13	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
14	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1
15	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
17	0	0	0	0	1	1	1	0	0	0	0	0	1	1	1	0	1	1	1	0	0	0
18	0	0	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0	0
19	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
20	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
21	0	0	0	1	1	1	1	1	0	0	0	1	1	1	1	0	1	0	1	1	0	0
22	1	0	1	1	1	1	1	1	0	0	0	1	1	1	1	0	1	0	1	1	1	1
23	0	0	0	1	1	1	1	1	0	0	0	1	1	1	1	0	0	0	0	1	1	1
24	1	1	1	1	1	1	1	0	1	1	0	0	1	1	1	0	0	0	1	1	1	0
25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
26	0	0	0	0	1	1	1	0	0	0	1	0	1	1	1	0	1	0	1	1	1	0
27	0	1	1	1	0	0	1	0	1	1	1	0	1	1	1	0	1	1	1	1	1	0
28	1	1	1	1	0	0	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	0
29	0	0	0	1	0	0	1	0	0	0	0	0	0	1	1	0	1	1	0	1	1	0

DISCUSSION

In the present study, 29 geographical populations belonging to three species of *Hypericum* of North region of Iran was examined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoretic data of the seed storage proteins presented in this study have shown that the dendrogram obtained from the studied species is not capable of the species recognition. We found findings the seed storage was often incongruent with the result of Faghir, *et al.* (2018) and Mahmoudi Otaghvari & al, (2015) and Bayat & al., (2015) for pollen data.

In previous studies, the micromorphology of pollen grains was performed in several species and their importance in plant taxonomy was emphasized (Faghir, *et al.* 2018; Mahmoudi Otaghvari & al,2015; Bayat & al., 2015).

Faghir, *et al.* (2018) pollen grains of ten species and two subspecies of the genus *Hypericum* in Iran belonging to four sections were studied using light and scanning electron microscopy. Palynological analysis of selected species of the genus *Hypericum* revealed important pollen morphological characters, especially pollen outline, numbers and types of apertures, colpus length; presence and absence of operculum; exine sculpturing type, pore shape, size and arrangements. These traits can be used for infrageneric classification, especially at sectional and species levels (Faghir, *et al.* 2018).

Different techniques including morphological, biochemical and especially molecular markers let scien-

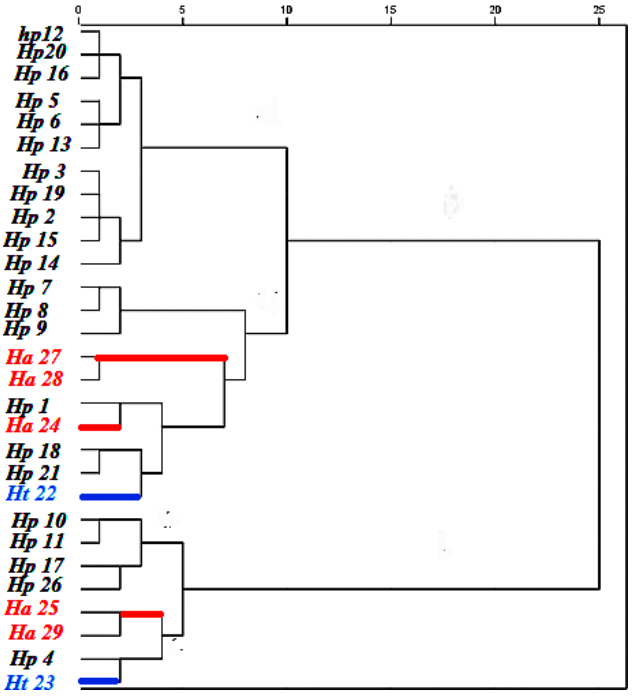

Figure 3. Phenogram by WARD method based on SDS-PAGE electrophoresis characters in *Hypericum* species. Note: Populations abbreviations are according to Table 1.

tists to study genetic variability of plants. As molecular markers present reproducible results regardless of environmental conditions, they have gained nowadays considerable attention for studies relating to the genetic diversity (Farooq and Azam 2002).

According to Morshedloo *et al.* (2015) genetic variability among ten wild populations of *H. perforatum* growing in different climatic regions of Iran via ISSR markers. They observed the studied populations were classified into four main groups which was, to the some extent, in accordance with their geographical origins. Also they recovered, ISSR markers revealed relatively a high level of genetic variability among Iranian *H. perforatum* populations suggesting that the ISSR technique is efficient and powerful for assessment of genetic diversity at the intraspecific level.

The present study also provide the way for use of molecular systematics within genus *Hypericum*. The taxa are not clearly separated on the basis of electrophoretic data of seed storage proteins. The results have revealed that *H. perforatum, H. tetrapterum* were closely related. A high Similarity Index is a reflex of genomic identity (J=0.66). The dendrogram showed close relationship and high protein similarity (J=0.66) between *H. perforatum, H. tetrapterum*. This is the first of its kind report on electrophoretic data of the seed storage proteins of three species of *Hypericum* of North region of Iran.

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