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## Molecular identification of yeast species associated with 'Hamei' – A traditional starter used for rice wine production in Manipur, India

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## ABSTRACT

In Manipur state of North-Eastern India, wine from glutinous rice using traditional solid state starter called 'Hamei' is particularly interesting because of its unique flavour. A total of 163 yeast isolates were obtained from fifty four 'Hamei' samples collected from household rice wine preparations in tribal villages of Manipur. Molecular identification of yeast species was carried out by analysis of the restriction digestion pattern generated from PCR amplified internal transcribed spacer region along with 5.8S rRNA gene (ITS1-5.8S-ITS2). Seventeen different restriction profiles were obtained from the size of PCR products and the restriction analysis with three endonucleases (Hae III, Cfo I and Hinf I). Nine groups were identified as *S. cerevisiae*, *Pichia anomala*, *Trichosporon* sp., *Candida tropicalis*, *Pichia guilliermondi*, *Candida parapsilosis*, *Torulaspota delbrueckii*, *Pichia fabianii* and *Candida montana* by comparing this ITS-RFLP profile with type strains of common wine yeasts, published data and *in silico* analysis of ITS sequence data available in CBS yeast database. ITS-RFLP profile of eight groups was not matching with available database of 288 common wine yeast species. The most frequent yeast species associated with 'Hamei' were *S. cerevisiae* (32.5%), *P. anomala* (41.7%) and *Trichosporon* sp. (8%). The identity of major groups was confirmed by additional restriction digestion of ITS region with Hind III, EcoRI, Dde I and Msp I. The genetic diversity of industrially important *S. cerevisiae* group was investigated using Pulsed Field Gel Electrophoresis (PFGE). Although most of the 53 strains of *S. cerevisiae* examined were exhibited a common species specific pattern, a distinct degree of chromosomal length polymorphism and variable number of chromosomal DNA fragments were observed with in the species. Cluster analysis showed seven major karyotypes (K1–K7) with more than 83% similarity. The karyotype pattern K1 was the most frequent (67.9%) among the strains from different samples. Other karyotypes K2–K7 were very unique with less than 80% similarity. Finally using mitochondrial DNA restriction analysis (mt-DNA RFLP), *S. cerevisiae* strains belonging to the major karyotype K1 were distinctly differentiated with highly polymorphic bands by Hinf I and Hae III endonucleases.

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## 1. Introduction

Rice wine from glutinous rice is a popular traditional alcoholic beverage in North-Eastern states of India. It is manufactured under non-sterile conditions at home scale using traditional solid rice flat cakes as starter. The principle of rice wine manufacturing consist of the saccharification of steamed rice starch by fungal enzymes under aerobic solid state fermentation and the moulded mass is mixed with water and is allowed to undergo submerged alcoholic fermentation by yeasts using traditional starter flat cakes (Blandino et al., 2003; Sujaya et al., 2004; Dung et al., 2007). 'Atingba' is an alcoholic beverage traditionally prepared in Manipur state of India from glutinous rice with starter called 'Hamei'. 'Hamei' is a natural starter (flat rice cake), similar to 'Ragi' of Indonesia, 'Budob' of Philippines, 'Chu' of China, 'Naruk' of Korea and 'Marcha' of Sikkim that has been traditionally

used for the preparation of rice wine (Tsuyoshi et al., 2005). The 'Hamei' cakes (Fig. 1B) are prepared from crushed raw rice with "Yangli" (*Albizia myriophylla*) bark powder @ 0.25 kg kg<sup>-1</sup> along with water to form dough like mass with moisture content 65–70%. This is inoculated by dry powdered starter 'Hamei' from previous batches, followed by thorough mixing. The inoculated dough is shaped into form flat cakes approximately 2–7 cm in diameter and 0.6–1.5 cm thickness. The prepared rice cakes are kept over rice husk (Fig. 1A) in the floor/ bamboo basket for 2–3 days at room temperature (20–30 °C). The desired state of fermentation is indicated by the swelling of cakes, alcoholic flavour production and yellowish coloration. These commercial undefined starters have been prepared during summer (May–July) and dried cakes have shelf life up to one year.

The "Hamei" is used by crushing the flat cake into powder, then mixing with cooked, cooled glutinous rice @ 5 cakes for 10 kg. The mixture is fermented under Solid State Fermentation in mud pots (Fig. 1C) covered with 'Hangla' (*Alocasia* sp.) leaves for 3–4 days during summer and 6–7 days in winter. This is followed by 2–3 days of

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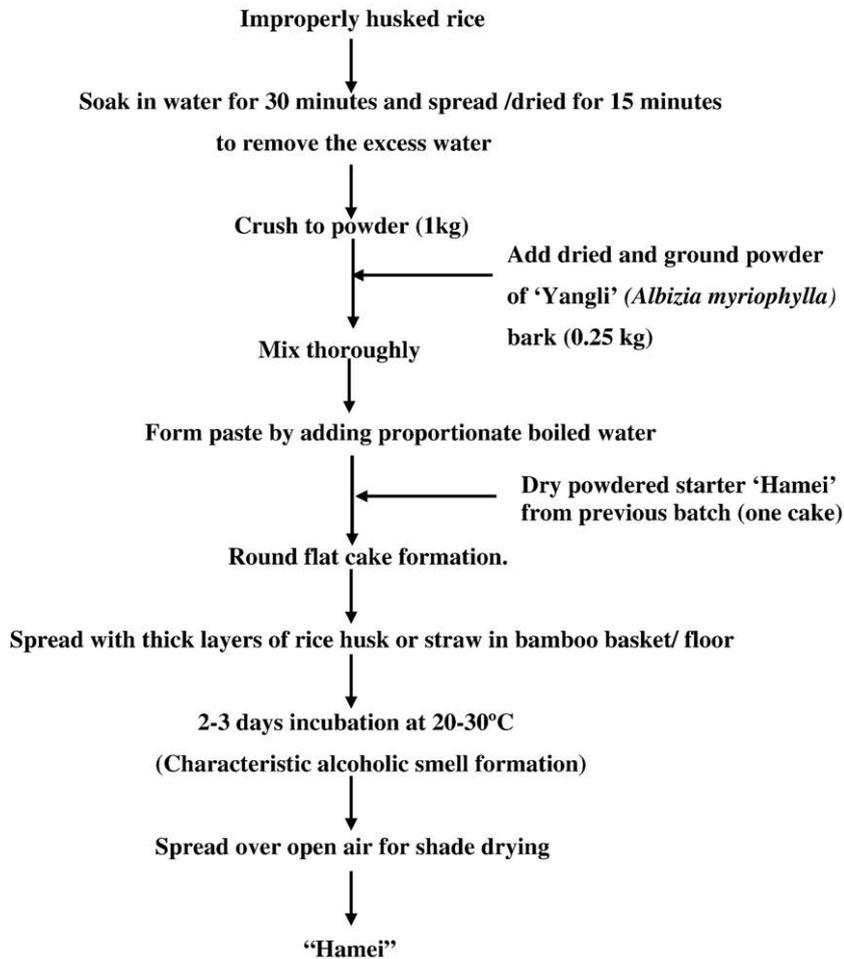


**Fig. 1.** 'Hamei' – traditional preparation over rice husk layered with gunny bags (A), dried 'Hamei' for storage & marketing (B) and solid state fermentation of glutinous rice inoculated with 'Hamei' powder for rice wine production in traditional earthen pots (C).

submerged fermentation in earthen pots. The filtered fermented beverage is called 'Atingba' and distilled clear liquor (using traditional assembly) is called 'Yu'. The recipes are being kept secret and passed on from generation by generation. This practice produces low yields of wine with variable quality where as wine producers are aware that the choice of starter influences the yield and quality of wine. The limited knowledge about the microbial composition of traditional starters and their effect on rice wine fermentation, obstacles development of defined mixed pure culture for industrialization of rice wine production (Dung et al., 2005).

Traditionally, yeasts have been identified based on morphological, physiological and biochemical characteristics (Jespersen, 2003). These methods are laborious and time consuming. Molecular methods based

on the analysis of polymorphism in DNA region that encodes the ribosomal RNA genes (5S, 5.8S, 18S and 26S) (Kurtzman and Robnett, 1998; Couto et al., 2005; Gonzalez et al., 2006) and the non-coding ITS (Internal Transcribed Spacers) (Sabate et al., 2002; Cadez et al., 2002) and IGS (Intergenic Spacer) regions (Diaz and Fell, 2000; Naumova et al., 2003) are being successfully used for the identification of many yeast species. In 1999, Esteve-Zarzoso et al., and Granchi et al., proposed a rapid and easy method for routine identification of yeast associated with fermented foods based on RFLP analysis of 5.8S rRNA gene and the Internal Transcribed Spacers (ITS1 and ITS2). Arias et al. (2002) compared different methodologies for the identification of yeast species and concluded that 5.8S rDNA-ITS-RFLP as the best method for rapid and accurate identification of yeasts species.



**Fig. 2.** Flow sheet for traditional 'Hamei' Preparation.

Comparative studies also demonstrated that the chromosome electrophoretic analysis using Pulsed Field Gel Electrophoresis (PFGE) (Canas et al., 1997; Sipiczki et al., 2001; Almedia et al., 2007) and mitochondrial DNA-RFLP (Querol and Barrio, 1990; Querol and Ramon, 1996) are able to differentiate strains of yeast species with industrial importance.

This investigation was aimed at the identification, differentiation and characterization of 163 indigenous yeasts isolates from 54 'Hamei' samples collected from different districts of Manipur, India. Yeast species were identified through analysis of the 5.8S-ITS-RFLP and the strain typing of yeast species with industrial importance was carried out using PFGE and mt-DNA RFLP.

## 2. Materials and methods

### 2.1. Samples collection

Indigenously producing 'Hamei' samples were aseptically collected during May–June, 2004 from different tribal villages (Khurkhul, Sekmai, Mahabalikhun, Dewlaland, Keikhu, Sangaithel choubok, Andro, Keinou, Jiribam, Sangaiprou, Kabuikhul, Irilbung) of Manipur and Silchar of Assam, India. They were brought into the laboratory and kept at 5 °C for microbial analysis. The information on the traditional

preparation of the products was documented (Fig. 2). 'Hamei' prepared in the laboratory by following the traditional method was also used for this study.

### 2.2. Isolation

Ten grams of powdered 'Hamei' sample was homogenized in 90 ml of 0.85% w/v sterile physiological saline. Two decimal dilutions ( $10^6$  and  $10^7$ ) were spread plated on plates of YEP agar (Yeast Extract 1%, Peptone 2%, Glucose 2%, Agar 2%, pH 6.5; Himedia, Mumbai, India) supplemented with ampicillin  $100 \text{ ng } \mu\text{l}^{-1}$  and plates were incubated at 28 °C. The colonies appeared were counted as colony forming unit (CFU)  $\text{g}^{-1}$  on dry weight of sample. The predominant representative colonies were selected based on colony morphology differences under microscope (Stereo binocular microscope SZ-ST, Olympus). Selected colonies were sub-cultured on new plates and purified by repeated streaking. A total of 163 isolates were obtained and maintained on YEP agar slants at 5 °C and in glycerol stock (30%) at –20 °C.

### 2.3. Yeast type cultures

Certified yeast strains of various species were obtained for controls from the Microbial Type Culture Collection (MTCC), Institute of

**Table 1**

Length in bp of the PCR amplified products of 5.8S rDNA-ITS region and ITS-RFLP obtained with three restriction endonucleases of the major species identified in this study

ITS-RFLP group	Species identification	Number of isolates	ITS size (bp)	Restriction digestion fragments size (bp)			Reference
				Hae III	Hinf I	Cfo I	
I	<i>Saccharomyces cerevisiae</i>	53	880	320+240+180+140	380+110	375+345	Esteve-Zarzoso et al., 1999
II	<i>Pichia anomala</i>	68	650	635	315	610	Granchi et al., 1999 and Esteve-Zarzoso et al., 1999
III	<i>Trichosporon</i> sp.	13	440	440	245+200	440	<i>In silico</i> analysis of ITS-rDNA sequence from CBS yeast database (Table 2)
IV	<i>Candida tropicalis</i>	4	530	450+90	265	280+245	Frutos et al., 2004
V	<i>Pichia guilliermondii</i>	3	630	400+125+95	310+280	260+220+50	Villa-Carvajal, 2006
VI	<i>Candida parapsilosis</i>	2	520	420+90	270+230+60	300+245	Guillamon et al., 1998
VII	<i>Torulospira delbrueckii</i>	2	845	845	410+380	320+210+130+100	University of Basilicata unpublished data and Granchi et al., 1999
VIII	<i>Candida montana</i>	2	525	400+120	245	265+215	Esteve-Zarzoso et al., 1999
IX	<i>Pichia fabianii</i>	1	655	550+100	300	260+170+60	Esteve-Zarzoso et al., 1999
X	Unidentified	1	770	645	250+190	610	–
XI	Unidentified	1	520	390+110	205+135	150+125+50	–
XII	Unidentified	1	845	785	395+310	640+70	–
XIII	Unidentified	1	810	710+100	380+295+240	630+245	–
XIV	Unidentified	1	815	710	234	310+205+115+100	–
XV	Unidentified	1	855	710+110	395+310	640+80	–
XVI	Unidentified	1	520	400+125	155+115+85	220+185+140	–
XVII	Unidentified	1	640	640	205+135	610	–
<i>Type strains</i>							
1.	<i>S. cerevisiae</i> MTCC 180		880	320+240+180+140	380+110	375+345	Type strains from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India.
2.	<i>Pichia anomala</i> MTCC 237		650	635	315	610	
3.	<i>Candida tropicalis</i> MTCC 184		560	450+100	250	285+245	
4.	<i>Candida intermedia</i> MTCC 1404		405	380+60	200+180	200+170+55	
5.	<i>Candida cacaioi</i> MTCC 1904		715	415+175+100	350+160+145	290+80	
6.	<i>Williopsis satunus</i> MTCC 638		630	310+130+90+60	300	470+70	
7.	<i>Wickerhamilla domerequiae</i> MTCC 2887		520	500	235	265+215	
8.	<i>Trigonopsis variabilis</i> MTCC 1354		580	460+95	280+240+120	280+150+125	

Microbial Technology (IMTECH), Chandigarh. The following strains were studied: *Candida tropicalis* MTCC184, *C. intermedia* MTCC1404, *C. cacaoi* MTCC1904, *Saccharomyces cerevisiae* MTCC180, *Pichia anomala* MTCC237,  *Williopsis saturnus* MTCC638, *Wickerhamilla domerequiae* MTCC2887 and *Trigonopsis variabilis* MTCC1354.

#### 2.4. ITS-PCR amplification

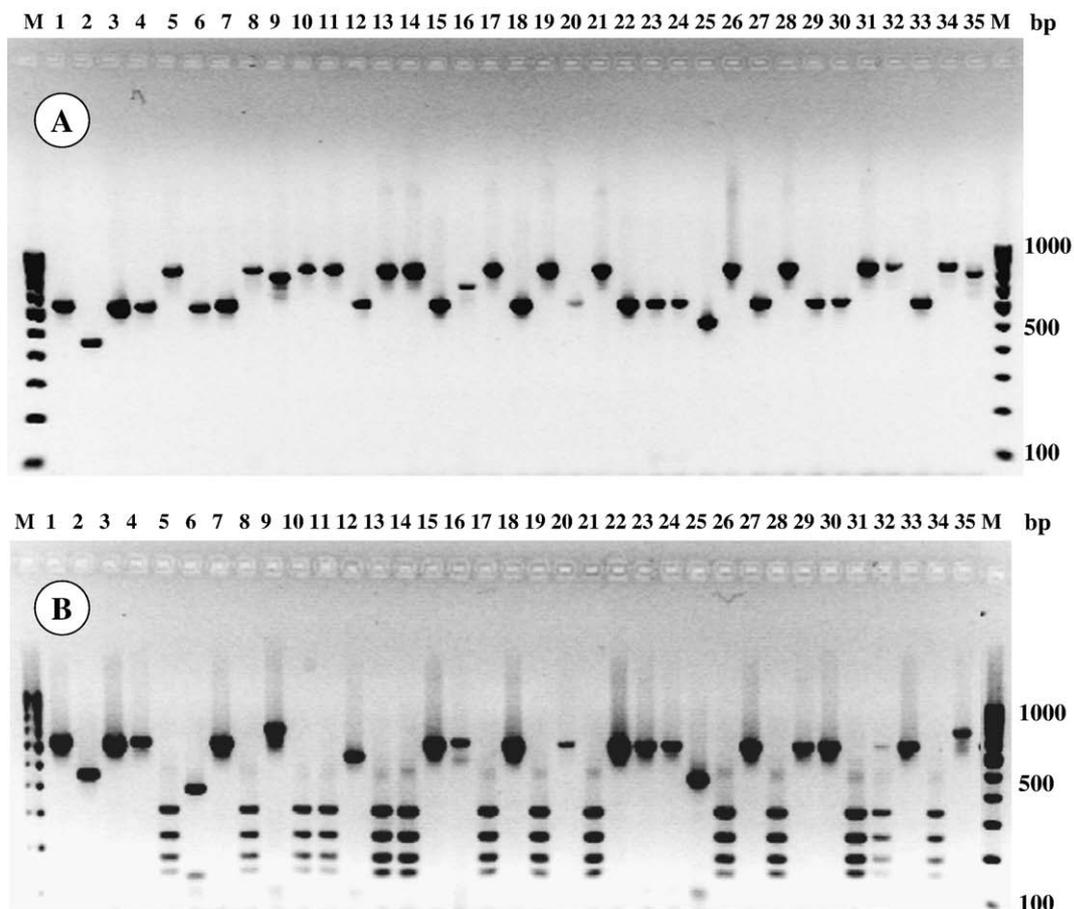
Yeast cells from 48 h old fresh colonies growing on YEP agar plate were collected with the sterile tip of toothpick and suspended in the PCR mixture and directly used for PCR analysis. The amplification of the ITS1-5.8S-ITS2 region was carried out in 25  $\mu$ l of reaction mixture containing 2.5  $\mu$ l of PCR buffer (10 $\times$ ) (Promega, A3511, USA), 1.0  $\mu$ l of 25 mM MgCl<sub>2</sub>, 0.5  $\mu$ l of 25 mM dNTP (Promega, U1240), 1  $\mu$ l each of forward and reverse primers (ITS1 5'-TCCGTAGGTGAACCTGCGG-3', ITS4 5'-TCCTCCGTTATTGATATGC-3') (200 pmol  $\mu$ l<sup>-1</sup>) (GeNei, BGC02) (White et al., 1990), 0.5  $\mu$ l (3U) of Taq DNA polymerase (Promega, M1901) and 18.5  $\mu$ l of autoclaved deionised water (MilliQ, Millipore India). The amplification was performed with a total of 36 PCR cycles in a thermal cycler (ICycler, Biorad, USA). The cycling program was started with an initial cell lysis at 95 °C for 10 min followed by 36 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30s and elongation at 72 °C for 1 min. The PCR was ended with a final extension at 72 °C for 7 min and the amplified product was cooled at 4 °C.

The amplified DNA fragment was separated by applying 3  $\mu$ l of each PCR product with 0.5  $\mu$ l of loading buffer (6 $\times$ ) (Promega, DV4371)

to 1 % agarose (Promega, V3125) gel containing 0.5  $\mu$ l ml<sup>-1</sup> ethidium bromide (Promega, H5041). DNA markers (GeNei, RMBD135; Promega, G5711) were included as standard for the calculation of the DNA fragments size. The gel was run in 0.5 $\times$  TBE buffer (89 mM I<sup>-1</sup> Tris (Promega, H5131), 89 mM Boric acid (Promega, H5003), 25 mM Na<sub>2</sub>-EDTA (Promega, H5031, pH 8.0) for 2 h at 100 V (Sub-Cell GT and 192, Biorad) and photographed using gel documentation system (Gel Doc EQ, Biorad). Band sizes were estimated by comparison against DNA ladders using Quantity One version 4.5.1 software (Biorad, USA).

#### 2.5. Restriction analysis

Four microliters of the PCR products were digested without further purification in 10  $\mu$ l reaction volume (1.0  $\mu$ l of 10 $\times$  buffer, 0.4  $\mu$ l of Restriction Enzyme(Promega), 0.2  $\mu$ l of BSA and 4.4  $\mu$ l of sterile deionised water), using the manufacturer's instructions and conditions with different restriction enzymes. The restriction enzymes used for all yeast isolates were Hae III, Hinf I and Cfo I (Esteve-Zarzoso et al., 1999; Granchi et al., 1999). The major groups were further confirmed by digesting with additional enzymes Dde I, Msp I, Hind III and Eco RI (Promega). The digestion reaction was incubated at 37 °C water bath overnight. RFLP products were analysed by horizontal electrophoresis in 2% (w/v) agarose gels. The DNA fragments were separated by applying 10  $\mu$ l of each digested PCR product with 1.5  $\mu$ l of loading buffer (Promega, DV4371) to 2% agarose gel (Promega, V3125) containing 0.5  $\mu$ l ml<sup>-1</sup> ethidium bromide (Promega, H5041). DNA



**Fig. 3.** ITS-RFLP grouping of yeast isolates associated with 'Hamei' by PCR amplification of ITS1-5.8S-ITS2 region (A) along with ITS-RFLP profile (B) using Hae III restriction endonuclease enzyme (Lanes 5, 8, 10, 11, 13, 14, 17, 19, 21, 26, 28, 31 and 32 belongs to *Saccharomyces cerevisiae* group of isolates PH2Y3, PH3Y3, A2Y2, A1Y7, A1Y5, A1Y6, H17Y1, H43Y2, H27Y6, PH1Y1, PH2Y1, PH3Y3A and PH2Y3A. Lanes 1, 3, 4, 7, 15, 18, 20, 22, 23, 24, 27, 29 and 30 belongs to *Pichia anomala* group of isolates H40Y1, A2Y6, PH3Y3, PH3Y3A, H43Y4, H43Y6, H43Y1, H43Y3, H27Y7, H27Y4, H27Y5, PH3Y14, AN4Y5 and PH3Y2A. Lane 2: *Trichosporon* sp. H24Y9; Lane 6: *Pichia guilliermondii* H101; Lane 12: *Pichia fabianii* A1Y8; Lane 25: *Candida tropicalis* H1. Lanes 9, 16 and 35 belongs to unidentified yeast isolates A2Y1, H43Y6 and A1Y3. Lanes 33 and 34 belongs to type strains *Saccharomyces cerevisiae* MTCC180 and *Pichia anomala* MTCC 237 respectively. Lane M: DNA size markers 100 bp (GeNei, RMBD135)).

markers 100 bp (GeNei, RMBD135) and 1 kb (Promega, G5711) size DNA ladders were included for the calculation of the DNA fragments size. The gel was run in 0.5× TBE buffer for 2 h at 80 V (Sub-Cell GT and 192, Biorad) and photographed using gel documentation system (Gel Doc EQ, Biorad). Gels were analysed using Quantity One version 4.5.1 software (Biorad, USA), which enabled the determination of molecular sizes of the restriction products.

## 2.6. Electrophoretic karyotyping

Karyotype analysis was performed with Pulsed Field Gel Electrophoresis (PFGE) (Gene navigator, Amersham Bioscience, Hong Kong) by following the protocol described by Schwartz and Cantor (1984) with some modification. *S. cerevisiae* isolates were grown in 5 ml of YEP broth and incubated at 28 °C overnight. Volume of cultures corresponding to 5 OD at 660 nm were harvested by centrifugation at 4000 rpm for 5 min at 4 °C, washed twice with 500 µl of 50 mM EDTA and resuspended in 200 µl of 50 mM EDTA. Spheroplast was prepared by adding 60 µl of lyticase (Sigma) 1 mg ml<sup>-1</sup> with 300 µl sample (for six plugs) and incubated at 37 °C for 5 min. Chromosomal DNA plugs of *S. cerevisiae* were prepared by mixing spheroplast suspension with equal volume of 1% low melting agarose (Agarose prep, Amersham) at 40 °C. The mixture was dispensed into molds to form agarose plugs and kept in refrigerator at 4 °C for 15 min for solidification. The plugs were incubated for overnight at 37 °C in a solution of 0.25 M EDTA and 5% mercaptoethanol. Subsequently the agar plugs were washed four times with 2 ml of 50 mM EDTA and incubated with solution containing 0.4 M EDTA, 10 mM Tris (pH 8.0), 1% SDS and 0.5 mg ml<sup>-1</sup> proteinase-K(GeNei) at 50 °C overnight. The plugs were washed twice with 2 ml of 50 mM EDTA and stored in 2 ml of 50 mM EDTA at 4 °C until used.

Electrophoresis was performed in 1.0% agarose (Agarose-PFGE, Amersham) and 0.5× TBE buffer at 9 °C and 180 V. Gels were run for 26 h with a switch time of 60s for 15 h followed by 90s for 11 h. *S. cerevisiae* MTCC 180 chromosome was used as control. For each run, PFGE marker (225–22,000 kb) from *S. cerevisiae* strain YPH80 (D4658, Sigma) was used as molecular weight marker. After electrophoresis, gels were stained with ethidium bromide solution (0.5 µg ml<sup>-1</sup>) and photographed using gel documentation system (Gel Doc EQ, Biorad). Banding patterns were evaluated by visual inspection and analyzed using Quantity One version 4.5.1 software (Biorad, USA). Isolates were considered as identical only if all bands were matched exactly. Isolates

with karyotype profiles differing only by the position of bands or by the presence or absence of one band were considered to be different strains. The computer program NTSYSpC software version 2.20f was used for generation of cluster analysis in a dendrogram based on the Dice algorithm and the Unweighed Pair group Method using Arithmetic averages (UPGMA).

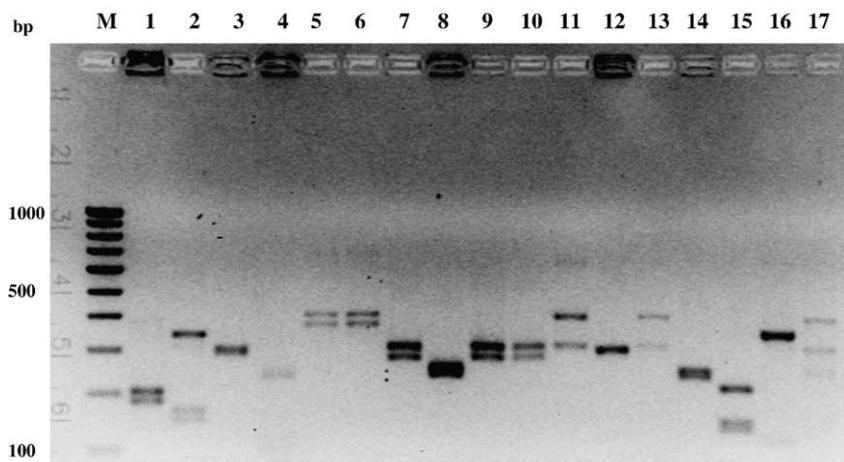
## 2.7. Mitochondrial DNA-RFLP

Five milliliters of overnight grown culture was transferred to 100 ml YEP broth in 250 ml conical flask and kept under 200 rpm at 28 °C for 2–3 h. Cells were harvested at 6000 rpm at 4 °C for 10 min and dissolved in 5 ml of TE buffer. Total DNA was isolated using the method described by Querol et al., 1992. Isolated DNA was quantified using spectrophotometer (ND-1000, Nano Drop) and freeze dried using speed vacuum concentrator assembly (Freeze dryer, Thermo) and the concentration was adjusted to 1 µg µl<sup>-1</sup> by adding sterile MilliQ water. DNA was digested with restriction enzyme (Hinf I, Rsa I and Hae III) in 20 µl reaction volume (2.0 µl of 10× buffer, 2.0 µl of restriction enzyme, 0.5 µl of BSA, 10 µl of DNA and 5.5 µl of sterile deionised water) according to the suppliers instruction. Restriction fragments were separated in 0.8% w/v agarose gel in 0.5× TBE buffer stained with ethidium bromide (0.5 µg ml<sup>-1</sup>) and documented using gel documentation system and analysed using Unweighed paired group average (UPGMA) cluster analysis based on Jaccard similarity index using PAST software version 0.82. One kilobyte DNA ladder (G5711, Promega) was served as the size marker.

## 3. Results

### 3.1. Molecular identification

In order to ascertain the dominant yeast flora present in the 'Hamei', a total of 163 yeast isolates from 54 'Hamei' samples were taken for this study. A region of the rRNA gene repeat unit, which includes two non-coding regions designated as the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene was amplified and digested by several restriction endonucleases. The pattern obtained for each isolate were compared with the pattern of the reference strains of the most common species present in wine (unpublished data from University of Basilicata, Potenza, Italy) and previously described in the literature (Guillamon et al., 1998; Esteve-



**Fig. 4.** ITS-RFLP grouping of yeast isolates using Hinf I restriction digestion (Lanes 1–4: *Candida intermedia* MTCC 1404; *Candida cacaui* MTCC 1904; *Williopsis saturnus* MTCC 638; *Wickerhamilla domerequiae* MTCC 2887; Lanes 5 & 6: *Torulospira delbrueckii* isolates S2Y1 & S2Y2; Lanes 7, 9 & 10: *Pichia guilliermondii* isolates S1Y3, H58 & H101; Lanes 8 & 14: *Candida montana* isolates H74b & H5; Lane 12: *Pichia fabianii* A1Y8; Lane 16: *Pichia anomala* A1Y2; Lanes 11, 13, 15 & 17: Unidentified isolates A2Y1, A1Y3, A2Y3 & S1Y1; Lane M: DNA size markers 100 bp (GeNei, RMBD135)).

**Table 2**  
*In silico* analysis of ITS-RFLP for *Trichosporon* spp. related with Group III using ITS sequence data available in CBS yeast database

ITS-RFLP compared	Accession number/strain	ITS size (bp)	Restriction fragment length (bp)					
			Hae III	Hinf I	Cfo I	Dde I	Msp I	Taq I
A. Group III ITS-RFLP ( <i>Trichosporon</i> sp.)	H24Y9	440	440	248	440	320	440	153
				202		150		70
B. CBS database								
1. <i>Trichosporon asahii</i>	AB018013	454	454	234 173	236 218	312 105	454	234 156 55
2. <i>T. asteroides</i>	AB018017	454	454	234 173	236 218	345 100	454	234 155 55
3. <i>T. caseorum</i>	AJ319758	450	450	236 180	233 217	325 100	450	238 87 66 59
4. <i>T. lactis</i>	AJ319759	451	451	236 171	234 217	342 100	451	237 154 59

Zarzoso et al., 1999; Granchi et al., 1999; Fernandez-Espinar et al., 2000; de Llanos et al., 2004; Villa-Carvajal et al., 2006).

The isolates showed different ITS-PCR product sizes, ranging from 440 bp to 880 bp (Table 1) (Fig. 3A). The PCR products digested with Cfo I, Hae III and Hinf I enzyme were analysed, whereby 17 different ITS-RFLP profiles were obtained (Figs. 3B and 4). A roman numeral designates each group (Group I to XVII). The size of the PCR products and the restriction of the major species identified in this study are shown in Table 1. For comparison, PCR-RFLP of the ITS region was simultaneously applied to the certified strains from MTCC, IMTECH, Chandigarh. Additionally, major groups' identity was confirmed using *in silico* analysis of ITS sequence data available in CBS yeast database ([www.cbs.knaw.nl/index.htm](http://www.cbs.knaw.nl/index.htm)). We were not able to identify all the isolates because some ITS-RFLP patterns did not match with any of the reference strains data published.

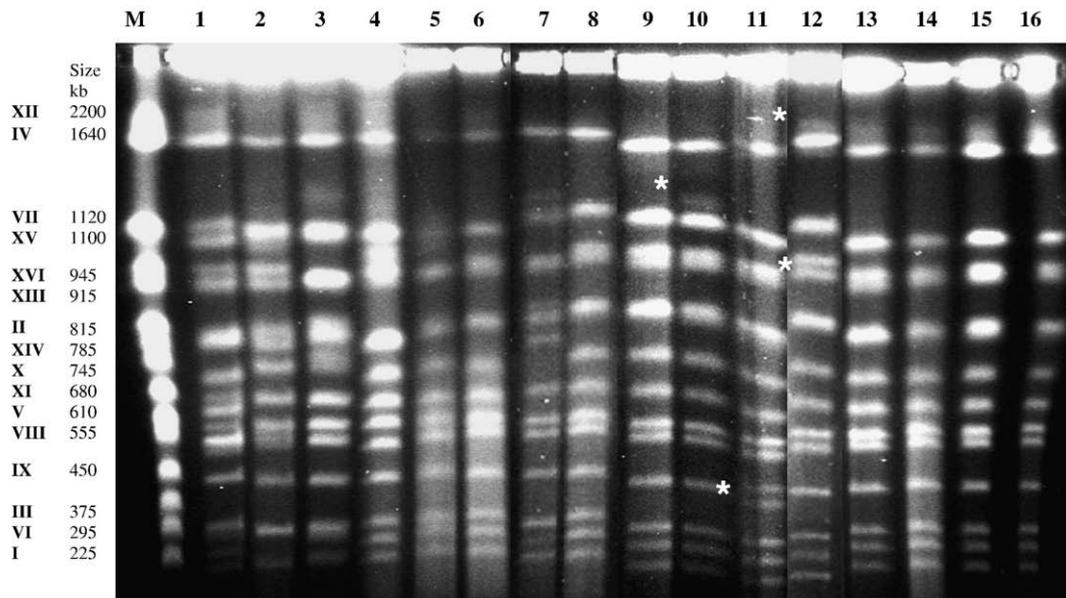
Nine of the major ITS-RFLP patterns were identified after comparing the molecular size of the restriction products with those previously described. The nine groups were *S. cerevisiae*, *P. anomala*, *Trichosporon* sp., *C. tropicalis*, *Pichia guilliermondii*, *Candida parapsilosis*, *Torulasporea delbrueckii*, *Candida montana* and *Pichia fabianii*. The most frequent yeasts associated with 'Hamei' were identified as *S. cerevisiae* (32.5%) and *P. anomala* (41.7%). The next frequently present species were *Trichosporon* sp. (8%) and *C. tropicalis* (2.5%). The remaining groups (group X to XVII) were not found to correspond with 288 species of common wine yeasts ITS-RFLP data collection of University of Basilicata, Potenza, Italy and Institute of Bioresources and Sustainable Development (IBSD), Imphal, India.

In order to confirm the identity of Group I (*S. cerevisiae*), additional restriction analysis with Hind III, Dde I, Msp I and Eco RI endonucleases were used (Hind III: 880 bp; Dde I: 760+120 bp; Msp I: 740 bp; Eco RI: 480+370 bp). Sixty eight isolates of the *P. anomala* group (Group II) exhibited larger ITS fragment (650–680 bp) than that previously reported (605–650 bp), although the same two primers were used. The band patterns of the isolate were totally coincident with *P. anomala* type strain MTCC 237 and reported by others using same three enzymes. The restriction enzyme Hae III showed only one fragment of 635 bp for the isolates same as *P. anomala* type strain MTCC 237. The enzyme Hinf I showed single cut site and the two fragments had the same size 315 bp and showed as single band. The pattern is in accordance with the results reported by Granchi et al., 1999 and Esteve-Zarzoso et al., 1999. We observed only a single band 610 bp for Cfo I digestion and not two fragments of 600+50 bp as reported previously. The reason why we observed that less than 50 bp is not possible to detect in agarose gel electrophoresis in the long run.

In order to find the identity of the Group III, ITS-PCR products size and ITS-RFLP patterns were compared with the ITS-DNA sequence data available in CBS yeast database using *in silico* analysis (Table 2). Colony, cell morphology (Fig. 5) and *in silico* analysis of ITS-RFLP profile of group III showed very high homology to *Trichosporon* sp. *Trichosporon* is a genus of anamorphic yeast (Basidiomycete, Hymenozymetes, Trichosporonales) with a distinct morphological characteristic of true mycelia (Fig. 5B). Group XI showed the same number and size of fragments as



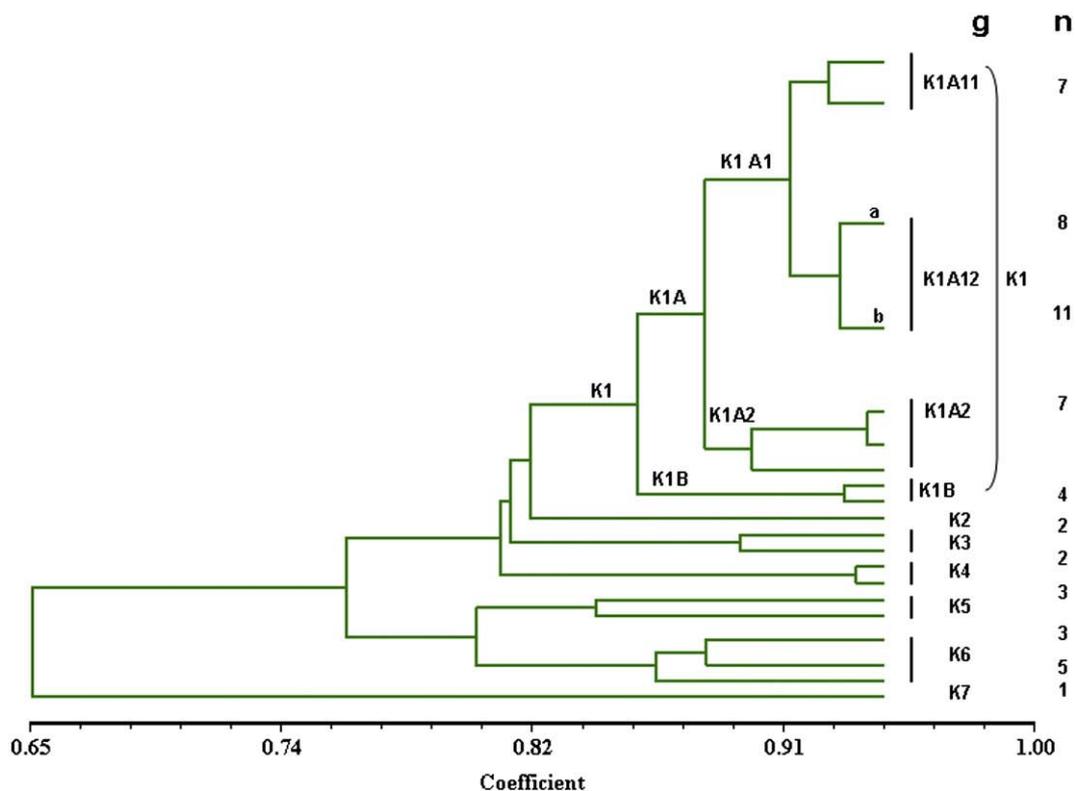
**Fig. 5.** Colony morphology (A), true mycelia with balistoconidia (B) and cells with germ tubes (C) of *Trichosporon* sp. H24Y9.



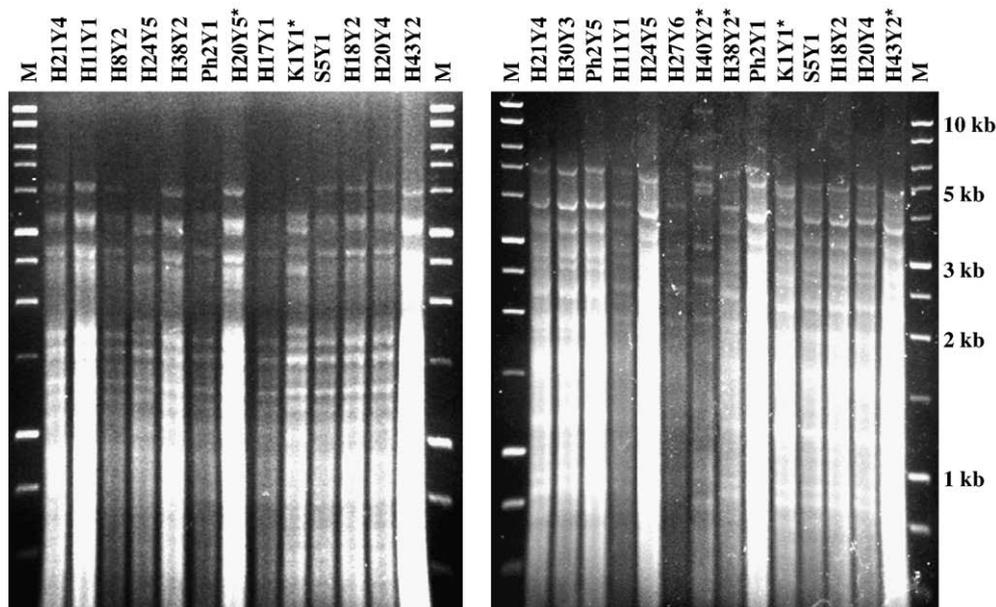
**Fig. 6.** Electrophoretic karyotypes of the prevalent *S. cerevisiae* strains in 'Hamei' *S. cerevisiae* karyotype K1 (Lanes 8–16: strains H38Y2, S5Y1, A1Y1, H20Y5, A1Y6, H27Y6, A2Y2, H18Y2 and H10Y3), karyotype K2 (Lanes 5 and 6: strains H20Y2 and PH2Y3A), karyotype K3 (Lane 7: strain K1Y5), karyotype K4 (lane 4: strain PH3Y1), karyotype K5 (lane 3: strain H18Y1), karyotype K6 (lane 2: strain H10Y4) and karyotype K7 (lane 1: strain H 40Y3). M – chromosomal size marker (*S. cerevisiae* strain YPH80, Sigma). I to XVI indicate chromosome numbers. \* indicates polymorphic bands in the major karyotype group K1.

*Issatchenkia orientalis* (Clemente-Jimenez et al., 2004) after digestion with Hae III and Hinf I, but larger size of fragments 200 and 180 bp instead of 150 and 125 bp when Cfo I was used. Group X was showed very close similarity with *Hanseniaspora valbyensis* (ITS 750 bp, Hae

III: 750 bp, Hinf I: 250+220+170+105 bp and Cfo I: 630+120 bp) (Esteve-Zarzoso et al., 1999). The size of ITS region for groups XII to XV (810–855 bp) were very close to *Torulaspora* spp (Esteve-Zarzoso et al., 1999).



**Fig. 7.** Simplified dendrogram based on UPGMA clustering of Dice similarity coefficient ( $S_D$ ) of PFGE karyotype profiles of *S. cerevisiae* group. Karyotype patterns were grouped into 7 clusters designated as K1 through K7 on basis of 83% similarity among the isolates used. At more than 85 to 95% similarity, major group K1 was subdivided into K1B, K1A2, and K1A12 & K1A11. 'n' – number of strains in the cluster. 'g' – karyotype groups.



**Fig. 8.** Examples of mt-DNA restriction pattern of major karyotype K1 using Hinf I (A) and Hae III (B). The lanes correspond to *S. cerevisiae* strain numbers. Strains with unique patterns are marked with an asterisk. M – Molecular weight marker (1 kb ladder from Promega).

### 3.2. Electrophoretic karyotyping

PFGE was employed to verify the diversity of industrially important *S. cerevisiae* isolates based on their chromosomal polymorphism. Fig. 6 shows the electrophoretic karyotypes of the prevalent *S. cerevisiae* strains. All karyotypes contained high number of chromosomal bands with molecular size ranging from 225 kb–22,000 kb. All the 53 isolates of *S. cerevisiae* analyzed were grouped into 32 electrophoretic karyotype (EK) profiles that were distinct from each other. The similarity coefficients between different EKs vary from 0.65 to 1.00. The dendrogram developed using Dice similarity coefficient (Fig. 7) showed a high similarity (more than 83%) among 18 EK profiles were grouped together as karyotype I (K1) with 36 *S. cerevisiae* isolates. The strains in K1 group displayed very similar chromosomal pattern differing only in faint variation of band position or the presence of doublets. These minor differences were reproduced in three times of repeated agarose gel electrophoresis for confirmation.

From the main cluster K1, four sub-clusters were defined by coefficient of similarity above 90% (K1B, K1A11, K1A12 and K1A2). K1A12 contained 19 isolates with two more sub-clusters labeled as K1A12a and K1A12b showing 95–100% similarity. Another large group K1A11 with 7 isolates showed more than 92% similarity among isolates. A third cluster (K1A2) with 7 isolates was grouped with a similarity below 90%. The remaining isolates were grouped into minor clusters with lower degree of similarity namely K2, K3, K4, K5 and K6 were showed less than 76% similarity.

The *S. cerevisiae* strains assigned as karyotype KA12 was further grouped into 'a' and 'b' where chromosome XVI (945 kb) and XIII (915 kb) were well separated for group 'b' but merged up together for group 'a'. Karyotype K1A11 showed similar to K1A12 but typically separating Chromosome IV band (22,000 kb) and Chromosome XII (16,400 kb). In other cases it was more or less merged together. Karyotype K2 is having size differences at chromosome X and XIV (800–850 kb). In K3, chromosome XIV was shifted far above (800 kb) than others. In K4, chromosome XVI and Chromosome XIII were widely separated along with additional band in between at 925 kb size. It was found that chromosomal modification in increased size at chromosome VI (320 kb) leads to upward shift resulted karyotype K6 and decreased size at chromosome III (350 kb) and increase size of chromosome I (250 kb) leads to K5. The only isolate of the cluster K7

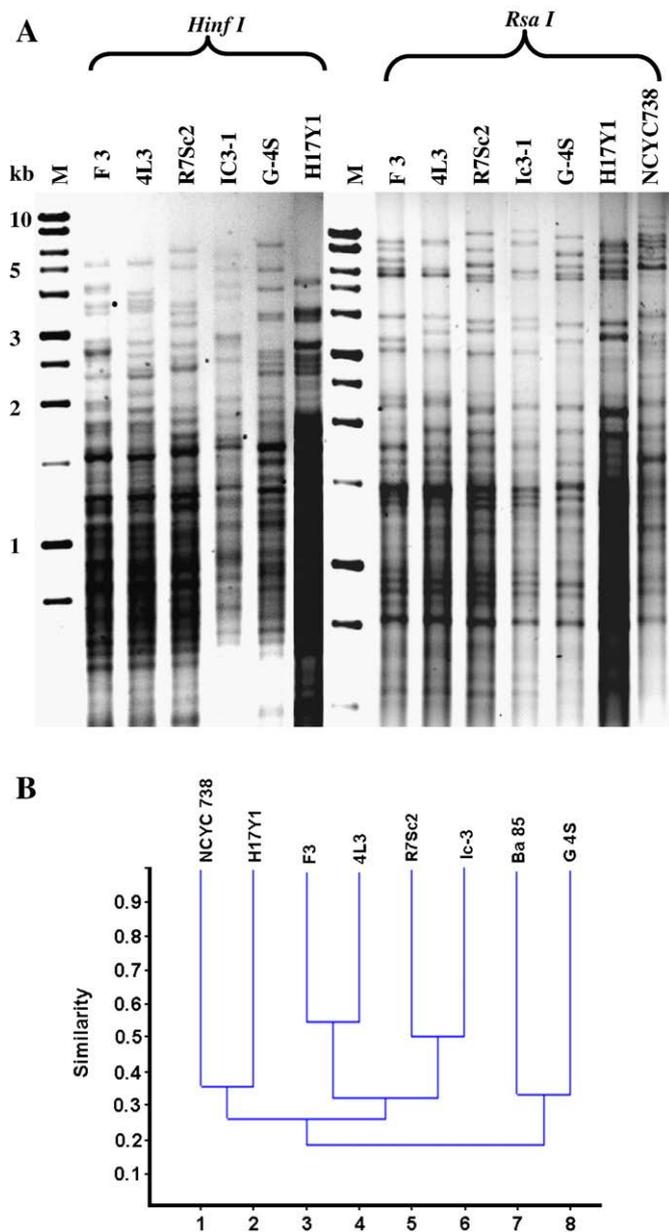
(*S. cerevisiae* strain H40Y3) showed the most dissimilar electrophoretic karyotype if compared to other isolates, which was isolated from Keiku village which is geographically separated hilly region inhabited by tribal.

### 3.3. Mitochondrial DNA-RFLP

Restriction analysis of mt-DNA was used to detect genetic variability among *S. cerevisiae* isolates. The mt-DNA-RFLP patterns generated by Hinf I and Hae III digests allowed differentiating the strains isolated from different villages of Manipur. Karyotype K1A12a distinctly differ from K1A12b in mt-DNA RFLP using Hinf I digestion (Fig. 8A). Interestingly Hae III digest was further differentiated this major mt-DNA-Hinf I-RFLP group with very high polymorphic bands (Fig. 8B). This suggested that the mt-DNA-RFLP using Hae III could be used to differentiate the similar karyotypes or mt-RFLP group formed by Hinf I. We also compared the mt-DNA restriction pattern of these *S. cerevisiae* strains of 'Hamei' with Italian grape wine strains from University of Basilicata Yeast Culture Collection. Six *S. cerevisiae* strains (F3, 4 L3, Ba85, R7Sc2, Ic3-1 and G4 S) were used for mt-DNA based digestion and compared with the dominant mt-DNA RFLP profile (*S. cerevisiae* strain H17Y1) of 'Hamei'. Type strain *S. cerevisiae* NCYC 738<sup>1</sup> was used as control. Restriction digestion using Hinf I and Rsa I showed a highly repeatable polymorphic mt-DNA-RFLP profile (Fig. 9A). From these results, it was concluded that each strain used for this study was unique and highly dissimilar with one another. UPGMA cluster analysis based on Jaccard similarity index generated two clear clusters with 25% similarity level (Fig. 9B). The major mt-DNA-RFLP pattern (strain H17Y1) was found to be 70% dissimilar with other strains studied and the closest pattern was with *S. cerevisiae* type strain NCYC738 with only 40% similarity.

## 4. Discussion

The average microbial load of 'Hamei' samples collected from tribal villages of Manipur and laboratory preparation using traditional method is presented in Table 3. Yeasts were present in the highest number (8 to 9 log CFU g<sup>-1</sup>) in all the samples studied. Mould population was 5 to 7 log CFU g<sup>-1</sup> and bacteria were not detected in most of the samples up to 10<sup>-3</sup> dilution. Most of the fungal isolates



**Fig. 9.** Comparison of the most frequent mt-DNA-RFLP pattern of *S. cerevisiae* (strain H17Y1) isolated from 'Hamei' with the *S. cerevisiae* strains of University of Basilicata Yeast Culture Collection (F3, 4 L3, R7Sc2, IC3-1, Ba85 & G-4S) from Italian grape wine (A) and UPGMA cluster analysis using Jaccard similarity index (B). The type strain *S. cerevisiae* NCYC 738 was used for control. M – Molecular weight marker (1 kb ladder from Promega).

were found to be the major source of amylolytic enzyme production with more than 4 cm clearing zone with in 48 h growth in starch agar flooded with iodine solution. *Mucor* spp. and *Rhizopus* spp. were the predominant moulds in 'Hamei' and their occurrence was with frequency of 75% and 12% respectively. Antimicrobial activity of *A. myriophylla* bark powder (Table 4) might be the reason for the reduced bacterial load in 'Hamei' by inhibiting amylolytic Gram positive bacteria.

This is the first report about yeast species associated with 'Hamei'. The restriction pattern analysis of ITS region using *Hae* III, *Hinf* I and *Cfo* I (most commonly used restriction endonucleases for wine yeast identification) permitted fast and effective identification of yeast species associated with 'Hamei'. Dlauchy et al. (1999) suggested *Msp* I, *Alu* I and *Rsa* I as alternate enzymes for yeast identification. *S. cerevisiae* identified were positioned in the *Saccharomyces sensu*

*stricto* group. Thus it is clear that this group contain the strains which are commonly used in alcoholic beverages production. The domination of *P. anomala* (41.7%) is a unique feature of 'Hamei'. This might be the reason for the association of unique flavour associated with Manipuri rice wine called 'Atingba'. In general, the non-*Saccharomyces* yeast like *P. anomala* is frequent cause of spoilage in fermented food and alcoholic beverages (Caggia et al., 2001). Huge differences in colony morphology and little differences in ITS region size (640–680 bp) showed the intraspecific heterogeneity in *P. anomala* isolates. Villa-Carvajal et al. (2006) suggested that ITS-RFLP patterns using *Hae* III, *Cfo* I and *Hinf* I failed to differentiate all *Pichia* species and that could be distinguished by use of alternative enzymes for digesting 5.8S-ITS-rDNA PCR product, sequence of 5.8S-ITS rRNA region or sequencing of the D1/D2 domain of the 26S rDNA gene for proper identification (Nisiotou and Gibson, 2005). *P. anomala* and *Trichosporon* sp. showed high amylolytic activity in starch agar plates. They might be playing major role in 'Hamei' samples lacking mould population for saccharification of glutinous rice during rice wine fermentation.

This is the first report of *Trichosporon* sp. association with rice wine starter. *Trichosporon* sp. are having ability to assimilate typical plant compounds like polygalacturate, gallic, tannic and cinnamic acids and it suggest that they actively take part in the mineralization of decaying plant materials (Middelhoven et al., 2001). The origin of *Trichosporon* sp. could be *A. myriophylla* bark, which is added during 'Hamei' preparation. Several *Trichosporon* sp. grow well with polygalactimate as sole carbon source. This is also suggesting the possible use of *Trichosporon* sp. for fruit juice clarification during wine fermentation. Molnar et al. (2004) reported that the ability of *Trichosporon mycotoxinivorans* sp. nov. to detoxify mycotoxins. This could be useful for detoxifying the possible mycotoxin released during saccharification of glutinous rice by moulds. *Trichosporon* sp. was previously reported from natural orange juice fermentation (Heras-Vazquez et al., 2003). The ITS-RFLP pattern of *Trichosporon* sp. group (Group III) is not exactly matching with the available 32 *Trichosporon* species ITS sequence data in CBS yeast database by *insilico* analysis (Table 2). Recent studies based on sequencing of the D1/D2 region of the large subunit of rDNA have shown great homology among seven species of *T. debeurmannianum*, *T. porosum*, *T. dermatis*, *T. cutaneum*, *T. moniliforme*, *T. coremiiforme*, *T. laibachii*, *T. jirovecii* and *T. sporotrichoides* (Heras-Vazquez et al., 2003) and suggesting for sequencing ITS and D1/D2 region could help species level identification of this *Trichosporon* sp. group.

The dominant yeast species associated with another Indian starter for rice wine from Himalayan region (Sikkim and Nepal) called 'Marcha' were identified as *S. bayanus*, *C. glabrata*, *P. anomala*, *Saccharomycopsis fibuligera*, *Saccharomycopsis capsularis* and *Pichia burtonii* (Tsuyoshi et al., 2005). Interestingly the domination of *S. cerevisiae* and *P. anomala* in Manipuri 'Hamei' is in agreement with Balinese rice wine starter 'ragi tape' and Vietnamese rice wine starter 'mem' (Dung et al., 2007; Sujaya et al., 2004). From this, it is hypothesized that the yeast species associated with rice wine starter used in Indo-Burma Biodiversity hotspot (includes Manipur, Vietnam and Indonesia of south eastern Asia) (<http://www.biodiversityhotspots.org>) are distinctly differ from the starter used in Himalayan regions including Sikkim and Nepal (Himalaya biodiversity hotspot). This is an evident for the cultural exchange of Manipur to other South East Asian countries.

Techniques such as chromosome karyotyping, PCR amplification of random (RAPD) or specific gene regions (rDNA or mt-DNA), restriction fragment length polymorphism (RFLP) studies and sequence of specific genes have been used to differentiate and identify various strains of yeast species (Fernandez-Espinar et al., 2000; Coton et al., 2006). Chromosomal karyotyping using PFGE was one of the first and reliable techniques employed to differentiate yeast strains (Briones et al., 1996). It is based on large genetic changes. While small changes

**Table 3**  
Microbial load of "Hamei" samples collected from different villages of Manipur

Si. no.	Place of collection	Number of samples	Size of the flat cake in cm. (Diameter × thickness)	Microbial load (Log CFU g <sup>-1</sup> of dry weight)		
				Fungi	Yeast	Bacteria
1.	Sekmai	8	2.6 × 1.1	7.31 <sup>a</sup> (6.04–7.70) <sup>b</sup>	8.15 (7.33–8.38)	–
2.	Khurkhul	3	4.2 × 1.5	6.93 (5.11–7.30)	8.19 (7.95–8.29)	–
3.	Mahabali Khun	3	4.7 × 0.8	5.56 (5.85–7.0)	7.91 (7.60–8.16)	–
4.	Muji Khun	3	4.7 × 1.1	7.33 (6.44–7.82)	9.20 (9.07–9.38)	–
5.	Dewlalaland	3	5.2 × 0.5	7.56 (7.02–7.88)	8.42 (8.08–8.51)	–
6.	Keikhu	3	4.5 × 1.1	7.35 (6.84–7.7)	8.55 (8.5–8.6)	8.52 (8.08–8.78)
7.	Andro	7	4.0 × 1.1	–	7.50 (5.10–7.64)	–
8.	Keinou	3	7.4 × 1.6	6.72 (6.33–7.09)	8.03 (7.11–8.10)	–
9.	Sangaithe Choubok	3	5.0 × 1.0	5.50 (4.70–6.21)	7.77 (7.62–8.24)	–
10.	Jiribam	3	3.5 × 0.6	–	8.06 (7.91–8.14)	–
11.	Sangaiprou Kabui	3	3.5 × 1.0	9.02 (7.91–9.07)	8.79 (8.08–8.83)	8.51 (7.78–8.55)
12.	Kabuikhun	3	7.2 × 0.7	5.72 (5.36–7.00)	8.56 (8.50–8.69)	–
13.	Iribbung	3	5.7 × 0.8	5.52 (5.23–6.10)	8.35 (7.90–8.43)	–
14.	Silchar	3	5.0 × 0.6	7.01 (5.71–7.43)	8.13 (8.09–8.48)	–
15.	Laboratory Preparations	3	4.8 × 1.0	6.71 (5.20–6.86)	8.48 (8.32–8.58)	–

<sup>a</sup>Data represent the mean of samples.<sup>b</sup>Ranges are given in parenthesis.

(point mutation), mt-DNA restriction analysis constitutes one of the most powerful tools in the investigation of intra specific diversity of industrially important *S. cerevisiae* strains. PFGE and mt-DNA restriction analysis of *S. cerevisiae* from 'Hamei' revealed different genotypic patterns, even in the same production site samples. There is some correlation between karyotype grouping and mt-DNA restriction analysis.

Electrophoretic karyotyping (EK) patterns of our *S. cerevisiae* isolates were comparable to the chromosomal number observed in previous studies (Canas et al., 1997; Sujaya et al., 2004). The modified PFGE run for 26 h at 9 °C with pulses of 60 s for 15 h and 90s for 11 h showed better chromosomal separation. The standard strain (*S. cerevisiae* YPH80) displayed 15 bands during electrokaryotyping. In this, chromosome XII and IV were migrated together as single band. In our study 53 isolates of *S. cerevisiae* with 32 EK pattern were grouped into 7 major groups. The high frequency of chromosomal difference occurring among strains has made electrophoretic karyotypes a highly discriminating technique to distinguish among isolates. Large chromosomal rearrangements such as reciprocal translocation during meiosis could be the reason for an association of more than two homologous chromosomes (Fischer et al., 2000). Transposons may be also the principal source of changes in chromosome structure in yeast that are growing under strong selection (Dunham et al., 2002). It was assumed that any two identical chromosome profiles corresponded to the same strains and if any difference was noticed between them, they were different strains (Canas et al., 1997). Isolates with karyotypic profiles that grouped in clusters with similarity values above 0.8 were considered to belong to the same group of strains as a result of micro evolution (Scoll, 2000).

The *S. cerevisiae* strains of K1 karyotypes showed similar PFGE pattern (Fig. 6), although individual bands had slightly different mobility due to chromosomal length polymorphism (Jemec et al., 2001). The homologous chromosomes that appeared as two bands (corresponding to the same chromosome) are diploid strain have also

been reported (Sujaya et al., 2004). Presence of these highly similar karyotypes supporting the possible clonal spread of this EK through marketing 'Hamei' samples from major production sites like Andro and Sekmai villages. Interestingly wine yeast isolates recovered within same village samples showed high karyotypic variation. This variation might be due to the location of Manipur (Indo-Burma Biodiversity hotspot). The predominant karyotype patterns K1 of 'Hamei' is highly similar to the predominant karyotype reported from 'ragi tape' used for Balinese rice wine 'brem' fermentation (Sujaya et al., 2004). This is also supporting the cultural link between Manipur and Indonesia during civilization. In future, microbial succession studies are required to find out the dominating *S. cerevisiae* karyotypes during rice wine fermentation.

mt-DNA restriction analysis undoubtedly constitute one of the most powerful tools in the investigation of intraspecific diversity of *S. cerevisiae* population in wine fermentation (Prametefaki et al., 2000; Lopez et al., 2001; Fernandez-Espinar et al., 2001). Wine yeast undergoes a selective process of adaptation to ethanol and is permanently exposed to its mutagenic effects which are specific for the mt-DNA (Torriani et al., 1999). *S. cerevisiae* isolates belonging to karyotype K1 were divided into two major clusters using Hinf I digestion. More homogenous distribution of mt-DNA fragments was found with Hinf I digestion of karyotype K1. Interestingly Hae III endonuclease showed heterogeneous distribution of fragments (six clusters) with in karyotype K1. This result suggested that Hae III could be more powerful in differentiating strains of same karyotype. Mitochondrial DNA restriction pattern of isolates of the same geographical origin were quite diverse. These results clearly underline the great diversity of *S. cerevisiae* isolated from geographically closer villages of Manipur. The combination of PFGE and mt-DNA RFLP approaches allowed a quick and reliable estimation of the frequencies of *S. cerevisiae* strains in different 'Hamei' samples (Prametefaki et al., 2000).

Twelve strains of *S. cerevisiae* (out of 53) colonies were showed varying degree of pinkish coloration in YEP plates. These may be due

**Table 4**  
Antimicrobial activity of *Albizia myriophylla* bark extracts

Si. no.	Microbial species tested	Diameter of zone of inhibition (mm/sensitive strain)			
		Petroleum ether extract	Chloroform extract	Methanol extract	Aqueous extract
1.	<i>Escherichia coli</i>	–*	–	–	–
2.	<i>Bacillus cereus</i>	9.5	7	5.5	–
3.	<i>Aspergillus flavus</i>	–	–	–	–
4.	<i>Candida albicans</i>	–	–	–	–

\* Indicates no sensitivity.

to mutagenic effects/ methionine deficient mutants. No correlation between pink coloration and karyotype or mt-DNA RFLP grouping was found. The observed polymorphism probably arise from point mutation or small deletion (Torriani et al., 1999). The association of specific yeast strains with specific metabolic characteristics/flavour will allow producing wines with particular desired features in each microclimatic areas/villages as well as in different rice wine producing household of the village.

Future studies are recommended with the aim of screening the *S. cerevisiae* strains which will be appropriate for glutinous rice fermentation and can improve the quality of traditional rice wine production.

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