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Characterization of *Ustilago tritici* and *U. nuda* on wheat and barley-II. Teliospore morphology : RAPD analysis

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Monokaryotic haploid hyphae of fourteen isolates of *Ustilago* spp. pathogenic on wheat and barley, were analysed by random amplified polymorphic DNA analysis (RAPD) using twenty five decamer primers. The results of the RAPD analyses showed that a high degree of variability exists among isolates of *Ustilago tritici.* Isolate Ut12 gave a distinct pattern and was separated from all others by UPGMA cluster analysis. Amplification products obtained with primers OPN5 and OPN6 were able to clearly separate *U. tritici* isolates. The study highlights the fact that DNA polymorphism can be used to separate *U. nuda* from *U. tritici* which could not be done on the basis of teliospore morphology.

Key words : RAPD, *Ustilago* spp., wheat, barley.

INTRODUCTION

Loose smut fungi, infecting wheat and barley are closely related filamentous basidiomycetes that are destructive pathogen. They cause heavy losses when susceptible varieties with infected seeds are grown under monocropping. The causal organisms of these cereals are biologically distinct but morphologically closely related and variously treated as different species, varieties or even physiological races (Hawksworth *et al.,*1995).

These fungi are identical in morphology of their teloispore, germination type, mode of infection and the fact that certain races can be hybridized on common host to form F1 teliospores (Nielsen,1987).

Recent developments in DNA- based technology including random- amplified polymorphic DNAs (RAPD) have provided suitable tools for rapid and detailed genetic analysis of higher organisms (Rafalski *et al.,* (1991); Williams *et al.,* (1990). The genetic diversity is assessed by amplification at low stringency with a single short primer of arbitrary sequence. The RAPD technique has been used to detect genetic variations among strains or isolates within a species (Cooks *et al.,* (1996) ; Hseu *et al.,* (1996) ; Body & Carris, (1997) ; Jeng *et al.,* (1997) ; Maurer *et al.,* (1997) ; Pie *et al.,* (1997) ; Jungechulsings & Tueznski, (1997).

The studies undertaken by various workers on teliospore morphology, indicate a high degree of relatedness of *U. tritici* and *U. nuda.* In the present investigations on definite criteria could be established to separate the two *Ustilago* species based on teliospore morphology (Sharifnabi *et al.,* 2000). Keeping in view, the present work was undertaken to characterize *U. tritici* and *U. nuda* based on DNA polymorphism to arrive at a certain conclusion for separation at the species level.

MATERIALS AND METHODS

Collection of isolates

Twenty isolates of *U. tritici* on wheat and five of *U. nuda* on barley were collected from various parts of Northern India viz., Punjab, Haryana, Himachal Pradesh, Uttar Pradesh, Delhi and it's vicinity during February and May, 1999 (Table 1). The teliospores samples were taken from infected heads and

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subjected to following investigations.

Cultural conditions

Monokaryotic haploid hyphae of these isolates were obtained as per method of Sharifnabi *et al.* (2000). Teliospores were surface sterilized and resuspended in sterile distille water and 150-250 ml of sterilized teliospore suspension was evenly spread on petri dishes containing 1.5-2 mm thick, 1.5% water agar and DL - aspartic acid (0.147 mg/ml water). The petridishes were incubated at 20°C for about 30 h and subsequently 1 square cm blockes of medium from these plates were transferred to plates with 1.5-2 mm thick, 1/5 of normal nutrient concentration of PDA (Potato Dextrose Agar) and incubated in refrigerator overnight. The squares were then transferred to another 1.5-2 mm thick layer of 1/5 PDA pre-warmed to 25°C and kept for 4-6 h. Monokaryotic haploid hyphae were obtained and isolated by microsurgery with very thin pasture pipettes and transferred to a thick layer of 1/5 PDA and kept at 20°C for two weeks. The mycelial mass production of the mycelium of haploid hyphae obtained by inclubation in PSB (Potato Sucrose Broth) in shaker incubator at 130 rpm at 20°C for 14-20 days and subsequentely mycelial growth in PSB were harvested by filtration through Whatman filter paper No. 41. These cultures were rinsed with sterile distilled water and frozen at 20°C. The frozen samples were used for DNA extraction.

DNA Extraction

Because of the slow growth habit of *Ustilago* spp, a modified procedure of the cetytrimethy-lammonium bromide (CTAB) method of Shi *et al.,* (1996) was followed. The frozen mycelium of each isolate was grounded in sterile morter and pestle using liquid nitrozen. The fine frozen powder was transferred to a sterile centrifuge tube. Pre-warmed at 65°C DNA extraction buffer [100mM Tris-HCI (pH 8.0, 50 mM EDTA (pH 8.0), 1.4M NaCI and 2% CTAB] was added to this and incubated in a waterbath at 65°C for 1 h. After incubation an equal volume of chloroform/isoamyl alcohol (24:1) was added and mixed gently for centrifugation at 10000 rpm, 25°C for 10 min. The aqueous phase was transferred to a new sterile tube and the DNA was precipitated with 0.6 volume of cold isopropanol and 0.1 volume of sodium acetate (3M, pH 5.2) for 25 min at room temperature. DNA was pelleted by centrifugation at 10000 rpm for 10 min at 25°C. The pellet was washed with cold 70% ethanol and resuspended in sterile water.

Purification of DNA

A stock solution of RNase-A was prepared @ 10 mg/ml in 10mM Tris-HCI pH 8.0 and 15mM sodium chloride. The solution was boiled for 10 min to destroy DNase. From the stock, 2 ml of RNase-A was added to the crude DNA sample and incubated at 37°C for 1 h. The DNA concentration of the samples and its purity was determined by taking ultraviolet absorbance at 260 nm and 280 nm in a spectrophotometer.

Random Amplification of Polymorphic DNA (RAPD) conditions

Thirty-four 10 mer oligonucleotides from sets P, S, M and N (Operon Techonologies, Inc, USA) were used as single primers for the amplification of sequences. The PCR reaction was performed in a Gene-Cycler (Bio-RAD, USA), in a 25µl volume containing 4µl of 1X reaction buffer (10mM Tris-HCl, pH 8.3 and 50mM KCl), 5mM $MgCl_2$, 0.5 unit of Taq DNA polymerase, 100µM each of dATP, dCTP, dGTP and dTTP (GENEl, Bangalore, lndia), 1µM of mer primer and 25ng of template DNA. The amplification conditions were : initial step of denaturation at 94°C for 2 min followed by 40 cycles of denaturation at 92°C for 1 min, primer annealing at 37°C for 1 min and extension at 72°C for 2 min, followed by an extended elongation step at 72°C for 5 min. Samples of 25µl PCR products were mixed with 3 µl of 10X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose, W/V) and spun briefly in a micofuge before loading (Sambrook *et al.,* 1989). The amplification products were analysed on 1.2% agarose gel containing 0.2 µg/ml of ethidium bromide in IX TAE buffer running at 60 volts for three hours and visualized under UV light and photographed on Polaroid 667 film under Ultra-Villet light. The DNA size marker used was λDNA double digested with *Eco*RI and *Hin*dIII.

Scoring and data analysis

Each amplification product was considered as RAPD marker. Gels were scored on the basis of the presence (1) or absence (0) of each band for all isolates. All amplifications were repeated at least twice and only reproducible bands were considered for analyse, jaccard's similarity coefficient values for each pairwise comparison between isolates were calculated (Jaccard, 1908) and a similarity coefficient matrix was constructed. This matrix was subjected to unweighted pair-group method for arithmetic average analysis (UPGMA) to generate a dendrogram using average linkage procedure. All the numerical taxonomic analysis were conducted using software NTSYS-pc, version 1.80 (Exeter software, NY).

RESULTS

The isolates of *U. tritici* and *U. nuda* were subjected to purification by obtaining monokaryotic haploid hyphae. The hyphae were then multiplied on potato socrose broth to raise mycelial mass for DNA extraction. However, only 12 isolates of *U. tritici* (Ut2, Ut3, Ut4, Ut6, Ut7, Ut8, Ut10, Ut11, Ut12, Ut15, Ut17 and Ut20) and two isolates of *U. nuda* (Un2 and Un4) yielded sufficient mycelium for DNA extraction and were further taken up for RAPD analysis.

Optimisation of RAPD markers

Good quality DNA of concentration 100 ng/µl was obtained from approximately 200 mg of frozen mycelial mass. Standardization of the PCR protocol was done by changing the variables like templage DNA, Taq DNA polymerase and magnesium chloride. The optimum amplification was obtained by using the reaction mix having 25ng templage DNA, 0.5 unit Taq polymerase, 5 mM $MgCl_2$, 1 µM primer and 100 µM dNTP in reaction volume of 25 µl.

Thirty four random 10 mer Operon primer from set of OPP, OPS, OPM and OPN were screeened and out of them twenty five were selected for doing RAPD analysis on the basis of reproducible and scoreable polymorphic bands obtained (Table 1).

The number of total bands as well as polymorphic bands obtained in case of *U. tritici* were much more than *U. nuda.* In case of *U. tritici* the total number of bands onbtained were 345 out of which 342 were polymorphic whereas in *U. nuda* out of 129 total bands only 98 gave polymorphic pattern. Maximum numbers (26) of bands were obtained with primer OPS5 in case of *U. tritici* and all of them were polymorphic. In case of *U. nuda* primer OPP2 and OPS8 gave 10 bands of which 8 were polymorphic for each of them. The size fragments otabined for *U. tritici* and *U. nuda* varied from 0.5 to 4 kb.

Table 1 : Primers used and the number of total and polymorphic bands produced in *Ustilago tritici* and *U. nuda.*

Prime	Sequence (3'–5')	*U. tritici*		*U. nuda*	
		Total bands	Ploymorphic bands	Total bands	Ploymorphic bands
OPM06	**CTGGGCAACT**	**15**	**15**	**6**	**0**
OPM07	CCGTGATCA	20	20	6	6
OPM13	GGTGGTCAAG	16	16	5	5
OPN02	ACCAGGGGCA	7	7	2	2
OPN05	**ACTGAACGCC**	**15**	**15**	**8**	**0**
OPN17	CATTGGGGAG	12	12	3	1
OPP01	GTAGCACTCC	11	11	3	3
OPP02	TCGGCACGCA	14	13	10	8
OPP03	CTGATAGGCC	12	12	5	4
OPP04	GTGTCTCAGG	13	13	6	6
OPP05	CCCCGGTAAC	6	6	1	1
OPP06	GTGGGCTGAC	12	12	8	6
OPP08	ACATCGCCCA	17	17	4	2
OPP09	GTGGTCCGCA	13	13	3	3
OPP10	TCCCGCCTAC	11	11	4	4
OPP16	CCAAGCTGCC	17	17	5	5
OPP19	GGGAAGGACA	13	12	9	8
OPP20	GACCCTAGTC	12	12	2	1
OPS01	CTACTGCGCT	10	10	3	3
OPS02	CCTCTGACTG	11	11	3	3
OPS03	CAGAGGTCCC	20	19	8	6
OPS05	**TTTGGGGCCT**	**26**	**26**	**8**	**7**
OPS06	GATACCTCGG	12	12	2	2
OPS08	TTCAGGGTGG	18	18	10	8
OPS09	TCCTGGTCCC	12	12	5	5
		345	342	129	98

UPGMA Cluster analysis

The date were obtained from RAPD analysis of 12 isolates of *U. tritici* and 2 isolates of *U. nuda* with 25 primers was subjected to UPGMA analysis. A dendrogram for these 14 isolates was prepared using the similarity coefficient of RAPD marker (Fig. 1). The dendrogram revealed the existence of variability among isolates of *U. tritici,* whereas isolates of *U. nuda* are grouped together in one cluster. Among the *U. tritici* isolates, Ut12 was found to be

separated from all other isolates of *U. tritici* and *U. nuda.* The rest of the *U. tritici* isolates were divided into two major cluster with Ut13 and Ut11 belonging to one and Ut12, Ut7, Ut4, Ut6, Ut8, Ut10, Ut15, Ut17 and Ut20 to the other cluster. These clusters were further divided into smaller clusters.

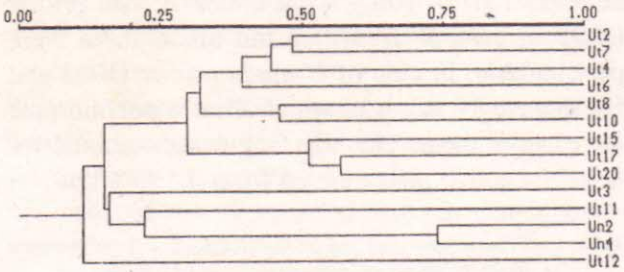


Fig. 1 : Dendrogram from RAPD analysis of *Ustilago tritici* (Ut) and *U. nuda* (Unisolates. Scale is distance by UPGMA method as implemented in NTSYS – pc.

UPGMA cluster analysis was also done for RAPD markers obtained with primer OPN5 and OPM6 for *U. tritici* and *U. nuda.* This was done because the two primers showed a very distinct pattern clearly differentiate *U. nuda* from *U. tritici* (Fig. 4). In case of *U. nuda* the banding pattern was monomorphic with both OPN5 and OPM6, whereas in *U. tritici* the primers gave polymorphic pattern. The dendrogram obtained separated completely *U. nuda* from all the other isolates of *U. tritici.* The cluster analysis revealed Un2 and Un4 to be exactly similar. In this dendrogram all the isolates of *U. tritici* including Ut12 belong to a separate cluster than *U. nuda.* Ut12 belongs to the same cluster as Ut3 and Ut11, but is separated from them at 20% level. Ut17 and Ut20 which are grouped in the same cluster in Fig. 1 appear as completely identical isolates in Fig. 2, except for the grouping of Ut8, Ut15 together in one cluster and Ut10 in another. The dendrogram obtained from *U. tritici* isolates was found to be similar to Fig.1 (Fig. 2).

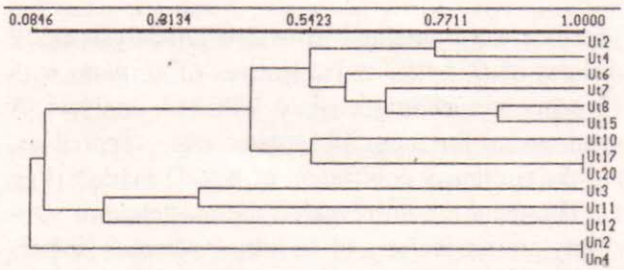


Fig. 2 : Dendrogram generated using UPGMA analysis demonstrating the relationships among *Ustilago tritici* and *U. nuda* based on RAPD data from primers OPN5 and OPM6.

It was obtained that even by using the data from only primer OPN5 we could differentiate *U. Nuda* isolates from *U. tritici,* Un2 and Un4 are grouped together but belong to a major clusted comprising of ohter *U.tritici* isolates, Ut3, Ut11 and Ut12 are separated from other *U. tritici* isolates as well as two isolates of *U,. nuda.* Isolates (Ut2 and Ut4), (Ut8 and Ut15), (Ut17 and Ut20) are identical to each other (Fig. 3).

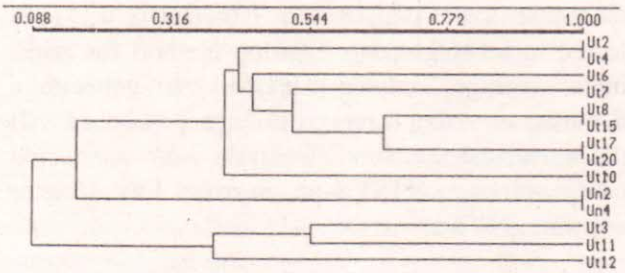
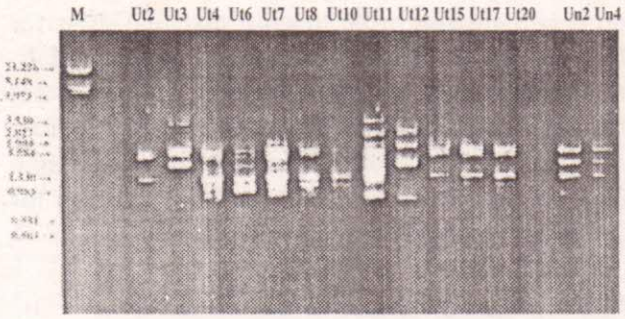
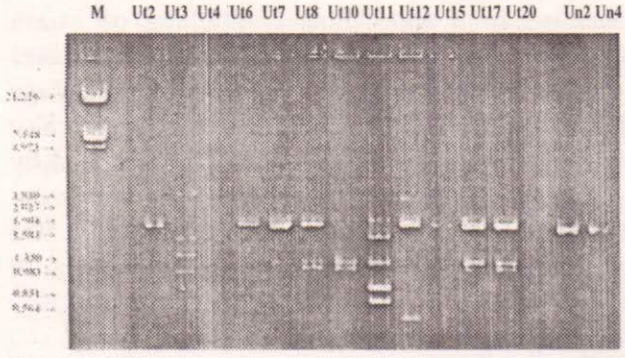


Fig. 3 : Dendrogram of RAPD profiles of *Ustilago tritici* and *U. nuda* isolates constructed using NTSYS-pe based on data analysis from primer OPN5.



OPN5 (a)



OPM6 (b)

Fig. 4 : RAPD analysis of 12 isolates of IUstilago tritici (Ut) and 2 isolates of *U. nuda* (Un) with primers OPN5 (a) and OPM6 (b). The products were separated in digested with *Eco*RI and *Hin*dIII.

DISCUSSION

Loose smut of wheat and barely is caused by the heterobasidiomycetous fungus *Ustilago* spp. A trinomial system to designate strains that are specialized on certain host species is impractical because of the multitude of possible *formae specialis* and the overlapping pathogenicity of each strain on several host species or genera (Nielsen, 1985). Kim *et al.* (1984) compared the detergent—soluble polypeptides patterns of *U. tritici* and *U. nuda* and found that they differed by 47 polypeptides, this high level off interspecific variation supports the concept of treating these two pathogens as separate taxon.

As *U. tritici* and *U. nuda* have identical teliospore and a similar biology, the two have been claimed to be same organism. This taxonomic treatment, based solely on teliospore morphology is untenable because it ignores the important differences (Kellerman & Swingle, 1889 ; Kim *et al.,* 1984 ; Kanehira & Shinohar, 1986 ; Vanky, 1994). These differences justify *U. tritici* and *U. nuda* as valid, though related species. *U. nuda* may have evolved from *U. tritici* (Nielsen, 1987).

The present investigation involving studies on teliospore morphology was not able to help us in separating *U. tritici* from *U. nuda* (Sharifnabi *et al.,* in press). This prompted us to undertake research in the area of using DNA polymorphism for separating the two. Similar appraoches have been undertaken for *Tilletia* spp. where again lack of sufficient morphological characters has resulted in grouping of species with morphologically similar species. Shi *et al.,* (1996) used 23 RAPD markers to divide 96 isolates of *Tilletia* spp. into groups with 12% similarity, The analysis of 16 isolates belonging to the *Tilletia fusca* complex using RAPD markers, and a subset of eight isolates using RFLP markers, supports the separation of the *Vulpia*-infecting variety *Fusca* from *Bromus*—infecting varieties *bromi—tectorum* and *guyotiana* (Boyed & Carris, 1997).

Different isolates of the two species subjected to RAPD analysis using 25 random decamer primer gave interesting information regarding variability among each of them and was also able to separate *U. tritici* from *U. nuda* isolates. *U. nuda* isolates gave identical banding pattern with primer OPN5 and OPM6 and this pattern was very distinct from the banding pattern of *U. tritici* isolates. The *U. tritici* isolates amongst themselves showed a high degree of variability. UT12 isolate from Kanpur, U.P. which hot separated from others by it's teliospore size, gave a distinct pattern and was separated from all other isolates by UPGMA cluster analysis. The clustering of isolates based on RAPD analysis does not bear any correlation with the geographical distribution of the isolates as well as the grouping based on teliospore morphology.

Therefore, in the light of these researches, the present work provides us a strategy to separate *U. tritici* from *U. nuda* and highlights the importance of the use of molecular approaches to differentiate species as an additional aid in taxonomic classification where other characters are either lacking or identical to each other. This tool leads to be exploited to it's fullest potential by separating the number of isolates in question as well as availability of the desired facilities.

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REFERENCES

Boyd, M.L., and Carris, L.M. (1997). Molecular relationships among varieties of the *Tilletia fusca (T. bromi)* complex and related species, *Mycol, Res.* **101** : 269-277.

Cooke, D.E.L. ; Kennedy, D.M. ; Guy, D.C. ; Russel, J. ; Unkles, S.E. and Duncan, J.M. (1996), Relatedness of group I species of *Phytophtora* as assessed by randomly amplified polymorphic DNA (RAPD) and sequence of ribosomal DNA. *Mycol. Res.* **100** : 297-303.

Hseu, R.S. ; Wang H.H ; Wang, H.F. and Moncalvo,. J.M. (1996). Differentiation and grouping of isolates of the *Ganoderma lucidum* complex by random amplified polymorphic DNA-PCR compared with grouping on the basis of internal transcribed spacer sequences. *Appli. Envirn. Microbio.* **62** : 1354-1363.

Jaccard, P. (1908). Nouvelles recherches sur la distibution florale. *Bull. Soc. Vaud. Sci. Nat.* **44** : 223-270.

Jeng, R.S. : Dumas, M. ; Liu, F.H. ; Wang, C.L. and Hubbes, M. (1997). DNA analysis of *Cylindrocladium floridamum* isolates from selected forest nurseries., *Mycol. Res.* **101 :** 285-291.

Jungehulsing. U. and Tudxynski, P. (1997). Analysis of genetic diversity in *Claviceps purpurea* by RAPD *markers.* Vycol. Res. **101** : 1-6.

Kanehira, T. and Shinohara, M. (1986). Comparison of electrophoretic patterns of enzymes from *Ustilago* spp. parasitic on barely, wheat and oats. *Ann. Phytopath. Soc. Japan.* **52** : 660-668.

Kellerman. W.A. and Swingle, W.T. (1989). Report on the loose smuts of cereals. Kansas Agric. Exp. Sta. Manhattan Ann. Rpt., pp. 213-288.

Kim, W.K. ; Rohringer, R. and Nielsen, J. (1984). Comparison of polypeptides in *Ustilago* spp. pathogenic on wheat, barely and oats : a chemotaxonomic study. *Can. J. Bot.* **62** : 1431-1437.

Maurer, P. ; Couteaudier, Y. ; Girard, P.A. ; Bridge, P.D. and Riba, G. (1997). Genetic diversity of *Beauveria bassiana* and relatedness to host insect range, *Mycol. Res.* **101 :** 159-164.

Nielsen. J. (1985). *Ustilago* spp. pathogenic on *Aegilops.* II. *Ustilago tritici. Can. J. Bot.* **63 :** 765-771.

Nielsen, J, (1987). Reaction of *Hordeum* species to the smut fungi *Ustilago nuda* and *U. tritici. Can. J. Bot.* **65 : 2024-2027.**

Pei, M.H. ; Whelan, M.J, ; Halford, N.G. and Royle, D.J. (1997). Distinction between stem and leaf- infecting forma of *Melapsora* rust on *Salix viminalis* using RAPD markers. *Mycol. Res.* **101 :** 7-10.

Rafalski, J.A. ; Tingey, S.V. and Williams, J.G.K. (1991). RAPD markers a new technology for genetic mapping and plant breeding. *AgBiotech. News Info.* **3 ;** 645-648.

Sambrook, J. ; Fritsch, E.F. and Maniatis, T. (1989). *Molecular cloning-A laboratory manual.* 2nd Edition. Cold Spring Harbor. NY, USA.

Sharifnabi, B. ; Mitter, N. and Sarbhoy, A.K. (2000). A technique for production of monokaryotic haploid hyphae from *Ustilago tritici,* causal agent of wheat loose smut. *Indian Phytopath.* **53 :** 101-102.

Sharifnabi, B. : Agarwal, D.K. and Mitter, N (in press) Characterization of Ustilago spp. pathogenic on wheat and barely : Teliospore morphology-I. Vanky. K. (1994). *European smut fungi.* Gustav Fischer Verlag. NY, USA, 570 pp.

Willams, J.G.K. ; Kubelik, A.R. ; Livak, K.J. ; Rafalski, J.A. and Tingey, S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful genetic markers. *Nucl. Acid Res.* **18 :** 6531-6535.

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