Automated rRNA intergenic spacer analysis (ARISA) was used to characterize bacterial (B-ARISA) communities from different land use systems. The 16S-23S intergenic spacer region from the bacterial rRNA operon was amplified from total soil community DNA for B-ARISA. Universal fluorescence-labeled primers were used for the PCRs, and fragments of between 200 and 1,200 bp. The PCR products are analyzed by an automated capillary electrophoresis system that produces an electropherogram, the peaks of which correspond to discrete DNA fragments detected by a laser-based fluorescence detection system. Methodological (DNA extraction and PCR amplification) and biological (inter and intrasite) variations were evaluated by comparing the number and intensity of peaks (bands) between electrophoregrams (profiles) and by principal component analysis. Our results showed that ARISA is a high-resolution, highly reproducible technique and is a robust method for discriminating between microbial communities. The current study aimed to test the hypothesis that both land-use change and soil type are responsible for the major changes in the bacterial community structure and functioning of the soil microbial community in regions of central Himalayas (India). Forest eco systems were highly diverse as compared to the agro ecosystem suggesting the intensive use harm to the microbial communities.

Key words: Soil DNA, Different land use systems, Central Himalayan region, Automated Ribosomal Intergenic Spacer Analysis
7.1 Introduction

Modern molecular biology leads to the development of culture-independent approaches for describing bacterial communities without bias. DNA fingerprinting allows the rapid assessment of the genetic structure of complex communities in diverse environments (Muyzer et al., 1998) and of the extent of changes caused by environmental disturbances (Engelen et al., 1998, Massol-Deya et al., 1997). Automated ribosomal intergenic spacer analysis (ARISA) is an automated, culture-independent technique suitable for analyzing structures of microbial environmental communities. This PCR-based technique, developed by Fisher and Triplett (Fisher et al., 2000), is based on the use of a fluorescent primer in the amplification of microbial ribosomal intergenic spacers, using DNA extracted from environmental samples as a template. A simple and reliable method is rRNA intergenic spacer analysis (RISA), which exploits the variability in the length of the intergenic spacer (IGS) between the small (16S) and large (23S) subunit tRNA genes in the rrn operon. This approach has been used successfully to assess the structure of soil bacterial communities (Ranjard et al., 2000). ARISA involves the use of a fluorescence-tagged oligonucleotide primer for PCR amplification and for subsequent electrophoresis in an automated system. This allows the bacterial community structure to be rapidly investigated even when there are a large number of samples. Due to the high resolution of the gels and the high sensitivity of fluorescence detection, the number of peaks detected is much higher on ARISA profiles than on RISA profiles. Similarly, differences in the intensity of the bands can be estimated precisely, which allows a finer comparison of the profiles.

In the present study, we evaluate the genetic fingerprint (profiles) of ten different land use systems in the central Himalayas.

7.2 Materials and methods

7.2.1 Study Site

Ten land use systems located in Almora region of Uttarakhand, India were selected for the study. Of this, four systems (organic farming, soybean-wheat, maize-wheat, fodder crops) are located at the experimental farm, Hawalbagh (29°36'N and 79°40'E at 1250...
mts above mean sea level) of Vivekananda Institute of Hill Agriculture, Almora and upland rice from Someshwar valley, which is 25 km away from the farm. The forest land use systems represent mixed forest (1800 mts amsl) undisturbed oak (2400 mts amsl) and pine (1800 mts amsl) forest of Binsar wildlife sanctuary (29°37' N and 79°20' E) and deodar forest of Jageshwar (29.65°N 79.58°E).

7.2.2 Soil and Weather Characteristics
The parent material of these soils consists of mica, schist, slates, sand stone, and calcium deficient granite and seynite rocks (Singh et al., 2000). Genetically these soils come under climatogenic podsolized grey-brown forest soils. All the systems were having acidic soil reaction except the soils of cultivated fields, which were slightly acidic. The climate is sub temperate, characterized by moderate summer (May–June), extreme winter (Dec–Jan) and general dryness, except during the southwest monsoon season (June–Sept).

7.2.3 Soil Sampling
Three composite soil samples from each site of two depths (0-15 cm and 15-30 cm) were collected in July 2009, 2010 and 2011. For making one composite sample, five soil cores were taken and mixed. Other workers [Patra et al., 2006] have also adopted pseudo-replication approach of sampling. The field moist soil samples were kept stored in refrigerator at temperature less than 4°C for preserving the enzyme activities till the analysis were over. All chemical results are mean of triplicate analysis and expressed on oven dry basis. Soil moisture was determined after drying at 105°C for 24 h.

7.2.4 Automated Ribosomal Intergenic Spacer Analysis
Soil ribosomal intergenic spacer analysis (RISA) was done according to Ranjard et al., 2001 total genomic DNA were extracted from 0.5 g sieved and soil using Power soil MO-BIO kit according to the manufacture recommendation. The intergenic spacers between the small- and large-subunit rRNA genes were amplified using the primers S-D-Bact-1522-b-S-20 (eubacterial rRNA small subunit, 5' TGCGGCTGGATCCCCCTCTT- 3') and L-D-Bact-132-a-A-18 (eubacterial rRNA
large subunit, 5'-CCGGGTTTCCCCATTCGG-3'). Amplified sequences which contained the IGS plus 150 bp corresponding to the 20 nucleotides of the S-D-Bact-1522-b-S-20 primer and about 130 bp in the 23S rRNA gene. Reaction mixtures (50 μl) for PCR contained 5 μl of 10x dilution buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 15 mM MgCl2, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.5% Tween 20 (vol/vol), 0.5% Nonidet P40 (vol/vol), 50% glycerol (vol/vol)), 1 μg T4 gene 32 protein (New England Biolabs), 0.5 μM of primers, 200 μM of each dNTP, 2.0 U of PR polymerase (Genei), and 100 ng of purified soil DNA. Amplification was performed in a Bio-Rad C1000 after a hot start at 94°C for 3 min, followed by 25 cycles consisting of 94°C for 1 min, 55°C for 30s, and 72°C for 1 min, extension of incomplete products for 5 min at 72°C. The volume of PCR reactions loaded on gel was calibrated in order to get a similar intensity per profile. And for more sensitivity the forward primer D-Bact-1522-b-S-20 (eubacterial rRNA small subunit, 5'-TGCGGCTGGATCCCCTCCTT-3'), 5' end was labeled with FAM dye and the PCR products were run under standard ABI310 denaturing electrophoresis conditions for 1 h each, with the POP-4 polymer, and the data were analyzed by using the GeneScan 3.1 software program (Perkin-Elmer).

7.2.5 Statistical Analysis
Principal component analysis (PCA), which requires that profiles be encoded on the basis of the presence or absence of bands and their intensity, was used to evaluate similarities between communities. To convert data obtained from the GeneScan software rapidly into a table summarizing band presence (i.e., peak) and Intensity (i.e., height or area of peak). PAST software is used to generate the PCA plot of the different land use system and to link the similarity between the ecosystems.

7.3 Results and Discussion
Electrophregrams of different land use profiles with peaks (bands) ranging from (i.e., a 50-bp IGS) to 1,200 bp (a 1,050-bp IGS) as extrapolated by the GeneScan 1,000-bp ROX standard were obtained. Before the automated sequencer analysis the profiles bands were analyzed on 1% agarose gel (Figure 7.1). Considering optimal (1 bp) and similar resolution power throughout the entire gel and a low fluorescence threshold (50
Chapter 7: Automated Ribosomal Intergenic Spacer Analysis

U of fluorescence intensity), between 189 and 265 bands were detected per profile. Two land use systems bacterial ribosomal intergenic spacer peaks were shown on figure 7.3. The bacterial communities from the ten sites were easily differentiated because the number and intensity of bands detected on the electrophoregrams were totally distinct. The structure of the profiles, characterized by the number and length distribution of major bands (peaks of highest relative fluorescence intensity), varied between soil types. PCA was performed on ARISA profiles to evaluate the between-site variation. Each site could be differentiated on the basis of Bacterial -ARISA fingerprinting (Fig. 7.2). There were more differences between sites than between independent repetitions for the same site. ARISA peaks using PCA and the first two principal components (PCs) accounted for 68 and 12.8 % of total variance. The forest ecosystem are clustered at one group and the organic farming shows close similarity with the mixed forest ecosystem. All the other agro ecosystems were clustered at one group. Ribosomal intergenic spacer analysis (RISA) was used to analyze the structure of microbial populations in soil (Bomeman and Triplett, 1997) by comparing the profiles after polyacrylamide gel electrophoresis. At most, only a few tens of bands were detected, suggesting a probable underestimation of diversity due to difficulty in identifying weak bands and resolving contiguous bands. The automated version of RISA, the ARISA, is a rapid and precise technique that allows microbial communities to be investigated and compared easily; highlighting the taxonomic diversity, evident from the marked variability in ribosomal spacer length, in the prokaryote genomes (Fisher and Triplett, 1997).

Fisher and Triplett developed a method for ARISA involving a capillary electrophoresis system. This system provides a rapid and reproducible method for estimating the diversity and composition of indigenous bacterial communities. The comparison of the B-ARISA profiles obtained from these soil samples showed that each was characterized by a specific pattern, which suggests a particular genetic structure of the bacterial communities. These results confirmed the potential of ARISA for characterizing and differentiating the genetic structure of soil bacterial communities.
Figure 7.1: A) Soil genomic DNA extraction and purification  B) & C) RISA profiles of the soil samples.

(A) Lane 1 = Maize wheat, Lane 3 and 4 = Organic farming, Lane 5 and 6 = fodder, Lane 7 and 8 = Pine, Lane 9 and 10 = Oak forest, Lane 11 and 12 = Mixed forest, Lane 13 and 14 = Deodar forest, M = Molecular weight marker 1kb

(B) Lane 1 = Control, Lane 2 and 3 = Mixed forest Chabatia, Lane 4 and 5 = Jageshwar, Lane 6 and 7 = Pine, Lane 8 and 9 = Binsar, Lane 10 and 11 = Organic farming, M = Molecular weight marker 100bp

(C) Lane 1 = Control, Lane 1 and 2 = Maize wheat, Lane 3 = Control Lane 4 and 5 = Soyabean wheat, Lane 6 and 7 = Mannan upland, Lane 8 and 9 = Fodder, Lane 10 and 11 = American block horticulture Chubatia, M = Molecular weight marker 100bp

** = 1 Percent Agarose gel
Figure 7.2: Ordination of ten different land-use system soils sites in function of mean of three subsequent years in the space defined by the PC1 and PC2 axis of the PCA analysis carried out with Automated Ribosomal Intergenic Spacer Analysis. Component 1 and 2 represent 68 and 12% of the variation in the data respectively.
Figure 7.3: RISA profile analysis by total lab 100 software: a) Maize wheat cropping system b) Organic farming.
Chapter 7: Automated Ribosomal Intergenic Spacer Analysis

7.4 Conclusion

This work presents a first tentative differentiation of genetic fingerprints of land use system in the central Himalayan region. Our results have shown that the forest ecosystem have similarity with the organic farming microorganism genetic community structure indicating the impact of long term use inorganic fertilizer and land use intensification on land use systems of the central Himalayan region. ARISA is a very effective and sensitive method for detecting differences between complex bacterial in comparison to RISA