JOURNAL OF CRUSTACEAN BIOLOGY, 29(3): 400-412, 2009

16S AND 28S rDNA SEQUENCES IN PHYLOGENETIC ANALYSES OF FRESHWATER PRAWNS (*MACROBRACHIUM* BATE, 1868) FROM TAIWAN

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ABSTRACT

Using 15 species of freshwater prawns (*Macrobrachium* Bate, 1868) from Taiwan as the study material, characters of mitochondrial 16S rDNA sequences (16S) and nuclear 28S rDNA sequences (28S) were examined. Their phylogenetic analyses were conducted with Bayesian (BI), maximum likelihood (ML), maximum parsimony (MP), and minimum evolution and neighbor-joining (MENJ) methods. The 16S extracted was smaller in sequence length, rich in adenine, poor in cyctosine, and biased strongly to transitions in the nucleotide substitutions, whereas the 28S extracted was larger, rich in guanine, and biased to transversions. In the separate analyses of the two genes, the phylogenetic trees derived from the 28S had appreciably higher topological resolution with deeper branching (less polytomies) and higher topological confidence with stronger phylogenetic signals than the 16S trees. The poor resolution and confidence of the 16S trees were attributable primarily to its poor sequence divergences associated with high transition/transversion (ts/tv) ratios and low α-values of the gamma distributions. The result was a severe convergence of taxa within a narrow range of small genetic distances, so that their bifurcation could not be determined unambiguously. The 28S was highly diverged and had larger genetic distances with low ts/tv ratios and high α-values, resulting in much less convergence of the taxa. The 28S tree reconstructed with BI produced the best topological resolution and confidence in the separate analyses. The partition homogeneity test indicated that the 16S and 28S data sets were congruent. Their combined analyses with ML, MP, and MENJ showed no improvement in both topological resolution and confidence from the separate analyses of the 28S. With BI the combined analysis produced mixed results; improved the estimates of phylogenies for some of the taxa but confused or even obscured for the others.

KEY WORDS: 28S rDNA, 16S rDNA, phylogeny, freshwater prawns, *Macrobrachium*, Taiwan

DOI: 10.1651/08-3069.1

INTRODUCTION

Mitochondrial genomes are maternally inherited through egg cytoplasm without recombination (David and Blackler, 1972). They have been considered to have a faster evolutionary rate than nuclear genomes (Brown et al., 1979) and regarded as a suitable study tool for systematics and molecular genetics (Moritz et al., 1987; Page and Holmes, 1998). The 16S rDNA sequences (16S) have been one of the most common segments of the mitochondrial genomes used in phylogenetic analysis. For crustaceans they have been used in the analysis of penaeid prawns (Palumbi and Benzie, 1991; Bouchon et al., 1994), freshwater crayfishes (Grandjean et al., 1998; Crandall et al., 1999; Shull et al., 2005), marine lobsters (Harding et al., 1997; Ovendon et al., 1997; Sarver et al., 1998; Machordom and Macpherson, 2004), fairy shrimps (Daniels et al., 2004), Caribbean sponge-dwelling snapping shrimps (Morrison et al., 2004), and freshwater prawns (*Macrobrachium* Bate, 1868) (Murphy and Austin, 2002, 2003, 2004, 2005; Liu et al., 2007).

A few recent studies indicated that the 16S produced poor resolution in reconstructed phylogenies for insects, perhaps due to its fast evolutionary rate (Misof et al., 2001; Hasegawa and Kasuya, 2006). The poor resolution was also found for phylogenies of freshwater prawns. Murphy and Austin (2005: fig. 3) made the phylogenetic analysis of 30 species of *Macrobrachium*, but it resulted in a poor resolution; their phylogenetic tree had a very high degree of polytomy represented by a single multifurcating node with the clades of 28 taxa, 93% of the total taxa under the study. Liu et al. (2007: fig. 2) increased the number of taxa to 38 but also obtained a poor resolution; their phylogenetic tree also had a high degree of polytomy represented by a multifurcating node with the clades of 37 taxa, 97% of the total taxa under the study.

Recently, nuclear 28S rDNA sequences (28S) have been used in phylogenetic studies of crustaceans (Crandall et al., 2000; Jarman et al., 2000; Shull, 2005). The 28S gene was found to have an evolutionary rate similar to or slower than that of the 16S gene (Fetzner and Crandall, 2001). It was able to clarify the relationships among distantly related taxa for water striders (Muraji and Tachikawa, 2000) and Odonata (Hasegawa and Kasuya, 2006), and also among closely related taxa for insects in Ichneumonoidea (Belshaw et al., 1998) and the hominoids (Gonzalez et al., 1990).

This study is intended to determine whether the 28S is a better tool than the 16S in phylogenetic analysis of freshwater prawns, by comparing their characters and evaluating topological resolution and phylogenetic signals of their reconstructed phylogenetic trees, using 15 species of *Macrobrachium* from Taiwan as the study material.

Many have criticized phylogenies derived from a single gene reflect only that particular gene (gene tree) rather than an overall evolutionary history of the organisms (species tree) (Chavarria and Carpenter, 1995; Brower et al., 1996;

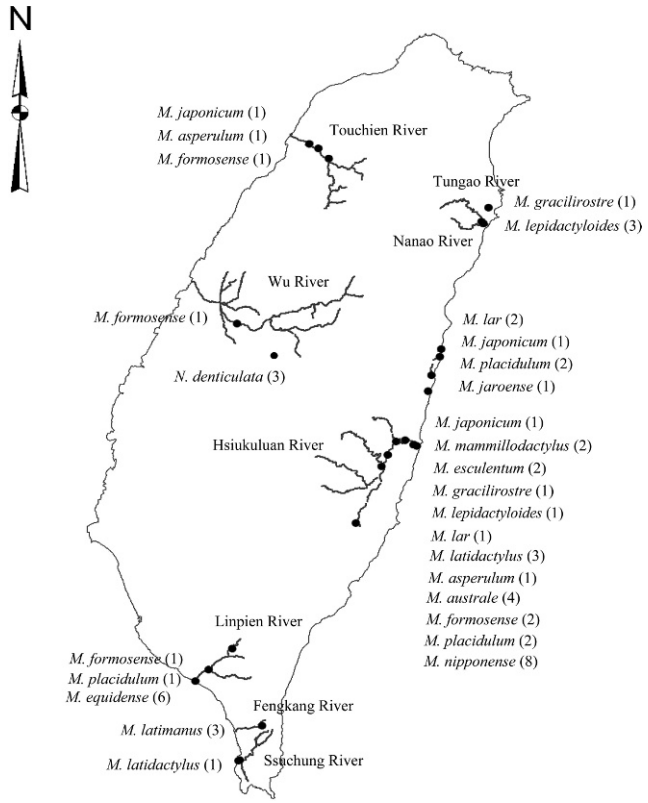

Fig. 1. Sampling locations of the freshwater prawns of Taiwan used for the mitochondrial 16S rDNA and nuclear 28S rDNA analyses (numbers of individuals collected in parentheses).

Nicholes, 2001). Combined analysis with DNA segments from different sources may be more meaningful but remains controversial (Wiens, 1998). It may increase phylogenetic signal, if phylogenetic relationships of the partitioned segments are congruent (Chavarria and Carpenter, 1995); but it may become ineffective (Lecointre and Deleporte, 2005) or even obscure the results of the separate analyses (Hasegawa and Kasuya, 2006), if the partitioned segments are incongruent or contradictory. This study also examined whether the combined analysis of the 16S and 28S sequences is favorable in the phylogenetic analysis of the prawns in the genus *Macrobrachium*.

Materials and Methods

Sources of DNA Sequences

There are a total of l6 species of *Macrobrachium* that have been reported from Taiwan (Hwang and Yu, 1982; Shy and Yu, 1998; Cai and Jeng, 2001). We collected 54 specimens of 15 species from the rivers around the island (Fig. 1). An exception was the endemic species *Macrobrachium shaoi* Cai and Jeng, 2001, that occurs only rarely in the Shuangchi River in the northern region and which we were unable to collect. All prawns collected were preserved in 95% ethanol solution and deposited at the Taiwan Endemic Species Research Institute.

For the prawns assembled, two specimens for each of the species were used for the 28S sequence extraction, with an exception of *Macrobrachium jaroense* (Cowles, 1914), which only had a single specimen collected. One of those two specimens was also used for the 16S sequence extraction. Another 16S sequence of each species was obtained from GenBank, except for *Macrobrachium mammillodactylus* (Thallwitz, 1892), which was not available from there. Thus, the above extracted 28S and 16S in this study had equal sample sizes of two sequences per species, with the exceptions of *M. jaroense* and *M. mammillodactylus* (Table 1).

Also, *Neocaridina denticulata* (de Haan, 1844) from Taiwan and *Paratya australiensis* Kemp, 1917 from Australia, both belonging to Atyidae, were used as the out-groups. The former was collected from the ecological pond of Taiwan Endemic Species Research Institute (TESRI), Chichi, Nantou, Taiwan, and the DNA sequences of the latter were obtained from GenBank (Table 1).

DNA Extraction, Amplification, and Alignment

For each of the prawn specimens collected, genomic DNA was extracted from muscles of the pleon with a commercial kit (GENTRA SYSTEMS, DNA purification from 5-1-10 mg marine invertebrate tissues kit). The portion of the 28S gene was amplified by PCR (polymerase chain reaction) with primer C1 (5′-ACCCGCTGAATTTAAGCAT-3′) and D2 (5′-TGGTCCGTGTTTCAAGAC-3′) (Hassouna et al., 1991). The 16Sar (5′-CGCCTGTTTATCAAAAACAT-3′) and 16Sbr (5′-CCGGTCTGAACTC AGATCACGT-3′) (Palumbi and Benzie, 1991) were used as the two primers to amplify a part of the 16S sequences. Double-stranded PCR products were obtained in a total reaction volume of 50 uL, containing 5 uL of 10× ExTaq buffer (Mg^{2+} added; TaKaRa), 5 uL of dNTP mixture (2.5 mM of each), 1 uM of each primer, 1 unit of Taq polymerase (TaKaRa Ex Taq™; TAKARA BIO. INC.), and 5 uL of DNA extract (100 ng).

For the 16S sequences, the target segment was amplified by PCR in the following temperature regime: the initial denaturizing step at 94°C for 5 min, followed by 35 cycles of denaturizing at 94°C for 1 min, an annealing temperature of 48–55°C for 1 min, an extension temperature of 72°C for 1 min, and then an additional extension of 72°C for 5 min. For the 28S sequences, the similar temperature profiles to those for the 16S were used, except that the annealing temperature was 58°C.

The DNA concentration was checked by comparing the brightness of the target segment with that of a band from standard marker (λ/HindIII; TOYOBO) on a 2% argarose/TAE gel containing ethidium bromide and visualized under UV light. PCR products were purified using Geneaid Genomic DNA Purification Kit (ORCUTIS SCIENTIFIC). All sequencing reactions followed the Perkin Elmer protocol, using a BigDye Terminator v3.1 Cycle Sequencing Kit with a DNA Analyzer (APPLIED BIOSYSTEMS). For each sample, sequencing was performed in both directions.

The forward and reverse sequence chromatograms were checked and edited manually using the programs of SeqMan 5.01 (DNASTAR) and BioEdit version 7.0.4.1 (Hall, 1999). Multiple alignments were performed using Clustal W (Thompson et al., 1994) with default parameter values (gap open penalty 10, gap extension penalty 0.1 in pairwise and 0.05 in multiple alignments) and all alignments were manually checked. All sequences of the 16S and 28S extracted in this study were deposited at the GenBank database (Table 1).

Sequence Character Analysis

Characters of nucleotide sequences of the 16S and the 28S extracted were examined with the exploratory data analysis, using MEGA version 3.1 (Kumar et al., 2004), DAMBE version 50.7 (Xia and Xie, 2001) and DnaSP 4.00 (Rozas et al., 2003). The genetic divergent distances among taxa were calculated using the best appropriated DNA substitution model obtained from Modeltest version 3.7 (Posada and Crandall, 1998). The distribution of pair-wise sequence divergences among the 15 taxa of *Macrobrachium* for the 16S and 28S sequences was established using SigmaPlot version 8.0.

True evolutionary relationships would be obscured in DNA sequence data sets if sites have become saturated by multiple substitutions (Swofford et al., 1996; Jesus et al., 2007). In order to assess the level of saturation about the 16S DNA and the 28S DNA, pair-wise sequences comparisons were performed by plotting the number of nucleotide substitutions per site for transitions and transversions, respectively, against the genetic divergent distances calculated from the best appropriated DNA substitution model, using PAUP* 4.0b10 (Swofford, 2002) and SigmaPlot version 8.0.

Instead of using forced linear regression function or natural logarithmic function on the substitution-distance relationships (Hasegawa and Kasuya, 2006; Liu et al., 2007), we used the quadratic function equation ($Y = a + b_1X + b_2X^2$, where Y is the number of substitutions per site, X is genetic divergent distance, a is constant, and b_1 and b_2 are the first and second degree regression coefficients, respectively). SigmaPlot version 8.0 was

Table 1. The sources of 16S rDNA and 28S rDNA sequences of freshwater prawns (*Macrobrachium*) used in this study (NCBI accession numbers: EU from this study, DQ from GenBank).

		Sequences from this study		Sequences from GenBank	
Species	Locations	16S rDNA	28S rDNA	16S rDNA	28S rDNA
M. asperulum	Hsiukuluan River	EU493137	EU008724	DQ194906	
	Touchien River		EU493102		
M. australe	Hsiukuluan River	EU493135	EU008719	DQ194904	
			EU518931		
M. esculentum	Hsiukuluan River	EU493145	EU008722	DQ194913	
			EU493113		
M. equidens	Linpien River	EU493149	EU008717	DQ194918	
			EU493126		
M. formosense	Hsiukuluan River	EU493150	EU008723	DQ194919	
	Touchien River		EU493104		
M. gracilirostre	Hsiukuluan River	EU493142	EU008728	DQ194924	
	Tungao River		EU493117		
M. japonicum	Hsiukuluan River	EU493141	EU008721	DQ194933	
			EU493109		
M. jaroense	Small creak	EU493146	EU008730	DQ194932	
M. latimanus	Ssuchung River	EU493143	EU008729	DQ194936	
			EU493114		
M. latidactylus	Hsiukuluan River	EU493140	EU008727	DQ194942	
	Ssuchung River		EU493105		
M. lar	Hsiukuluan River	EU493136	EU008720	DQ194939	
	Small creak		EU493107		
M. lepidactyloides	Nanao River	EU493138	EU493118	DQ194929	
	Hsiukuluan River		EU008725		
M. mammillodactylus	Hsiukuluan River	EU493148	EU008732		
			EU493096		
M. nipponense	Hsiukuluan River	EU493147	EU008731	DQ194951	
			EU493122		
M. placidulum	Hsiukuluan River	EU493139	EU008726	DQ194928	
			EU493099		
Neocaridina denticulata	Nantou, Taiwan	EU493134	EU518932		
Paratya australiensis	Australia			DQ478566	AY374160

used to calculate the best fitted equation by eliminating the constant and the regression coefficients, if they were not significantly different from zero (t-tests). When $b_2 = 0$, the relationship is a linear function, $b_2 > 0$, acceleration in substitutions; and $b_2 < 0$, deceleration in substitutions.

When a substitution-distance relationship is expressed as a quadratic function equation, b_1 is not the substitution rate (number of substitutions per site per distance) of the sequences, and b_2 is neither acceleration rate nor deceleration rate of the substitutions. In order to obtain substitution rates of transitions and transversions, the middle point between the minimum range and the maximum range of the genetic divergent distances was set as the boundary between close taxa and distant taxa. For each of the close taxa and distant taxa, a linear function equation was established for the relationship to obtain the slope as the substitution rate. The amount of increase in substitution rate from close taxa to distant taxa was considered as the acceleration rate, while the amount decreased as the deceleration rate. Also, the total substitution rate [total number of substitutions (transitions + transversions) per site per distance] of each of the genes was calculated for close taxa and distant taxa, respectively. The total substitution rate for the close taxa was considered as the evolutionary rate of the gene. It was the initial rate with no or very little influence of deceleration or acceleration in the substitutions.

Separate Phylogenetic Analyses

Phylogenetic analyses of the 16S and the 28S were separately conducted with the Bayesian (BI), maximum likelihood (ML), maximum parsimony (MP), and minimum evolution-neighbor-joining (MENJ) methods.

Both MP and ML were implemented using PAUP* 4.0b10 (Swofford, 2002). MP was performed with equal weightings for all characters, and gaps were treated as missing. The appropriated DNA substitution model for ML was obtained via testing alternative models of evolution using Modeltest version 3.7 (Posada and Crandall, 1998). A heuristic search was conducted for both MP and ML, using the tree bisection and reconnection branch swapping option (TBR) with 100 stepwise random additions of taxa. Nonparametric bootstrap support for nodes was estimated with 1000 and 100 heuristic bootstrap pseudo-replicates for MP and ML, respectively. MENJ was implemented using PAUP* 40b10 with the appropriated DNA substitution model selected by Modeltest, while nonparametric bootstrapping was consisted of 1000 pseudo-replicates.

BI was implemented with MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001). It was conducted with random starting trees and four MCMC chains, ran 2×10^6 generations, and sampled every 2000 generations, using general models previously identified with Modeltest. The resulted 1000 sampled trees were used to estimate the Bayesian posterior probabilities with Metropolis-couple, Markov chain Monte Carlo (MC-MCMC) analysis. In all searches, stationarity of the Markov Chain was determined by the point when sampled negative log-likelihood values against the number of generations reaching a stable mean equilibrium value. The ''burn-in'' data sampled from the generations proceeding to the stable mean value were discarded. The burn-in value was 250 and all data collected at stationarity were used to estimate posterior nodal probabilities and a summary of phylogeny.

In consensus phylogenetic trees, the majority consensus was set at 50% bootstrap value for ML, MP, MENJ and 50% Bayesian posterior probability for BI. When the values or probabilities were less than 50%, the lineages were collapsed under a multifurcating node (polytomy), indicating bifurcation of lineages in the clade could not be determined unambiguously (Graur and Li, 2000). Topology of the consensus trees, and their topological resolution and confidence were compared between the 16S and the 28S.

Topological resolution was calculated as the percentage of the number of bifurcating nodes observed in a consensus tree to the maximum number of such nodes assumed to be produced by the tree. As binary splitting is the common mode of the allopatric speciation process, in the case of the 15 taxa data sets of this study, the maximum number of the bifurcating nodes that could be produced by the tree was 14, i.e., 100% topological resolution, or the higher the topological resolution, the less the polytomies.

Phylogenetic signal is a tendency for evolutionarily related organisms to resemble each other (Blomberg et al., 2003). In phylogenetic analysis, it is

represented by the Bayesian posterior probability or bootstrap value, and considered as an estimate of clade probabilities. In this study, the Bayesian posterior probability at 0.95 was set as the significant level of clade confidence. It was a close estimate of true clade probability, suggesting that bifurcation of the lineages was strongly supported (Reeder, 2003). This 0.95 criterion was also applied to the bootstrap values as the significant level for ML, MP, and MENJ trees.

Topological confidence is a close estimate of the resemblance of the topology of a reconstructed phylogenetic tree to that of the tree with a true evolutionary history. It is an estimate of the phylogenetic support of the tree. In this study, it was calculated as the percentage of the number of bifurcating nodes with the significant clade confidence (posterior probabilities or bootstraps at or higher than 0.95) in a consensus tree to the maximum number of such nodes assumed to be produced by the tree (14 nodes as 100% topological confidence for the 15 taxa data sets). The higher the topological confidence, the higher the number of strongly supported bifurcating nodes and the stronger the phylogenetic support.

Combined Phylogenetic Analysis

In combined analysis, difference in substitution rates between partitioned segments (genes) may produce conflicting phylogenetic signals, leading to incongruent results. The incongruence length difference (ILD) (Faris et al., 1995) quantifies phylogenetic congruence between two partitioned segments. In this study it was implemented with the partition homogeneity method in PAUP*4.0b10 with 1000 replicates. The combined analysis of the 16S and 28S was also conducted with ML, MP, MENJ, and BI. Their topological resolution and topological confidence of reconstructed trees were determined. The methods and software used were similar to those used in the separate analyses. The topology, resolution, and confidence of the combined analyses were compared with those of the separate analyses.

Results

Sequence Characters

Characters of the 16S and the 28S extracted are shown in Table 2. As compared to the 16S, the 28S data were 30% larger in alignment length and were 53% greater in both variable and parsimoniously informative sites. Also, there was a significant difference in the percentage composition of nucleotide contents (adenine, thymine, cytosine, and guanine) between the 16S and the 28S data (χ^2-value = 9.7, *d.f.* = 3, $p < 0.05$). The 16S was richest in adenine (28.5%) and thymine (35.8%) and poorest in cytosine (12.4%), while the 28S was richest in guanine (35.4%) with the remaining three nucleotides almost even in the composition.

A significant difference was also found in relative frequencies of nucleotide substitutions (adenine-guanine, cytosine-thymine, adenine-cytosine, adenine-thymine, cytosine-guanine, and guanine-thymine) (χ^2-value = 31.4, *d.f.* = 5, $p < 0.05$). The 16S sequences had a high frequency of transitional substitutions that occupied 64.9% of the total substitutions, while the 28S had a high frequency of transversional substitutions, occupying 59%. The transition/transversion (ts/tv) ratio was 1.85 for the 16S sequences and 0.69 for the 28S; the former was 2.7 fold higher than the latter (Table 2).

Sequence Divergences

Sequence divergences calculated from the best-fit models selected by Modeltest as the divergent distances of the 16S and 28S sequences among 15 taxa of *Macrobrachium* (interspecific distances) and two out-groups are shown in Table 3, and their frequency distributions are compared in Figure 2. For 16S, the interspecific distances ranged between 0.018 and 0.303 with an average of 0.174. The distant range (distance between the maximum range and the minimum range) was 0.285. For 28S, the interspecific distances were between 0.064 and 0.533 at an average of 0.293. The distant range was 0.469. The 28S data was 68.4% higher in the average and 64.6% larger in the distant range than the 16S data. The smallest interspecific distance was between *Macrobrachium formosense* Bate, 1868, and *Macrobrachium nipponense* (De Haan, 1849), 0.064 for the 28S and 0.018 for the 16S; the former distance was 72% larger than the latter.

Furthermore, both 16S and 28S were slightly skewed toward right (larger distances) in the frequency distribution with the mode at 0.18 and an amplitude of 36% for the 16S, and at 0.2 with 22% for the 28S. With the small distant range of the 16S, there was a severe convergence of the frequencies of occurrence at the distances of 0.12 and 0.24 in the distance-frequency distribution, which accounted for 82% of the total occurrences. Because the frequencies of the distances are proportional to the frequencies of the occurrences of taxa, it could conclude that there was a convergence of about 12 taxa within that particular small distant range. For the 28S data, the frequencies of divergent distances were more evenly distributed; we saw no severe convergence of taxa within any particular small distant range as that for the 16S data (Fig. 2).

Table 2. Statistics for the data sets of 16S rDNA and 28S rDNA sequences extracted for 15 species of freshwater prawns from Taiwan.

	16S rDNA	28S rDNA
Characters		
Total alignment length (bp) (including gaps)	535	768
Variable sites (bp)	141	300
Parsimony informative sites (bp)	133	284
Nucleotide composition (mean %)		
Adenine (A)	28.5	20.5
Thymine (T)	35.8	22.2
Cytosine (C)	12.4	21.9
Guanine (G)	23.3	35.4
Substitutions		
Total number	18895	41720
Relative frequency (%)		
Transitions (ts)		
A-G	37.9	18.4
C-T	27.0	22.6
Transversions (tv)		
A-C	1.8	6.3
A-T	18.8	10.0
C-G	1.0	20.3
G-T	13.4	22.4
ts/tv ratio	1.85	0.69

Substitution-Divergence Relationships

Relationships between the number of nucleotide substitutions per site and sequence divergent distances for transitions and transversions of the 16S and the 28S sequences were found to be curvilinear. They were expressed as quadratic function equations with remarkably different regression coefficients (Fig. 3).

Table 3. The genetic divergent distances calculated from the best-fit models selected by Modeltest among 15 species of *Macrobrachium* from Taiwan and 2 outgroup species (28S rDNA below diagonal; 16S rDNA above diagonal).

Taxon	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.
1. *M. australe*		0.211	0.192	0.153	0.198	0.120	0.177	0.159	0.191	0.194	0.267	0.181	0.201	0.173	0.190	1.513	2.124
2. *M. equidens*	0.239		0.161	0.303	0.256	0.206	0.251	0.166	0.206	0.221	0.257	0.255	0.182	0.137	0.240	1.530	2.106
3. *M. formosense*	0.245	0.201		0.184	0.217	0.135	0.232	0.124	0.160	0.223	0.203	0.188	0.018	0.129	0.237	1.906	2.118
4. *M. japonicum*	0.205	0.254	0.217		0.156	0.143	0.171	0.180	0.149	0.144	0.241	0.181	0.177	0.189	0.119	1.872	5.484
5. *M. lar*	0.158	0.297	0.295	0.216		0.101	0.210	0.181	0.132	0.163	0.213	0.169	0.193	0.174	0.137	1.586	2.070
6. *M. latimanus*	0.132	0.222	0.196	0.180	0.144		0.131	0.134	0.120	0.128	0.202	0.129	0.114	0.125	0.140	1.490	2.211
7. *M. placidulum*	0.336	0.362	0.438	0.331	0.360	0.300		0.207	0.183	0.111	0.203	0.265	0.198	0.192	0.158	1.758	2.539
8. *M. asperulum*	0.306	0.340	0.210	0.265	0.418	0.282	0.515		0.143	0.178	0.221	0.166	0.125	0.124	0.187	1.525	1.847
9. *M. esculentum*	0.209	0.229	0.150	0.207	0.269	0.205	0.379	0.221		0.139	0.246	0.123	0.138	0.122	0.148	1.699	1.943
10. *M. gracilirostre*	0.315	0.388	0.338	0.289	0.365	0.305	0.168	0.380	0.339		0.239	0.164	0.175	0.172	0.074	1.218	1.938
11. *M. lepidactyloides*	0.271	0.322	0.332	0.265	0.279	0.233	0.170	0.412	0.298	0.164		0.285	0.212	0.242	0.155	1.944	2.538
12. *M. latidactylus*	0.301	0.291	0.200	0.233	0.360	0.246	0.479	0.266	0.117	0.432	0.380		0.181	0.154	0.174	1.508	2.438
13. *M. nipponense*	0.243	0.203	0.064	0.219	0.303	0.211	0.424	0.212	0.145	0.351	0.353	0.208		0.119	0.196	1.652	1.867
14. *M. mammillodactylus*	0.329	0.291	0.252	0.295	0.377	0.343	0.533	0.331	0.257	0.444	0.421	0.309	0.234		0.173	1.687	2.323
15. *M. jaroense*	0.255	0.304	0.252	0.240	0.224	0.190	0.238	0.325	0.289	0.172	0.146	0.343	0.275	0.408		1.581	3.010
16. *N. denticulata*	0.877	1.097	1.168	1.145	1.016	0.953	1.009	2.298	1.160	1.369	1.304	1.861	1.062	1.833	1.009		0.816
17. *P. australiensis*	1.275	2.384	1.771	1.699	2.028	1.191	1.746	1.784	1.391	1.483	1.778	2.028	1.766	2.177	1.396	0.383	

The 16S had the first-degree regression coefficient (b_1) of 0.44 for transitions that was 43.2% higher than 0.25 for transversions; whereas the 28S had 0.34 for the transitions that were 17.6% lower than 0.40 of the transversions. The differences suggested that the nucleotide substitutions were initially strongly biased toward the transitions in the 16S but almost equal between the transitions and transversions in the 28S (Fig. 3).

The second-degree regression coefficients (b_2) of the quadratic equations were negative for transitions and transversions for both 16S and 28S, suggesting the presence of deceleration in both transitions and transversions, the common characters of both genes. The deceleration in transitions was faster than that in transversions. With the increase in the distances, the transition-distance line and the transversion-distance line became fairly parallel for the 16S but split farther apart for the 28S (Fig. 3).

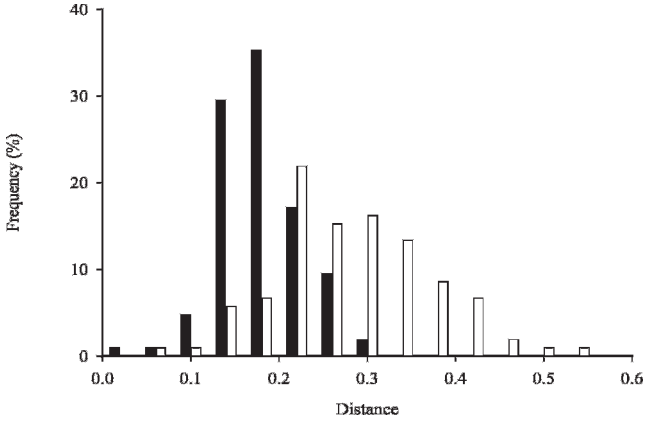

Fig. 2. Frequency distributions of genetic divergent distances calculated from the best-fit models selected by Modeltest among 15 *Macrobrachium* species of Taiwan for the 16S rDNA sequences (solid columns) and the 28S rDNA sequences (open columns).

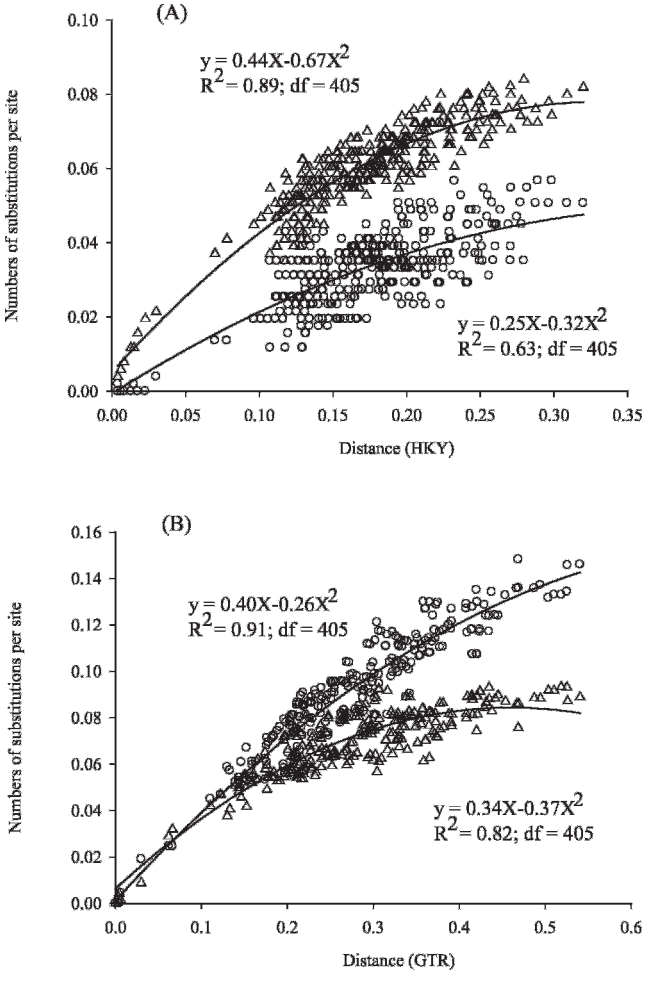

Fig. 3. Relationships between numbers of substitutions per site and the generic divergent distances calculated form the best-fit models selected by Modeltest for transitions (open triangles) and transvertions (open circles) of the the 16S sequences (A) and the 28S sequences (B) of 15 species of Taiwanese *Macrobranchium* prawns.

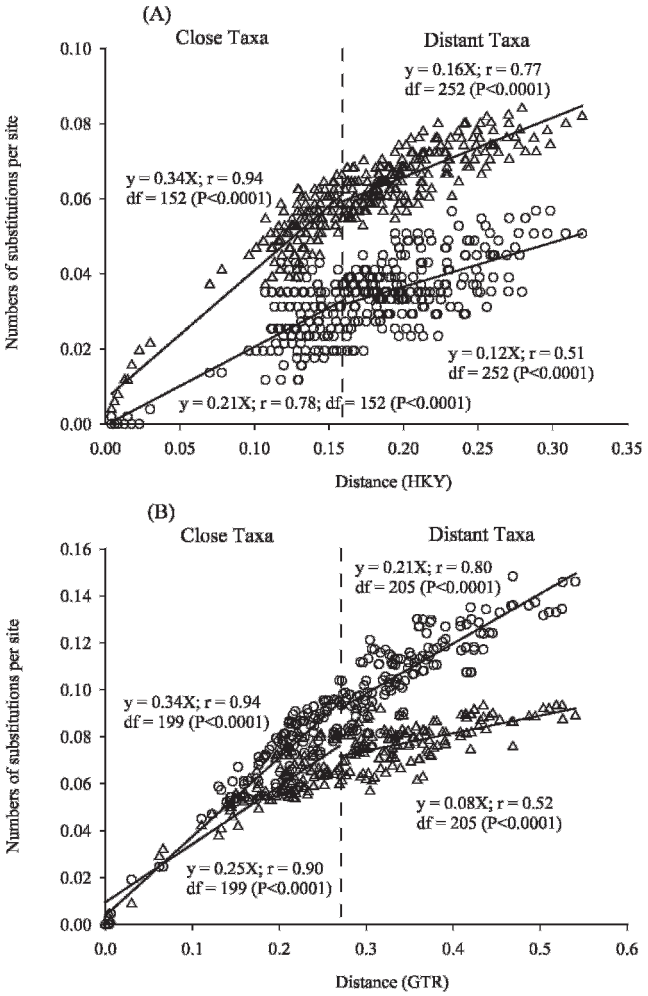

Fig. 4. Linear function relationships between numbers of substitutions per site and the genetic divergent distances calculated from the best-fit models selected by Modeltest for transitions (open triangles) and transversions (open circles) of the 16S sequences (A) and the 28S sequewnces (B) for close and distance taxa (distance at 0.16 for 16S and 0.27 for 28S as the boundaries) of 15 species of Taiwanese *Macrobranchium* prawns.

Substitution Rates

The linear function equations between numbers of substitutions per site and divergent distances for transitions and transversions of the 16S and the 28S sequences for close taxa and distant taxa are shown in Figure 4. Their substitution rates and evolutionary rates are in Table 4.

The 16S data had faster transition rates than transversion rates for both close taxa and distant taxa. The transition rate was 1.6 times faster than the transversion rate for close taxa, but only 1.3 times faster for distant taxa. The deceleration rate in the transitions was much faster than that for transversions (Table 4). In contrast to the 16S data, the 28S had faster transversion rates than transition rates for both close taxa and distant taxa, but the former was 1.4 times faster than the latter for close taxa but 2.6 times faster for the distant taxa.

The total substitution rate (transition rate + transversion rate) of the 16S sequences was 0.55, slightly slower than 0.59 of the 28S sequences for close taxa. In other words, the evolutionary rate (the initial total substitution rate) of the 16S was slightly (7%) slower than that of the 28S. For distant taxa, the total substitution rate was 0.28 for the 16S that was fairly equal to 0.29 for the 28S (Fig. 4).

Table 4. Substitution rates of transitions and transversions of the 16S and 28S rDNA sequences for close taxa and distant taxa of 15 species of *Macrobranchium* from Taiwan.

Substitution	Close taxa	Distant taxa	Deceleration (−)*
16S			
Transitions	0.34	0.16	−0.18
Transversions	0.21	0.12	−0.09
28S			
Transitions	0.25	0.08	−0.18
Transversions	0.34	0.21	−0.13
Total			
16S	0.55**	0.28	−0.27
28S	0.59**	0.29	−0.30

*Substitution rate of distant taxa minus substitution rate of close taxa.
**Evolutionary rate.

Gamma Distribution

For DNA sequences data sets of the 15 Taiwanese species of *Macrobrachium* and 2 species of the out-group, the most appropriate model for the 16S data was the HKY + I + G (Hasegawa et al., 1985) with the gamma distribution shape parameter $\alpha = 0.2441$ and a proportion of invariable sites of 0.2207. For the 28S data, the best-fit model for evolution of the nucleotide substitution was GTR + I + G (Rodriguez et al., 1990) with the gamma distribution shape parameter $\alpha = 0.6856$ and a proportion of invariable sites of 0.2840. The 28S has three fold higher in the α-value than that of 16S.

Separate Phylogenetic Analyses

The phylogenetic trees derived from the 16S with ML, MP and MENJ (Fig. 5) had poor topological resolution and topological confidence (Table 6). The resolution was 21.4–28.6%, which was about a quarter of the maximum resolution of the tree could havae produced, and the confidence was 0 to 7.1%, less than one tenth of the maximum possible confidence. All 15 taxa of *Macrobrachium* were collapsed into a single multifurcating node that had clades of 11 to 12 lineages. There was no strongly supported clade in the ML tree and merely a single strongly supported clade of closely related taxa, *M. nipponense* and *M. formosense* in the MP and MENJ trees.

The 16S tree reconstructed with BI greatly improved the topological resolution and confidence over those with ML, MP, and MENJ. The resolution improved about two fold to 64.3% and the confidence about three fold to 21.4%. There were five taxa in two strongly supported clades: the one containing *M. nipponense* and *M. formosense* with *Macrobrachium asperulum* (von Martens, 1868) as the basal lineage, and the other containing *Macrobrachium esculentum* (Thallwitz, 1891) and *Macrobrachium latidactylus* (Thallwitz, 1891). The 16S tree with BI resolved the phylogenetic

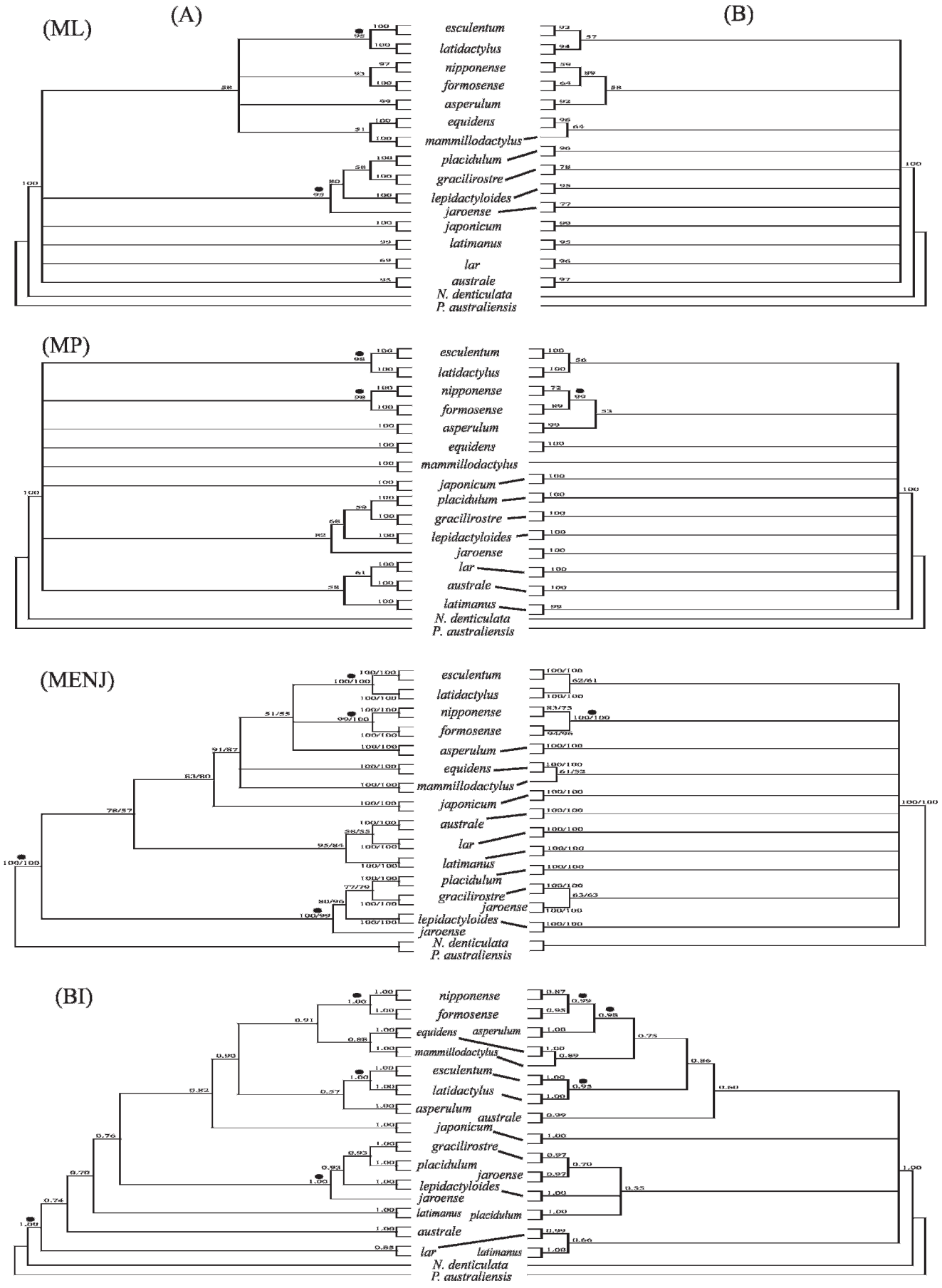
(ML)
(A)
(B)
esculentum
latidactylus
nipponense
formosense
asperulum
equidens
mammillodactylus
placidulum
gracilirostre
lepidactyloides
jaroense
japonicum
latimanus
lar
australe
N. denticulata
P. australiensis
(MP)
esculentum
latidactylus
nipponense
formosense
asperulum
equidens
mammillodactylus
japonicum
placidulum
gracilirostre
lepidactyloides
jaroense
lar
australe
latimanus
N. denticulata
P. australiensis
(MENJ)
esculentum
latidactylus
nipponense
formosense
asperulum
equidens
mammillodactylus
japonicum
australe
lar
latimanus
placidulum
gracilirostre
jaroense
lepidactyloides
jaroense
N. denticulata
P. australiensis
(BI)
nipponense
formosense
equidens
asperulum
mammillodactylus
esculentum
latidactylus
asperulum
australe
japonicum
gracilirostre
placidulum
jaroense
lepidactyloides
jaroense
latimanus
placidulum
australe
lar
latimanus
N. denticulata
P. australiensis

Table 5. Topological resolution (numbers of the bifurcating nodes with 50% consensus in parentheses) and topological confidence (numbers of the bifurcating nodes with 95% consensus in parentheses) of the phylogenetic trees reconstructed from the 16S, 28S and combined (16S + 28S) sequences of 15 species of *Macrobranchium* of Taiwan with the maximum likelihood (ML), maximum parsimony (MP), minimum evolution and neighbor-joining (MENJ), and Bayesian methods.

Sequences	ML	MP	MENJ	BI
16S				
Topological resolution	28.6 (4)	21.4 (3)	28.6 (4)	64.3 (9)
Topological confidence	0	7.1 (1)	7.1 (1)	21.4 (3)
28S				
Topological resolution	42.9 (6)	50.0 (7)	71.4 (10)	100.0 (14)
Topological confidence	21.4 (2)	14.3 (2)	28.6 (4)	28.6 (4)
16S + 28S				
Topological resolution	42.9 (6)	28.6 (4)	71.4 (10)	92.9 (13)
Topological confidence	7.1 (1)	14.3 (2)	21.4 (3)	42.9 (6)

relationships for a few close taxa but could not resolved most of the close taxa and all of the distant taxa.

On the other hands, the phylogenetic trees derived from the 28S with ML, MP and MENJ (Fig. 5) had topological resolutions of 42.9–71.4% and the topological confidences ranged between 14.3–28.6%. These values were about two fold higher than those of the 16S trees (Table 5). However, like the 16S trees, all 15 taxa were still collapsed into a single multifuracting node with the clades of 6 and 8 lineages in the ML and MP trees and into two multifurcating nodes in the MENJ tree. For the latter, there were only three strongly supported clades: one containing *M. nipponense* and *M. formosense*, one containing *M. esculentum* and *datidactylus*, and the other containing *Macrobrachium placidulum* (de Man, 1892), *Macrobranhium gracilirostre* (Miers, 1875), *Macrobrachium lepidactyloides* (de Man, 1892), and *Macrobrachium jaroense* (Cowles, 1914).

The 28S tree reconstructed with BI, the topological resolution increased to 100% (Table 5), indicating that the bifurcation of all 15 taxa under the study were determined unambiguously. The tree produced the best resolution for both close taxa and distant taxa. However, the topological confidence was 28.6% (4 nodes strongly supported), a slight improvement from the 16S tree that had 21.4% (3 nodes strongly supported).

Combined Phylogenetic Analysis

The partition-homogeneity test showed that there was no significant heterogeneity between the 16S and 28S data sets ($p, 0.19 > 0.05$), suggesting that they were phylogenetically congruent. The best-fit model for evolution of the nucleotide substitution of the combined sequences (16S + 28S) was GTR + I + G with the gamma distribution shape parameter $\alpha = 0.4965$ and a proportion of invariable sites of 0.2502. The results of the combined phylogenetic trees reconstructed with ML, MP, MENJ, and BI are shown in Figure 6, and their topological resolutions and confidences are shown in Table 5. As mentioned previously, the 28S trees had appreciably higher resolutions and confidences than those of the 16S trees. The comparison was made between the combined analyses and the 28S separate analyses.

As compared to the 28S tree, the topological resolution of the combined tree was similar for ML and MENJ, but lower for MP, whereas the topological confidence was similar for MP, but lower for ML and MENJ. Both resolutions and confidences varied among the methods but showed no improvement in the combined trees and remained as poor as those of the 28S trees.

When BI was used, the combined tree decreased the topological resolution to 92.9% from 100% of the 28S tree, but increased the topological confidence to 42.9% from 28.6%. In terms of the number of the nodes, the differences were still very minor: only one node for the former and two nodes for the latter. However, the tree topologies and phylogenetic signals differed between the combined tree and the 28S tree, particularly at two internal nodes with *M. asperulum* and *M. japonicum* (Figs. 5, 6).

In the 28S tree, *M. asperulum* was the basal lineage of the clade with *M. esculentum* and *M. latidactylus* with weak support (posterior probability, 0.57) (Fig. 5). In the combined tree, *M. asperulum* shifted its phylogenetic position to the clade of *M. formosense* and *M. nipponense* with nearly strong support (0.94) (Fig. 6). With this shift the clades of the above three taxa and the clade of *Macrobrachium equidens* (Dana, 1852) and *M. mammillodactylus* became strongly supported, instead of two strongly supported nodes in the 28S tree. The combined analysis improved the phylogenies of these 7 taxa.

On the other hand, in the 28S tree *M. japonicum* was the basal lineage of the internal node with the clades containing 7 terminal nodes, *M. asperulum*, *M. latidactylus*, *M. esculentum*, *M. mammillodactylus*, *M. equidens*, *M. formosense*, and *M. nipponense* (Fig. 5). In the combined tree, *M. japonicum* shifted its phylogenetic position to the other internal node with the clades containing *M. jaroense*, *M. lepidactyloides*, *M. gracilirostre*, and *M. placidulum* (Fig. 6). The Bayesian posterior probabilities were 0.82 for the 28S tree and 0.86 for the combined tree. The phylogenetic signals of both 28S and combined trees had the moderate support and their difference was small, causing confusion for the phylogenetic position of *M. japonicum*. In addition, the phylogenetic signals for the

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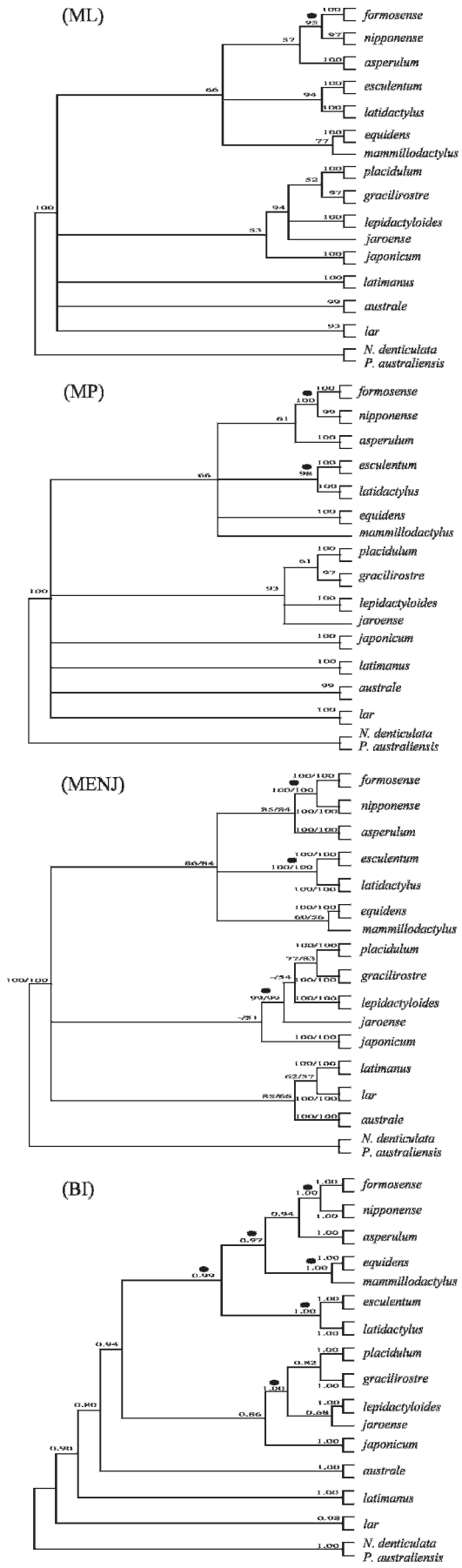
Fig. 5. Consensus phylogenectic trees reconstructed from the 28S rDNA sequences (A) and the 16S rDNA sequences (B) for 15 species of Taiwanese *Macrobranchium* prawns with *Neocaridina denticulate* and *Paratya australiensis* as the outgroups, using maximum likelihood (ML), maximum parsimony (MP), minmum evolution and neighbor-joining (MENJ) (numbers near lines, bootstrap values), and Bayesian (BI) (numbers above lines, Bayesian posterior probabilities) methods (solid circles, the bifurcating nodes with and above 95% consensus).



three bifurcating nodes of the above four taxa also decreased in the combined tree from the 28S tree. The combined analysis confused the phylogenetic position of *M. japonicum* and obscured the phylogenies of some of its associated taxa.

For the remaining taxa, the phylogenetic signals increased at the nodes with *M. australe* and *M. latimanus* but decreased at the node with *M. lar* in the combined tree (Fig. 6) from the 28S tree (Fig. 5). For the latter node, Bayesian posterior probability decreased to less than 0.5 (not supported) in the combined tree from 1.00 (strongly supported) in the 28S tree, severely obscuring the result of the separate analysis for the taxa.

The combined analysis with BI produced mixed results: improvement in phylogenies for some of the taxa, but confusion or even obscurity for the others

Discussion

Characters of DNA sequences have been known to affect topological resolution in phylogenetic analysis. The most common such character is site saturation by multiple substitutions that may cause obscurity in true relationships of taxa in evolution (Swofford et al., 1996; Jesus et al., 2007). However, there is the lack of saturation of transitions for the 16S of the freshwater prawns (Murphy and Austin, 2005; Liu et al., 2007). In our study, there was deceleration in transitions in both 16S and 28S sequences, but it did not reach a level of the saturation (Fig. 3).

Increasing a sequence length increases the number of phylogenetic informative sites that improves the resolution of phylogenetic analysis (Miyamoto et al., 1990; Chang et al., 2005). It is correctly said that increasing a sequence length provides more data in the analysis, the better the chance to obtain a better phylogeny. In this study, the 16S extracted sequence was much smaller than the 28S extracted sequence length (Table 2). Liu et al (2007, Fig. 3) combined the 16S sequences and the COI sequences to make the combined sequences 37% larger in the length and 47% more in the total parsimonious information sites than those of the 28S. The combined sequences improved the resolution of the phylogenetic tree derived from the 16S, but many of the internal multifurcating nodes were unresolved and the phylogenic relationships of many taxa remained obscure. Obviously, transitional saturation and sequence length were not the primary factors for the 16S to produce poor resolution in the phylogenetic analysis for the freshwater prawns (Fig. 5).

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Fig. 6. Consensus phylogenectic trees reconstructed from the combined 16S rDNA and 28S rDNA sequences for 15 species of Taiwanese *Macrobrachium* prawns with *Neocaridina denticulata* and *Paratya australiensis* as the outgroups, using maximum likelihood (ML), maximum parsimony (MP), minmum evolution and neighbor-joining (MENJ) (numbers near lines, bootstrap values), and Bayesian (BI) (numbers above lines, Bayesian posterior probabilities) methods (solid circles, the bifurcating nodes with and above 95% consensus).

Murphy and Austin (2005) attributed this poor resolution in phylogenetic analysis of freshwater prawns from the 16S to a rapid and explosive radiation in their early evolutionary history, which might have occurred during the Oligocene or early Miocene. They speculated that was a period with the proper environments (possibly low sea levels and short water distances among land masses) for rapid dispersion and diversification of amphidromous prawns in the Southeast Asia and farther to Australia, the western Pacific islands, India, Africa, and even Atlantic coasts of Tropical America (Chen et al., 2009).

The rapid radiation created numerous taxa within a short time period. The 16S exhibits small genetic distance range, resulting in a severe convergence of the taxa within the range (Fig. 2) so that any bifurcating branching patterns could not be determined unambiguously, as expressed by the presence of severe polytomies in the phylogenetic trees (Fig. 5). In contrast, the 28S data had larger genetic distance ranges that enabled accommodation of the taxa without severe convergence (Fig. 2), and thus resulting in higher resolution with fewer polytomies in the phylogenetic analysis (Fig. 5, Table 5).

Phylogenetic information in DNA sequences is built up by nucleotide substitutions over time. Due to the limited number of character states, phylogenetic signals also erode by similar process. Several evolutionary factors can potentially mislead phylogeny estimations (Conant and Lewis, 2001), such as heterogeneity in substitution rates among lineages (Felsenstein, 1978) and sites (Navidi et al., 1991; Reeves, 1992; Sidow and Steel, 1992; Yang, 1993), transition/transversion bias (Kimura, 1980; Wakeley, 1993), nonindependence of sites within a gene (Goldman and Yang, 1994; Schoniger and Von Haeseler, 1995; Muse, 1995, 1996), and non-stationarity of nucleotide frequencies across lineages (Loomis and Smith, 1990; Burggraf et al., 1992; Hasegawa and Hashimoto, 1993; Lockhart et al., 1994; Galtier and Gouy, 1995, 1998).

The ts/tv ratio is a parameter used by some of the models in phylogeny estimation. It is intended to reflect that nucleotide substitutions are not all equally alike among the DNA sequences (Purvis and Bromham, 1997). The ts/tv ratio is an important aspect of modeling sequence evolution. It expresses relative probabilities of different types of nucleotide changes, and thus, it is needed in order to correct measures of genetic distances (Wakeley, 1996). It is generally assumed that the ts/tv ratio is higher than 1:2 in animal nuclear genomes, if all substitutions were equally alike, while the relative transition rate is even much higher in mitochondrial DNA (Wakeley, 1996; Keller et al., 2007). In this study, the 28S data set had the ts/tv ratio at 1:1.45 closer to 1:2 that would have more uniform substitution rate than 1:0.54 of the 16S.

The gamma distribution has been proposed to model variable rates at sites (Jin and Nei, 1990; Li et al., 1990; Tamura and Nei, 1993). The distribution involves a shape parameter α of which the value is inversely related to the extent of rate variation at sites. Sullivan et al (1995) and Misof et al (2001) demonstrate that there is extreme among-site variation in mitochondrial 12S sequences, which has a highly skewed distribution of the rates and makes the sequences particularly susceptible to the misleading effects of non-independency and other nonrandom noise in phylogenetic analysis.

In our study, the 16S data set had the α-value of 0.2207 much lower than 0.6856 of the 28S data set. The lower α-value indicated higher among-site variation rates (Page and Holmes, 1998). The 16S had the α-value less than 0.5, the presence of extremely high among-site rate variation (Tateno et al., 1994; Sullivan et al., 1995), whereas the 28S had the value higher than 0.5, indicating very low among-site rate variation. The higher among-site rate variation of the 16S as indicated by its low α-value suggested that most sites of its sequences were invariable, but a few had very high substitution rates. Consequently, it was less informative, and thus the divergences among sequences were smaller (Yang, 1996). The α-values were inversely related to ts/tv ratios (Yang, 1996). In other words, low α-value and high ts/tv ratio were one of the sequence characters of the 16S data that is associated with the low genetic divergent distances for poor resolution in its phylogenetic analysis as compared to that of the 28S.

The combined analysis of different DNA segments has been often tested, but the results are controversial (Brower et al., 1996; Wiens, 1998; Nicholes, 2001; Lecointre and Deleporte, 2005; Hasagawa and Kasuya, 2006). A combined analysis is effective if the partitioned components of the data sets are congruent with consistent phylogenetic signals (Chavarria and Carpenter, 1995; Hasagawa and Kasuya, 2006). In this study, the partition-homogeneity test showed that the 16S and 28S data sets were congruent; nevertheless, the combined analysis with ML, MP, MENJ showed no improvement from the 28S, but with BI produced mixed results. For the latter the analysis did not consistently improve the resolution of the phylogenetic relationships of all taxa, but improved that of some of the taxa, and confused or even obscured the others. Obviously, in this instance, the combined analysis is not the first choice in this phylogenetic analysis; the separate analyses should be done first (Lecointre and Deleporte, 2005; Hasegawa and Kasuya, 2006). To find the most plausible phylogenetic hypothesis, a mutual reference should be made among the results of the separate and combined analyses (Hasegawa and Kasuya, 2006).

In conclusion, the 16S tree produced poor topological resolution and topological confidence, and thus, the 16S is not a proper tool for phylognetic analysis of our species of *Macrobrachium*. The 28S tree greatly improved the resolution and confidence over that of the 16S tree. The best resolution was found for the 28S tree with BI. The combined analysis with BI produced mixed results. Although the topological confidence of 28S and combined trees were better than that of the 16S tree but still remained poor; less than 50% of the nodes were strongly supported. For clarifying the phylogenetic history of *Macrobrachium*, future studies should search for specific gene or genes that have adequate ts/tv ratio and α-value for the focal taxa. In this aspect, nuclear genes might be more promising.

Acknowledgements

This study was financial supported by Taiwan Endemic Species Research Institute. The authors are grateful to Messrs R. C. Jang, M. H. Shen, H. P. Chen, K. W. Wang, Y. R. Syu, and Y. H. Yu for field collections, and Ms Y. J. Chen for DNA extractions.

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RECEIVED: 31 July 2008.
ACCEPTED: 2 December 2008.