J Mol Evol (1996) 43:517–522

JOURNAL OF MOLECULAR EVOLUTION

Phylogenetic Analysis of Veneridae (Bivalvia): Comparison of Molecular and Palaeontological Data

A. Canapa,[1] I. Marota,[2] F. Rollo,[2] E. Olmo[1]

[1] Istituto di Biologia e Genetica, Facoltà di Scienze, Università di Ancona, via Brecce Bianche, I-60131 Ancona, Italy
[2] Dipartimento di Biologia Molecolare, Cellulare e Animale, Università di Camerino, I-62032 Camerino, Italy

Received: 11 March 1996 / Accepted: 6 May 1996

Abstract. An approximately 400-bp-long portion of the 16s rRNA gene sequence has been determined for the venerid clams *Chamelea gallina* (Chioninae), *Dosinia lupinus* (Dosiniinae), *Pitar rudis, Callista chione* (Pitarinae), *Tapes decussatus, T. philippinarum, Venerupis* (= *Paphia*) *aurea* (Tapetinae), and *Venus verrucosa* (Venerinae). Neighbor-joining and maximum parsimony trees support the results of traditional classification methods at the subfamily level but do not support the concept of a genus *Tapes.* The transversion divergence rate estimated on the basis of the palaeontological record for the *C. gallina/V. verrucosa* separation and for the Pitarinae is very close (0.14–0.16% per Myr, respectively) to that of ungulates and cetaceans, while the Tapetinae exhibit a much higher (0.36% per Myr) rate.

Key words: Veneridae — Bivalvia — mtDNA — Molecular clock — 16s rDNA

The Veneridae is a large and diverse family of Bivalvia, including more than 500 living species, that are classified into approximately 12 subfamilies, with 50 extant and 55 extinct genera. The members of this family live in various marine ecosystems and are characterized by having three cardinal teeth in each valve, and sometimes up to three anterior teeth. Valves show concentric sculpture ranging from smooth to pronounced, and sometimes radial and divaricate sculptures as well.

Venerid taxonomy is controversial and several discrepancies among different systematic papers have been observed (Keen 1969; Fisher-Piette and Vukadinovic 1977). Mechanisms of adaptation to different environments and lifestyles may have strongly influenced the evolution of Veneridae, leading to several cases of parallelism in conchological characters of distantly related species and to extensive conchological diversification between closely related species (Harte 1992).

Recently, methodological approaches such as enzyme electrophoresis (Borsa et al. 1992), karyological analysis (Borsa and Thiriot-Quiévreux 1990; Insua and Thiriot-Quiévreux 1992), radio-immunoassay (Harte 1992), and highly repetitive DNA analysis (Passamonti et al. 1994; Canapa et al. 1993), have been used to study the taxonomy of Veneridae. Although most of these studies are limited to Tapetinae the results obtained have provided interesting contributions to clarifying some controversial aspects of venerid taxonomy. Moreover, they have confirmed that the systematics of this clam family, based only on morphological characters, may be influenced by evolutionary convergence phenomena.

Where morphological or physiological evidence of systematic relationships is unclear, genetic characters may provide accurate and unambiguous indicators of taxonomic divergence (Wilson et al. 1985). The mitochondrial gene for 16s ribosomal RNA has proved a powerful tool in phylogenetic studies and has provided information on the systematics of terrestrial and marine vertebrates (Allard et al. 1992; Milinkovitch et al. 1993) and on the systematics of marine invertebrates (Cunningham et al. 1992; Geller et al. 1993; Rumbak et al. 1994; Bucklin et al. 1995). To verify if this approach can con-

Correspondence to: E. Olmo

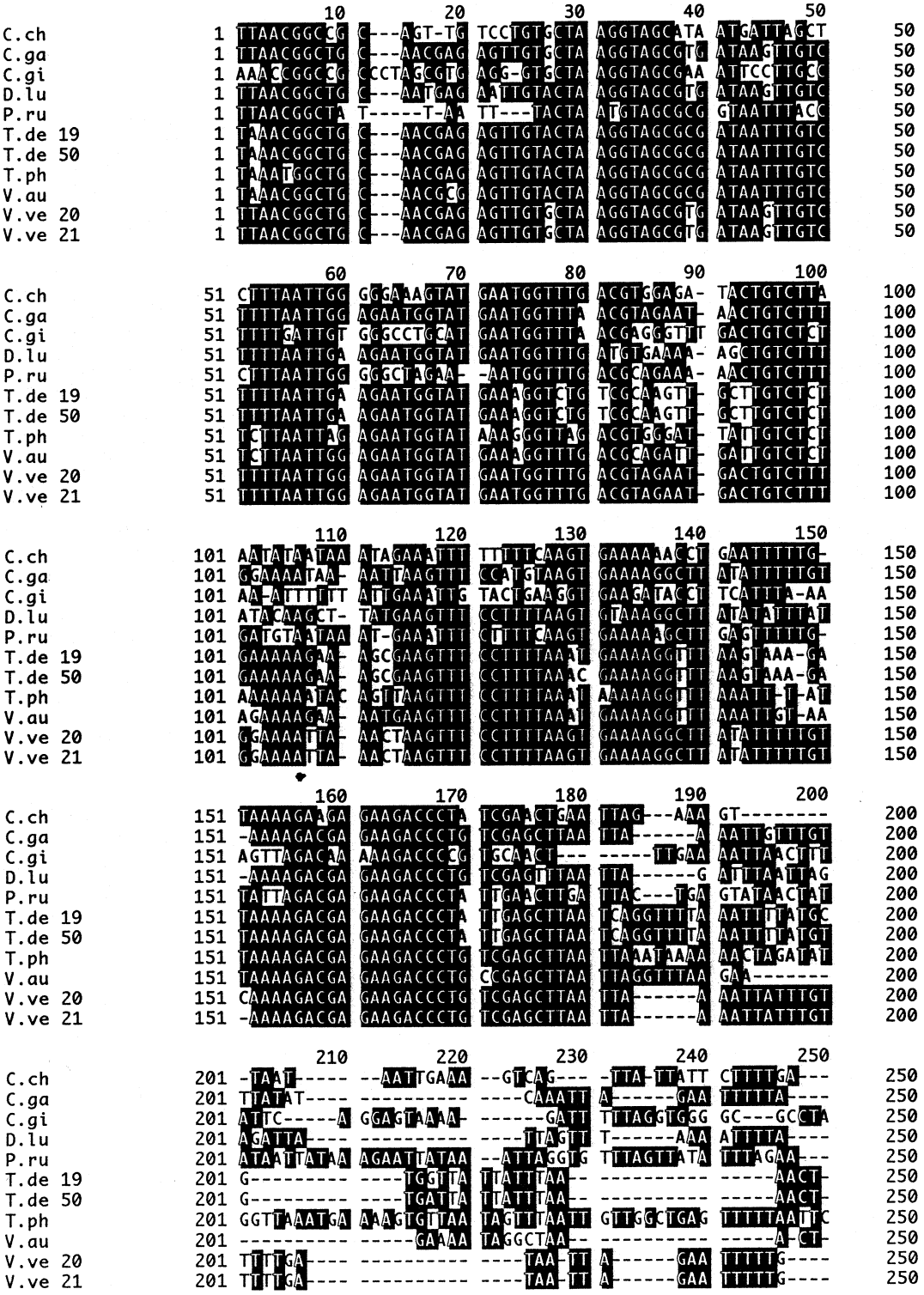

Fig. 1. Alignment of 16s rDNA sequences of *C. chione* (C.ch), *C. gallina* (C.ga), *C. gigas* (C.gi), *D. lupinus* (D.lu), *P. rudis* (P.ru), *T. decussatus* (T.de), *T. philippinarum* (T.ph), *V. aurea* (V.au), *V. verrucosa* (V.ve). The specimens C.ch, C.ga, D.lu, P.ru, and V.au were collected along the coast near Ancona (Italy), V.ve specimens come from the Maliston Gulf near Split (Croatia); the T.de specimens come from the Gulf of Naples (Italy) and T.ph specimens were collected along the coast near Goro (Ferrara, Italy). Semipurified mitochondrial DNA was prepared according to Geller et al. (1993). One-half gram of clam foot is homogenized in 2 ml TEK (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1.5% NaCl). The homogenate is then centrifuged at 1,000*g* for 20 min and the supernatant is collected, diluted with 2 vol TEK, and finally centrifuged at 18,000*g* for 30 min at 4°C. The mitochondrial pellet is resuspended in 0.5 ml TEK and, following treatment with 0.5% Nonidet P-40 at 0°C, extracted by 1 vol of phenol-chloroform. Eventually, the DNA is precipitated by ethanol. Semipurified mitochondrial DNA was PCR amplified using the universal 16AR (5′-CGCCTGTTTAACAAAAACAT-3′) and 16BR (5′-CCGGTTTGAACTCAGATCACGT-3′) oligonucleotide primers (Palumbi et al. 1991) under the following reaction conditions: 94°C, 1 min; 55°C, 30 s; 72°C, 1 min. Amplified DNA was directly sequenced in both senses using a manual procedure employing Taq polymerase (''Cycle Sequencing'') and ^{33}P as the radioactive label. The alignment was performed using the Higgins-Sharp algorithm (CLUSTAL4) contained in the MacDNASIS (Hitachi) package, set at the default parameters. The voucher specimens are lodged in the Museum of the Faculty of Sciences of Ancona University.

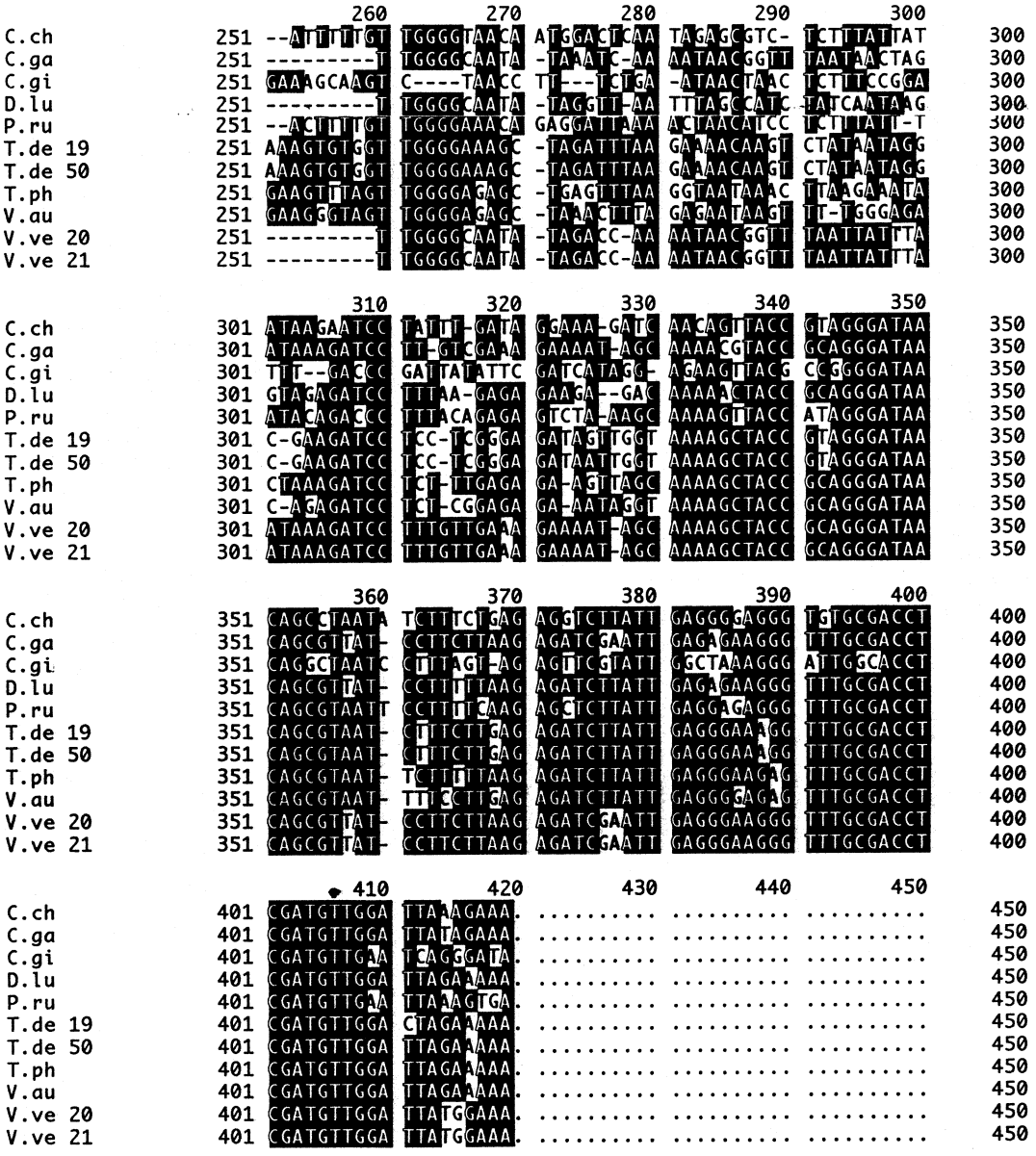

Fig. 1. Continued.

tribute to clarify the systematics and phylogeny of Veneridae, we have determined the nucleotide sequence of an approximately 400-bp-long portion of the 16s rRNA gene in *Chamelea gallina* (Linné, 1758) (Chioninae), *Dosinia lupinus* (Linné, 1758) (Dosiniinae), *Venus verrucosa* (Linné, 1758) (Venerinae), *Tapes decussatus* (Linné, 1758), *T. philippinarum* (Adams & Reeve, 1850) (usually assigned to *Ruditapes*), *Venerupis (= Paphia) aurea* (Gmelin, 1791) (Tapetinae), *Pitar rudis* (Poli, 1795), and *Callista chione* (Linné, 1758) (Chioninae). *Chione, gallina, verrucosa,* and *decussatus* are, respectively, the type species of the genera *Callista, Chamelea, Venus,* and *Tapes (Ruditapes)*. The sequences have been used to construct phylogenetic trees (utilizing different methods) and the results have been compared with the palaeontological data.

Sequences of the 16s rRNA gene fragment (not including the primer sequence) from the above-mentioned species and from *Crassostrea gigas* (Ostreida:Bivalvia), are shown in Fig. 1. In the case of *T. philippinarum, T. decussatus,* and *V. verrucosa,* two specimens for each species were analyzed. The two *T. philippinarum* specimens showed identical sequences, while a single base-pair insertion/deletion distinguishes the two *V. verrucosa.* The largest difference (five transitions) was observed in the case of *T. decussatus.*

Phenetic analysis of the sequence data for each pair of taxa is presented in Table 1. In general, the number of pairwise substitutions is rather high, ranging from approximately 15% (between *V. aurea* and *T. philippinarum*) to 42.% (between *D. lupinus* and *C. gigas* and between *C. gigas* and *T. decussatus*). An exception is

Table 1. Pairwise distance-matrix for the 16s ribosomal RNA gene fragment. Below diagonal, percent difference values; above diagonal, transition/transversion ratio (abbreviations as in Fig. 1)

	C.ch	C.ga	C.gi	D.lu	P.ru	T.d19	T.d50	T.ph	V.au	V.v20	V.v21
C.ch	0	1.2	0.6	1.4	1.3	1.4	1.4	1.3	1.6	1.3	1.3
C.ga	28.6	0	0.7	1.2	1.2	1.3	1.3	1.4	1.2	1.6	1.6
C.gi	39.4	38.9	0	1.0	0.7	1.0	1.0	0.9	1.0	0.8	0.8
D.lu	30.5	18.7	42.6	0	1.3	1.5	1.3	1.2	1.5	1.3	1.3
P.ru	23.2	30.0	41.2	29.7	0	1.2	1.2	1.2	1.4	1.3	1.2
T.d19	35.3	22.5	42.4	24.6	33.3	0	–[a]	2.3	2.7	1.3	1.3
T.d50	35.3	22.0	41.5	24.3	32.5	1.3	0	2.3	2.9	1.3	1.3
T.ph	33.0	21.6	41.4	22.5	33.3	20.2	20.2	0	1.7	2.0	1.8
V.au	32.3	20.8	38.3	23.3	32.8	16.4	15.8	15.3	0	1.3	1.2
V.v20	26.7	6.3	37.6	18.7	28.5	21.9	21.3	19.6	20.2	0	–[b]
V.v21	26.5	6.3	37.4	18.7	28.3	21.7	21.1	19.4	19.9	0.0	0

[a] 5 transitions/0 transversions
[b] 0 transitions/0 transversions

represented by the genera *Chamelea* (Chioninae) and *Venus* (Venerinae), which show 6.3% base substitutions only. In contrast, the two species of the genus *Tapes* considered in this study (*T. decussatus* and *T. philippinarum*) show 20% sequence divergence, which is higher that between *V. aurea* and *T. decussatus* (approximately 16%) and *V. aurea* and *T. philippinarum* (approximately 15%). It may be worth mentioning that *T. decussatus* is a northeast Atlantic/Mediterranean species while *T. philippinarum* is a northwest Pacific species, each of which had been geographically isolated for a very long time before the latter was introduced into the Mediterranean recently. We have excluded from our analysis the central region of the examined sequences, because it appears very variable. However, very similar results were obtained when also including this region in the analysis.

In general, mitochondrial sequences from metazoans show a transition bias (Brown et al. 1982; Rumbak et al. 1994). A ratio of transitions to transversions of about ten indicates that the sequences are far from saturation. Once transitions have become saturated, the transversions continue to accumulate approximately linearly with time (Miyamoto and Boyle 1989). The data in Table 1 (above diagonal) exhibit transition/transversion rates very close to one, sometimes even lower than one, indicating that we are deep within the saturation zone.

According to Keen (1969), the Veneridae family probably is polyphyletic in origin; subfamily divisions do not necessarily reflect genetic relationships, but are adopted for convenience in arrangement. More recent studies, on the other hand, indicate that Veneridae have a monophyletic origin but are by far more deeply divided than shown by the morphological analyses (Harte 1992).

All the taxa considered in the present investigation show a similar (37–42%) divergence from *C. gigas.* Sequence divergence detectable within the Veneridae is constantly lower, though in the case of *T. decussatus* and *C. chione* (35% sequence divergence), not much lower. The even distance of all taxa from *C. gigas* may be attributable to transition saturation.

A phylogenetic tree calculated using neighbor-joining is shown in Fig. 2. We can observe that *D. lupinus* is basal to the Tapetinae. This early dicotomy, however, is only weakly supported (bootstrap value = 54). A maximum parsimony (branch and bound) tree (Swofford 1993) shows the same topology. If different methods of parsimony tree construction (e.g., heuristic search) are used, the dicotomy collapses to polytomy. All the trees are also consistent with the inclusion of *T. decussatus, T. philippinarum,* and *V. aurea* into one (Tapetinae) subfamily. Neither tree supports the concept of a genus *Tapes* as, in both cases, *V. aurea* clusters with *T. decussatus.* In contrast to the previous results, transversion analysis using neighbor-joining presents the phylogeny of the venerid subfamilies as essentially polytomic (Fig. 3). This tree confirms that the concept of a genus *Tapes* has no phylogenetic basis. In conclusion, our data strongly support the idea (Insua and Thiriot-Quiévreux 1992) that the Tapetinae form a heterogeneous group and *T. decussatus* and *T. philippinarum* are probably much more genetically distant than assumed so far on the basis of morphology.

Early members of the superfamily Veneracea are found in the Lower Cretaceous, i.e., they are about 100–135 million years (Myr) old (Keen 1969). The fossil record, on the other hand, indicates a broad range of ages for the different genera considered in the present study (Table 2), with the oldest taxon represented by *Callista,* the youngest by *Venerupis,* while *Chamelea* and *Venus* are of intermediate age.

The transversion divergence rate for mitochondrial ribosomal genes of ungulates and cetaceans has been computed to be about 0.14% per Myr (Allard et al. 1992; Milinkowitch et al. 1993). If we apply this rate to the separation *C. gallina/P. rudis,* we obtain a figure (100 Myr) which fits very well with the indication of a Lower Cretacean origin of the Veneracea. However, previous reports have shown that animal mitochondrial DNA (mtDNA) can display variable rates of sequence evolution among taxa (Rand 1994). Different evolutionary

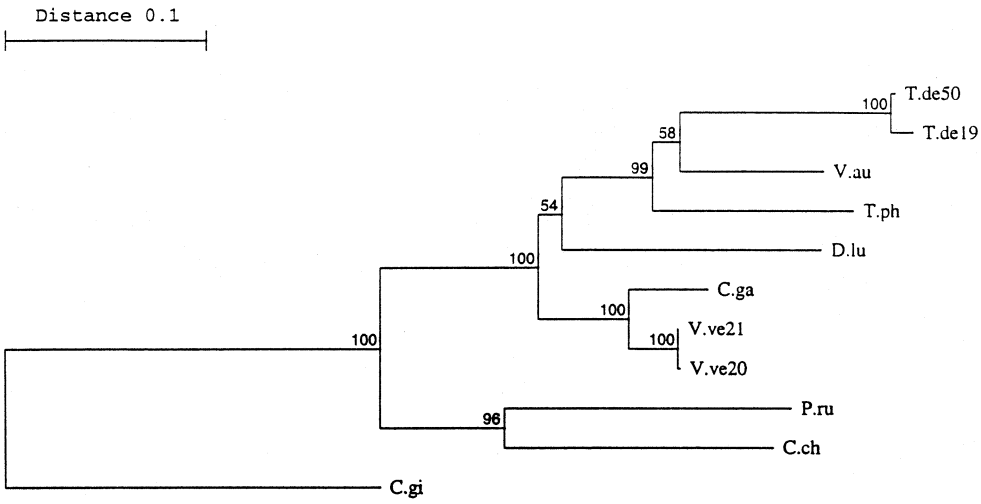

Fig. 2. Neighbor-joining (Saitou and Nei 1987) tree constructed with pairwise distances calculated following the application of Kimura's (1980) two-parameter correction for multiple substitutions. The tree was produced using TREECON (Van de Peer and De Wachter 1993). The *numbers* represent the percentage of 100 bootstrap replications in which a given node appeared. *Abbreviations* as in Fig. 1.

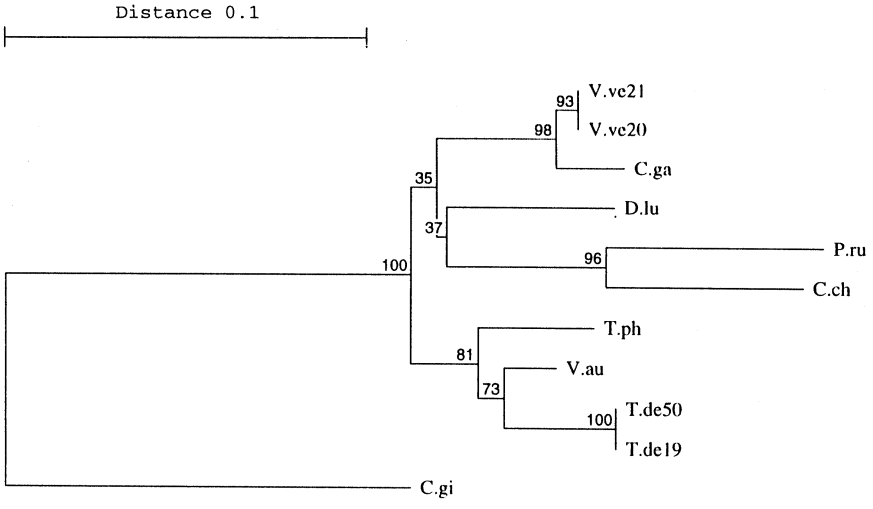

Fig. 3. Neighbor-joining (Saitou and Nei 1987) tree calculated on the basis of the sole transversions. The tree was produced using TREECON (Van de Peer and De Wachter 1993). The *numbers* represent the percentage of 100 bootstrap replications in which a given node appeared. *Abbreviations* as in Fig. 1.

Table 2. The fossil record of the venerid species considered in the present study

Subfamily	Species	Oldest fossil record[a]
Chioninae	*C. gallina*	Oligocene (25–40 Myr ago)
Dosiniinae	*D. lupinus*	Miocene (11–25 Myr ago)
Pitarinae	*Pitar* sp.	Eocene (40–60 Myr ago)
	C. chione	Paleocene (60–70 Myr ago)
Tapetinae	*Tapes* sp.	Miocene (11–25 Myr ago)
	T. decussatus	Miocene (11–25 Myr ago)
	Venerupis sp.	Pliocene (1–11 Myr ago)
Venerinae	*V. verrucosa*	Oligocene (25–40 Myr ago)

[a] According to Keen (1969)

rates of ''molecular clocks'' are also found in the nuclear genes for ribosomal RNA of several Bivalvia (Rice et al. 1993). The transversion divergence rate computed for *Callista* and *Pitar* (0.16% per Myr) on the basis of the age of the first fossil record is not much different from that of ungulates and cetaceans. A remarkably similar transversion divergence rate (0.14% per Myr) is also found if we consider the *V. verrucosa/C. gallina* separation. These figures, however, double if we consider *T. decussatus/V. aurea* and *T. decussatus/T. philippinarum* (0.31% and 0.36% per Myr, respectively).

References

Allard MW, Miyamoto MM, Jarecki L, Kraus F, Tennant MR (1992) DNA systematics and evolution of the artiodactyl family Bovidae. Proc Natl Acad Sci USA 89:3972–3976

Borsa P, Thiriot-Quiévreux C (1990) Karyological and allozymic characterization of *Ruditapes philippinarum, R. aureus* and *R. decussatus* (Bivalvia: Veneridae). Aquaculture 90:209–227

Borsa P, Jousselin Y, Delay B (1992) Relationships between allozymic heterozygosity, body size, and survival to natural anoxic stress in the palourde *Ruditapes decussatus* (Bivalvia: Veneridae). J Exp Mar Biol Ecol 155:169–181

Brown WM, Prager EM, Wang A, Wilson AC (1982) Mitochondrial DNA sequences of primates: tempo and mode of evolution. J Mol Evol 18:225–239

Bucklin A, Frost BW, Kocher TD (1995) Molecular systematics of six *Calanus* and three *Metridia* species (Calanoida: Copepoda). Mar Biol 121:655–664

Canapa A, Nisi Cerioni P, Olmo E, Sabelli B, Capriglione T (1993) Composizione del genoma e DNA altamente ripetuti in *Chamelea gallina.* Atti del 55°congresso U.Z.I.Torino 27 settembre 1993:275

Cunningham CW, Blackstone NW, Buss LW (1992) Evolution of king crabs from hermit crab ancestors. Nature 355:539–542

Fischer-Piette E, Vukadinovic D (1977) Suite des révisions des Veneridae (Moll. Lamellibr.) *Chioninae, Samaranginae* et complément aux *Vénus.* Mém Mus Nat Hist Nat, Paris, Série A, 106:1–182

Geller JB, Carlton JT, Powers DA (1993) Interspecific and intrapopulation variation in mitochondrial ribosomal DNA sequences of *Mytilus spp.* (Bivalvia: Mollusca) Mol Mar Biol Biotechnol 2:44–50

Harte ME (1992) A new approach to the study of bivalve evolution. Am Malac Bull 9:199–206

Insua A, Thiriot-Quiévreux C (1992) Karyotypes of *Cerastoderma edule, Venerupis pullastra* and *Venerupis rhomboides* (Bivalvia, Veneroida). Aquat Living Resour 5:1–8

Keen AM (1969) Veneridae. In: Moore RC (ed) Treatise of invertebrate paleontology. Geological Society of America and University of Kansas Press Lawrence, pp N671–N688

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Milinkovitch MC, Orit G, Meyer A (1993) Revised phylogeny of whales suggested by mitochondrial ribosomal DNA sequences. Nature 361:346–348

Miyamoto MM, Boyle SM (1989) The potential importance of mitochondrial DNA sequence data to eutherian mammal phylogeny. In: Fernholm B, Bremer K, Brundin L, Jornvall H (eds) The hierarchy of life. Elsevier, New York, pp 437–450

Palumbi S, Martin A, Romano S, Mc Millan WO, Stice L, Grabowski G (1991) The simple fool's guide to PCR (ver. 2) (unpublished manuscript)

Passamonti M, Mantovani B, Scali V (1994) Struttura genetica e rapporti filetici di alcune specie di *Tapes e Paphia* (Molluschi, Bivalvi). Atti del Convegno congiunto A.G.I. 26-30 September 1994, Montesilvano, (PE) p 484

Rand DM (1994) Thermal habit, metabolic rate and the evolution of mitochondrial DNA. TREE 9:125–130

Rice EL, Roddick D, Singh RK (1993) A comparison of molluscan (Bivalvia) phylogenies based on palaeontological and molecular data. Mol Mar Biol Biotechnol 2:137–146

Rumbak E, Reid DG, Thomas RH (1994) Reconstruction of phylogeny of 11 species of *Littorina* (Gastropoda: Littorinidae) using mitochondrial DNA sequence data. Nautilus (Suppl) 2:91–97

Saitou N, Nei M (1987) The Neighbor-Joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Swofford DL (1993) PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1. Laboratory of Molecular Systematics, Smithsonian Institute, Washington

Van de Peer Y, De Wachter R (1993) TREECON: a software package for the construction and drawing of evolutionary trees. Comput Appl Biosci 9:177–182

Wilson AC, Cann RL, Carr SM, George M, Gyllesten UB, Helm-Bychowski KM, Higuchi RG, Palumbi SR, Prager EM, Sage RD, Stoneking M (1985) Mitochondrial DNA and two perspectives on evolutionary genetics. Biol J Linn Soc 26:375–400