

Full Length Research Paper

***Trichoderma* species variety examination based on internal transcribed spacer**

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Accepted 21 December, 2007

The phylogeny of *Trichoderma* and the phylogenetic relationships of its species was investigated by maximum parsimony analysis and distance analysis of DNA sequences from multiple genetic loci 18S rDNA sequence analysis suggests that the genus *Trichoderma* evolved at the same time as *Hypomyces* and *Fusarium* and thus about 110 Myr ago 28S rDNA sequence analysis shows that the genus *Trichoderma* is part of a monophyletic branch within the *Hypocreaceae*. Most isolates of the genus *Trichoderma* were found to act as mycoparasites of many economically important aerial and soil-borne plant pathogens. *Trichoderma* has attained importance as a substitute for chemical pesticides and hence an attempt was intended to corroborate the positive relatedness of molecular and morphological characters. Two fungal strains, *Trichoderma koningii* T_k-5201/CSAU and *Trichoderma virens* T_{vi}-4177/CSAU were isolated from a soil sample collected from CSA Farm, Kanpur district of Uttar Pradesh, India. The universal primers (internal transcribed spacer, ITS) were used for the amplification of 18S rRNA gene fragment and strains were thus characterized with the help of ITS marker. It is proposed that the identified strains *T. koningii* T_k-5201/CSAU and *T. virens* T_{vi}-4177/CSAU be assigned as the type strains of a species of genus *Trichoderma* based on phylogenetic tree analysis together with the 18S rRNA gene sequence search in Ribosomal Database Project, small subunit rRNA and large subunit rRNA databases. The sequence was deposited in GenBank with the accession numbers KC800923 and KC800924, respectively. Thus an integrated approach of morphological and molecular markers can be employed to identify a superior strain of *Trichoderma* for its commercial exploitation.

Key words: 18S ribosomal RNA gene, *Trichoderma*, phylogenetic analysis, internal transcribed spacer (ITS), DNA sequencing, GenBank.

INTRODUCTION

Species of the deuteromyceteous genus *Trichoderma* are cosmopolitan and typically soil-borne or wood-decaying fungi (Samuels, 1996; Esposito and Da Silva, 1998).

Meyer and Plaskowitz (1989) and Meyer (1991) demonstrated diversity in conidium ornamentation and mito-chondrial DNA and plasmids among strains having warted conidia, and they hinted at a need for taxonomic revision. *Trichoderma*, commonly available in soil and

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root ecosystem, has gained immense importance since last few decades due to its biological control ability against several plant pathogens. Some strains of

Trichoderma like *Trichoderma harzianum*, *Trichoderma atroviride*, *Trichoderma viride*, *Trichoderma virens* and *Trichoderma koningii* are efficient bio-control agents which have the ability to inhibit pathogen growth in the soil, hence improving the overall health of the plant. Antagonistic microorganisms such as *Trichoderma* reduce growth, survival of pathogen by different mechanisms like competition antibiosis, mycoparasitism, hyphal interactions and enzyme secretion. Such microorganisms are now commercially available and used in crop management and practices. The use of *Trichoderma* species as biological control agents has been investigated for over 70 years, but it is only relatively recent that strains have become commercially available.

Accurate and definitive fungal identification is essential for correct disease diagnosis and treatment of associated fungal infections. Characterization of fungal species using classical methods is not as specific as the genotyping methods. Genotypic techniques involve the amplification of a phylogenetically informative target, such as the small-subunit (18S) rRNA gene (Woese et al., 1977). rRNA is essential for the survival of all cells, and the genes encoding the rRNA are highly conserved in the fungal and other kingdoms. The sequences of rRNA and proteins comprising the ribosome are highly conserved throughout evolution because they require complex inter- and intra-molecular interactions to maintain the protein-synthesizing machinery (Sacchi et al., 2002; Hillis et al., 1991; Woese, 1987). *Trichoderma* spp. are common soil inhabitants and are effective in providing biocontrol of soil borne pathogens due to antagonistic behavior. The major aspect of successful biological control strategies includes the production, formulation and delivery system of bio-agents. The internal transcribed spacer (ITS) region of the rDNA is perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematic study at species level, and even within species. Many scientists (Ospina-Giraldo et al., 1998; Kubicek et al., 2000; Kulling-Gradinger et al., 2002; Lee et al., 2002; Kindermann et al., 1998) attempted the phylogenetic analysis of whole genus of *Trichoderma* using sequence analysis of the ITS region of rDNA.

In this study, the method of isolation and identification of an unknown fungus from CSA Farm, Kanpur district using 18S rRNA gene sequence as reported in bacterial rRNA gene (Srivastava et al., 2008) was used to cha-

racterize the strains $T_K-5201/CSAU$ and $T_{Vi}-4177/CSAU$ as members of the *Trichoderma* spp. The soil sample has received great attention from public due to its potential for biodiversity and biological conservation. The ITS region of the rDNA is perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematic study at species level and even within species (Nirenberg, 1976).

MATERIALS AND METHODS

Isolation and Identification of *Trichoderma*

Soil samples were collected from various experimental fields of Indian CSA Farm, Kanpur district. Isolate of *Trichoderma* species was identified in potato dextrose agar (PDA) with low sugar medium (Hiney et al., 1992). The identification of *Trichoderma* isolates were confirmed both by morphological and molecular characters (ITS), and deposited in Indian Type Culture Collection (ITCC), IARI, Pusa, New Delhi that allotted the Accession Numbers 5201 and 4177 to T_K and T_{Vi} strains, respectively.

Agarose gel electrophoresis

Ten microlitre of the reaction mixture was then analyzed by submarine gel electrophoresis using 1.0% agarose with ethidium bromide at 8 V/cm and the reaction product was visualized under Gel Doc/UV trans-illuminator.

Internal transcribed spacer (ITS) region

The ITS regions of the rDNA repeat from the 3' end of the 18s and the 5' end of the 28s gene were amplified using the two primers, ITS A and D which were synthesized on the basis of conserved regions of the eukaryotic rRNA gene (White et al., 1990). The polymerase chain reaction (PCR) amplification reactions were performed in a 50 μ l mixture containing 50 mM KCl, 20 mM Tris HCl (pH 8.4), 2.0 mM MgCl₂, 200 μ M of each of the four deoxynucleotide triphosphates (dNTPs), 0.2 μ M of each primer, 40 ng/ μ l of template and 2.5 U of Taq polymerase. The cycle parameters included an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min and primer extension at 72°C for 3 min and a final extension for 10 min at 72°C. Amplified products were separated on 1.2% agarose gel in TAE buffer, pre-stained with ethidium bromide (1 μ g/ml) and electrophoresis was carried out at 60 v for 3 h in TAE buffer. One Kb ladder (MBI, Fermentas) was used as a marker. The gel was observed in a trans-illuminator over ultra violet light. The desired bands were cut from the gel with minimum quantity of gel portion using QIAGEN gel extraction kit.

Nucleotide sequencing and *in silico* analysis

The sequencing of the PCR product was carried out in automated Sequencer at Genome - Bio, Pune, India. Related sequences were searched using Basic Local Alignment Search Tool (BLAST) programme from the Gen-Bank database (<http://www.ncbi.nlm.nih.gov/blast/>) (Altschul et al., 1997). The multiple sequence alignment and pairwise alignment were made using BioEdit version 5.09 (Hall, 1999). The neighborhood-joining bootstrap tree was created using CLUSTAL W 1.6 matrix by the CLUSTAL X programme ver. 1.81 [Thompson et al., 1997].

RESULTS

Confrontation assay *in vitro*

Biocontrol agents are widely regarded as "natural" and non-threatening products, although risk assessments must clearly be carried out on their effects on non-target organisms. Moreover, knowledge concerning the beha-



Figure 1. *Trichoderma koningii* T_K-5201/CSAU and *T. virens* T_{Vj}-4177/CSAU strain in PDA medium.

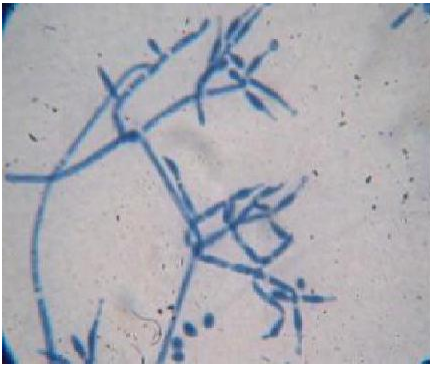


Figure 2. *Trichoderma koningii* T_K-5201/CSAU and *T. virens* T_{Vj}-4177/CSAU strain under microscope.

viour of such antagonists is essential for their effective use. The morphological and microscopic figures 1 and 2 of *T. koningii* and *T. virens* are given below.

Molecular characterization

Rapid identification of bioagents is very necessary and important in the pathological laboratory to take decision for installment of commercialized bioformulation. The rRNA based analysis is a central method in pathology used not only to explore microbial diversity but also to identify new strains. The genomic DNA was extracted from isolated

fungal strains *T. koningii* T_K-5201/CSAU and *T. virens* T_{Vj}-4177/CSAU. The universal ITS1 primers 20F and ITS4 primers 22R were used for the amplification and sequencing of the 18S rRNA gene fragment. A total of 206 and 635 bp of the 18S rRNA gene were sequenced and used for the identification of isolated fungal strain. Subsequently, 18S rRNA gene sequence based phylogenetic tree showing the relationships bet-

ween the test strains T_K-5201/CSAU and T_{Vj}-4177/CSAU and selected representatives of the genus *Trichoderma* is given in Figure 3. It is evident from the phylogenetic analysis of 18S rRNA gene that the isolates T_K-5201/CSAU and T_{Vj}-4177/CSAU represent a genomic species in the genus *Trichoderma*. Comparison of test strain against known sequences of SSU rRNA and LSU rRNA databases showed that the gene sequence of isolate T_K-5201/CSAU and T_{Vj}-4177/CSAU has 90% sequence similarity (Score=546 bits, Expect=0.0) with 18S rRNA gene sequence of *Trichoderma* (Genbank Acc. No.: KC800923 and KC800924). Thus, data shows that the isolate T_K-5201/CSAU and T_{Vj}-4177/CSAU is a member of the genus *Trichoderma*.

Phylogenetic resolution of *Trichoderma* sp. genotypes

Similarity rank program classifier available at the Ribosomal Database Project (Wang et al., 2007) classified

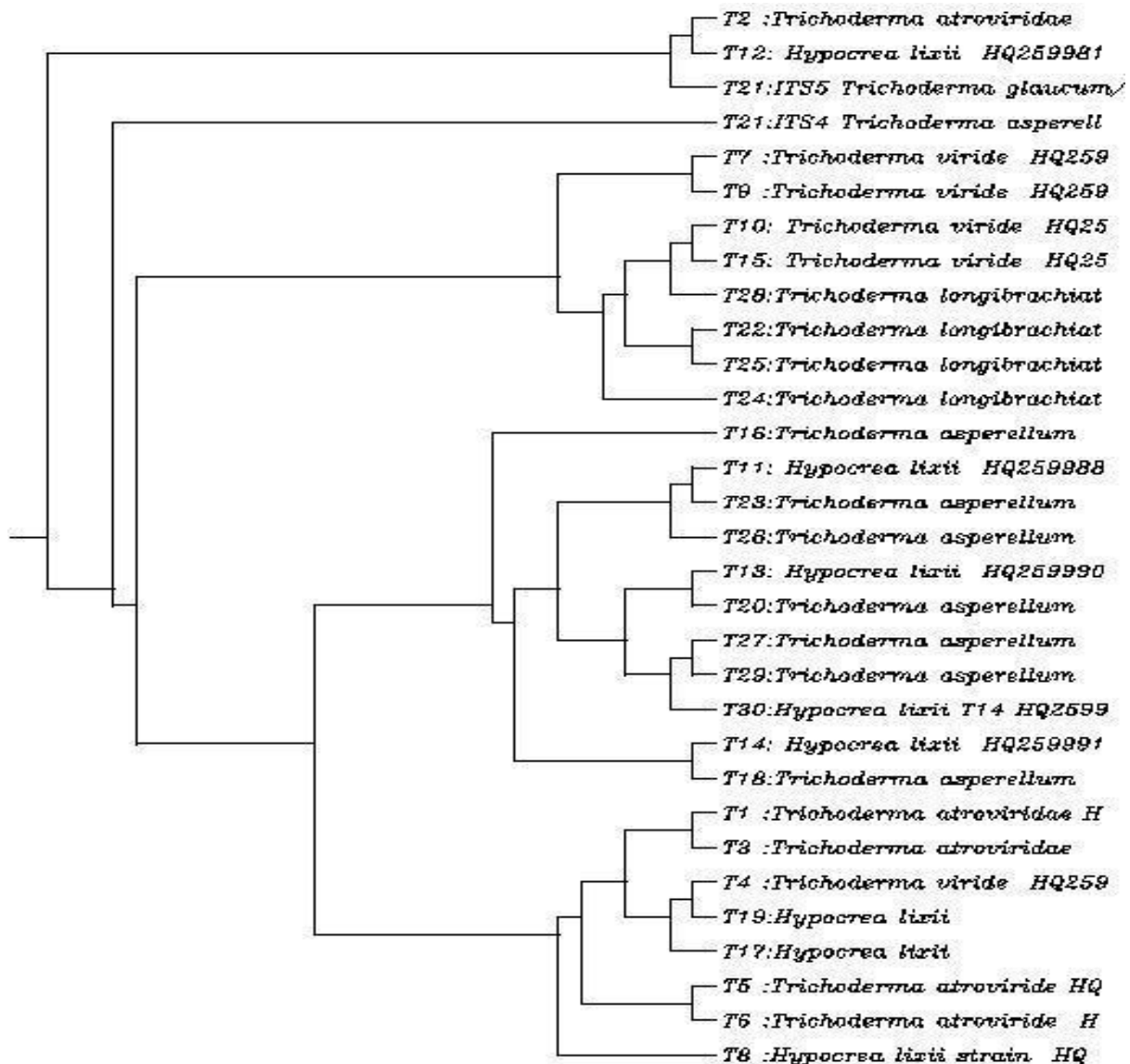


Figure 3. Phylogenetic and molecular variability analysis of *Trichoderma* species.

the isolate *T_K-5201/CSAU* and *T_{Vj}-4177/CSAU* as a novel genomic species of the genus *Trichoderma* with a confidence threshold of 90% (Figure 3). The 18S rRNA gene sequence of isolate *T_K-5201/CSAU* and *T_{Vj}-4177/CSAU* was deposited in GenBank and allotted the accession numbers KC800923 and KC800924, respectively.

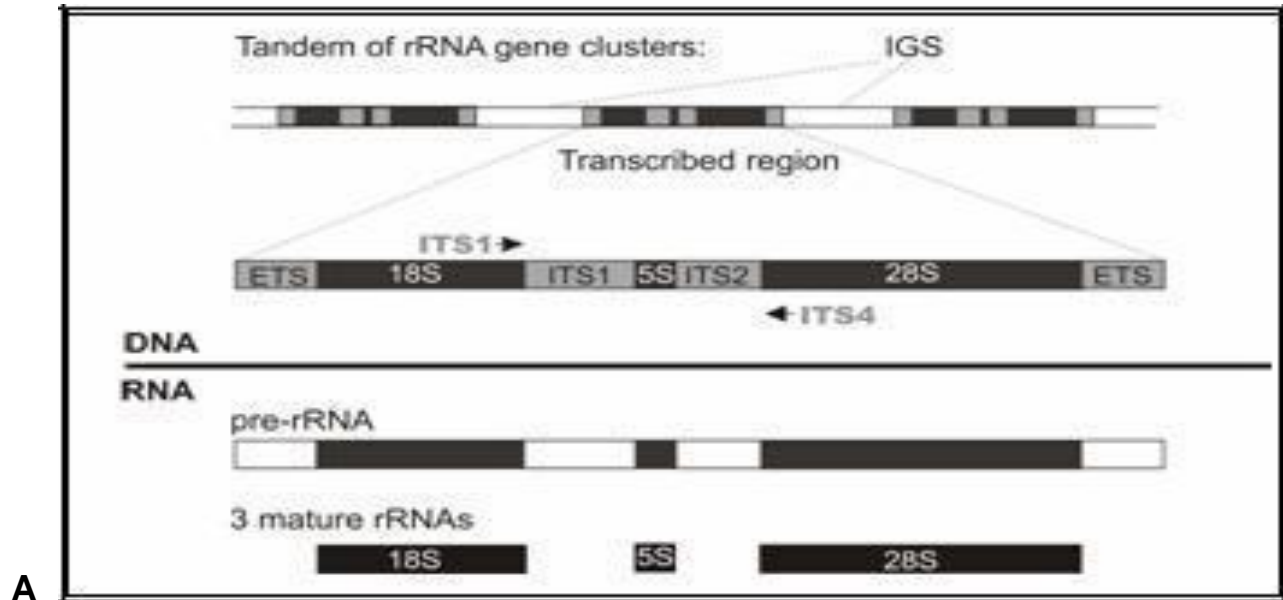
DISCUSSION

Molecular analysis using internal transcribed spacer (ITS) region

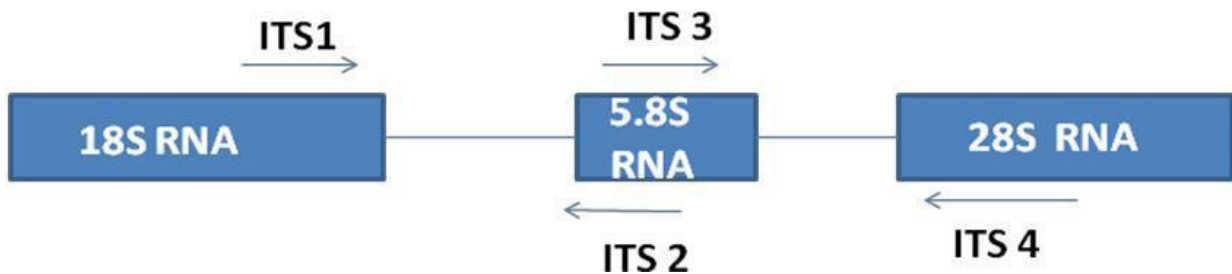
Ribosomal RNA (rRNA) sequence analysis has been well-documented as a means of determining

phylogenetic relationships in all of the major organismal domains. Variable sequences within the mature small subunit (SSU) and large-subunit (LSU) rRNA genes have been found to be appropriate for assessing subgeneric relationships in many eukaryotes. One of these variable regions, the D2 region of the LSU, has been used to determine phylogenetic relationships in a number of pathogenic fungal genera (Logrieco et al., 1995). The ITS region of the rDNA operon, which is more variable than the D2 region, has proven useful in distinguishing relationships at the species level (Kusaba et al., 1995).

The genetic variability within 69 bio-control isolates of *Trichoderma* collected from different geographic locations and culture collections and their phylogenetic analysis were done with the help of the sequence data obtained



A



B

Figure 4. Location of Internal Transcribed Spacer sequence 1 and 2 (A) and the position of primers for PCR based amplification of the region (B).

from the inter transcribed spacer 1 (ITS1) region of ribosomal DNA and a fragment of the translation elongation factor 1 (*tef1*) and reported that more than 50% of the potential bio-control strains were grouped within *Trichoderma* section *Pachybasium* (Heramosa et al., 2004).

Trichoderma isolates with different biocontrol capabilities and identification by molecular methods were further characterized into three main clades by sequence analysis. Consequently, a reliable phylogenetic tree was constructed containing isolates belonging to the *T. harziunum* clade (Maymon et al., 2004).

In eukaryotes, the genes encoding ribosomal RNAs are organized in arrays which contain repetitive transcriptional units involving 16 - 18S, 5.8S and 23 - 18S rRNAs, two transcribed intergenic spacers ITS1 and ITS2 and two external spacer sequences (5' and 3' ETS). These

units are transcribed by RNA polymerase I and separated by non-transcribed intergenic spacers (IGS) as represented in Figure 4. The product of RNA polymerase I is processed in the nucleolus, where ITS1 and ITS2 are excised and three types of rRNAs are produced. In eukaryotic genomes, the ITS regions vary greatly in size and sequence. In *S. cerevisiae* the ITS1 spans 361 bp and ITS2 is 232 bp long, the PCR and subsequent analysis of amplified rDNA using restriction endonucleases were employed in different studies to achieve efficient interspecies discrimination in medical and food mycology. The ITS regions have important biological meaning in rRNA processing. The structures of analyzed ITS1 and ITS2 contain four or three helical arms. The changes in size and sequence of these regions are then biologically permissible as long as they do not disturb the formation of secondary structures which facilitate the rRNA

processing. It thus presents a simple method for determination of inter- and intra-species variability in fungal isolates.

Molecular phylogenetic analyses of biological control strains of *Trichoderma* (*Ascomycetes*, *Hypocreales*) that have warted conidia are traditionally identified as *T. viride*, the type species of *Trichoderma*. However, two morphologically distinct types of conidial warts (I and II) have been found. Since each type corresponds to a unique mitochondrial DNA pattern, it has been questioned whether *T. viride* comprises more than one species. Combined molecular data (sequences of the ITS-1 and ITS-2 regions and part of the 18S rRNA gene along with results of restriction fragment length polymorphism analysis of the endochitinase gene and PCR fingerprinting), morphology, physiology, and colony characteristics distinguish type I and type II as different species. Type I corresponds to "true" *T. viride*, the anamorphic of *Hypocrea rufa*. Type II represents a new species, *T. asperellum*, which is, in terms of molecular characteristics, close to the neotype of *T. hamatum*.

Analysis of ITS1-5.8S-ITS2 region of the cDNA showed that approximate 600 bp and size variation was observed. Restriction analysis of this region showed that inter and intra -specific polymorphism (Latha et al., 2002). *Trichoderma* has attained importance for substitute of chemical pesticides and hence an attempt was intended to corroborate the positive relatedness of molecular and morphological characters. A fungal strain of *Trichoderma longibrachiatum* 28CP/7444 was isolated from a soil sample collected from Barabanki district of Uttar Pradesh, India. The universal primers were used for amplification of the 28S rRNA gene fragment and strain characterized by using 28S rRNA gene sequence with the help of ITS marker. It is proposed that the identified strain *T. longibrachiatum* 28CP be assigned as the type strain of a species of the genus *Trichoderma* based on phylogenetic tree analysis together with the 28S rRNA gene sequence search in ribosomal database project, small subunit rRNA and large subunit rRNA databases. The sequence was deposited in GenBank with the accession number JX978541. Thus, an integrated approach of morphological and molecular markers can be employed to identify a superior strain of *Trichoderma* for its commercial exploitation (Shahid et al., 2013; Cornelia et al., 2002; Gary et al., 2010; Muthu Kumar et al., 2011).

Conclusion

Most of the *Trichoderma* species are morphologically very similar and were considered as a single species for many years. Since new species were discovered, a consolidated taxonomical scheme was needed, proposed and defined nine morphological species aggregates. DNA methods brought additional valuable criteria to the taxonomy of *Trichoderma* which are being used today for

studies that include identification and phylogenetic classification. *Trichoderma* strains used as biocontrol agents can act in the following ways as: a) Colonizing the soil and/or parts of the plant, occupying a physical space and avoiding the multiplication of the pathogens; b) producing cell wall degrading enzymes against the pathogens; c) producing antibiotics that can kill the pathogens; d) promoting the plant development and e) inducing the defensive mechanisms of the plant. Antifungal formulations based on *Trichoderma* strains and proteins require, as in the case of chemical fungicides, a costly and sound registration process previous to their commercialization.

ACKNOWLEDGEMENTS

The authors are grateful to the financial support granted by the ICAR under the Niche Area of Excellence on "Exploration and Exploitation of *Trichoderma* as a antagonist against soil borne pathogen" running in Biocontrol Lab, Department of Plant Pathology, C.S.A University of Agriculture and Technology, Kanpur-208002, UP, India.

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