Indian J Microbiol (Oct–Dec 2015) 55(4):357–365
DOI 10.1007/s12088-015-0538-4

ORIGINAL ARTICLE

Thermus parvatiensis RL^T sp. nov., Isolated from a Hot Water Spring, Located Atop the Himalayan Ranges at Manikaran, India

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Received: 3 May 2015 / Accepted: 5 June 2015 / Published online: 14 June 2015

Abstract A Gram negative, yellow pigmented, rod shaped bacterium designated as RL^T was isolated from a hot water spring (90–98 °C) located at Manikaran in Northern India. The isolate grows at 60–80 °C (optimum, 70 °C) and at pH 7.0–9.0 (optimum pH 7.2). Phylogenetic analysis of 16S rRNA gene sequences and levels of DNA–DNA relatedness together indicate that the new isolate represents a novel species of the genus *Thermus* with closest affinity to *Thermus thermophilus* HB8^T (99.5 %) followed by *Thermus arciformis* (96.4 %). A comparative analysis of partial sequences of housekeeping genes (HKG) further revealed that strain RL^T is a novel species belonging to the genus *Thermus*. The melting G+C content of strain RL^T was calculated as 68.7 mol%. The DNA–DNA relatedness value of strain RL^T with its nearest neighbours (>97 %) was found to be less than 70 % indicating that strain RL^T represents a novel species of the genus *Thermus*. MK-8 was the predominant respiratory quinone. The presence of characteristic phospholipid and glycolipid further confirmed that strain RL^T belongs to the genus *Thermus*. The predominant fatty acids of strain RL^T were iso-$C_{17:0}$ (23.67 %) and iso-$C_{15:0}$ (24.50 %). The results obtained after DNA–DNA hybridization, biochemical and physiological tests clearly distinguished strain RL^T from its closely related species. Thus, strain RL^T represents a novel species of the genus *Thermus* for which the name *Thermus parvatiensis* is proposed (=DSM 21745^T= MTCC 8932^T).

Keywords Hot spring · *Thermus parvatiensis* · Manikaran · Thermophile

Introduction

The genus *Thermus* was established by Brock and Freeze in the year 1969 by the description of *Thermus aquaticus* [1]. Ever since the isolation of *T. aquaticus*, efforts were being made to isolate more thermophilic bacteria as they have great biotechnological potential due to the presence of various thermostable proteins. Several species of the genus *Thermus* have been isolated from natural and artificial thermal environments such as hydrothermal areas, hot water taps, self-heating compost piles and rock surfaces [2]. At the time of writing there were thirteen published species belonging to this genus including *T. aquaticus* [1], *T. filiformis* [3], *T. scotoductus* [4], *T. thermophilus* [5], *T. brockianus* [6], *T. oshimai* [7], *T. igniterrae* [8], *T. antranikianii* [8], *T. kawarayensis* [9], *T. islandicus* [10], *T. arciformis* [11], *T. composti* [12], *T. caliditerrae* [13].

In an effort to understand the microbial diversity at the hot water spring of Manikaran (90–98 °C), in Kullu District of Himachal Pradesh situated in northern part of India, we isolated a novel species of the genus *Thermus*. The hot water spring is located at 1700 m above sea level (31° 20′ 25″ to 32° 25′ 0″ north latitude and 76° 56′ 30″ to 77° 52′ 20″ east

Vatsala Dwivedi and Kirti Kumari have contributed equally to this work.

Sequence Deposited The GenBank accession number for 16S rRNA gene sequence of strain RL^T (=MTCC 8932^T= DSM 21745^T) is EU017402.

Electronic supplementary material The online version of this article (doi:10.1007/s12088-015-0538-4) contains supplementary material, which is available to authorized users.

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longitude). The pH of the water was neutral (7.0). The water of the hot spring also contained calcium carbonate, the hard crust of which could be seen at the bottom of the spring.

The present study was designed to establish the taxonomic status of *Thermus parvatiensis* strain RL^T isolated from hotwater spring located at Manikaran, India. A polyphasic taxonomic study showed that this isolate belongs to a new species, for which we propose the name *Thermus parvatiensis* sp. nov.

Materials and Methods

Selective Isolation, Maintenance and Culture Conditions

In order to isolate strain RL^T, water samples from the hot water spring were collected, serially diluted and plated on LB (Luria–Bertani) agar, NA (Nutrient Agar), YM (Yeast Extract–Malt Extract) agar and Polypeptone-Yeast Extract agar (0.4 % yeast extract, 0.8 % polypeptone, 0.2 % NaCl, 0.1 % glucose in distilled water, pH 7.2) plates. Routine cultivation of strain RL^T was done on polypeptone-yeast extract agar. Unless otherwise mentioned, cultures were incubated at 60 °C in a humidified oven. A flask containing distilled water was kept in the oven to replace the water loss by evaporation. Agar plates were prepared by adding powdered agar (final concentration 2 %) to the polypeptone-yeast extract medium. The agar plates were incubated at 60 °C. Cultures were stored at −80 °C in 20 % v/v glycerol for long term storage.

For comparative analysis two strains *T. thermophilus* $HB8^T$ and *T. thermophilus* HB27 were procured from the DSMZ culture collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany).

16S rRNA Gene Sequence Comparison

16S rRNA gene sequence analysis of strain RL^T was carried out as described by Prakash et al. [14]. A continuous stretch of 16S rRNA gene (1408 bp) of strain RL^T was obtained. This sequence was compared with those deposited in the GenBank [15] and EzTaxon e-server [16]. The full length 16S rRNA gene sequences of all the validly published species closely related to RL^T were retrieved from GenBank and EzTaxon. For the construction of a phylogenetic tree *Meiothermus ruber* showing 93.47 % sequence similarity to strain RL^T was taken as an outgroup. Sequence similarity analysis and multiple sequence alignment were performed with Clustal_X version 1.81b [17]. Trimming of terminal nucleotides that were not common to all sequences was carried out manually. Phylogenetic analysis was carried out using the MEGA software package version 6 [18]. The method of Jukes and Cantor [19] was used to calculate evolutionary distances. Phylogenetic tree was constructed by the neighbor-joining method [20]. Statistical evaluation of the tree topology based on 1000 resamplings was done using the bootstrap option in the MEGA software.

Chemotaxonomic and Morphological Properties

Isolate RL^T was examined for chemotaxonomic and morphological properties considered to be typical of the genus *Thermus*. Cell morphology and motility of strain RL^T were examined by phase contrast microscopy during the exponential growth phase in polypeptone-yeast extract medium. Cell dimensions were determined with transmission electron microscope (Morgagni, 269D TEM, Fei, The Netherlands). Quinones were extracted from 200 mg dry cell mass with a 10 % aqueous solution of 0.3 % (w/v) NaCl in methanol and petroleum ether at a ratio of 1:1. The upper phase was collected and dried in a rotavapor (Buchi rotavapor R-114, Switzerland). The residue was dissolved in 100 µl acetone. The extract was run on a TLC plate (Silica gel 60 F254, 20 × 20 cm, Merck, Germany) using petroleum ether and diethyl ether (85:15, v/v). Purified menaquinones were dissolved in di-ethyl ether and analysed by reverse phase TLC according to Collins and Jones [21]. Cultures for fatty acid analysis of strain RL^T, *T. thermophilus* $HB8^T$ and *T. thermophilus* HB27 were harvested on polypeptone-yeast extract agar after 2 days. Fatty acid methyl esters analysis of all the three strains was carried out at Royal Life Sciences Ltd, Secunderabad, India. Fatty acid methyl esters (FAME) were analyzed from 2 to 4 loops of inoculum of culture nearly at the same phase of growth. The inoculum was scraped from a petridish and subjected to saponification, methylation and extraction using the method of Miller [22] and Kuykendall et al. [23]. Identification and quantification of fatty acid methyl esters, as well as numerical analysis of the fatty acid profiles, were performed automatically by using the Sherlock Microbial Identification System (MIDI, USA).

Polar lipids were extracted from 100 mg of lyophilized cell culture and were analyzed with the help of two-dimensional TLC using 9 × 9 cm silica-gel F254 plates (Merck) in accordance with the method described by Bligh and Dyer [24].

Biochemical and Tolerance Characteristics

Biochemical tests for enzyme activities and the utilization of substrates as sole carbon source were carried out by using API 20 NE Biolog according to the manufacturers' protocol. Gram staining test was performed using Gram staining kit (HiMedia, Mumbai, India). Oxidase activity was tested using reagents from bioMérieux, France. Catalase activity was tested by adding 3 % (*v*/*v*) hydrogen peroxide solution

Table 1 Differential morphological and physiological characteristics of strain RL^T and members of *Thermus thermophilus*

Characteristics	1	2	3
Habitat	Hot spring (India)	Hot spring (Japan)	Hot spring (Japan)
Colour	Yellow	Yellow	Yellow
Temperature (°C)	80	80	80
Salinity (NaCl %)	1	1	1
Oxidase	+	+	−
Catalase	+	+	+
Nitrate reduction	−	−	−
Indole production	−	−	−
Glucose fermentation	−	+	+
Arginine dihydrolase	−	−	−
Hydrolysis of			
Gelatin	+	+	+
Caesin	+	+	+
Esculin	−	+	+
Tween 20	+	+	+
Tween 80	+	−	+
Urease	−	−	−
L-Arginine	−	−	−
Assimilation of			
D-glucose	+	+	+
L-arabinose	+	+	+
D-mannose	+	+	+
D-mannitol	+	+	+
D-maltose	+	+	+
Adipic acid	+	+	+
Malic acid	+	+	+
Citrate	+	+	+
Capric Acid	+	+	+
Phenylacetic acid	+	+	+

(All data obtained from current study) All species are Gram negative, rod-shaped. Distinguishing characteristics were that strain RL^T was unable to ferment glucose or hydrolyse esculin

1, *T. parvatiensis* RL^T; 2, *T. thermophilus* $HB8^T$; 3, *T. thermophilus* HB27; +, positive; −, negative

to colonies grown on polypeptone-yeast extract medium [25]. To determine the physiologically optimal conditions for growth, strain RL^T was incubated in polypeptone-yeast extract broth for 2 days at various temperatures (28, 37, 45, 55, 60, 70, 80 °C), pH (1.0–10.0 at increment of 1 pH units) and NaCl concentrations (w/v 0, 1, 2, 3, 4, 5,6, 7, 8, 9, 10). Tests were carried out as described earlier [26]. Antibiotic sensitivity was tested as follows: (µg antibiotic per disc in parentheses) ampicillin (10), chloramphenicol (30), gentamicin (10), kanamycin (30), oxytetracycline (30), rifampicin (5), tetracycline (30), vancomycin (30), ciprofloxacin (5), amikacin (30) and nalidixic acid (30). Hydrolysis of tween-20 and tween-80 was tested according to the Arden-Jones et al. [26]. Differential biochemical characteristics of strain RL^T with closely related member of the genus *T. thermophilus* $HB8^T$ and HB27 are given in the species description listed in Table 1.

Multilocus Sequence Analysis

The diversity in housekeeping genes (HKG) (*dnaK*, *glnA*, *recA*, *atpD*, *gap*, *rpoB*, *pnp*, *thrC*, *gyrB*) was analysed. These sequences were retrieved from the draft genome sequence of strain RL^T [27] (Accession No. AIJQ00000000) and other genomes published. For this purpose genome sequence of strain RL^T [27], and seven other strains belonging to the genus *Thermus* whose complete genome sequences are available (*T. thermophilus* $HB8^T$, *T. thermophilus* HB27, *T. thermophilus* SG0.5JPP17-16, *T. scotoductus* SA-01, *Thermus* sp. CCB_US3_UF1 *T. aquaticus* Y51MC23, *Deinococcus radiodurans* R1) were retrieved from NCBI database for comparative analysis. Sequences were subjected to homology search using BLAST program of the National Centre for Biotechnology Information. (http://www.ncbi.nlm.nih.gov). The accession numbers of all the genes used in the analysis are

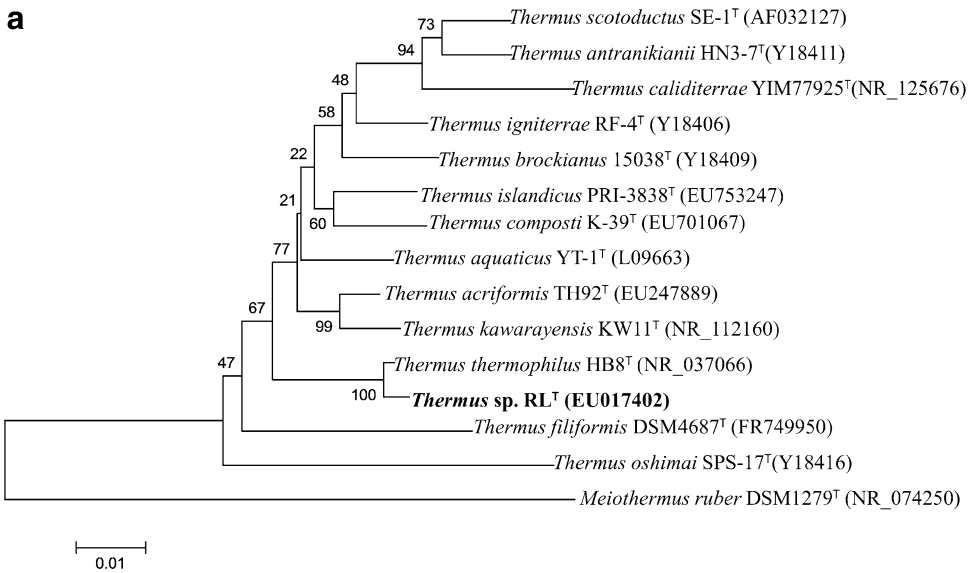


Fig. 1 **a** Evolutionary relationships of taxa. Phylogenetic tree based on nearly complete 16S rRNA gene sequence data showing the evolutionary relationship of strain RL^T and members of representative genus *Thermus*. The tree was constructed by using neighbor-joining [19] method of MEGA6 software and rooting was done by using *Meiothermus ruber* as the outgroup. *Scale bar* 0.01 nucleotide substitution per 1000 nucleotide position. The GenBank accession number for the 16S rRNA gene sequence of each strain is shown in *parenthesis*. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.34242405 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes–Cantor method and are in the units of the number of base substitutions per site. The analysis involved 15 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1327 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. **b** NJ trees [19] obtained from nucleotide sequences of *dnaK* (*i*), *glnA* (*ii*), *recA* (*iii*), *atpD* (*iv*), *gap* (*v*), *rpoB* (*vi*), *pnp* (*vii*), *thrC* (*viii*) and *gyrB* (*ix*). Sequence accession numbers are provided in Supplementary Table S1. Only bootstrap values above 40 % are shown. *Deinococcus radiodurans* R1 was used as the outgroup. *Scale bar* 0.02 nucleotide substitution per 1000 nucleotide position

listed in Supplementary Table S1. Further phylogenetic analysis of the HKG was carried out using the same procedure as done for 16S rRNA gene analysis.

Determination of Mean Base Composition of DNA and DNA–DNA Reassociation Studies

In order to calculate the degree of binding among the strains RL^T, $HB8^T$ and HB27, DNA was extracted according to the procedure described earlier [28]. The degree of DNA reassociation was determined spectrophotometrically from the initial renaturation rates [29]. The renaturation rates were measured in 0.1× SSC by using a Perkin Elmer Lambda 25 UV/Vis spectrophotometer and PTP-1 Pettier System of Perkin Elmer. The optimal renaturation temperature used in each case was calculated from the GC content [29].

DNA–DNA hybridization was carried out between strains RL^T and closely related *T. thermophilus* strains ($HB8^T$ and HB27). Total genomic DNA of all the three closely related strains was extracted, purified and hybridization was done by following the protocol as described earlier [30, 31]. The amount of bound DNA probe was calculated by using scintillation counter (1450 LSC & Luminescence counter Wallac Microbeta Trilux, PerkinElmer, USA). Percentage relatedness was calculated on the basis of data obtained (mean of the three replicates) by DNA–DNA hybridization.

The DNA G+C content of strain RL^T was also calculated by in silico analysis of the draft genome published [27].

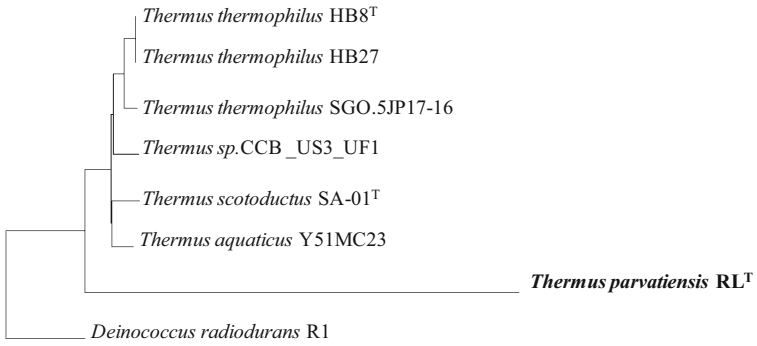
Results and Discussion

Strain RL^T formed rod-shaped cells short filaments in liquid and on solid media (Supplementary Fig. S1). Cells are non-motile, non-sporulating and appear as short rods, an average rod measured 3 × 0.5 µm. The colonies of strain RL^T showed optimum growth on polypeptone-yeast extract within 36 h of incubation. Colonies of strain RL^T were yellow coloured, circular and smooth.

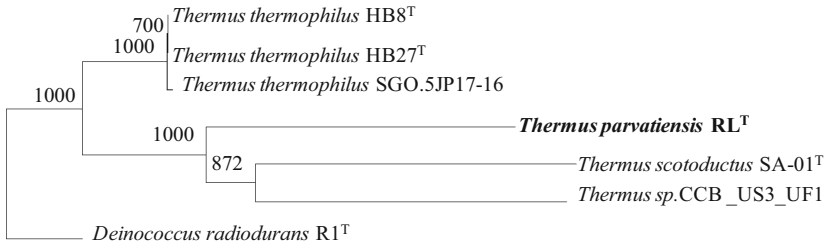
16S rRNA genes showed highest homology to *T. thermophilus* $HB8^T$ (99.5 %) followed by *T. arciformis* (96.4 %). Levels of 16S rRNA gene sequence similarity between strain RL^T and the type strains of other recognized *Thermus* species were in the range 93.92–99.5 %. Strain RL^T falls in the clade containing members exclusively belonging to the genus *Thermus* (Fig. 1a). The delineation

b

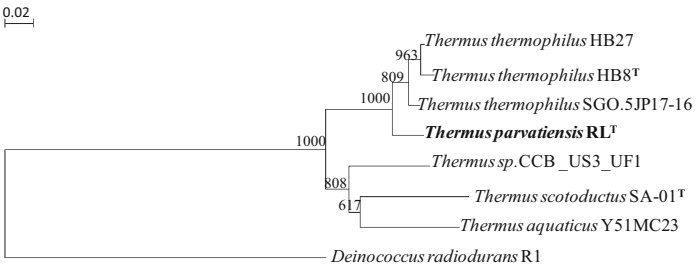
i)



ii)



iii)



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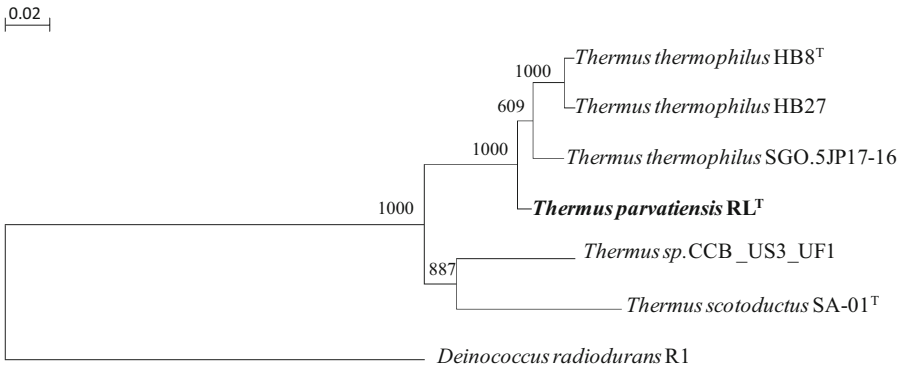
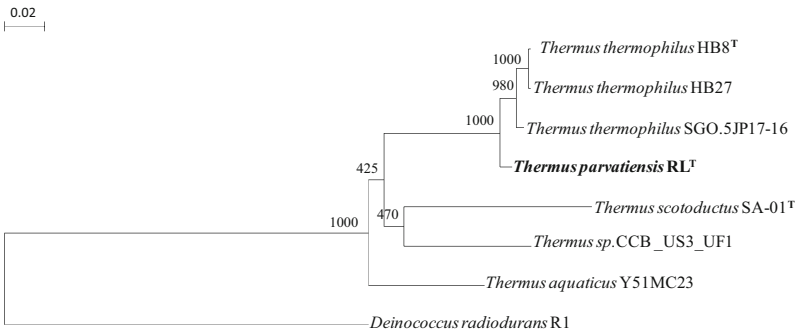


Fig. 1 continued

v)



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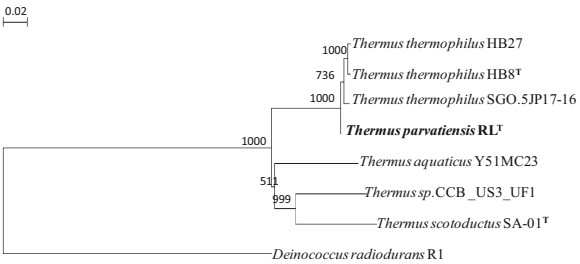
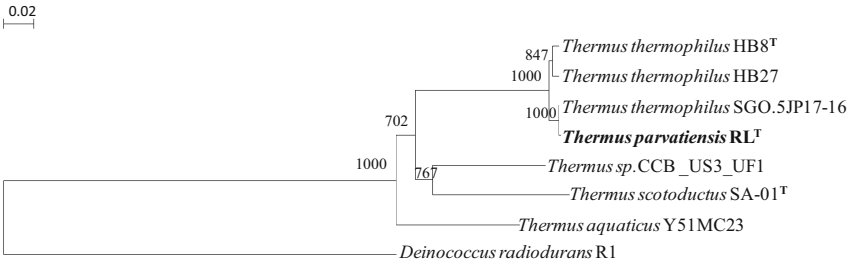
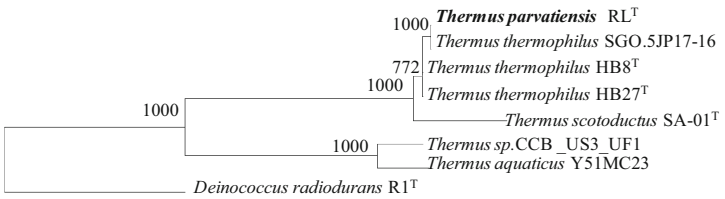


vii)



viii)



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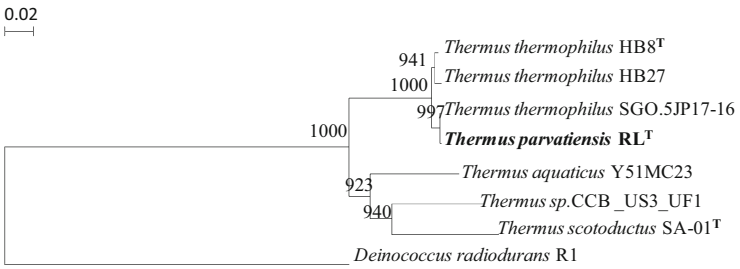

Fig. 1 continued

Table 2 Comparative analysis of housekeeping genes of strain RL^T with its nearest neighbours based on 16S rRNA gene analysis

Housekeeping genes	1	2
dnaK	92.3	92.1
glnA	94.2	94.2
recA	95.9	96.2
atpD	98.2	97.9
gap	97.8	97.8
rpoB	97.6	97.7
pnp	98.7	98.7
thrC	98	98
gyrB	98.8	98.7

The nucleotide sequence of all the housekeeping genes were retrieved from NCBI database. The accession no. are listed in Supplementary Table S1

1, *T. thermophilus* $HB8^T$; 2, *T. thermophilus* HB27

Table 3 DNA reassociation values between strain RL^T and its closely related species *T. thermophilus* $HB8^T$ and HB27

Strain	1	2	3
1	100	47.8	56.67
2	47.8	100	87.4
3	56.67	87.4	100

1, *T. parvatiensis* RL^T; 2, *T. thermophilus* $HB8^T$; 3, *T. thermophilus* HB27

of a novel species only on the basis of 16S rRNA gene sequence analysis is critical [32]. 16S rRNA gene sequence analysis often lacks resolving power at and below the species level. Several studies have reported bacteria that represent different species with identical or nearly identical 16S rRNA gene sequences [33–35]. Analyses of HKG further confirm that the strain RL^T is distinct from *T. thermophilus* $HB8^T$. Comparative gene sequence analysis between sequenced strains of the genus *Thermus* with those of strain RL^T revealed that similarity of genes *dnaK* and *glnA* is less than 95 % while all other HKG analysed show similarity less than 99 % (Table 2; Fig. 1b). The conclusion drawn from the phylogenetic tree of the 16S rRNA gene was supported by the comparative analysis of the HKG studied, that strain RL^T is a novel species of the genus *Thermus.*

The predominant fatty acids of the three strains tested were iso-$C_{15:0}$ and iso-$C_{17:0}$ and the next most prominent fatty acids were anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. The fatty acid profile of strain RL^T showed both qualitative and quantitative differences as compared to other closely related strains (Supplementary Table S2) further suggesting that RL^T is a novel species of the genus *Thermus* (Supplementary Table S2).

Menaquinone 8 was the predominant respiratory lipoquinone detected in the three strains which confirms that this strain belongs to the genus *Thermus*.

The predominant polar lipids of strain RL^T were phospholipid (PL1) and glycolipid (GL2) (Supplementary Fig. S2), which again confirms that strain RL^T belongs to the genus *Thermus* [2].

The chemotaxonomic and morphological properties of isolate RL^T were seen to be consistent with its classification in the genus *Thermus*. Strain RL^T was found to be gram stain negative. It was unable to hydrolyse esculin and ferment glucose whereas both these properties were found in its closest related species *T. thermophilus* $HB8^T$ and HB27. Strain RL^T was sensitive to all antibiotics tested whereas $HB8^T$ was sensitive to all except nalidixic acid and rifampicin. These results suggested that strain RL^T is a new species of the genus *Thermus*.

Degree of binding of strain RL^T with representative strains of *T. thermophilus* $HB8^T$ and HB27 were low. DNA–DNA binding value of strain RL^T with $HB8^T$ was 47.8 % and with HB27 was 56.7 %. However, the degree of binding between DNA of *T. thermophilus* $HB8^T$ and *T. thermophilus* HB27 was as high as 87.74 %. Thus, there are marked differences between strain RL^T and the other two closely related strains (Table 3) further suggesting that strain RL^T is a novel species of the genus *Thermus*.

The mean DNA–DNA relatedness values of strain RL^T was found to vary from 47 to 55 % with its nearest neighbours (50 % with *T. thermophilus* $HB8^T$ and 49 % with *T. thermophilus* HB27). The DNA–DNA hybridization values were below the threshold value of 70 % (Supplementary Table S3), as is recommended for the delineation of bacterial species [36]. The G+C content of strains RL^T, $HB8^T$ and HB27 were 68.7, 68.04 and 69.4 respectively. All these data further confirm that strain RL^T represents a novel species of the genus *Thermus*.

Description of *Thermus parvatiensis* sp. nov

Thermus parvatiensis RL^T (par.va.ti.en'sis. N.L. masc. adj. parvatiensis, of or belonging to the river Parvati). Strain RL^T forms yellow pigmented colonies that are 1–2 mm in diameter. The cells are Gram-negative, non-motile and non-spore forming. Growth occurs between 60 and 80 °C; the optimum growth temperature for strain RL^T is 70 °C. The optimum pH is between 7.0 and 9.0. Strain RL^T could tolerate only up to 1 % NaCl concentration and did not survive on further increasing salt concentration. Strain RL^T also tested positive for production of oxidase and catalase. Strain RL^T was found sensitive to all the antibiotics tested. Strain RL^T assimilated D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium

gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. However the strain RL^T did not ferment glucose. Neither did strain RL^T hydrolyse esculin nor urease. Nitrate reduction and indole production was found to be negative (Table 2). Protease activity was qualitatively determined by zone of clearance on milk-casein plates at 70 °C and was found to be positive. Analysis of the HKG of strain RL^T to its closely related and taxonomically characterized neighbours namely *T. thermophilus* $HB8^T$ and HB27 revealed differences. The G+C content of the DNA of strain RL^T was 68.7 %. Degree of binding of RL^T with two other representative strains of *T. thermophilus* namely $HB8^T$ and HB27 was 48.7 % and 56.67 % respectively. The major fatty acids are iso-$C_{15:0}$ (24.50 %), iso-$C_{17:0}$ (33.67 %), anteiso-$C_{15:0}$ (11.89 %) and anteiso-$C_{17:0}$ (13.09 %). Polar lipid analysis of strain RL^T revealed presence of phospholipid PL-2 and glycolipid GL-1 that is consistent with genus *Thermus*. This bacterium was isolated from a hot water spring of Manikaran, India. 16S rRNA gene sequence is deposited in GenBank under the sequence accession number EU017402. The type strain of the species is RL^T (MTCC 8932^T, DSM = 21745).

Acknowledgments This research was supported by funds from Department of Biotechnology (DBT), National Bureau of Agriculturally Important Microorganisms (NBAIM, ICAR), DU/DST-PURSE Grant, Government of India. KK, VD, SKG, RK, CT, PL, NN, AKS, RK, AN, NG gratefully acknowledge University Grants Commission (UGC), Council for Scientific and Industrial Research (CSIR), Department of Biotechnology (DBT) and Indian Council for Agricultural Research (ICAR) for providing research fellowships. We thank SAIF-DST (Sophisticated Analytical instrumentation Facility, Department of Science and Technology, Department of Anatomy, AIIMS) for providing the transmission electron facility. We also thank Dr. J. P. Euzeby (Ecole Nationale Veterinaire, Toulouse, France) for etymological advice.

Conflict of interest The authors state that they have no conflict of interest.

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