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DNA sequence evidence for speciation, paraphyly and a Mesozoic dispersal of cancellothyridid articulate brachiopods.

C. LÜTER* AND B. L. COHEN

University of Glasgow

Institute of Biomedical and Life Sciences

Division of Molecular Genetics

Pontecorvo Building

56 Dumbarton Road

Glasgow, G11 6NU

Scotland, U. K.

Corresponding author:

B. L. Cohen

e-mail: b.l.cohen@bio.gla.ac.uk

Fax: +44 141 330 4878

* Present address:
Museum für Naturkunde der Humboldt-Universität zu Berlin
Institüt für Systematische Zoologie
Invalidenstrasse 43
D-10115 Berlin
Germany

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Abstract.

Because the classification of extant and fossil articulate brachiopods is based largely upon shell characters observable in fossils, it identifies morphotaxa whose biological status can, in practice, best be inferred from estimates of genetic divergence. Allozyme polymorphism and restriction fragment length polymorphism of mitochondrial DNA (mtDNA RFLP) have been used to show that nuclear and mitochondrial genetic divergence between samples of the cancellothyridid brachiopods *Terebratulina septentrionalis* from Canada and *T. retusa* from Europe is compatible with biological speciation, but the genetic distances obtained were biased by methodological limitations. Here, we report estimates of divergence in 12S rDNA mitochondrial sequences within and between samples of these brachiopods. The sequence-based genetic distance between these samples (5.98 ± s.e.m. 0.07%) is at least ten times greater than within them and, since they also differ in a complex life-history trait, their species status is considered to be securely established.

Divergence levels between 12S rDNA genes of three other cancellothyridids, *Terebratulina unguicula* from Alaska, *T. crossei* from near Japan, and *Cancellothyris hedleyi* from near Australia are higher than between the two North Atlantic species, and the mean nucleotide distance between all these cancellothyrids is similar to the mean distance between species of *Littorina* (Mollusca: Gastropoda). Sequences of both 12S and 16S genes from cancellothyridids and other short-looped brachiopod species show neither saturation nor lineage-specific rate differences and, when analysed with different outgroups, either separately or together, yield one unexpected, but well-supported, tree with Alaskan *T. unguicula* basal and *C. hedleyi* nested within *Terebratulina*, i.e. these genera are paraphyletic. A geologically dated divergence between Antarctic and New Zealand species of the short-looped brachiopod *Liothyrella* is used to calibrate the rate of 12S evolution at ca. 0.1% per MY, and this rate is used to infer that *T. septentrionalis* and *T. retusa* have been diverging for ca. 60 MY and that they and *T. unguicula* have been diverging from their last common ancestor for ca. 100 MY. This indicates a Mesozoic origin for the present-day distribution of cancellothyridids and the basal position of *T. unguicula* suggests a possible North Pacific centre of origin, with separate Atlantic and Pacific radiations. The inclusion of *Cancellothyris* within *Terebratulina* also shows that adult shell characters such as umbo, foramen and symphytium shape, whilst probably indispensible for the practical classification of fossils, are not reliable guides to genealogy.

Introduction

Brachiopods attributed to the family Cancellothyrididae first appeared in the Jurassic and became abundant in the Cretaceous and Tertiary. Cancellothyridid genera recognized in Recent seas include *Agulhasia*, *Cancellothyris*, *Chlidonophora*, *Cnismatocentrum*, *Eucalathis*, *Murravia* and *Terebratulina* (Cooper 1973). Of these genera, only *Terebratulina* is both widely distributed and relatively common, on hard substrates from ~5m down to at least the continental shelf-break. Some members of this family fall into a well-supported molecular clade (Cohen et al. 1998a; Cohen et al. 1998c; Cohen 2001).

The reproductive biology of articulate brachiopods appears to favour speciation: larvae are typically lecithotrophic and short-lived, and larval brooding is not uncommon; larvae are generally rare in near-shore plankton samples and, with one exception, virtually unknown in oceanic plankton (Peck and Robinson 1994; Stanwell-Smith et al. 1999). Thus, short-range dispersal is thought to predominate. In many articulate brachiopods including *Terebratulina*, low dispersal potential is apparent: populations are generally disjunct, young animals commonly settle on the shells of older ones and in localities with restricted water exchange, such as fjords, local populations may be very dense, reaching thousands of individuals per square metre. Moreover, regional endemicity is common (Richardson 1997). It therefore appears that the distribution of globally widespread genera such as *Terebratulina* reflects a long history of dispersal involving some combination of slow, current-driven, predominantly coastwise spread along continental shelves, conditioned by plate tectonic and other complex earth-history, vicariance and marine environmental processes, leading to isolation by distance and speciation. However, since brachiopod classification is necessarily based on shell characters that can be determined in fossils, and these characters are rarely clear-cut, designated brachiopod species may not always represent reproductively isolated populations.

There have been few reports of genetic divergence assays within and between brachiopod population samples. In the first such study, no differentiation was detected by allozyme analysis between samples of the inarticulate brachiopod *Lingula* from three widely separated sites around Northern Australia (Hammond and Poiner 1984), and a similar result has been reported for northern Pacific samples (Kusumi et al. 1994). More recently, however, re-analysis of the allozyme data and DNA sequence-based analyses revealed substantial differentiation, amounting to cryptic speciation, between some northern Pacific populations of *Lingula* (Endo et al. 2001). However, these brachiopods have long-lived, pelagic juveniles, unlike articulate brachiopods, which typically lack a pelagic, planktotrphic stage.

In articulates, genetic differentiation between morphospecies has been studied only in *Terebratulina septentrionalis* from the Atlantic coast of Canada and *T. retusa* from western Europe. Morphologically, these forms were separable only by principal components analysis of the shell rib ornament, but allozyme and mitochondrial DNA restriction fragment length polymorphism (mtDNA RFLP) analyses found deep divergence and strongly suggested that *T. septentrionalis* and *T. retusa* do, indeed, belong to genetically isolated populations so divergent as to justify biological species status. Around western Europe, however, no

significant differentiation was found amongst SCUBA-collected samples of *T. retusa* (Cohen et al. 1991a; Cohen et al. 1993). In these studies the quantities of tissue homogenate and mtDNA available from individual animals were limited, and this made it necessary to preselect informative allozyme loci and restriction enzymes, leading to some overestimation of divergence values. Thus, whilst comparisons within the studies were valid, the results could not be compared with those obtained from other organisms.

Here, we revisit this problem and use polymerase chain reaction (PCR)-based sequence analyses of segments of the mitochondrial 12S rDNA gene to obtain more broadly comparable estimates of divergence within and between population samples of *Terebratulina retusa* and *T. septentrionalis*. These analyses are based in part on the same individual animal DNAs used in the earlier, RFLP analyses, supplemented by additional specimens from the same or similar samples. The new results confirm that divergence between these morphospecies is at least ten times greater than divergence within them and therefore support genetic isolation and biological speciation.

Since the relevant mitochondrial rDNA sequences show neither saturation nor lineage-specific rate differences, we also use data from a brachiopod species pair (*Liothyrella* spp.) and of brachiopod genera separated by a geologically datable vicariance event (isolation of the continental shelves of New Zealand and Antarctica), to approximately calibrate the rate of molecular evolution, and we use this to infer the probable divergence times of *Terebratulina* spp. These results are compared with the predictions of three phylogeographic hypotheses, leading to the conclusion that the root causes of the present-day diversity pattern of cancellothyridids probably lies in the biogeography of the Mesozoic era. By comparison with mammals and a crab, brachiopod 12S gene sequences appear to evolve slowly, but by comparison with *Littorina* spp., speciation is associated with similar levels of molecular divergence (Rumbak et al. 1994; Reid et al. 1996).

In addition to using 12S mitochondrial rDNA sequences to quantify divergence within and between Atlantic *Terebratulina*, we also use concatenated 12S and 16S rDNA sequences to infer the genealogical relationships of some Atlantic and Pacific cancellothyridids. The results are unexpected: not only does Alaskan *T. unguicula* branch basally, but there is paraphyly; the morphologically and geographically distinct, South Pacific species *Cancellothyris hedleyi* clusters amongst *Terebratulina* spp. in a well-supported clade containing Atlantic and Pacific subclades. These results suggest how and when the present-day distribution of cancellothyridids came about and imply that some taxonomically important brachiopod adult shell characters do not accurately reflect genealogy.

Materials and methods

Details of specimens and sequences are given in Table 1. DNAs re-used from earlier work (Cohen et al. 1991b; Cohen et al. 1991a) were selected for volume and amplifiability, not for RFLP haplotype. DNAs were

extracted and templates amplified by PCR and prepared for automated sequencing as described (Cohen et al. 1998a; Cohen 2000). New sequences were obtained with 'universal' primers 12F1091, 12R1478, 16F2510 and 16R3080 (Kocher et al. 1989; Palumbi et al. 1989), which amplify respectively about 400 base-pairs (bp) of mitochondrial small subunit (12S) ribosomal DNA, and about 600 bp of the mitochondrial large subunit (16S) rDNA. Unambiguous sequences were read from both strands of these templates, generally with at least three-fold redundancy and, after discarding ragged ends and adding alignment gaps, 336 12S and 498 16S sites were available for comparison.

The starting-point for the present work was an earlier 12S alignment (EMBL DS32096, Cohen et al. 1998c) To this were added a sequence from *Terebratulina crossei* and 19 more sequences from North Atlantic *Terebratulina* spp., whilst sequences of all other taxa except the short-looped brachiopods *Liothyrella* spp. were removed. These data were aligned without ambiguity by inspection, using a small number of single alignment gaps (at one point two gaps for some taxa). New 16S sequences were obtained from 9 individual cancellothyridids, and these too were readily alignable one with another, with one region of possible ambiguity involving 6 nucleotides. Each 16S sequence was then concatenated with the 12S sequence from the same specimen, the boundary being marked by an added 'N' site. Also included were the corresponding 12S and 16S portions of the complete mtDNA genomes of a *T. retusa* from Sweden (Stechmann and Schlegel 1999) and of two outgroup brachiopods, *Laqueus rubellus* (Noguchi et al. 2000) and *Terebratalia transversa* (Helfenbein 2001). These two divergent outgroup sequences created 167 sites of potentially ambiguous alignment which were excluded from analyses involving the outgroups. Site exclusions were implemented as PAUP exclusion sets (Swofford 2000); and the working alignment, together with exclusion details, has been deposited in the EMBLALIGN database (Cohen et al. 1998b; Stoesser et al. 1998), accession number ALIGN_00188. This file is extensively annotated and a NEXUS file comparable to that used for the analyses reported here should be readily reconstructable by simple text-editing (Maddison et al. 1997; Cohen et al. 1998b). Individual sequence files and voucher shells have also been deposited (Table 1). Parsimony and distance-based phylogenetic analyses were performed with PAUP*4b8 and maximum likelihood analyses with PUZZLE 5.0 (Strimmer and von Haeseler 1996) as described (Figure 1, legend). Relative rate tests were performed with RRTree (Robinson et al. 1998) and statistical analyses with MINITAB 10 (Minitab Inc., State College, PA, USA).

table 1 about here

Results

Within- and between-species divergence in *Terebratulina retusa* and *T. septentrionalis*: comparison of RFLP and sequence results.

The earlier allozyme and RFLP-based analyses used samples collected from single sites in the Bay of Fundy, Canada (*T. septentrionalis*) and the Firth of Lorne, Scotland (*T. retusa*) respectively (Cohen et al. 1991b;

Cohen et al. 1993). The Canadian sample was less variable than the Scottish one (6 mitochondrial haplotypes in 30 individuals *versus* 36 haplotypes in 41 individuals) and the two samples shared no haplotype (0/42). In the allozyme analysis, only 9 of 31 electromorphs were shared. Moreover, there were clear differences in allelic composition: e.g. hexokinase in the Canadian sample was fixed for an allele not recognized in Scottish (or other European) samples (Cohen et al. 1993), yielding an estimated allozyme distance of 0.318. Mitochondrial nucleotide difference estimated by a procedure appropriate to mapped restriction site data (some of the sites were mapped) was 28.9 ± 6.9% (Cohen et al. 1991b), but a later, unpublished calculation using a procedure appropriate for unmapped sites (Miller 1991) gave a much lower distance (12.1± 6.1%) with the same data. RFLP nucleotide divergence within Scottish and other European population samples, like most between-population divergence estimates, did not differ significantly from zero, i.e. geographic differentiation was not detected from Scandinavia to the Mediterranean (Cohen et al. 1993, Tables 2 and 3).

In other previous work, analyses of an alignment of 12S rDNAs from a wide taxonomic range of short-looped brachiopods including cancellothyridids gave evidence of strong phylogenetic signal and stationary base composition (Cohen et al. 1998c). In the new 12S alignment described here, phylogenetic signal was high (g1 from 10,000 random trees and 80 informative characters = -0.86, $P < 0.01$, Hillis and Huelsenbeck 1992) and base composition again showed no heterogeneity ($P = 1.00$, 84 d. f.). Since ingroup distances were not large and saturation wasabsent, i.e. scatter-plots of the numbers of pairwise transitions *versus* transversions or of transitions *versus* '*p*' distances were close to linear (not shown), a simple distance correction (Kimura 1980) was adopted for divergence measurement (Table 2).

table 2 about here

For the present analyses the *Terebratulina septentrionalis* were collected from two Canadian sites, Bay of Fundy and Bonne Bay, separated by ca. 600 miles (~1000 km) but the sequences were all identical (except for one ambiguous nucleotide base in one individual). Eight of the *T. retusa* specimens came from two sites in Scotland, Firth of Lorne (with Loch Fyne) and Loch Duich, separated by about 60 miles (100 km) and chosen because the RFLP data had suggested slight haplotype differentiation, although significant divergence was not found (Cohen et al. 1993, Tables 2 and 3). Corresponding 12S nucleotide distances (%) are: within Lorne 3.75 ± s. e. m. 0.78 (N = 10); within Duich 4.54 ± s. e. m. 2.12 (N = 3); between Lorne and Duich 3.02 ± s. e. m. 0.757 (N = 19). The 12S sequence of the *T. retusa* specimen from Sweden was identical to that of a Lorne animal, consistent with previous RFLP findings for a Norwegian sample (Cohen et al. 1993). Thus, the sequence data agree with the earlier RFLP results in finding no significant divergence between Scottish (and other European) localities. Sequence and RFLP data also agree in finding much less variation in the Canadian 12S sequences than in the Scottish ones (1 haplotype in ten individuals *versus* 7 haplotypes in 9 individuals). From the data in table 2, mean 12S Kimura distance between the Canadian and Scottish sequences (5.98%) was about ten times greater than within the Scottish (0.61%) but about one fifth of the values previously estimated by the RFLP 'mapped sites' method and about one half of the RFLP 'unmapped sites' distance. Divergence between *Terebratulina septentrionalis* and *T. retusa* concatenated 12S and 16S

sequences was about 10% greater than divergence between 12S sequences alone (not shown). This reflects the faster evolution-rate of the 16S gene (Hillis and Dixon 1991).

Estimation of cancellothyridid phylogeny and divergence time

Preliminary estimates of phylogeny were made with 12S sequences alone, using short-looped non-cancellothyridid *Liothyrella* as outgroup. In these analyses (not shown) *Cancellothyris hedleyi*, from deep water near Australia, was expected to be genetically furthest from the sampled *Terebratulina* spp., but it unexpectedly clustered within the *Terebratulina* clade and *T. unguicula* from Alaska was instead the unexpectedly basal taxon. Therefore, to increase the available data and to test for incongruence with the 12S result, 16S sequences were obtained. However, both sets of data gave the same topology when analysed separately or together, and they were not statistically heterogeneous (partition heterogeneity test, 100 branch and bound replicates, 10 taxa, $P = 0.49$).

In case the unexpected topology was a rooting artefact it was further tested by the use of outgroup sequences from two, more distant, long-looped articulate brachiopods. Inclusion of these sequences is not ideal however, because base composition of the mtDNA genome in the long-looped clade differs from that in short-looped brachiopods including cancellothyridids (Stechmann and Schlegel 1999; Noguchi et al. 2000; Saito et al. 2000; Helfenbein 2001). In agreement, we found that amongst the short-looped (i.e. cancellothyridid plus *Liothyrella*) 12S or 16S sequences, pairwise base frequencies did not differ significantly (heterogeneity chi-squared tests, $P = 0.67 - 0.99$), but base frequencies did differ significantly between any pair of short-looped ingroup and long-looped outgroup taxa ($P \leq 0.01$). Nevertheless, interspersed, conserved motifs made it possible to align the sequences with the addition of a moderate number of gaps. Secondary structure information (which might have been problematical because of the base composition differences) was not used. Between the conserved motifs positional homology was sometimes uncertain because of alignment gap position ambiguity and/or base composition divergence and 167 alignment-ambiguous sites were therefore excluded from analysis when the long-looped outgroups were used, leaving 667 characters of which 409 were constant and 147 parsimony-informative. Exclusion of the alignment-ambiguous sites reduced base composition differences between long- and short-looped taxa. For example, before exclusion 12S+16S from *Laqueus* and *Cancellothyris* were very significantly different (pairwise chi-squared test for heterogeneity, $P = 0.000023$, d. f. = 3), but after exclusion the difference, though still significant, was much smaller ($P = 0.010$). Thus, divergence in base composition is concentrated in the more variable, less readily aligned, regions; the more conserved motifs that facilitate alignment naturally differ less in base composition. After excluding the potentially misaligned sites strong phylogenetic structure was present ($g_1 = -2.26$, $P < 0.01$, 10,000 random trees, Hillis and Huelsenbeck 1992) and partition heterogeneity was absent ($P = 0.49$, 100 branch and bound replicates). Saturation was not detected (not shown) and relative rate tests confirmed the absence of rate differences between lineages (pairwise exact probabilities for four ingroup lineages 0.21 - 0.66, long-looped outgroup), indicating that a molecular clock approach to divergence time inference was worth exploring. The topology of relationships inferred from the 12S + 16S alignment rooted with long-looped brachiopods (Figure 1) confirmed the unexpected findings from 12S alone: *T. unguicula* is basal and *C. hedleyi* nests

within the *Terebratulina* clade. Furthermore, neither inclusion of the potentially misaligned sites nor exclusion of the long-looped outgroups altered the topology of maximum parsimony exhaustive search or LogDet minimum evolution ingroup trees (not shown). Thus, the base composition differences and site exclusions were phylogenetically unimportant. Note that the inferred relationship of *T. crossei* with *C. hedleyi* is provisional (Figure 1, legend).

figure 1 about here

Subsequent to the analyses so far described, further evidence was obtained that supports the observed paraphyly. First, *C. hedleyi* D1150 was one of three specimens collected together and because the shell of this animal was not readily available for re-examination, having been previously deposited as a taxonomic voucher (Cohen et al. 1998a), the shell of a second animal from the same collection (Glasgow accession D1149) was examined. Its morphology closely matched illustrations of *C. hedleyi* (Moore 1965, Figure 684). Second, DNA was extracted and 12S and 16S sequences were obtained from the third specimen of *C. hedleyi* (D1423), the taxonomic identity of which had earlier been independently confirmed. These sequences differed from those of D1150 by one single-base insertion, three transitions and one probable transversion and they clustered with D1150 (Figure 1).

Discussion

RFLP and sequence-based divergence estimates

Because RFLP analyses sample sites spread over the entire organelle genome they are expected to give a broadly reliable view of mitochondrial genetic divergence, especially when performed with mapped sites in purified mtDNA. However, the original analyses (Cohen et al. 1991b), necessarily performed by Southern blotting, were unable to detect fragments smaller than ca. 500 base pairs, used few mapped restriction sites, and were complicated by size variation in the Canadian mtDNAs, bias due to preselection of informative restriction enzymes, and uncertainty over the most appropriate method of distance calculation. By contrast, the gene sequences reported here give reliable divergence estimates for the analysed regions but yield no overall picture. However, since 12S and 16S rRNA genes are amongst the least variable mtDNA regions (Jacobs et al. 1988; Hillis and Dixon 1991), they are likely to underestimate overall divergence, suggesting that the RFLP 'unmapped sites' distance (12.1 ± 6.1%) may have been a reasonable overall estimate. The results reported here compare well with divergence estimates in the same 12S segment of *Littorina*, a molluscan genus with a similar geographical distribution and in which at least some species also have low dispersal potential (Reid et al. 1996). For *Terebratulina* (including *Cancellothyris*), the mean between-species 12S rDNA divergence is 9.1 ± 0.5% (from Table 2) whilst the equivalent divergence for eleven species of *Littorina* is 7.3 ± 0.4% (Rumbak et al. 1994, Table 2). Thus, by mitochondrial 12S sequence comparison, speciation in *Terebratulina* and *Littorina* is associated with similar levels of divergence.

Systematics of North Atlantic *Terebratulina*

The long-standing morphology-based taxonomic separation of *Terebratulina retusa* and *T. septentrionalis* (Davidson 1886-1888; Wesenberg-Lund 1941) is now unambiguously supported by both nuclear (allozyme) and mitochondrial genetic evidence of substantial divergence (this paper and Cohen et al. 1991a). In addition to these molecular differences and a difference in the spatial density of shell ribs, the species also differ in a complex reproductive trait: *T. septentrionalis* larvae are brooded before release (Webb et al. 1976), whereas brooding has not been reported in *T. retusa*. These data comprehensively refute the suggestion that these species are synonymous (Emig 1990). Whilst it is likely that today, western Atlantic populations are exclusively referable to *T. septentrionali*s, it is less certain that eastern Atlantic ones are exclusively *T. retusa*. It has been suggested that a morphological cline (or at least intermediate forms) may exist between Greenland and near-Arctic Europe (Wesenberg-Lund 1938; Wesenberg-Lund 1940b; Wesenberg-Lund 1940a; Wesenberg-Lund 1941), though this has been discounted on indirect morphometric grounds (Curry and Endo 1991). The latter authors have also reported that morphometrically unambiguous *T. septentrionali*s occurs in Scandinavian waters, but this suggestion needs to be tested by molecular analysis; until that is done it cannot be discounted that such specimens are morphological outliers of *T. retusa* rather than members of a relict *T. septentrionalis* population, as suggested. If genetically identified Scandinavian *T. septentrionalis* could be confirmed to coexist with *T. retusa*, it would be evidence of reproductive isolation between sympatric forms, and hence additional evidence of biological speciation.

Rate of molecular evolution and phylogeography of Atlantic and Pacific cancellothyridids

Since nucleotide substitution in the mtDNA regions studied appears to occur without saturation, and relative rates of change do not differ significantly between the lineages examined, it is reasonable to assume that genetic distances between lineages grow in rough proportion to time since isolation, i.e. a local, approximate molecular clock obtains. No directly relevant node currently exists for which geological dates and genetic distance co-exist, but clock-rate can be indirectly calibrated and the calibration roughly checked as follows:

Palaeontological evidence (Bitner 1996) shows that shortly after the Cretaceous there was considerable faunal overlap between Antarctica (Seymour Island) and New Zealand; in the Eocene, ca. 50 MYa, nine brachiopod genera were shared. However, geophysical evidence indicates that by 70 - 90 MYa tectonic movements were separating New Zealand from Antarctica (Stevens 1989; Sutherland 1999 and D. E. Lee and R. Sutherland, personal communications, 2000), whilst a cooling event at the Eocene/Oligocene boundary (ca. 36.5 MYa) reduced the Antarctic brachiopod fauna. Furthermore, in the Miocene, a circum-polar current effectively isolated the two provinces (Barker 2001). Thus, it is reasonable to suppose that isolation by distance allowed genetic divergence between conspecific Antarctic and New Zealand brachiopods to start ca. 70 - 90 MYa and that movement-apart, the cooling event and the circumpolar current later caused the separation to develop into an absolute barrier to larval exchange. Thus, *Liothyrella*, the principal brachiopod genus plentiful today in both Antarctic and New Zealand waters can be used to roughly calibrate the rate of genetic divergence.

Mean distance between New Zealand and Antarctic *Liothyrella* 12S genes is 7.14 ± 0.52% (from Table 2), indicating that divergence between them accumulated at approximately 0.1% per million years. This estimate might be too low if New Zealand waters were in fact colonised later than 70 - 90 MYa, for example by high-dispersal larvae like those recently recognized in *L. uva* (Peck and Robinson 1994) and it would rise to ca. 0.35% per MY if the onset of *Liothyrella* divergence was delayed until establishment of the circumpolar current. However, these factors will be discounted because long-lived larvae of *L. neozelanica* are not known, New Zealand *Liothyrella* differ morphologically from Antarctic ones, and long-term isolation is established by the genus-level endemicity of the New Zealand brachiopod fauna (Dawson 1971; Foster 1974; Dawson 1990).

The estimated *Liothyrella* divergence-rate appears slow when compared with some other organisms. For example, in mammalian mtDNA genomes the entire 12S gene shows roughly 0.7% divergence per MY between sister taxa (Pesole et al. 1999), i.e. about seven times faster than the portion of this gene sequenced in *Liothyrella*. Similarly, the Jamaican land crab 16S gene (Schubart et al. 1998) diverges about five times faster than *Liothyrella* 16S (calculated on the assumption that the cancellothyridid differential applies, i.e. 16S rates are ~1.4 times 12S rates). Thus, both comparisons indicate that the brachiopod sequences described here may evolve slowly, in keeping with the unusually deep phylogenetic resolution obtained from brachiopod 12S sequences (Cohen et al. 1998c).

Indirect support for the 12S rate calibration and Antarctic-New Zealand divergence time inferred above has been obtained as follows: cytochrome oxidase I (*cox*1) mtDNA sequences have been reported from a wide range of long-looped brachiopods (Saito 1998; Saito et al. 2000; Saito and Endo 2001; Saito et al. 2001) including *Magellania fragilis* and *M. joubini* from the Weddell Sea and representatives of the New Zealand forms *Calloria inconspicua*, *Gyrothyri mawsonis* and *Terebratella sanguinea*, two of which are endemic. Reasonably well-constrained minimum divergence times inferred from the fossil record for other long-looped taxa gave a calibration curve usable up to at least 112 MYa (Saito and Endo 2001) and the divergence-time of the Antarctic and New Zealand taxa (based on 1095 nt common to them all) was obtained by interpolation (Lüter & Cohen, unpublished analyses). Estimates of nucleotide divergence between *Magellania* spp. and the New Zealand genera using different corrections for unseen substitutions, using all codon sites, or using second position transversions alone, give divergence-time estimates ranging widely around 70 MYa, whilst aminoacid sequence-based analyses placed divergence at 43 MYa. Mean pairwise aminoacid sequence divergence between the three New Zealand genera was 77% of that between them and *Magellania* spp. Thus, these analyses are broadly consistent with the biogeographical history described above and indirectly confirm the inferred *Liothyrella* 12S rate calibration.

Accepting the *Liothyrella* 12S rate of ca. 0.1% per MYas a guide, the divergence observed between *Terebratulina retusa* and *T. septentrionalis* (5.98%, Table 2) must have accumulated over ca. 60 MY, whilst the lineage leading to *T. unguicula* appears to have diverged from that leading to the two Atlantic species ca. 100 MYa (9.2% divergence, Table 2). These estimated divergence times may be compared with the predictions of three hypotheses for the historical origin of *Terebratulina* populations: (1) that like many

molluscs, North Atlantic populations originated by *trans*-Arctic migration from the North Pacific (Vermeij 1991), or (2) that the Recent North Atlantic distribution resulted from northward migration of warm-adapted *T. retusa* replacing cold-adapted *T. septentrionalis* during a relatively short Holocene period of changing climate (Curry and Endo 1991), or (3) that the Recent distribution in the Atlantic and Pacific is a relic of a world-wide Mesozoic circulation associated with a broad, mid-latitude Tethys (Cohen et al. 1998c) The first hypothesis predicts that divergence between *Terebratulina unguicula* and *T. septentrionalis* (and perhaps also *T. retusa*) should have been initiated no earlier than the time of opening of the Beringian passage, 4.8 - 5.5 MYa (Marincovitch and Gladenkov 1999), which is clearly incompatible with the ~100 My divergence implied by the molecular data. It is also incompatible with the low diversity of the sampled *T. septentrionalis* populations. The second hypothesis does not explicitly predict the time of divergence between *T. retusa* and *T. septentrionalis*, except perhaps to place it between the time when the North Atlantic opened and the Holocene. It is also incompatible with low divergence in *T. septentrionalis*. The third hypothesis predicts that divergence between the Atlantic and Pacific cancellothyridids analysed here must have occurred between the Bajocian (170 MYa, Smith et al. 1994) and closure of the Panama seaway (3.1 MYa, references in Schubart et al. 1998). Since this is compatible with the inferred divergence-time between *T. unguicula* and the Atlantic species (~100 MYa), we suggest that the global spread and divergence of ancestral cancellothyridids took place by coastwise spread during the Mesozoic existence of a broad, mid-latitude seaway (Smith et al. 1994) and that this was followed by separate Atlantic and Pacific radiations. The basal tree position of Alaskan *T. unguicula* suggests that the northern Pacific might have been the centre of origin, though a much wider geographic sample of cancellothyridids should be examined before this is accepted. This hypothesis is compatible with the observed low diversity amongst Canadian *T. septentrionalis* if the latter lies near the end of a north- and west-trending chain of stepping-stone colonisations in the North Atlantic and it predicts that fossils of this species will be absent from all except relatively recent North American marine deposits.

Implications of paraphyly amongst cancellothyridids

The evidence presented here clearly establishes that *Cancellothyris* clusters within the *Terebratulina* clade. These genera have been differentiated by adult shell characters including 'umbo short, massive, suberect; foramen large, entire, epithyridid; symphytium narrow' (in *Cancellothyris*) *versus* 'umbo suberect; foramen incomplete, mesothyridid to permesothyridid; deltidial plates disjunct' (in *Terebratulina*) (Moore 1965, pages H807-810). Thus, if the relationships implied by the gene tree reported here are truly genealogical, the genealogical value of the differentiating adult shell characters must be questioned. This conclusion is similar to one drawn from the analysis of mitochondrial *cox1* gene sequences amongst laqueoid brachiopod genera and species (Saito 1998; Saito et al. 2001). In the analysis of nuclear-encoded 18S rDNA gene trees however, the brachiopod shell was thought to be a generally reliable indicator of genealogy, although the emphasis here was at higher taxonomic levels (Cohen et al. 1998a).

Reduction in the taxonomic weight that may be placed on widely-used, traditional adult shell characters means that much brachiopod taxonomy at generic and species levels (at least) may not satisfy the aim that classifications should be genealogical (Darwin 1859) and highlights the conflict of interest between practical

classification (e.g. of a collection of fossils) and the theoretical underpinnings of systematics. Since fossil collections do not permit molecular analyses, and the ontogenetic approach may also be unavailable, practical brachiopod classification may continue to employ adult shell characters, but should come to be viewed in a new light.

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Table 1. Brachiopod specimens and sequences. Details of collector, etc. are given only for specimens first reported here.

classifi-cation[a]	binomial	Glasgow accession (identified by)[b]	GenBank (G) and Natural History Museum (N) accessions	collector (locality)	reference
L	*Laqueus rubellus*	-	G: AB035869	-	(Noguchi et al. 2000)
L	*Terebratalia transversa*	-	G: AF331161	-	(Helfenbein 2001)
C	*Cancellothyris hedleyi*	D1150	G: AF334234	-	(Cohen et al. 1998)
C	*C. hedleyi*	D1423 (CHCB)	G: AF334215, AF334233 N: XB4458	L. A. Marsh, W. Australia Museum (Off Meelup, Geographe Bay, 21 m)	new
S	*Liothyrella uva*	D930, D1024, D1072	-	-	(Cohen et al. 1998)
S	*L. neozelanica*	DNZ289, DNZ290	-	-	(Cohen et al. 1998)
L	*Terebratalia transversa*				(Helfenbein 2001)
C	*Terebratulina crossei*	D1275	G: AF334214, AF334232 N: not submitted	Y. Endo (Otsuchi Bay, northern Japan, 65 m)	new
C	*Terebratulina retusa*	D389, D390, D391 (GBC)	G: AF334225, AF334219, AF334226, AF334238. N: ZB4477 (representative)	A.S.G.Curtis (L. Duich)	new
C	*T. retusa*	D482, D486, D497	G: AF334227, AF334228, AAF334229, N: as above	A.S.G.Curtis (L. Fyne)	new
C	*T. retusa*	D677, D678	G: AF034231, AF034262 N: as above		(Cohen et al. 1998)
C	*T. retusa*	-	G: AJ245743 N: no shell available		(Stechmann and Schlegel 1999)
C	*Terebratulina septentrionalis*	D163, D164	G: AF034233, AF034234 N: no shell available		(Cohen et al. 1991;Cohen et al. 1998)
C	*T. septentrionalis*	D168,D196, D200	G: AF334220, AF334216, AF334221. N: no shell available		(Cohen et al. 1991;Cohen et al. 1998)
C	*T. septentrionalis*	D1366, D1367, D1368, D1391, D1392 (MR)	G: AF334217, AF334218, AF334222, AF334223, AF334224, AF334235, AF334236, AF334237, N: ZB4561 (representative)	M. C. Rhodes (S. Head, Bonne Bay, Newfoundland)	new
C	*Terebratulina unguicula*	D1404, D1411 (SL)	G: AF334212, AF334213, AF334230, AF334231 N: ZB4560 (representative)	S. Walker (Clover Passage, N. of Ketchikan, Alaska)	new

Table 2. Cancellothyridid brachiopods. Mean 12S rDNA pairwise Kimura genetic distances ± s.e.m and numbers of comparisons (n). Since all *Terebratulina septentrionalis* individuals were identical, distances involving this taxon were calculated using a single representative sequence.

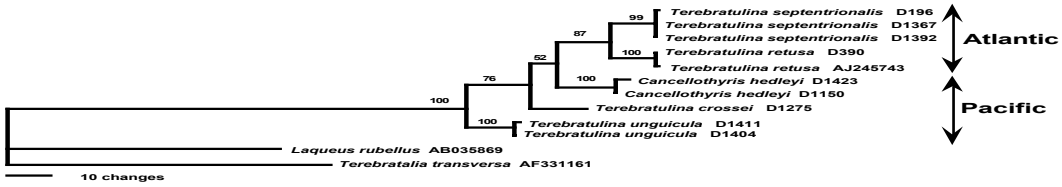
	Terebratulina unguicula	*Cancellothyris hedleyi*	*Terebratulina crossei*	*Terebratulina septentrionalis*	*Terebratulina retusa*	*Liothyrella uva*	*Liothyrella neozelanica*
Terebratulina unguicula	0.00000 (1)						
Cancellothyris hedleyi	0.10745 ± 0.00101 (2)	0.00924 (1)					
Terebratulina crossei	0.09408 ± 0.00000 (2)	0.07712 ± 0.00172 (2)	—				
Terebratulina septentrionalis	0.08893 ± 0.0000 (2)	0.09751 ± 0.00173 (2)	0.07694 (1)	0.00000 (1)			
Terebratulina retusa	0.09567 ± 0.0086 (18)	0.11510 ± 0.00161 (18)	0.09885 ± 0.0094 (9)	0.05983 ± 0.00072 (90)	0.00614 ± 0.00182 (36)		
Liothyrella uva	0.18414 ± 0.00560 (6)	0.19501 ± 0.00447 (3)	0.19617 ± 0.00745 (3)	0.20575 ± 0.00740 (30)	0.21539 ± 0.00231 (27)	0.03158 ± 0.00676 (3)	—
Liothyrella neozelanica	0.12288 ± 0.00225 (4)	0.17390 ± 0.00196 (4)	0.15711 ± 0.00347 (2)	0.17733 ± 0.00279 (2)	0.17492 ± 0.00144 (18)	0.07139 ± 0.00519 (6)	0.003422 (1)

FIGURE CAPTION

Figure 1. Phylogeny of cancellothyridid brachiopods based on concatenated 12S and 16S mitochondrial rDNA sequences. Parsimony exhaustive search tree (L = 362 steps, CI = 0.876, RI = 0.836) with branch and bound bootstrap support frequencies (%). Neighbor-joining bootstrap and quartet puzzling trees differed only by uniting *Cancellothyris hedleyi* and *Terebratulina crossei* into a single clade which, like all other nodes, was strongly supported.

In the alignment of 835 sites, 1 site was excluded because it was an added gene boundary marker, 167 were excluded as alignment-ambiguous, 409 were constant, 111 were variable but parsimony-uninformative and 147 were parsimony-informative. Parsimony exhaustive search found the single most parsimonious tree shown, identical in topology to the parsimony branch and bound bootstrap consensus tree. Search details were: all characters equally weighted and unordered, gaps treated as missing and zero-length branches collapsed. Bootstrap support values are based on 500 pseudoreplicates with 50% resampling, retaining groups compatible with the 50% majority rule tree.

For neighbor-joining tree construction Kimura and LogDet distances were used with minimum evolution optimization, invariant character frequency set to zero and rates across sites equal. Gamma-distributed variable rate parameter space was explored for Kimura distances with 4 classes and α values 0.2 - 0.5. Maximum likelihood trees were constructed by quartet puzzling (1000 steps) with HKY and TN distances and all free parameters estimated from the data. In both cases the rate distribution shape parameter estimates were close to 0.5.



[a] L, long-looped outgroup; C, cancellothyridid short-loop; S, non-cancellothyridid short-loop.

[b] Identification of specimens by: CHCB, Dr C.H.C.Brunton, Natural History Museum, London; GBC, Dr G. B. Curry, University of Glasgow; DEL, Dr D. E. Lee, University of Otago; MR, Dr Melissa Rhodes; SL, S. Long, Natural History Museum, London.