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Molecular evolution and morphological speciation in North Atlantic brachiopods (*Terebratulina* spp.)

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Morphological and molecular differentiation of western and eastern North Atlantic brachiopods were examined by morphometric analysis of six shell characters (n = 144), allozyme electrophoresis at six nuclear gene loci (n = 485), and estimation of nucleotide difference by digestion of mitochondrial DNA (mtDNA) with nine restriction endonucleases (n = 96). Principal components analysis associated 16.1% of morphometric variation with shape differences, resulting in clear morphometric discrimination. Allozyme genotype frequencies indicated sampling of large populations in genetic equilibrium. Heterozygosity was 0.174 and 0.141 for the Canadian and Scottish samples, respectively. Mitochondrial DNA nucleon (haplotype) diversity, $\hat{h}$, was 0.53 and 0.99 for the Canadian and Scottish samples, respectively. Allozyme genetic distance (0.318) and mtDNA net nucleotide difference ($\hat{d}$ = 0.289 ± 0.069 (SD)) were both high. The samples shared only 9 of 31 allozyme electromorphs, 13 of 132 restriction fragments, 1 or 2 of 67 restriction fragment patterns (''mitomorphs''), and none of 42 composite nine-enzyme mtDNA phenotypes (''mitotypes''). Canadian mtDNAs varied in size (modal size 15.83 kilobase pairs (kbp), range 15.2–16.7 kbp) but quasi-continuous and discrete variation were not distinguished. Scottish mtDNAs did not vary in size (mean size, 15.72 ± 0.13 kbp). Overall results are consistent with the classical assignment of Canadian and Scottish *Terebratulina* to separate morphospecies, *T. septentrionalis* and *T. retusa*, and suggest divergence at least 11–18 million years ago. There is no evidence of genetic exchange between the populations sampled. These conclusions are consistent with the known biogeography and life history of these articulate brachiopods.

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La différenciation morphologique et moléculaire des brachiopodes de l'ouest et de l'est de l'Atlantique Nord a été étudiée par analyse morphométrique de six caractéristiques de la coquille (n = 144), par électrophorèse des allozymes à six locus nucléaires (n = 485) et par estimation des différences dans les nucléotides par digestion de l'ADN mitochondrial au moyen de neuf endonucléases de restriction (n = 96). L'analyse des composantes principales a révélé que 16,1% de la variation morphométrique était associée à des différences de forme, résultant en une discrimination morphométrique nette. La fréquence des allozymes chez les divers génotypes reflète la représentation de grandes populations en équilibre génétique. Les résultats suivants ont été obtenus : hétérozygotie : canadien 0,174, écossais 0,141; diversité des nucléons (haplotypes) d'ADN mitochondrial, $\hat{h}$: canadien 0,53, écossais 0,99. La distance génétique entre les allozymes (0,318) et la différence globale entre les nucléotides d'ADN mitochondrial ($\hat{d}$ = 0,289 ± écart type = 0,069) se sont avérées élevées. Les échantillons n'avaient en commun que 9 des 31 électromorphes d'allozymes, 13 des 132 fragments obtenus par restriction, 1 ou 2 des 67 modèles de fragments obtenus par restriction (« mitomorphes ») et aucun des 42 phénotypes d'ADN mitochondrial composés de 9 enzymes (« mitotypes »). L'ADN mitochondrial des brachiopodes canadiens subit des variations de taille (taille modale 15,83 kbp, étendue 15,2–16,7 kbp), mais il n'a pas été possible de faire la différence entre la variation quasi-continue et la variation vraiment discrète. L'ADN mitochondrial des brachiopodes écossais ne subit pas de variations de taille (taille moyenne 15,72 ± 0,13 kbp). Dans l'ensemble, les résultats confirment la classification classique des *Terebratulina* canadiens et écossais en morphoespèces distinctes, *T. septentrionalis* et *T. retusa*, et indiquent que la divergence s'est probablement produite il y a 11–18 millions d'années. Rien ne permet de croire à l'existence d'échanges génétiques entre les populations étudiées. Ces conclusions corroborent celles d'études antérieures sur la biogéographie et la biologie de ces brachiopodes articulés.

[Traduit par la rédaction]

Introduction

As noted by Foster (1974), variation within and between populations of Recent brachiopods has been little studied, and this dearth of knowledge has largely prevented the use of Recent taxonomic information as a basis for interpreting fossil species. Moreover, the morphology of Recent brachiopods does not lend itself to certainty in classification; virtually every feature varies noticeably within a species, either during ontogeny or between individuals within populations or between populations. Furthermore, in some forms the variation in shell shape can be as great as that between putative species (Foster 1974; Dubois 1916). For these reasons it seems desirable to undertake studies of brachiopods that are based on methods which estimate genetic variation at the genomic, nucleotide-sequence level and are therefore capable of generating potentially unambiguous evolutionary information (Zuckerkandl and Pauling 1965). In this report we describe the levels of nuclear and mitochondrial genetic divergence within and between samples of *Terebratulina* from the east coast of Canada and the west coast of Scotland. The results reveal genetic differences at least at species grade, in agreement with classical morphotaxonomy. A similar result was recently obtained for morphospecies of Bryozoa (Jackson and Cheetham 1990).

Terebratulina was selected for this study because it is the

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TABLE 1. Geographical origin of *Terebratulina* spp. sampled

Localities where populations were sampled	Geographical coordinates	No. of animals	
		Allozymes	mtDNA
Canada			
Haddock Ledge, Deer Island	45°00′N, 66°55′W	133	40
Scotland			
Firth of Lorne	56°24′N, 05°33′W	243	19
Insh Island	56°18′N, 05°39′W	87	18
Sound of Jura	56°05′N, 05°35′W	22	19
Total for Scotland		352	56

most accessible and best-studied brachiopod genus in the North Atlantic (Brunton and Curry 1979). These brachiopods are sexually reproducing, diploid, sessile filter-feeders, with separate sexes. Individuals become sexually mature after 2 or 3 years and may survive and breed for at least 7 years, reaching a maximum length of about 25 mm (Noble *et al.* 1976; Curry 1982). Fertilisation is external and the larvae hatch from the egg after a short period of development, settling permanently within a few days (Morse 1873; Noble *et al.* 1976; Webb *et al.* 1976). As a result, *Terebratulina* is gregarious, but patchily distributed, and populations of these animals may be expected to show macrogeographical genetic differentiation.

Materials and methods

Specimen collection

Brachiopods were collected by divers in June 1985 from the Bay of Fundy, Canada, and transported by air to Scotland in chilled seawater. Scottish samples were collected between 1984 and 1988 by dredge or by divers. For the purposes of this report, data from three neighbouring Scottish collection sites will be pooled (Table 1) on the basis of allozyme data described below.

Allozyme electrophoresis

Soft tissues were scraped from within the shells of individual animals, added to 200 μL (ca. 1 tissue-volume) ice-cold homogenisation buffer (10 mM Tris–HCl, pH 7.5, 1 mM NAD, 1 mM NADP), frozen, and stored at −70°C. Before analysis, homogenates were thawed on ice and centrifuged (12 000 × *g*, 5 min). Aliquots of supernatant were loaded on Whatman 3MM insert papers into 11% Connaught starch gels alongside electromorph standards. After electrophoresis at 6 V/cm for 4 h at 5°C in citrate–borate discontinuous buffer (Poulik 1957), 1.5 mm thick slices were cut and stained by standard methods (Harris and Hopkinson 1976; Shaw and Prasad 1970).

Mitochondrial DNA restriction fragment analysis

DNA preparations were made by the proteinase K method (Maniatis *et al.* 1982), using scraped-out soft tissues, with the exception of shells less than 6 mm long, which were digested whole after removal of epibionts. Alcohol-precipitated, dried DNA was redissolved in 25 μL or more of TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA), according to yield. Purification of mtDNA by density gradient ultracentrifugation (Lansman *et al.* 1981) was precluded by the small size of the animals.

Restriction endonucleases from commercial sources were used under conditions specified by the manufacturer but with the addition of 4 mM spermidine. Nine restriction enzymes, AccI, AvaI, AvaII, BglII, DraI, EcoRV, PstI, PvuII, and SstI, were identified as informative because they gave three or more fragments per digest in most individuals. Restriction fragments were resolved by electrophoresis in 0.7, 1.0, or 1.2% agarose gels, using 1× TBE buffer (Maniatis *et al.* 1982). After electrophoresis, fragments were transferred to Hybond-N (Amersham), using alkaline capillary transfer as recommended by the manufacturer, with depurination. Prehybridisation and hybridisation were in 50% formamide, 5× SSC (0.15 M NaCl + 0.015 M sodium citrate), at 42°C, with blocking agents (Maniatis *et al.* 1982). The hybridisation mix contained at least 10^6 cpm/mL (1 cpm = 0.0167 Bq) of heat-denatured probe DNA (λ Tr65, see below), labelled with [^{32}P]dNTP to >5 × 10^8 dpm/μg (1 dpm = 0.0167 Bq) (Feinberg and Vogelstein 1983). Nonspecifically bound probe was removed by washing twice for 10 min in 2× SSC, 0.1% SDS, and twice for 30 min in 0.5× SSC, 0.1% SDS, all at room temperture. Hybridised probe was detected by autoradiography (Maniatis *et al.* 1982). Lambda-DNA restriction fragments of known size were included in each gel and the sizes of unknown fragments were estimated by interpolation (Schaffer and Sederoff 1981). Mean deviation between estimated and known sizes of λ fragments was 0.86% ± 0.1%.

To clone the *Terebratulina* mitochondrial genome, preparations enriched for mtDNA (cyt-DNA; Lansman *et al.* 1981) were made from oocytes of Scottish *Terebratulina*. In one such preparation, 2.9 × 10 oocytes were recovered from 18 ripe females by washing the pallial sinuses with 2× TSM (60 mM Tris–HCl, pH 7.5, 300 mM NaCl, 3 mM $MgCl_2$). Preliminary experiments showed that the insert of SpP144, a plasmid clone of sea urchin large-subunit ribosomal mtDNA sequence (Jacobs *et al.* 1983), hybridised specifically with three components of *Terebratulina retusa* cyt-DNA. These corresponded closely in electrophoretic mobility with closed circular, linear, and concatenated forms of sea urchin mtDNA. (This hybridisation was carried out in 4× SET (1.2 M NaCl, 4 mM EDTA, 0.4 M Tris–Cl, pH 7.5) at 56°C and washed in 1× SET at 50°C.) Amongst several restriction enzymes tested, BamHI was found to convert the three *Terebratulina* components to a single component corresponding to linearised mtDNA. Therefore, the 9–20 kilobase pairs (kbp) fraction of a BamH1 digest of cyt-DNA was ligated into the BamH1 site of the λ vector EMBL3. A library of about 50 000 recombinant phages was screened by plaque hybridisation with ^{32}P-labelled SpP144 and strongly positive plaques were isolated and purified. One recombinant, designated λ Tr65, with an insert of 15.8–16.5 kbp, was selected for use as probe. The cloned segment was established to be entire *Terebratulina* mtDNA by the identity (save at known polymorphic sites) of single- and double-digest fragments produced from it and from the unfractionated DNA of pooled oocytes and of individual animals. In particular, several enzymes (e.g., KpnI) that cleave a single restriction site in the Tr65 insert also linearise the homologous sequence in *Terebratulina* cyt-DNA to give a product that comigrates with both the insert and authentic (Jacobs *et al.* 1983) linearised sea urchin mtDNA (data not shown).

Each characteristic mtDNA fragment pattern generated by a restriction enzyme (a "mitomorph") was labelled alphabetically in order of identification. The composite mitomorph phenotype of each individual, in alphabetical order of restriction enzymes, constitutes its mitochondrial haplotype or "mitotype." Within-population diversity was summarised quantitatively by Nei and Tajima's (1981) nucleon (haplotype) diversity ($\hat{h}$). Because the data involved some mapped restriction sites, nucleotide difference ($\hat{d}$) was estimated using eq. 8 of Nei and Li (1979). In view of the large between-samples nucleotide distance and the unrealistic assumptions (such as absence of selection and equal nucleotide base frequency) involved in such calculations, the possible underestimation of $\hat{d}$ by a factor of up to 2 is acceptable. Nucleotide difference calculations incorporated results from every individual for which data from at least five out of the nine informative restriction enzymes were available. Recognition-site length weighting (Nei 1987, Table 5.7) was used and the resulting between-populations nucleotide difference estimate was corrected for polymorphism (Nei and Li 1979). Size variants of mtDNA were not included in the nucleon diversity and nucleotide difference calculations. The combined stochastic and sampling variance of $\hat{d}$ was estimated by the method of Takahata and Nei (1985).

Scottish sample mtDNAs were screened for size variation as follows: each was digested with BamHI and SmaI, which cut the mtDNA of most individuals into two fragments of ca. 8 kbp, differing in size by ca. 350 base pairs (bp) and therefore clearly, but only slightly, separated in

electrophoresis. Phage λ EMBL3 right arm (9.21 kbp), which migrates to a position close to the two mtDNA moieties, was added to each sample before electrophoresis. After blotting and probing with λ Tr65, the relative mobilities of the λ right arm and the two Bam–Sma mtDNA fragments provided a sensitive test for size variation involving gain or loss of ca. 150 bp or more.

Morphometric analysis

During the initial stages of the investigation of morphometric differentiation, a wide range of shell parameters was measured on representatives of the Canadian and Scottish shells, and these data were subjected to principal components analysis. The eigenvector values of this exploratory analysis revealed that most of the discrimination related to ribbing density and to the dimensions of the pedicle foramen relative to shell size. By this means a subset of six measurements that could be used to reveal most of the measurable morphometric differentiation was identified. These six parameters were determined as follows. Length, width, and height of complete shells were measured with dial calipers accurate to 0.1 mm. Length and maximum width of the pedicle foramen were determined using a binocular microscope equipped with a graticule, again to an accuracy of 0.1 mm. Ribbing density was determined by counting the number of ribs in a 5-mm median sector situated 10 mm from the umbo of the dorsal valve. Because the ribs are relatively highly elevated from the shell surface, they can have a variable cross section, periodically bulging along their length; this indirect measure of rib dimension was more accurate and reproducible than direct measurement of the transverse wavelength of individual ribs.

Results

Nuclear gene variation

Three hundred and fifty-two Scottish and 133 Canadian animals were analysed for six enzyme loci: naphthyl esterase (*Est*), glutamate oxalacetate transaminase (*Got*), glucose phosphate isomerase (*Gpi*), hexokinase (*Hex*), malic enzyme (*Me*), and tetrazolium oxidase (*To*). One monomorphic locus (*To*) provided diagnostic alleles, and one locus (*Hex*) was monomorphic in Canadian animals but polymorphic in Scottish animals (Table 2). The activities of two enzymes, mannose phosphate isomerase (MPI) and 6-phosphogluconate dehydrogenase (6PGDH), were polymorphic and classifiable in Canadian animals but undetectable in Scottish animals. A further eight polymorphic enzyme activities, representing 10 presumed gene loci, were detected, but none could be resolved well enough to discriminate betweeen all presumed homozygotes and heterozygotes. These loci, which were not studied further, included adenosine monophosphate kinase, catalase, α-glycerophosphate, glucose-6-phosphate, isocitrate and malate dehydrogenases, glutamate oxalacetate transaminase-2, phosphoglucomutase-1 and -2, and leucyl-alanyl peptidase-1 and -2. The between-samples differences in MPI and 6PGDH, together with independent confirmation of many of our results (P. King, personal communication), suggest that the number of resolved allozyme systems was limited as a result of factors specific to *Terebratulina* (also confirmed by an anonymous reviewer). Hammond and Poiner (1984) noted similar difficulties with the larger (inarticulate) brachiopod *Lingula*. However, in *Freileia* (a very small articulate brachiopod) and *Liothyrella* (a large articulate), as well as *Coptothyris*, many enzyme loci have been successfully analysed (Ayala *et al.* 1975; Valentine and Ayala 1975; Balakirev and Manchenko 1985).

Mean heterozygosity in the Canadian sample was 17.4%. In the Scottish sample it was 14.1%. When the data from the Scottish sample were subdivided according to collection site (Table 1), allele frequencies did not differ significantly (rare alleles pooled, contingency χ^2 test: P = 0.2–0.1), and these subsamples were therefore pooled (Table 2). Tests for departure from Hardy–Weinberg equilibrium (with rare alleles pooled) showed nonsignificant deviations for *Got* and *Gpi* in both Canadian and Scottish samples. Only *Hex* in the Scottish sample gave a significant deviation (P = 0.05–0.02), owing to a deficiency of heterozygotes, which probably arose from residual misclassification due to closely similar mobilities of electromorphs. Overall, the allozyme data indicated that in *Terebratulina*, as in *Freilia* and *Liothyrella* (Ayala *et al.* 1975; Valentine and Ayala 1975), the samples could have been drawn from large, diploid and panmictic, sexually reproducing populations. There was major genetic divergence between the Canadian and Scottish samples: of 31 electromorphs recognised, only 9 were common to both samples. Nei's minimum genetic distance (Nei 1987) was 0.318, but with so few loci examined, this value is very approximate.

TABLE 2. Allozyme allele frequencies at six loci in Canadian and Scottish specimens of *Terebratulina* spp.

Locus	Allele	Canadian population (n = 133)	Scottish population (n = 352)
Est	*100*	1.000	1.000
Me	*100*	1.000	1.000
To	*100*	0.000	1.000
	120	1.000	0.000
Got	*86*	0.086	0.037
	89	0.000	0.003
	90	0.169	0.008
	92	0.226	0.010
	97	0.143	0.000
	98	0.004	0.000
	100	0.346	0.937
	102	0.004	0.000
	105	0.000	0.001
	108	0.023	0.003
Gpi	*86*	0.846	0.001
	90	0.075	0.000
	93	0.000	0.001
	97	0.079	0.000
	100	0.000	0.925
	102	0.000	0.001
	105	0.000	0.026
	108	0.000	0.044
Hex	*90*	0.000	0.192
	92	0.000	0.007
	94	0.000	0.028
	95	0.000	0.003
	96	0.000	0.176
	98	0.000	0.045
	100	1.000	0.584
	101	0.000	0.001
	102	0.000	0.026

NOTE: Loci are as follows: *Est*, naphthyl esterase; *Me*, malic enzyme; *To*, tetrazolium oxidase; *Got*, glutamate oxalacetate transaminase; *Gpi*, glucose phosphate isomerase; *Hex*, hexokinase. Alleles were numbered according to the mobility of their electromorphs (measured to the nearest millimetre, under standard conditions) relative to the commonest allele, designated *100*

Restriction-site variation in mitochondrial DNA

The size of the cloned *Terebratulina* mtDNA (15.8–16.5 kbp) and its comigration with sea urchin mtDNA, as well as the mean

TABLE 3. Mitotypes (composite phenotypes) produced by nine restriction endonucleases in Canadian and Scottish samples of *Terebratulina* spp. and nucleon diversity estimates ($\hat{h}$)

Population	Mitotype	Number observed	Frequency	$\hat{h}$
Canada				
($n = 30$)	CELCDDBEJ	21	0.700	
	CELCDDBEL	4	0.133	
	CETCDDBEJ	2	0.066	
	FEMCDDBEJ	1	0.033	
	CENCDDBEJ	1	0.033	
	CESCDDBEJ	1	0.033	0.53
Scotland				
($n = 41$)	AADABAAAA	4	0.097	
	AAEABCAAB	1	0.024	
	AAFABAAAD	1	0.024	
	AAGABAAAA	1	0.024	
	AAHABAAAF	1	0.024	
	AAIABAAAA	1	0.024	
	AAWABBAAA	1	0.024	
	ABAABAAAA	1	0.024	
	ABDABAAAA	1	0.024	
	ABDABAAAB	1	0.024	
	ABDABAAAF	1	0.024	
	ABIABAAAA	2	0.049	
	ABIABAAAF	2	0.049	
	ABIABAAAG	1	0.024	
	ABIABAAAN	1	0.024	
	ABIACAAAA	1	0.024	
	ABIAHAAAA	1	0.024	
	ABIABAACF	1	0.024	
	ABIBBAAAA	1	0.024	
	ABPABAAAA	1	0.024	
	ABRABAACA	1	0.024	
	AB@ABAAAA	1	0.024	
	ACDABAABA	1	0.024	
	ACFAAAAAD	1	0.024	
	ACIABAAAA	1	0.024	
	ACIABAAAF	1	0.024	
	ACVABAFAA	1	0.024	
	AIDABAAAA	1	0.024	
	AJGABCAAA	1	0.024	
	AJHAACAAD	1	0.024	
	AJKEBCAAA	1	0.024	
	ALIAHACAA	1	0.024	
	BCKABCAAA	1	0.024	
	DDOAACAAA	1	0.024	
	IAAABAAAA	1	0.024	
	NCHABCAAA	1	0.024	0.99

NOTE: For each individual, the morphs observed are listed in alphabetical order of restriction enzymes used: *Acc*I, *Ava*I, *Ava*II, *Bgl*II, *Dra*I, *Eco*RV, *Pst*I, *Pvu*II, *Sst*I. Thus, the mitotype designation CELCDDBEJ indicates the presence of the C morph for *Acc*I, the E morph for *Ava*I, etc. Partially determined mitotypes of 10 Canadian and 15 Scottish specimens are not shown, but additional morphs occurring in these specimens are listed in the Appendix. @ represents *Ava*II mitomorph AF.

summed fragment size of 54 Scottish mitomorphs (15.72 ± 0.133 kbp; see the Appendix), indicate that *Terebratulina* mtDNA falls within the size range typical for Metazoa. No example of restriction-site heteroplasmy was observed.

Estimation of nucleon diversity was based on 30 Canadian and 41 Scottish individuals for which a result was obtained with all nine restriction enzyme digests (Table 3). In the Canadian sample (6 mitotypes amongst 30 individuals, $\hat{h} = 0.53$), nucleon diversity was lower than in the Scottish sample, which had a

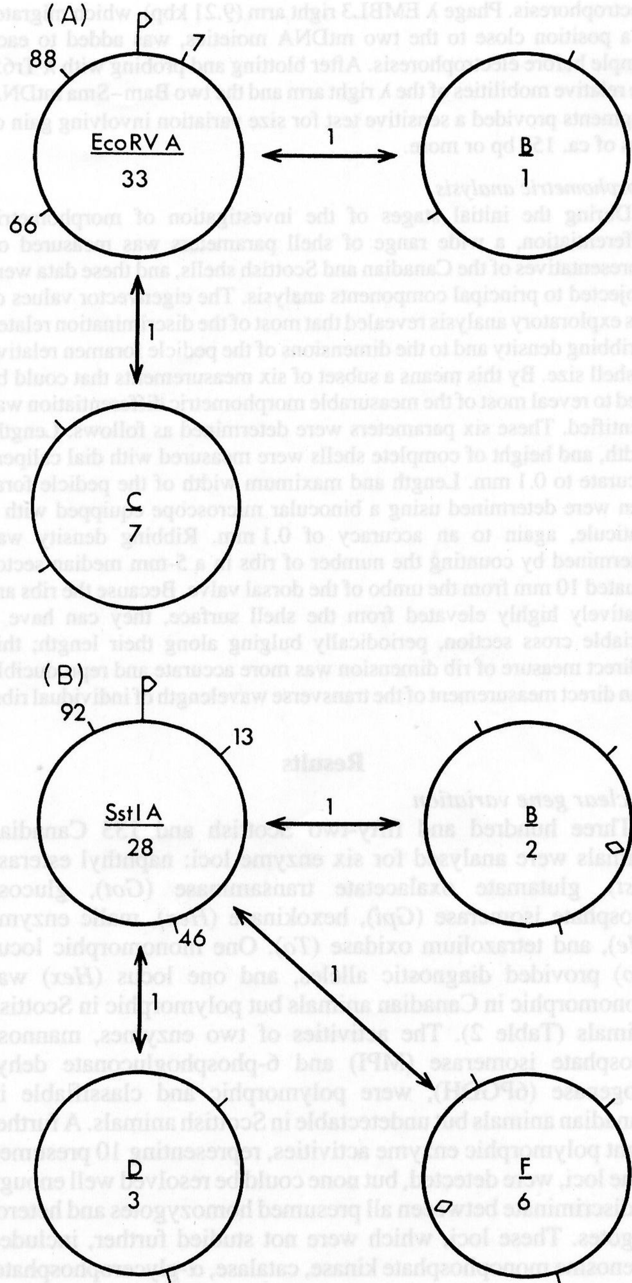

FIG. 1. Mutational interrelationships of *Eco*RV A, B, and C mitomorphs (A) and *Sst*I A, B, D, and F mitomorphs (B) of *Terebratulina retusa*. The mitochondrial genome was divided into 100 map units starting from the unique *Bam*H1 site (flag symbol), and the relative positions of mapped sites were calculated on the basis of a genome size of 16.0 kbp. Additional sites, which were mapped only to a fragment, are indicated (◇). The number of observed individuals with each mitomorph is indicated within each diagram.

notably high level of diversity (36 mitotypes amongst 41 individuals, $\hat{h} = 0.99$).

In the restriction fragment analysis about 43 fragments of 0.5 kbp or larger were scored per individual. In addition to the 30 Canadian and 41 Scottish individuals referred to above, results were obtained from at least five of the nine different restriction enzymes on a further 10 Canadian and 15 Scottish individuals (data not shown). Nucleotide divergence estimates based on all these data were as follows: within the Canadian sample, 0.96%

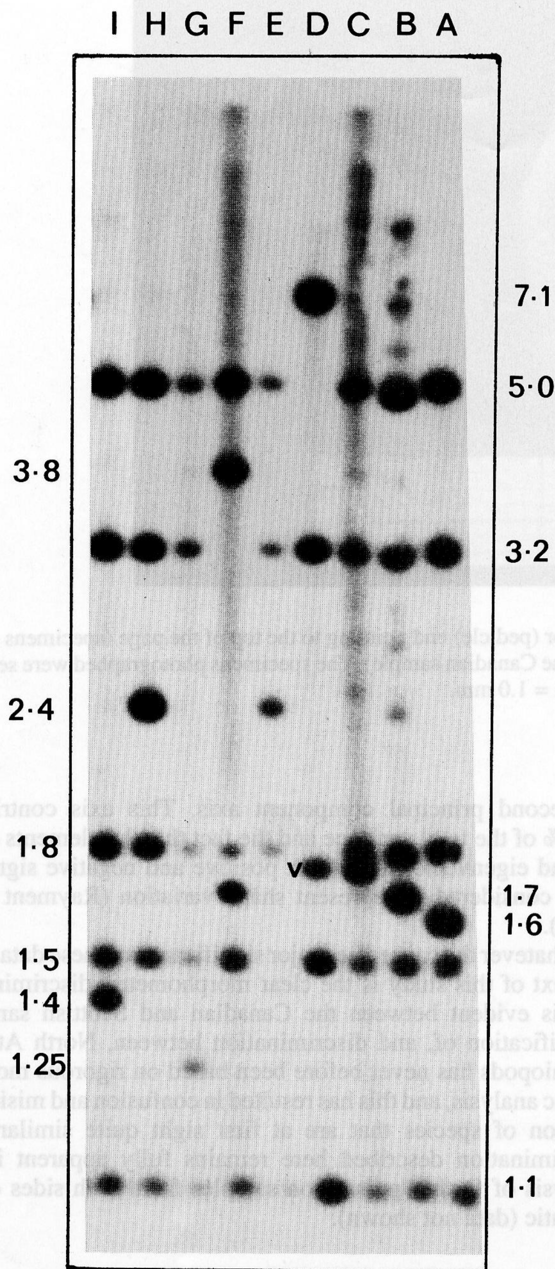

FIG. 2. Fragment patterns of nine *Dra*I mitomorphs in *Terebratulina* spp. The sizes of the fragments are indicated in kilobase pairs. The size-variable fragment in the Canadian mitomorph *Dra*I D is labelled "v." *Dra*I E, F, G, and I did not occur amongst the specimens referred to in this paper.

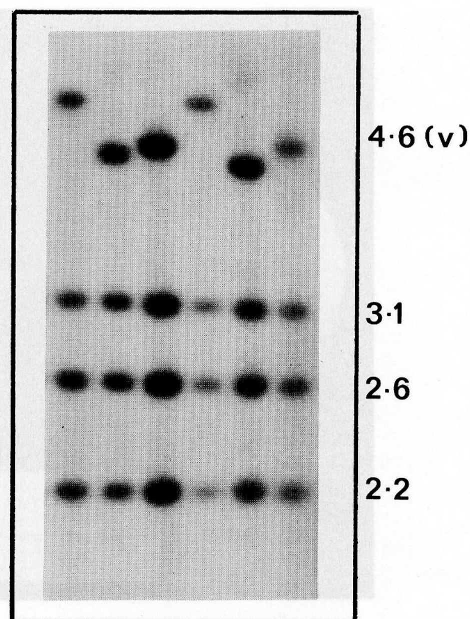

FIG. 3. Fragment patterns of the *Ava*II L mitomorph in six individuals from the Canadian sample of *Terebratulina* spp. At least four different size variants of the 4.6-kbp fragment occur amongst these individuals.

± 4.9%; within the Scottish sample, 1.6% ± 2.3%; between the Canadian and Scottish samples, 28.9% ± 6.9%. Exemplifying this high level of between-samples divergence, Canadian and Scottish animals shared 0 of 42 mitotypes (Table 3), not more than 2 of 67 mitomorphs, and 13 of 132 fragments (see the Appendix for the shared mitomorphs occurring in individuals not listed in Table 3). Because of the high between-samples divergence, no comparison of Canadian and Scottish mtDNA restriction maps is presented, but within the Scottish sample, most *Eco*RV, *Pst*I, *Pvu*II, and *Sst*I mitomorphs could be derived from the commonest mitomorph by the gain or loss of one site (Fig. 1 and the Appendix).

Despite the high overall divergence, Canadian and Scottish *Dra*I mitomorphs (e.g., B and D) share fragments amounting to at least 12.9 kbp (Fig. 2 and the Appendix). This strong similarity probably reflects restriction-site conservation rather than convergence or coincidence. It therefore implies conservation of genetic organisation between these sites and, hence, probably over most of the mitochondrial genome.

Size variation of mitochondrial DNA

In the Canadian sample, one fragment (Fig. 3), presumably containing the control or D-loop region, was observed to vary in size between individuals in all nine digests (Appendix) and to be heteroplasmic in a few individuals (not shown). Similar size variation has now been observed in many organisms (reviewed by Moritz *et al.* 1987). For 50 individual brachiopods the mobility of the variable fragment was determined (to the nearest millimetre, equivalent to ca. 200 bp) in AvaII and BamHI digests (fragment size ca. 5 and 6 kbp, respectively). The distribution of fragment sizes was consistent with either a discrete distribution into at least nine classes differing by 150- to 200-bp steps or with a quasi-continuous distribution (data not shown). In the same 50 animals the modal mitochondrial genome size was found to be 15.83 kbp (range 15.2–16.7 kbp). Sample exhaustion precluded further analysis.

Size variation in the Scottish sample is suggested by the *Dra*I A, B, C, G, H, and I fragment patterns (Fig. 2), which can be explained if one fragment (1.6 kbp in *Dra*I A; probably homologous with the size-variable fragment in the Canadian mitomorph *Dra*I D) ranges in size from 1.25 to 2.4 kbp. Alternative restriction-site gain–loss hypotheses are possible. To choose between size- and site-variation explanations, all autoradiographs were carefully searched for cross-digest size differences, and the migration of the *Bam*HI–*Sma*I moieties of all available samples was compared with an internal λ-DNA standard (data not shown). No clear case of size variation was found and it therefore seems most likely that these *Dra*I mitomorphs result from restriction-site gains or losses. Because of sample exhaustion no

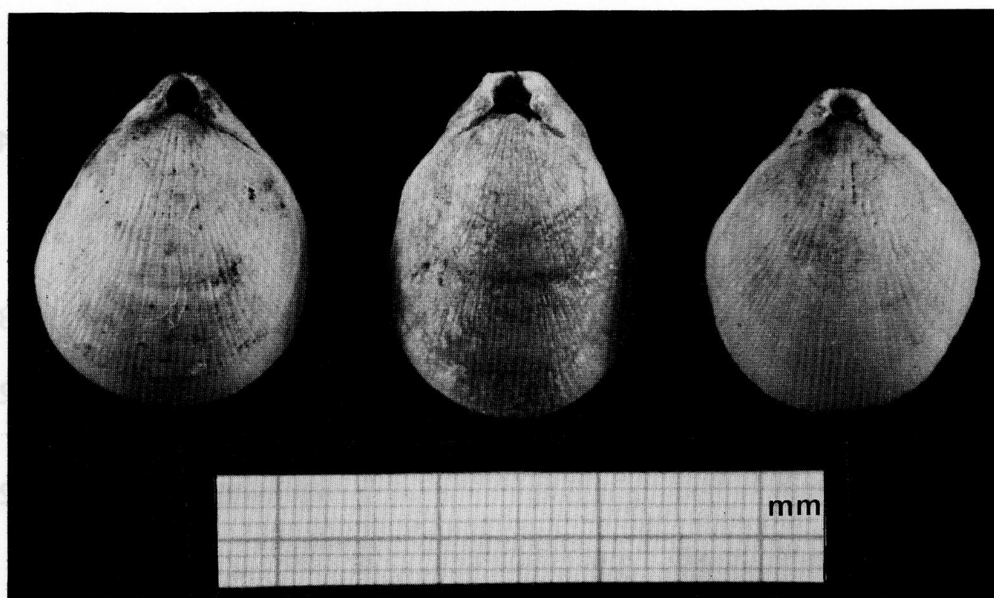

FIG. 4. Morphology of *Terebratulina* shells, seen from the dorsal side, posterior (pedicle) end pointing to the top of the page (specimens on the left and right are from the Scottish sample and the specimen in the centre is from the Canadian sample). The specimens photographed were selected only for comparable size and freedom from epibionts. One scale grid subdivision = 1.0 mm.

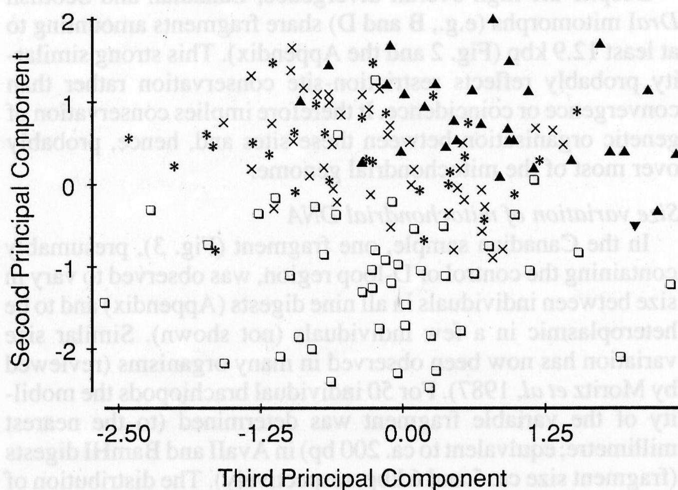

FIG. 5. Principal components analysis of six morphometric parameters on 144 specimens of *Terebratulina* spp. from various locations. □, Canada ($n = 43$); ×, Firth of Lorne ($n = 42$); ▲, Insh Island ($n = 29$); *, Sound of Jura ($n = 30$).

search was possible for the relatively small fragments expected according to the site-variation hypothesis.

Morphometrics

Shells of Canadian and Scottish *Terebratulina* are illustrated in Fig. 4 and representative results of principal components analysis of the morphometric data are shown in Fig. 5. The analysis of the brachiopod data falls into a "classic" pattern in which the first principal component axis contributes a high percentage of the variability (67.9%) and contains elements that, having the same sign and relatively similar magnitudes, have been interpreted as reflecting size variation (Rayment *et al.* 1984). A clear discrimination is obtained between the single Canadian population and the three Scottish populations along the second principal component axis. This axis contributes 16.1% of the total variance and the fact that the elements of the second eigenvector have both positive and negative signs has been considered to represent shape variation (Rayment *et al.* 1984).

Whatever the cause, the major significance of these data in the context of this study is the clear morphometric discrimination that is evident between the Canadian and Scottish samples. Identification of, and discrimination between, North Atlantic brachiopods has never before been based on rigorous morphometric analysis, and this has resulted in confusion and misidentification of species that are at first sight quite similar. The discrimination described here remains fully apparent in the analysis of further population samples from both sides of the Atlantic (data not shown).

Discussion

The main objective of our analysis of nuclear gene (allozyme) variation in *Terebratulina* was to establish the broad nature of the population genetic structure in these brachiopods. Although based on few loci, the results make it clear that both Canadian and Scottish samples, as expected on the basis of life-history criteria, could have been drawn from large, panmitic, sexually reproducing populations and that genetic distance between them is compatible with separation at the species level or above (Thorpe 1982, 1983). The observed level of heterozygosity is not unusual for marine invertebrates (Nevo *et al.* 1984), and other articulate brachiopods analysed by comparable methods have given broadly similar results (Ayala *et al.* 1975; Valentine and Ayala 1975; Balakirev and Manchenko 1985). No estimate of between-populations nuclear genetic distance has previously been reported for articulate brachiopods.

The analysis of mtDNA restriction fragment length polymorphism was based on nine 5- and 6-base restriction enzymes

because this was the largest number of digests for which DNA from many individuals was available. The marked difference between the Canadian and Scottish samples in nucleon diversity ($\hat{h}$) may reflect differences in relative isolation, effective population size, and (or) dispersal potential. The Canadian sample was from an island site within a large tidal embayment and its larvae are reportedly brooded (Webb *et al.* 1976; Witman and Cooper 1983). By contrast, brooding has never been reported in Scottish *Terebratulina*, which were collected from a coastal region with strong longshore tidal currents.

The net nucleotide difference between the mtDNA of the Canadian and Scottish samples ($\hat{d}$ = 28.9 ± 6.9%) is much larger than has been associated with different demes, populations, or subspecies of any organism (Vawter and Brown 1986; Tegelström *et al.* 1988) and is above the range of accurate estimation (Nei and Li 1979). Similarly, the allozyme genetic distance appears to be high (Thorpe 1983). Taken together, the nuclear and mitochondrial genetic divergence data appear to rule out the possibility of continuous gene flow between the sampled populations and are entirely consistent with their being representative of different species, i.e., of populations that have been effectively isolated for a long time, cannot interbreed, and have also diverged morphologically, as demonstrated by the morphometric analysis, and physiologically, as indicated by the difference in larval brooding. If the rate of mtDNA nucleotide substitution were 1% per million years in each lineage, as in vertebrates (Brown *et al.* 1979), the observed nucleotide difference (itself almost certainly underestimated) predicts mitochondrial genome cladogenesis to have occurred at least 11–18 million years ago. This time period is very uncertain, pending data on the rate of mtDNA evolution in brachiopods.

The conclusion that the Canadian and Scottish samples of *Terebratulina* differ genetically to at least the extent typical of species agrees with the classical systematics of North Atlantic *Terebratulina*, according to which the Canadian sample corresponds to *T. septentrionalis*, whilst that from Scotland belongs to *T. retusa* (Davidson 1886–1888). In fact, *Terebratulina* specimens from Iceland eastwards at least to the Barents Sea and southwards to the Iberian Perninsula and the western Mediterranean Sea have generally been allocated to *T. retusa* (Wesenberg-Lund 1938, 1941; Logan 1979), whilst those from Greenland westwards and south to the Gulf of Maine have been assigned to *T. septentrionalis*. Our conclusion disagrees with the suggestion (Emig 1990) that these species might be synonymous, since the observed between-species nucleotide difference is 10–20 times greater than that within either species.

Wesenberg-Lund (1938; 1940*a*, 1940*b*, 1940*c*, 1941) remarked that because of morphological overlap between *T. septentrionalis* and *T. retusa* from distant sites, some individuals collected from east Greenland, Iceland, and the Faroe Islands could not be unambiguously assigned to a species. We were unfortunately not successful in obtaining samples of *Terebratulina* from the northern North Atlantic, so whether these species are genetically disjunct or overlap or intergrade in that region remains unknown at present. However, a morphometric reanalysis of relevant museum collections (G.B.C., work in progress) is expected to clarify this question in the light of the data presented here. It is to be hoped that samples from these regions will eventually become available for genetic study. Nevertheless, the present data clearly permit the conclusion that classical morphotaxonomy of brachiopod species does indeed reflect molecular genetic divergence in at least end-members of this species-pair.

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Appendix: Sizes of mtDNA fragments (kbp) produced by each of nine restriction endonucleases on mtDNAs of 40 Canadian and 56 Scottish *Terebratulina* specimens

***Acc*I**

A	B	C	D	I	N
5.2	5.2	4.1	4.7	5.2	5.2
2.25	2.25	2.8	2.25	2.15	2.25
2.15	1.75	2.1	2.15	1.75	1.9
1.75	1.2	1.95 (*v*)	1.75	1.2	1.75
1.2	1.0	1.8	1.2	0.78	1.2
0.78	0.78	1.25	0.78	0.68	0.9
0.72	0.72	0.9	0.72		0.78
0.68	0.68	0.53	0.68		0.72
					0.68
14.73	13.58		14.23	11.76	15.38

***Ava*I**

A	B	C	D	E	I	J	K	L	M
4.4	6.4	4.4	4.4	4.0	4.4	4.4	5.1	6.4	4.4
4.0	4.4	4.0	4.0	3.3	4.0	2.9	4.4	4.4	4.0
2.9	2.8	2.9	2.1	2.7 (*v*)	2.1	2.0	2.9	2.9	2.9
1.9	1.2	2.2	1.9	2.2	2.0	1.65	1.3	1.1	2.2
1.2	1.0	1.9	1.15	1.8	1.9	1.2	1.25	1.0	1.5
1.0	0.7	0.9	1.0	1.7	1.2	1.0	1.0	0.8	0.9
0.9			0.9		0.75	0.9	0.9		
			0.7		0.7				
			0.55						
16.3	16.5	16.3	16.7		17.05	14.05	16.85	16.6	15.9

***Ava*II**

A	B	D	E	F	G	H	I	K	L
8.3	8.3	8.3	6.9	6.6	7.6	4.6	8.3	4.0	4.6 (*v*)
3.7	4.0	3.7	3.7	3.7	3.7	3.7	6.1	3.7	3.1
2.2	2.4	2.2	2.1	2.1	2.2	3.2	2.2	3.1	2.6
2.1	2.1	1.2 (2)	1.2	1.4	2.1	2.1		2.2	2.2
			1.15	1.15		1.2		2.1	0.85
									0.7
									0.65
									0.6
16.3	16.8	15.4	15.05	14.95	15.6	14.8	16.6	15.1	

AvaII *(continued)*

M	N	O	P	R	S	T	W	AA	AF
4.6 (*v*)	4.6 (*v*)	8.3	8.3	12.6	4.6 (*v*)	4.6 (*v*)	8.3	6.9	8.3
3.1	2.6	3.7	3.7	2.2	3.1	3.1	2.25	3.7	4.4
2.6 (2)	2.2	1.3	1.7		2.6	2.6	2.1	2.2	2.2
0.7	0.85	1.2	1.2		1.7	2.2	1.35	1.4	1.75
0.65	0.7	1.05			1.25	1.05	1.2	1.2	
0.6	0.65				0.85	0.7			
	0.6				0.6	0.65			
						0.6			
		15.55	14.9	14.8			15.2	15.4	16.65

***Bgl*II**

A	B	C	E	M
7.5	7.5	10.5 (*v*)	6.0	9.3
3.1	3.2	6.5	3.1	3.1
2.8	2.8		2.8	2.8
1.45	1.45		1.7	1.25
1.25	1.25		1.45	
			1.25	
16.10	16.20		16.30	16.45

***Dra*I**

A	B	C	D	H
5.0	5.0	5.0	7.1	5.0
3.2	3.2	3.2	3.2	3.2
1.8	1.8	1.8 (2)	2.0 (*v*)	2.4
1.6	1.7	1.5	1.5	1.8
1.5	1.5	1.1	1.1	1.5
1.1	1.1	0.65	0.65	1.1
0.65	0.65	0.5	0.5	0.65
0.5	0.5			0.5
16.95	15.45	15.50		16.05

***Eco*RV**

A	B	C	D	E
9.4	9.4	13.0	13.0	9.4
3.6	7.2	3.6	3.6 (*v*)	2.4
3.3				2.0
				1.0
				0.98
16.3	16.6	16.6		15.78

***Pst*I**

A	B	C	F
11.0	6.5 (*v*)	11.0	11.0
2.65	4.8	2.95	2.3
0.96	2.85	0.96	0.96
14.61		15.91	14.26

***Pvu*II**

A	B	C	E
11.0	7.5	11.0	12.0 (*v*)
2.9	4.2	4.6	2.0
1.6	2.9		
	1.6		
15.5	16.2	15.6	

***Sst*I**

A	B	D	F	G	L	J	N
7.8	7.8	11.0	6.3	7.8	5.0	11.0 (*v*)	6.3
5.15	3.4	5.15	5.15	5.15	4.8	5.0	5.15
3.4	3.1		3.4	2.9	4.2		4.9
	1.85		1.4	0.8	1.85		
16.35	16.15	16.15	16.25	16.65			16.35

NOTE: Upper case letters identify different mitomorphs (missing letters belong to mitomorphs found in population samples not reported here). Size-variable fragments are marked *v*, and the size given is the approximate average size of the variable fragments in the population sample. No variable fragment could be identified in the *Sst*I L mitomorph because the mtDNA of the three individuals of this type did not differ detectably in size. The approximate total observed fragment size is given for all Scottish mitomorphs, but because the sizes assigned to variable fragments are averages, total sizes are not given for Canadian mitomorphs. The number 2 marks nonstoichiometric components, interpreted as two comigrating fragments.