Pulsed-Field Gel Electrophoresis of Genomic Digests of *Thermus* Strains and Its Implications for Taxonomic and Evolutionary Studies

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Pulsed-field electrophoresis (PFGE) of the *SspI* genomic digests of 14 *Thermus* isolates showed that each one had a unique restriction enzyme digestion pattern. A group of New Zealand strains showed some shared bands, but each isolate gave essentially a unique fingerprint. In addition, evolutionary distances between *Thermus* strains estimated by using PFGE restriction fragment length polymorphisms (PFGE-RFLPs) correlate well with those based on small-subunit rRNA sequence data. As a consequence, the phylogenetic trees constructed on the basis of PFGE-RFLPs and those constructed by using small-subunit rRNA sequences generally agree. On the basis of the evolutionary distances estimated by using PFGE-RFLPs, the estimated average genomic rate of divergence for *Thermus* spp. is approximately 0.27% per million years.

Many strains of thermophilic bacteria isolated from hot springs located around the world have been classified as belonging to the genus *Thermus*, but the relationships among these strains have not been well established. Only three species of *Thermus* have been validly described so far (3, 11, 15): *Thermus aquaticus*, originally isolated from hot springs in Yellowstone National Park; *Thermus filiformis*, a single strain isolated in New Zealand; and *Thermus ruber*, isolated from many locations, and distinguished from other *Thermus* strains by red pigmentation and a lower growth temperature. "*Thermus thermophilus*" (20) is thought to be a separate species, though it has not been formally accepted. Several *Thermus* isolates show high DNA-DNA homology with HB8, the "*T. thermophilus*" type strain (28). Whether all the *Thermus* isolates obtained are taxonomic variants of the three nominal species or are species themselves is the source of some debate.

A number of studies have attempted to resolve the status of *Thermus* strains. Phenetic studies have been performed to classify *Thermus* isolates from New Zealand (10), Iceland (4), and Portugal (22) on the basis of microbiological and phenotypic characters. In addition, a phenetic classification of 131 *Thermus* strains isolated from hot springs around the world has been performed (12). On the basis of analysis of 68 phenotypic characteristics, eight distinct groups of *Thermus* strains were defined at the level of similarity separating the three recognized species. Classifications based on numerical taxonomy, however, often fail to identify groups that share close evolutionary relationships, and it is precisely these relationships that many systematists feel should form the basis of any classification.

Therefore, another approach to resolving the problems with the classification of the *Thermus* strains is to use procedures explicitly designed to uncover the evolutionary relationships or phylogeny of the taxa in question. In a recent paper, Saul et al. (23) used small-subunit (SSU) rRNA sequences of 20 *Thermus* strains to reconstruct their phylogenetic history. The study showed that of the 20 strains, there were four primary clades

that were separated from each other at a statistically significant level.

While molecular sequences often provide reliable estimates of phylogeny, there are other more accessible sources of information that are as reliable, depending, of course, on the extent to which the taxa are evolutionarily divergent (8). Recently, several workers have used pulsed-field gel electrophoresis (PFGE) to compare genomic restriction digests amongst bacterial strains. This method is equivalent to the identification of restriction fragment length polymorphisms (RFLPs) and can allow a unique DNA fingerprint to be obtained for a bacterial isolate. PFGE-RFLPs have been used successfully to distinguish among isolates of several bacteria, including *Listeria* species (9), *Lactococcus* strains (14, 27), and *Pseudomonas aeruginosa* strains (7). To date, however, no one has reported on the possibility of using this method for phylogenetic reconstruction and classification.

In this paper, we describe the comparison and phylogenetic analysis of PFGE fingerprints from 14 *Thermus* strains. We show that (i) PFGE-RFLPs can successfully discriminate between different strains of *Thermus*; (ii) the evolutionary distances between *Thermus* strains estimated by using PFGE-RFLP data are significantly correlated with those obtained from SSU rRNA sequences; (iii) PFGE-RFLPs produce phylogenetic trees that are independently corroborated by SSU rRNA data; and finally, (iv) on the basis of the evolutionary distances estimated from PFGE-RFLPs, we can obtain an estimate of the average rate of nucleotide substitution across the entire *Thermus* genome of 0.18 to 0.36 nucleotide substitutions per site per million years.

MATERIALS AND METHODS

Fingerprinting of *Thermus* strains by PFGE. Intact chromosomal DNA in agarose gel plugs was prepared from 14 *Thermus* strains previously isolated from hot springs in Japan, the United States, New Zealand, and Fiji: "*T. thermophilus*" HB8, "*T. thermophilus*" HB27, *T. aquaticus* YT-1, *T. aquaticus* YVII-51B, *Thermus* sp. strain Rt41A, *Thermus* sp. strain OK6, *Thermus* sp. strain T. sp., *Thermus* sp. strain T351, *Thermus* sp. strain W28, *Thermus* sp. strain W28P, *Thermus* sp. strain Tok3, *Thermus* sp. strain Tok20, *Thermus* sp. strain Tok21, and *Thermus* sp. strain Fiji3. DNA was prepared as previously

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described (1) either by the *Thermus* Rt41A proteinase method modified by use of 45 U of proteinase per 6 gel plugs (2), or by the standard proteinase K method of Smith et al. (24).

The genome of "*T. thermophilus*" HB8 has been mapped previously by PFGE techniques (2), and during map construction, several restriction endonucleases were screened for infrequent cleavage of the 69% G+C genome. Since the endonuclease *Hpa*I generated only six fragments with the "*T. thermophilus*" HB8 genome, it was tested in this study for rare cleavage of other *Thermus* genomes. However, most of the *Thermus* chromosomes were cleaved by *Hpa*I into too many fragments of similar sizes to be adequately resolved by PFGE (data not shown) and after further screening the enzyme *Ssp*I was chosen for this study.

One DNA gel plug was equilibrated with 200 μ l of *Ssp*I buffer (Gibco BRL/Life Technologies, Auckland, New Zealand) at 4°C for 20 min. The buffer was replaced, and DNA was digested with 20 U of *Ssp*I for 16 h in a 100- μ l reaction volume. After digestion, buffer was removed and each gel plug was equilibrated with 1 ml of Tris-EDTA buffer at 4°C for at least 20 min prior to electrophoresis.

One-fifth of each of the gel plugs was loaded onto a 1% agarose MP (Boehringer-Mannheim, Auckland, New Zealand) gel, and PFGE was performed in 0.5 $\times$ TBE buffer (0.045 M Tris-borate, 0.001 M EDTA [pH 8.0]) at 14°C with a Bio-Rad Chef DR-II system. Lambda DNA oligomers (FMC BioProducts, Rockland, Maine) were used as a size standard. Two sets of electrophoretic conditions were sufficient to separate all of the *Ssp*I fragments for each strain. Most of the fragments were resolved with a pulse time of 20 s for 24 h (Fig. 1A), but fragments larger than 390 kb were separated with a ramped pulse time of 40 to 80 s for 24 h (Fig. 1B). Fragment sizes were measured by comparison with the λ ladder. Band sizes were analyzed by using a digitizer linked to a Macintosh computer running a software program using interpolation of band sizes based on a cubic spline formula. The program did not generate a straight-line relationship over the entire size range of the λ markers. Bands were sized only in the linear portion of the calibration curve, and the pulse time was altered to shift different bands into the linear range.

Phylogenetic analyses. Although it has been customary to perform phylogenetic analyses on restriction fragment or fingerprint profiles using the presence or absence of bands as characters (for an example, see reference 6), this procedure is fraught with problems due, in large part, to the nonindependence of fragments (26). We have used an alternative approach to estimate the evolutionary distance (expressed as the rate of nucleotide substitutions per site) between pairs of taxa from the patterns of band sharing. Nei and Li (18) showed that for two genomes of comparable sizes, the expected proportion of shared fragments is given by: $F \approx G^2/(3 - 2G)$, where $G = e^{-sr}$, and s is the number of bases recognized by the restriction enzyme, r is the rate of nucleotide substitution per site, and t is the time of divergence. Given the RFLP profiles of any two taxa, x and y , we can estimate F using the following equation: $\hat{F} = 2n_{xy}/n_x + n_y$, where n_x and n_y are the numbers of bands in x and y , respectively, and n_{xy} is the number of bands shared by x and y . Because the estimation of F relies on the fact that two taxa share at least one band in common, evolutionary distances between *Thermus* strains with no bands in common cannot be estimated.

Once F has been estimated, G can be calculated by an iterative algorithm (18), such that $G_{i+1} = [F(3 - 2G_i)]^{0.25}$, where G_k is the estimate of G at the k th iteration. Let $G_1 = F^{0.25}$, and choose a convergence criterion, ϵ , such that if $|G_{i+1} - G_i| < \epsilon$, then G_{i+1} is taken as the best estimate of G . In our

FIG. 1. PFGE analysis of *Ssp*I restriction fragments from *Thermus* genomic DNA. Samples were prepared and electrophoresed as described in Materials and Methods. (A) PFGE was performed at 200 V for 24 h with a pulse time of 20 s. Lanes: 1, lambda DNA concatemer size standard; 2, *T. aquaticus* YVII-51B; 3, *T. aquaticus* YT-1; 4, *Thermus* sp. strain T351; 5, *Thermus* sp. strain T. sp.; 6, *Thermus* sp. strain OK6; 7, *Thermus* sp. strain Rt41A; 8, *Thermus* sp. strain W28P; 9, *Thermus* sp. strain W28; 10, *Thermus* sp. strain Tok21; 11, *Thermus* sp. strain Tok20; 12, *Thermus* sp. strain Tok3; 13, *Thermus* sp. strain Fij3; 14, *T. thermophilus* HB27; 15, *T. thermophilus* HB8. (B) PFGE was performed at 200 V with a ramped pulse time of 40 to 80 s over 24 h. The lanes are as in panel A.

analysis, ϵ was set at 0.0001. Having obtained G for any two taxa x and y , the evolutionary distance between x and y , $d(x,y)$, can be estimated as $d(x,y) = -(2 \ln G)/r$.

The neighbor-joining tree reconstruction method (21) was used to construct a phylogenetic tree of all taxa for which there was a complete set of pairwise evolutionary distances based on PFGE-RFLP data. Neighbor joining has been shown to perform better than other distance-based tree-building methods with simulated data under a range of different models of evolution (17).

RESULTS

Fingerprinting of *Thermus* strains by PFGE. The number of bands detected ranged from 6 (for "*T. thermophilus*" HB8 and W28P) to 13 (for *T. aquaticus* YT-1). Table 1 shows the number of bands generated for each strain and their approximate sizes estimated by comparison with a ladder of lambda DNA oligomers. The sum of the restriction fragment sizes for each strain gave an estimate of the genome size. As reported previously, the genome of "*T. thermophilus*" HB8 was determined to be approximately 1.8 Mbp (2). The genomes from

TABLE 1. Restriction fragments produced by *SspI* cleavage of *Thermus* genomic DNA

Strain	Genome size (Mbp)	No. of <i>SspI</i> bands	Fragment size (kb) ^a												
			1	2	3	4	5	6	7	8	9	10	11	12	13
<i>T. thermophilus</i> HB8	1.8	6	101	134	259	290	348	664							
<i>T. thermophilus</i> HB27	2.0	9	11	37	90	111	160	237	257	275	782				
Fiji3	2.1	8	81	181	221	228	285	317	376	404					
Tok3	2.1	8	29	44	55	128	221	338	433	841					
Tok20	2.1	11	16	23	28	38	48	114	149	232	242	561	697		
Tok21	2.1	11	13	21	27	30	35	109	116	237	243	570	665		
W28	2.1	9	34	44	52	103	154	227	234	527	729				
W28P	1.3	6	24	42	42	131	344	673							
Rt41A	2.2	9	17	40	109	131	152	210	234	545	731				
OK6	2.2	10	18	25	42	67	110	154	237	246	544	719			
<i>T. sp.</i>	1.9	8	17	39	110	132	150	234	715						
T351	2.0	9	17	25	42	110	131	151	235	536	714				
<i>T. aquaticus</i> YT-1	1.5	8	14	23	73	127	218	239	324	519					
<i>T. aquaticus</i> YVII-51B	2.0	13	21	29	54	91	101	110	125	142	161	218	257	300	354

^a Average of at least two determinations.

most of the other strains were similar in size (measured at 1.9 to 2.1 Mbp). However, the sums of the fragment sizes for both *T. aquaticus* YT-1 and *Thermus* strain W28P were much smaller: 1.5 and 1.3 Mbp, respectively. Whether these differences in calculated chromosome size are genuine or are an artifact resulting from incomplete restriction endonuclease digestion or poor separation of bands by PFGE is unknown but could be resolved by comparing calculated genome sizes after cleavage by a variety of rarely cutting endonucleases. All of the *Thermus* strains tested produced a characteristic and reproducible PFGE band profile. Most of the strains showed quite different banding patterns, with a small number of shared bands. The New Zealand strains Rt41A, *T. sp.*, and T351 showed strikingly similar banding patterns, despite the fact that they were isolated from different locations.

Phylogenetic analysis. Table 2 shows the evolutionary distances between all the taxa examined. Note that for some pairs of strains, no distance has been estimated since the strains in question share no bands in common. We have also calculated the evolutionary distances on the basis of the SSU rRNA sequences reported by Saul et al. (23) of those *Thermus* strains used in this study (Table 3) by the maximum-likelihood method described by Kishino and Hasegawa (13). The maxi-

mum-likelihood method takes into account differences in rates of transitions and transversions as well as the different equilibrium frequencies of each nucleotide. As such, this is the most appropriate method for species such as *Thermus* with a bias towards a higher G+C content. The maximum-likelihood method is implemented in DNADIST, a computer program that is part of J. Felsenstein's Phylogenetic Inference Package (available from J. Felsenstein, University of Washington, Seattle, Wash.). A close look at the two distance matrices shows that there is a close linear correlation between the distances estimated from PFGE-RFLP data and those from SSU rRNA sequences, particularly between those of more closely related taxa (Fig. 2). The Pearson correlation coefficient between the two sets of distances is 0.88. Mantel's test (25) confirmed that this correlation is highly significant ($P < 0.006$).

The neighbor-joining tree obtained from PFGE-RFLP data is shown in Fig. 3. The tree obtained by Saul et al. (23), pruned to show only those taxa in common with those in this study, is also shown, and it is apparent that there is broad agreement between the two trees. Note that in the study by Saul et al. (23), while there is strong bootstrap support (5) for the divergence of *Thermus* strain HB27 and the strains Tok20, Tok3, W28, Rt41A, T351, and OK6, there is weak or no support for the

TABLE 2. Evolutionary distances based on PFGE-RFLPs^a

Strain	Expected no. of nucleotide substitutions per site														
	YVII-51B	YT-1	T351	T. sp.	OK6	Rt41A	W28P	W28	Tok21	Tok20	Tok3	Fiji3	HB27	HB8	
YVII-51B	0.0000														
YT-1	0.6164	0.0000													
T351	0.4737	0.3739	0.0000												
T. sp.	0.6164	0.5079	0.1194	0.0000											
OK6	0.6532	0.5546	0.1588	0.2050	0.0000										
Rt41A	0.6352	0.5318	0.1396	0.1194	0.2246	0.0000									
W28P	0.8605	0.7336	0.4825	0.4555	0.7888	0.4825	0.0000								
W28	0.6352	—	0.2870	0.2658	0.3072	0.2870	0.7621	0.0000							
Tok21	0.9592	—	0.8821	0.8605	0.9027	0.8821	0.8140	0.5967	0.0000						
Tok20	0.9592	—	0.3264	0.3072	0.3449	0.3264	0.8140	0.2434	0.3626	0.0000					
Tok3	0.4555	0.7888	0.3739	0.3509	0.5546	0.3739	0.4555	0.3739	0.5762	0.4167	0.0000				
Fiji3	—	—	—	—	—	—	—	—	—	—	—	0.0000			
HB27	0.4737	—	0.8379	0.8140	0.8605	0.8379	—	0.5546	0.5967	0.5967	0.8140	—	0.0000		
HB8	—	—	—	—	—	—	0.6705	—	0.8140	—	—	—	—	0.0000	

^a The lower triangular evolutionary distance matrix used PFGE-RFLP data. —, nonestimable distances. See Materials and Methods for information on the methods used to estimate the distances.

TABLE 3. Evolutionary distances based on SSU rRNA sequences^a

Strain	Expected no. of nucleotide substitutions per site						
	T351	OK6	Rt41A	W28	Tok20	Tok3	HB27
T351	0.0000						
OK6	0.0007	0.0000					
Rt41A	0.0013	0.002	0.0000				
W28	0.0034	0.0041	0.0048	0.0000			
Tok20	0.0041	0.0048	0.0054	0.0034	0.0000		
Tok3	0.0054	0.0061	0.0068	0.0034	0.0013	0.0000	
HB27	0.0584	0.0592	0.0599	0.0577	0.0577	0.0562	0.0000

^a The lower triangular evolutionary distance matrix used SSU rRNA sequence data. Evolutionary distances based on SSU rRNA sequences have been calculated only for taxa used in this study. See Materials and Methods for information on the methods used to estimate the distances.

precise patterns of divergence of the last six of these strains. It appears that the lack of support is due largely to the fact that there are insufficient differences between these SSU rRNA sequences of strains to discriminate effectively between them. Any incongruence, therefore, between the PFGE-RFLP tree and the SSU rRNA tree may be an effect of chance.

DISCUSSION

It is clear from our study that PFGE-RFLP profiles can successfully discriminate between different strains of *Thermus*, with each strain being characterized by a unique pattern of bands. Our analysis has also shown that it is also possible to use PFGE-RFLP data to construct a matrix of evolutionary distances that correlate well with those obtained from an independent data set, namely, SSU rRNAs. Therefore, it is possible to use PFGE-RFLP profiles to construct phylogenies of groups of closely related taxa. Apart from demonstrating the ability of PFGE-RFLPs to successfully recover phylogenetic information, our analysis also highlights the fact that there is an upper limit to the evolutionary distances that PFGE-RFLP profiles can be used to accurately estimate. In the simplest case, this limit is determined by the presence of at least one shared band between any two taxa. As stated above, if two taxa share no bands in common, then the evolutionary distance between the two is nonestimable. The presence or absence of shared restriction fragments amongst related taxa, in turn, is likely to be due to the expected number of sites for any given restriction enzyme along the genome and the degree of evolutionary divergence. It is reasonable to assume that the more restriction sites there are along the length of the genome, the more likely it will be for two taxa to share at least one fragment in common. Therefore, the results that we report for the upper

FIG. 2. Scatterplots showing relationships between evolutionary distances estimated with PFGE-RFLPs and SSU rRNA sequences. (A) Relationship between PFGE-RFLP evolutionary distances in the range 0.0 to 0.1 and SSU rRNA evolutionary distances. (B) Relationship between PFGE-RFLP evolutionary distances in the range 0.09 to 0.15 and SSU rRNA evolutionary distances. Distances are plotted in percent divergence per million years.

FIG. 3. Phylogenetic trees estimated with PFGE-RFLP data and SSU rRNA data. The phylogenetic tree on the left was estimated by the neighbor-joining tree-building method, with the evolutionary distances calculated between all pairs of taxa in this study that shared at least a single band in common. The phylogenetic tree on the right is a subtree of that obtained by Saul et al. (23) pruned to show only those taxa that were used in this analysis.

limits of PFGE-RFLP evolutionary distance estimation are specific to our experimental conditions and the restriction enzyme used.

The relationship between PFGE-RFLP evolutionary distances and SSU rRNA evolutionary distances also suggests that the degree of evolutionary divergence affects the accuracy of evolutionary distance estimation. Returning to Fig. 2 and 3, it is apparent that the correlation between the larger PFGE-RFLP distances and SSU rRNA (correlation coefficient, $r = 0.36$) is not as strong as that between the smaller PFGE-RFLP distances and SSU rRNA ($r = 0.64$). Although this result may be an artifact of different sample sizes, it is consistent with the conjecture that PFGE-RFLPs are not efficient estimators of evolutionary distance over longer periods of divergence. This observation could be due to the fact that over greater evolutionary distances, the number of bands shared between two strains may be as few as one or two. Any estimate based on one or two shared bands conceivably could increase the sampling variance of the estimate.

What may be the upper time limit to the estimation of evolutionary distances with PFGE-RFLP data? Assume that the average rate of sequence divergence between two SSU rRNA sequences is 1% per 50 million years (29) or 0.02% per million years. Note that the divergence between *Thermus* strain HB27 and the other strains accounts for the large PFGE-RFLP distances shown in Fig. 2B. Assume that these distances approach the upper limits of PFGE-RFLP distance estimation. If we estimate the time of divergence between HB27 and the other strains from the SSU rRNA evolutionary distance, we obtain an average evolutionary distance of 0.0581 or a divergence rate of 5.81%; this corresponds to an expected time of divergence of 290 million years. Therefore, a divergence of about 290 million years approaches the upper limits of evolutionary distance estimation from PFGE-RFLPs under the experimental conditions we have described above. Interestingly, Hillis and Moritz (8), in their review of the appropriateness of different types of molecular data to resolve phylogenies over different time scales, have noted that restriction analysis is "marginally appropriate or appropriate under limited circumstances" with phylogenies that span 50 to 500 million years. Our value for the upper limits of PFGE-RFLP resolution falls in this range.

On the other hand, our results suggest that PFGE-RFLPs are able to resolve the evolutionary relationships of closely related taxa that would otherwise be unresolved with SSU rRNA data. It is possible that by using both PFGE-RFLPs and gene sequences a composite phylogenetic tree of the organisms in question can be constructed. We are investigating ways in which this may be done.

PFGE-RFLP data can also be used to estimate the average genomic rate of divergence if we assume that *SspI* restriction sites are distributed throughout the *Thermus* genomes. We do this by calculating the rate of sequence divergence relative to SSU rRNA. In Fig. 2, the slopes of the regression lines between PFGE-RFLP distances (taken as the dependent variable) and SSU rRNA distances (the independent variable) give values indicating how much faster the average genomic rate of divergence is relative to SSU rRNA. However, as both sets of distances are measured with error, the slope of the line is most appropriately estimated by a geometric mean regression (see reference 16 for a description of the technique). The slope of the regression line between the smaller PFGE-RFLP distances and SSU rRNA is 9.21, whereas that of the line between the larger PFGE-RFLP distances and SSU rRNA is 17.89. The average of these two values is 13.55, indicating that the average genomic rate of divergence is 13.55 times faster than that of

SSU rRNA for the *Thermus* genomes. Taking the SSU rRNA rate of divergence to be 0.02% per million years (see the explanation above), the average genomic rate of divergence based on our data is estimated to be 0.27% per million years (or a range of between 0.18 and 0.37% per million years, if the two different slopes obtained above are used).

We can also estimate the average genomic rate of divergence with data from the study by Ochman and Wilson (19). They estimated the rate of silent substitution of nucleotides in bacterial coding sequences at 0.7 to 0.8% per million years (midrange, 0.75% per million years) and the rate of amino acid substitution at 0.035 to 0.04% per million years (midrange, 0.0375% per million years). If we crudely assume that most of the bacterial genome is structured as coding sequences, that the majority of amino acid substitutions are the result of nucleotide substitutions in the first and second codon positions (and therefore each position has a divergence rate of 0.01875% per million years) and that all silent substitutions occur in the third codon position, then we can estimate the total rate of divergence as follows: $2/3 \times 0.01875 + 1/3 \times 0.75 = 0.2625\%$ per million years. This value is strikingly close to our estimate of 0.27% as the average genomic rate of divergence, thus giving us greater confidence in the applicability of PFGE-RFLPs to studies of molecular evolution and phylogenies.

It appears that the PFGE-RFLP fingerprint analysis is valuable as a taxonomic aid in distinguishing between isolates of *Thermus*. More pertinent to bacterial systematics, perhaps, is the possibility that PFGE-RFLP data can be used to reconstruct the phylogeny of closely related strains of *Thermus*. We have shown that evolutionary distance models constructed with PFGE-RFLPs correlate well with those estimated by using SSU rRNA sequences. However, our analysis also suggests that there are limits to the ability of PFGE-RFLP data to estimate evolutionary distances accurately; these limits are likely to be a function of the restriction enzyme used and the degree of divergence between the taxa studied. Finally, we have shown that the estimation of the average genomic rate of nucleotide substitution using our PFGE-RFLP data agrees remarkably well with estimates derived from other studies.

ACKNOWLEDGMENTS

This work was supported by grants from the University Grants Committee, the University of Auckland Research Grants Committee, and Pacific Enzymes Ltd.

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