Hierarchical (mm- to km-scale) Environmental Variation Affecting Skeletal Phenotype Of A Marine Invertebrate (*Electra pilosa*, Bryozoa): Implications For Fossil Species Concepts

By: **Steven J. Hageman** and Christopher D. Todd

Abstract

A comparative record of morphological change of fossil specimens through geologic time can provide insights into the rates and patterns of microevolution and speciation. However, questions concerning the extent to which environmental conditions influence skeletal morphology, and potentially confound recognition of genetic change, can be most meaningfully addressed with living taxa. The marine encrusting bryozoan, Electra pilosa (L.), was used to assess the magnitude of environmental effects on zooecium-level skeletal morphology at different spatial scales. The latter included environmental effects or factors ranging in level from (1) micro-environmental (variation within and among immediately adjacent colonies), (2) meso-environmental (small-scale colony positional effects among colonies within a common habitat), and (3) macro-environmental (morphological variation among colonies from recognizably different environmental settings). Macro-environmental influence on zooecial morphology for colonies among localities (10 km-scale) can be detected by comparison of colonies from wave-protected/tidal-dominated versus open coast/wave-dominated settings, and this accounted for approximately 7.5% of the observed morphological variation. Meso-environmental variation - that is, small-scale (10 (super 1) to 10 (super 2) m) systematic (nonrandom) differences that would go undetected in a geologic setting - had a minimal deterministic influence on zooecial morphology, and accounted for approximately 2.5% of observed variation. Variation among colonies from the same site was highly significant. Much of this morphologic variation (approximately 30%) is attributable to genotypic variation among colonies, but micro-environmental sources cannot be excluded (10 (super 1) to 10 (super 2) cm-scale). Variation within colonies, accounting for approximately 60% of the observed morphological variation, can be further partitioned into Micro-environmental differences, approximately 40% (10 (super 1) to 10 (super 2) mm-scale) associated with spatiotemporal position, and the Life History of individual modules (approximately 20%). Environmental levels (factors) that are associated with significant morphological effects can also be recognized by their sedimentological properties, which can be preserved in the geologic record. Thus, results from this and similar studies have relevance for and can potentially be directly applied to studies of fossil organisms.

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Hierarchical (mm- to km-scale) environmental variation affecting skeletal phenotype of a marine invertebrate (*Electra pilosa*, Bryozoa): Implications for fossil species concepts

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A B S T R A C T

A comparative record of morphological change of fossil specimens through geologic time can provide insights into the rates and patterns of microevolution and speciation. However, questions concerning the extent to which environmental conditions influence skeletal morphology, and potentially confound recognition of genetic change, can be most meaningfully addressed with living taxa. The marine encrusting bryozoan, *Electra pilosa* (L.), was used to assess the magnitude of environmental effects on zooecium-level skeletal morphology at different spatial scales. The latter included environmental effects or factors ranging in level from (1) micro-environmental (variation within and among immediately adjacent colonies), (2) meso-environmental (small-scale colony positional effects among colonies within a common habitat), and (3) macro-environmental (morphological variation among colonies from recognizably different environmental settings).

Macro-environmental influence on zooecial morphology for colonies among localities (10 km-scale) can be detected by comparison of colonies from wave-protected/tidal-dominated versus open coast/wave-dominated settings, and this accounted for ~7.5% of the observed morphological variation. Meso-environmental variation – that is, small-scale (10^1 to 10^2 m) systematic (nonrandom) differences that would go undetected in a geologic setting – had a minimal deterministic influence on zooecial morphology, and accounted for ~2.5% of observed variation.

Variation among colonies from the same site was highly significant. Much of this morphologic variation (~30%) is attributable to genotypic variation among colonies, but micro-environmental sources cannot be excluded (10^1 to 10^2 cm-scale). Variation within colonies, accounting for ~60% of the observed morphological variation, can be further partitioned into Micro-environmental differences, ~40% (10^1 to 10^2 mm-scale) associated with spatio-temporal position, and the Life History of individual modules (~20%). Environmental levels (factors) that are associated with significant morphological effects can also be recognized by their sedimentological properties, which can be preserved in the geologic record. Thus, results from this and similar studies have relevance for and can potentially be directly applied to studies of fossil organisms.

1. Introduction

Most fossil taxa are identified to the species level on the basis of preserved phenotype, usually comprised of skeletal hard parts. The phenotype of an organism is produced as a result both of the genetic composition of the organism and the influences of the environmental conditions under which the organism develops and grows. Paleontologists are potentially disadvantaged in studies of microevolution because they cannot (routinely) access the genetic composition of fossil specimens. However, the effects and likely extent or importance of environmentally induced variation can be tested directly in taxonomic groups with extant members.

The overall goal of this study was to evaluate the extent to which consequences of environmental variation (at scales that would not be readily evident in the rock record to a paleontologist, e.g. within sedimentary or biofacies) affect the expression of skeletal morphology. That is, how much "hidden" ecophenotypic variation among fossil populations should paleontologists expect to routinely encounter and potentially "mistake" for genetic variation in their interpretation of the fossil record? Bryozoa provide an excellent study group to address questions regarding sources of phenotypic variation because their clonal, modular growth allows for the analytical partitioning of morphological variation into its environmental and genetic sources (Beklemishev, 1969; Boardman et al., 1970; Abbott, 1973; Boardman et al., 1973; Schopf, 1976; Boardman et al., 1983). Each bryozoan colony is a single

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genotype comprised of modular individuals and therefore variation in size and shape of each individual zooecium within a colony is environmentally influenced over the short time and small space within which that portion of the colony grows (Boardman et al., 1970; Schopf, 1976). Such micro-environmentally induced variation within the colony can result from the interplay of external and internal biological and physical sources of variation within colonies (e.g. pathogens, subcolony-scale heterogeneity of substrate; Hageman et al., 2011). By contrast, potential within-colony variation that is not environmentally induced includes all that variation which is developmentally programmed (astogenetic) and that ontogenetic portion of development shared by all individuals, which is manifested as a gradient from immature growing edges to mature proximal regions (Boardman et al., 1970; Schopf, 1976).

The primary question addressed in the present study was: at which level of a spatial hierarchy (Table 1) is environmentally-induced variation most significant to the skeletal phenotype of a representative bryozoan species? That is, can micro-environmental variation within and among colonies (mm- to cm-scale) have as much, or more, effect on the phenotype as meso-environmental heterogeneity (10 to 100 m-scale) or macro-environmental variation (10 to 100 km-scale)? This question arises from previous studies of bryozoans in which environmental effects were identified, but the scale at which their influence had the greatest effect on morphology either was not clear, or was not fully accounted for as environmentally induced variation (Farmer and Rowell, 1973; Taylor and Furness, 1978; Key, 1987; Hageman, 1994, 1995; Hageman et al., 2002; Hageman and Sawyer, 2006). Accordingly, the focus of the present study was to partition sources of environmental variation within a spatial hierarchy. Thus, we ask: (1) what is the magnitude of systematic (nonrandom) micro-environmental variation within a single colony (mm to cm-scale) relative to different colonies that had grown in close spatial proximity (<m-scale)? (2) What is the magnitude and significance of meso-environmental variation among closely spaced colonies (m-scale) relative to groups of colonies in the same environment, but located tens of meters to 1.5 km apart? (3) What is the systematic variation among groups of colonies at one locality relative to those at a geographically different locality (habitat) 10s to 100s km apart? and (4) How does systematic variation among groups of colonies from natural environments compare with that from specimens grown in controlled, stable laboratory conditions? An important final question relates to how these sources of variation will be interpreted when they are not accounted for directly; i.e., when levels of the hierarchy are pooled by choice, or as a consequence of paleoenvironmental resolution. Thus, we ask: (5) are significant sources of environmentally-induced morphologic variation associated with environmental effects that cannot be determined independently in the geologic record based on grain size, sorting, composition and sedimentary structures? Although phenotypic variation within and among sedimentary facies that could be discriminated in the rock record were not tested directly in this study, implications of hierarchical scale in modern environments may serve as a proxy for variation within and among the detectable limits of paleoenvironments discernible in the geologic record (Table 1).

In the present study, physical distance is an appropriate proxy for environmental hierarchy (Table 1), but in other settings significant macro-environmental effects can exist over very different scales. Thus, Table 1 cannot be generalized too broadly and a clear and appropriate definition of categories is necessary for any study of environmentally induced morphologic variation. The broader relevance of these five questions lies in their possible consequences for the development of concepts relating to phenotype-based species recognition, and therefore to fossil record studies of microevolutionary patterns and the processes that may have led to speciation. The present results showed sufficient evidence of environmental influence being relevant in its extremes, but there was virtually none at scales that would go unrecognized by paleontologists, or that would compromise paleontological studies of micro-evolution and speciation.

2. Materials and methods: common to all specimens

Four data sets were applied in this analysis. Data Set-1 (ClaSei) and Data Set-2 (RonPoi) provide for a study of Meso-environmental variation (Table 1, level-IIa) in the bryozoan *Electra pilosa* (Linné, 1767) from sample sites within two study regions – each comprising ~1.5 to 2.0 km-scale transects, through successively smaller sales (Table 1, levels IIb and IIIa) – down to micro-environmental variation (Table 1, level-IIIb) among cm-scale patches within colonies of a single genotype. Data Set-3 (EnvGrd) permitted the study of hierarchical environmental variation in *E. pilosa* both within and among macro-environments (Table 1, levels Ia and Ib) at a scale 10s to 100s of km within and among two environmental gradients of wave-protected to wave-exposed settings along the west coast of Scotland.

Nested analysis of variance was applied to Data Sets 1–3 to evaluate the relative importance of environmental effects at different hierarchical levels. Details of ANOVA models are given below and summarized in the corresponding Supplementary Data 1–3. For each of the three ANOVA models, the Sum of Squares was calculated by three independent methods; (i) in Excel using equations from primary sources (Doncaster and Davey, 2007; Sokal and Rohlf, 2012), (ii) using SuperANOVA™ (v. 1.11), and (iii) JMP Pro v. 9.0.0. The results conformed to six decimal places. F-values were then calculated for each level and evaluated for significance. The variance components, and percentage of the total variance represented by each level in the ANOVA model, were calculated using the Bayesian method in JMP (variability/gauge chart) v. 9.0.0. Because some of the variance component percentages were small, the average value for an effect level within a model was calculated across the five morphological variables using an arcsine transform of the original percentages (Supplementary Data 1–3).

Data Set-4 comprised the first three data sets, with two additional localities on the west coast of Scotland (Fig. 2) and an eleventh suite of data denoted as locality GatLab. The latter were conspecific specimens of *E. pilosa* cultured in a controlled laboratory setting at the University of St Andrews (Bayer et al., 1994; Hageman et al., 2009, highest food level, D). All Data Sets are available in Supplementary Data 1–4.

The morphological characters that are used in modern systematics for species differentiation of *Electra* and the closely related genus

Table 1
Definition of environmental scales as used in this study and the potential to recognize equivalent levels in the geologic record.

Hierarchical environmental level	Scale (radius between samples)	Modern record (this study)	Potential geologic record
Ia. Macro-environmental	10^1 to 10^2 km	Geographical distance (*among* sea lochs)	Different *or* same sedimentary facies
Ib. Macro-environmental	10^1 km	Geographical distance (*within* sea lochs)	Different sedimentary facies
IIa. Meso-environmental	10^1 to 10^2 m	Unrecognized potential variation within an environment (stations)	*Not* differentiable geologically
IIb. Meso-environmental	10^1 m	Unidentified sources within a meso-environment (substations)	*Not* differentiable geologically
IIIa. Micro-environmental	10^1 to 10^2 cm	Unidentified sources within a meso-environment (among colonies at arms reach)	*Not* differentiable geologically
IIIb. Micro-environmental	10^0 to 10^2 mm	Unidentified sources within a micro-environment (*within* colonies)	Identifiable in (partially) complete specimens but not small fragments

Einhornia (Winston and Hayward, 2012; Nikulina et al., 2013a, 2013b) are based largely on the presence/absence of discrete conditional states of features. These include: (1) colony budding and branching patterns, (2) zooid shape, (3) relative size of opesia (opening) and gymnocyst (frontal skeleton) to each other and to the overall zooid, (4) presence/absence of internal pits in the gymnocyst, (5) features (rims, lips, textures) related to the margin of the opesia, (7) features related to the operculum (shape, size, mineralization) and color of the living tissue, neither of which are available in fossils, and, most importantly for this group, and (8) the typically discrete/characteristic nature of spines (position, size, shape, orientation, calcified vs. chitinous). All the foregoing were evaluated for each specimen in all data sets and were found to be invariant, thus confirming the morphological identity of all as *Electra pilosa*.

2.1. Laboratory methods and data collection

Field specimens of *Electra pilosa* (Fig. 1) were collected from macroalgal fronds cut to a size that included one to three colonies. These were rinsed in freshwater and preserved in 70% ethanol. Alcohol-preserved specimens were returned to the laboratory in Boone, North Carolina, USA, where colonies of *E. pilosa* were digitally photographed while still attached to their original algal substratum. The algal frond and *E. pilosa* colony were kept flat by placing them between two bound glass slides (7.0 × 4.0 × 0.05 cm). The slides encasing the algal frond and colony were immersed in a large petri dish with 70% ethanol, filled to just cover the upper glass slide with no internal air bubbles. An Olympus DP10 digital camera mounted on an Olympus SZX-12 stereo microscope was used to acquire images of the colonies and zooecia for measurement. The suite of morphometric zooecial characters that was measured included opesia width (OW), zooecium width (ZW), opesia length (OL), zooecium length (ZL), and zooecium area (ZA) (Fig. 1c). These measures, and the environmental setting of the inter- to sub-tidal biotope of western Scotland (Fig. 2) were common to all three data sets.

Molecular data which would enable the calculation of genetic distances were not available for the present specimens. However, Nikulina et al. (2007) recorded 11 mitochondrial 16S rDNA haplotypes of *Electra pilosa* from 17 colonies sampled around the coast of Denmark. The geographic distance between collection sites for those colonies (Helgoland, North Sea to Kattegat, Western Baltic) ranged from <10 m to ~250 km (Nikulina et al., 2007). Among the 17 colonies, two sympatric haplotype groups were recognized, with up to nine mutational steps within groups and 43 steps between groups, but these still were regarded as a single species compared to molecular and morphological differences with other congeners included in their study (*E. posidoniae* Mediterranean; *E. scuticifera* New Zealand (Nikulina et al., 2007; Nikulina, 2008a)). Thus, while the present specimens of *E. pilosa* from western Scotland can be expected to exhibit some degree of variation among genotypes, there is no expectation of geographically dictated separation of populations. Being a malacostegan bryozoan with long-lived swimming larvae (up to two months), *E. pilosa* is likely to display relatively high levels of gene interchange over the distances studied (Yoshioka, 1982).

2.2. Selection of zooecia for measurement

Zooecia were selected for measurement using the following criteria. (1) Zooecia were as close to the growing edge of the colony as possible, but showed complete formation of the zooecium. Thus all zooecia measured were of closely similar ontogenetic development. (2a) A Patch (3 columns [A–C] × 4 rows [1–4]) of 12 healthy, vigorous zooecia was chosen such that each zooecium could be identified by its row/column designator, e.g., A1 or C3 (Fig. 1a). (2b) Each Patch was of an established budding axis (not the result of lateral budding) and included no column bifurcations. (2c). Each Patch also was the result of budding into originally unrestricted space; thus, there was no crowding due to other epibionts, edges/holes in algal frond, or self crowding due to interaction with other primary budding columns of the same colony. (3a) Five zooecia (A1, A4, B3, C1, and C4) were measured for each patch (Fig. 1a), the objective being to maximize the distance among zooecia within a patch (Hageman et al., 1999, 2002). (3b) If one or more of the zooecia in step 3a did not meet the criteria for measurement, then other zooecia within the patch were substituted. Rarely, adjacent zooecia outside of the primary patch were required, e.g. column D, or row 5. (4) For some analyses, two patches of five zooecia per colony were required, in which case a second patch (Patch-B) was selected as a region on the colony orientated ~180° to the growth direction from the first, providing that it met the criteria listed above. Five zooecia were measured from Patch-B using the criteria listed above.

2.3. Data collection

For each colony a low magnification (×5) image was taken as a reference map. For each patch selected within a colony, two photographs (LowMap [×12×], HighMap [×24]) were taken, to allow for identification of the patch position within the colony and in order to label the individual zooecia within each Patch. Each zooecium to be measured was photographed to near full screen size in the final image. The image analysis program NIH Image v. 1.61 was used to measure characteristics of the zooecia, (1 μm = 0.62 pixels) recorded in millimeters.

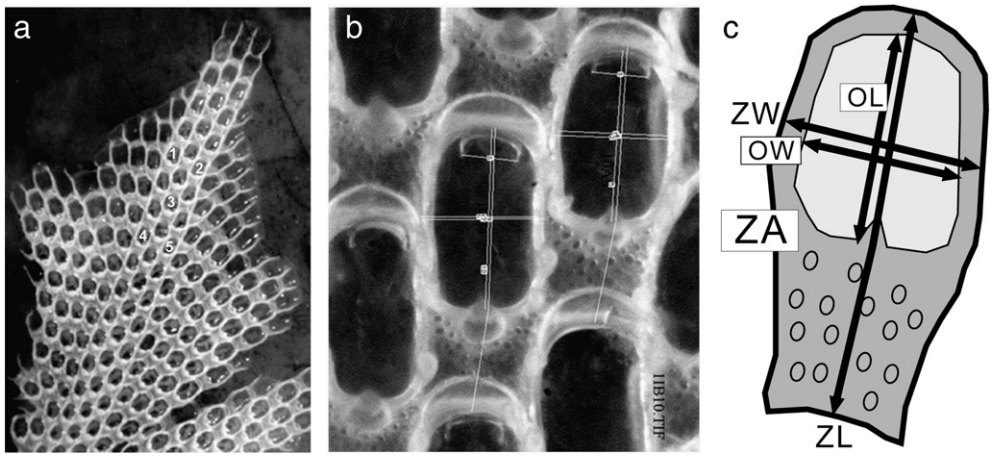

Fig. 1. The Bryozoa *Electra pilosa* (Linné, 1767), with maps of zooecia selected for measurement. (a) Low magnification of a colony (5×), (b) example image from which measurements were made, (70×), and (c) zooecium with placement of measurements shown (100×).

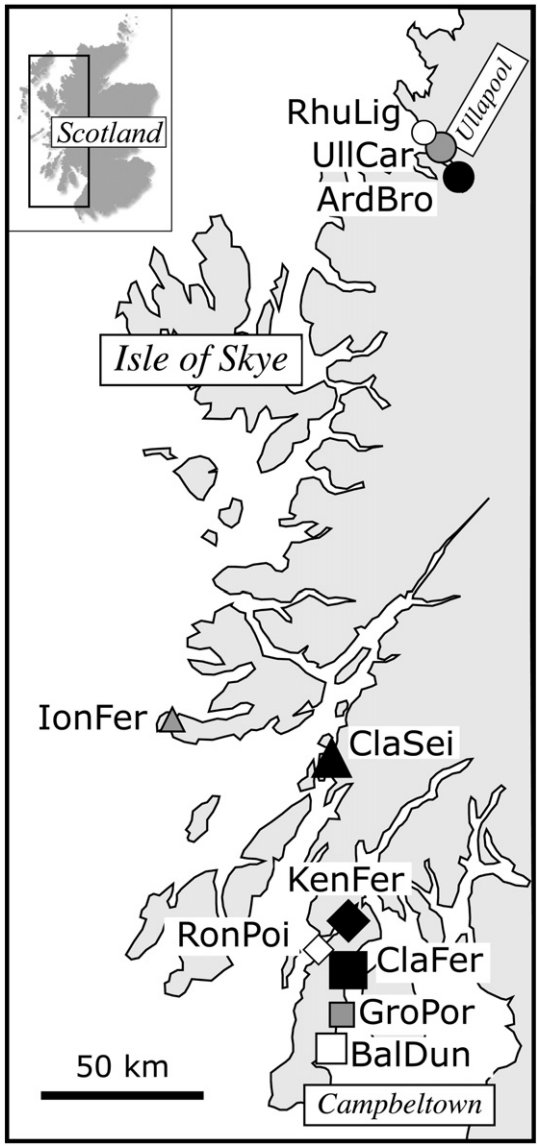

Fig. 2. A. Map of western Scotland sample localities; RhuLig (Rhue Light House), UllCar (Ullapool Caravan Park), ArdBro (Ardcharnich Loch Broom), IonFer (Iona Ferry), ClaSei (ClaSei), KenFer (Kennacraig Ferry), RonPoi (Ronachan Point), ClaFer (Claonaig Ferry), GroPor (Grogport), BalDun (Ballochagair Dun). Circular symbols in the north, square symbols in the south, triangles and diamonds in the central. Black symbols are the most protected localities (terrestrial influence), white symbols most exposed (open sea), gray symbols intermediate. Large symbols have the greatest morphological variation within a locality (average coefficient of variation > 13.0) and small symbols are the least variable localities (average coefficient of variation < 9.0).

3. Materials and methods: Data Set-1, meso-environment (Clachan Seil)

Data Set-1 permitted an analysis of systematic (nonrandom) meso-environmental variation across a spatial hierarchy from a scale of ~1 km among colonies, down to ~1 cm within colonies (Fig. 3a, Table 1, levels IIa, IIb, IIIa, IIIb).

3.1. Characterization of Clachan Seil meso-environment

Clachan Seil (ClaSei) is a narrow, shallow tidal rapid, open on both ends but with elevated tidal sills at each end (Todd et al., 1988) connecting the Firth of Lorne to the north with Seil Sound (Fig. 3a), and the Sound of Jura to the south (Fig. 2). Because of the sills, the ebb tidal height never falls below a set level within the rapid and infralittoral organisms are permanently immersed. Clachan Seil separates the Isle of Seil from the Argyll mainland (Fig. 3a), and the tidal rapid is protected from the open ocean by Seil island itself and a number of the Inner Hebrides islands (Luing, Lunga, Scarba, Jura, Shuna). Hydrographically, the rapid is characterized by intense tidal currents (up to 0.9 m s^{-1}, Todd, 1998), with a maximum tidal amplitude of ~2 m (Todd and Turner, 1986; Todd et al., 1988). Clachan Seil is 1.3 km long and ranges from 30 to 120 m wide (Todd et al., 1988). *Laminaria digitata* (Hudson) Lamouroux dominates the permanently immersed infralittoral, while *Fucus serratus* L., *Ascophyllum nodosum* (L.) le Jolis and *Himanthalia elongata* (L.) Gray characterize the lower intertidal. The unilaminate, encrusting bryozoan *Electra pilosa* is abundant both on algal and rock substrata, but was collected for this study from *L. digitata* because this kelp provided the most uniform surface among all specimens.

ClaSei: Clachan Seil (56° 19.06′ N, 5° 34.97 W), narrow channel between the mainland and Seil island, from Seil Sound to the Firth of Lorn.

3.2. Experimental design (Clachan Seil)

The ANOVA model (four levels nested, all levels random) was derived from Sokal and Rohlf (2012, p. 281, Box 10.1), as summarized in Supplementary Data 1, and formulated as:

Level: number of categories (category designations, spatial scale, environmental level from Table 1)

Station: n = 3 (I, II, III, separation 0.5 to 1.5 km, meso-environment IIa)
Substation: n = 3 (a, b, c, separation 10 to 30 m, meso-environment IIb)
Colony: n = 5 (1–5, separation ~0.5 m, micro-environment IIIa)
Patch: n = 2 (A, B, separation ~1 cm, micro-environment IIIb)
Zooecium: n = 6 (1–6, separation 1 to 5 mm).

Parameters for the nested ANOVA for Data Set-1 (Clachan Seil) are given in Supplementary Data 1, with the null hypotheses as:

Ho1. no difference among the three stations (meso-environment IIa);

Ho2. no difference among the three substations within each station (meso-environment IIb);

Ho3. no difference among the three colonies within each substation (micro-environment IIIa);

Ho4. no difference among the two patches within each colony (micro-environment IIIb).

3.3. Sampling protocol (Clachan Seil)

Specimens were collected 1 Aug 2000 during the lowest neap tide in the central part of the channel. Fronds of *Laminaria digitata* with live, full-grown but discrete colonies of *Electra pilosa* were cut free with scissors and stored individually in labeled sample bags. Colonies selected for potential study were distinct and separate, and each included the ancestrula, but multiple colonies could exist on a single algal frond. Each substation comprised multiple but separate fronds collected within two arms length (<2 m). A minimum of ten colonies was collected and preserved per substation, three of which were later selected randomly for study. Three substation sites, separated by 20 m along the length of the channel, were collected per station (Fig. 3a).

The three stations in Clachan Seil represent closely comparable environments, as characterized by the presence of *Laminaria* (intermediate water depth of 1–1.5 m at low neap tide, Fig. 3a). No systematic environmental effects attributable to station position were predicted a priori.

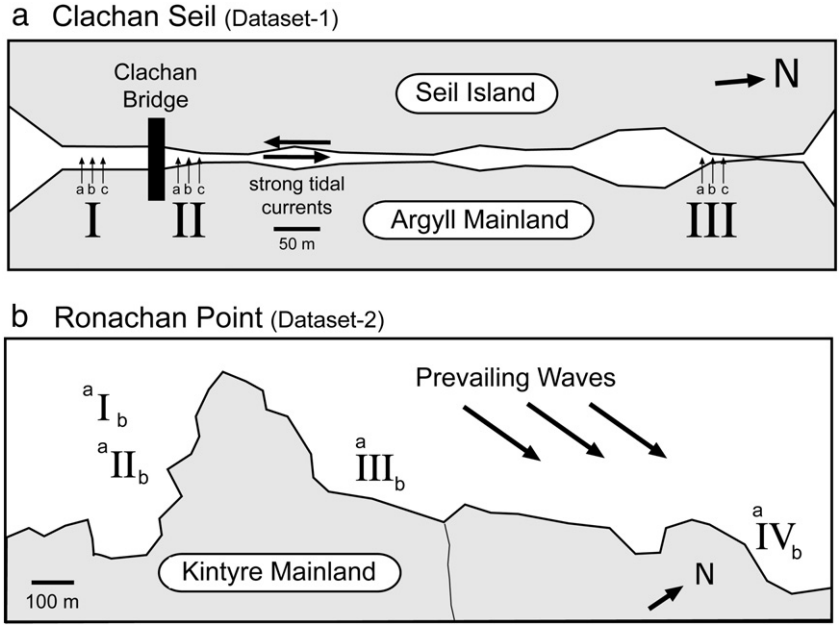

Fig. 3. Schematic maps of the two primary sample areas A. Clachan Seil (ClaSei) and B. Ronachan Point (RonPoi). See Fig. 2 for locations. Large Roman numerals are sample station numbers and letters a–c are sample substations labels (Tables 2–3).

4. Materials and methods: Data Set-2, meso-environment (Ronachan Point)

The purpose of Data Set-2 was to test for systematic (nonrandom) environmental variation within a spatial hierarchy from a second site at a scale of ~1 km among colonies, to ~1 cm within colonies (Fig. 3b, Table 1, levels IIa, IIb, IIIa, IIIb).

4.1. Characterization of Ronachan Point meso-environment

Ronachan Point (RonPoi) is a minor promontory on the northwest coast of the Kintyre Peninsula (Fig. 2) and is a variably rocky to sandy shore. This section of coast is protected from storm waves and the prevailing southwesterly winds by the Isle of Gigha which is 4 km offshore, but the RonPoi shore site still is wave-dominated. Rocks and boulders in the shallow sublittoral are covered with *Fucus serratus*, which are encrusted by *Electra pilosa* and other epibionts. Several rocks and boulders ~10–20 m offshore are exposed during the lowest neap tide and these served as the collection site. Specimens of *E. pilosa* were collected from four stations (*F. serratus* on boulders) in paired substations on the seaward and leeward sides of the boulders (Fig. 3b).

RonPoi: Ronachan Point (55° 44.27′ N, 5° 35.99 W), northeast shore of the Sound of Gigha, below the car park, south of Dunskeig Bay.

4.2. Experimental design (Ronachan Point)

The ANOVA model (four levels nested; position level (either side of rock), fixed; all other levels random) was derived from Sokal and Rohlf (2012, p. 289, Box 10.3), as summarized in Supplementary Data 2, and formulated as:

Level: number of categories (category designations, spatial scale, environmental level from Table 1)

Position: n = 2 (exposed, protected, separation ~1 m; meso-environment IIb)
Station: n = 4 (I, II, III, IV, separation 0.5 to 2.0 km; meso-environment IIa)
Colony: n = 4 (1–4, separation ~0.5 m; micro-environment IIIa)
Patch: n = 2 (A, B separation ~1 cm; micro-environment IIIb)
Zooecium: n = 6 (1–6, separation 1 to 5 mm).

Parameters for the nested ANOVA for Data Set-2 (RonPoi) are given in Supplementary Data 2, with the null hypotheses as:

Ho1. no difference among the two positions (meso-environment IIb);

Ho2. no difference among the four stations within each position (meso-environment IIa);

Ho3. no difference among the four colonies within each substation (micro-environment IIIa);

Ho4. no difference among the two patches within each colony (micro-environment IIIb).

4.3. Sampling protocol (Ronachan Point)

Specimens were collected 25 May 2001 during the lowest neap tide. Fronds of *F. serratus* with live, full-grown but discrete colonies of *E. pilosa* were cut free with scissors and stored individually in labeled sample bags. Each substation comprised multiple but separate fronds collected from opposite sides of a boulder (<1.0 m). A minimum of ten colonies was collected and preserved per substation, four of which were later selected randomly for study. Four substation sites were selected on the basis of their similarity in size, location and occurrence of *F. serratus* and *E. pilosa* (Fig. 3b).

The four stations at Ronachan Point represent comparable environments as characterized by the presence of *Fucus serratus* (Fig. 3b). No systematic environmental effects based on station position were predicted a priori; however, it was suspected that the wave-exposed versus wave-protected sides of boulders (Position a- and b-) may comprise an effect in the analysis.

5. Materials and methods: Data Set-3, macro-environments (Environmental Gradient)

Data Set-3 permitted the assessment of systematic (nonrandom) environmental variation of selected characters in *Electra pilosa* among environments within the environmental limits of its local distribution. This gradient from innermost protected inland = ArdBro, *to* middle =UllCar *to* outermost exposed = RhuLig (Fig. 2) was repeated ~150 km to the south, with a comparable gradient of

inner-protected ClaFer, GroPor, to outer-exposed BalDun (Fig. 2). These data thus allow for comparison of macro-environments at levels Ia and Ib (Table 1).

The ANOVA model (three factors, crossed and then nested; location factor, fixed; all other factors random) was derived from Doncaster and Davey (2007, p. 111, Model 3.4(i)), as summarized in Supplementary Data 3 and formulated as:

Region: n = 2 (northern vs. southern, separated ~150 km, macro-environment Ia).
Position: n = 3 (inner, middle, outer; separated ~25 km, macro-environment Ib).
Region × Position: n = 6 (interaction, macro-environments Ia & Ib)
Colony [within Region × Position]: n = 6 (separated 1 m to 10 m within position, meso-environments IIa & IIb)
Residual = zooecium (within colony): n = 6 (separated 2 mm to 2 cm, within algal frond).

Parameters for the ANOVA for Data Set-3 (EnvGrd) are given in Supplementary Data 3, with the null hypotheses as:

Ho1. no difference among the two regions (north and south).

Ho2. no difference among the three positions (inner, middle, outer) within each area.

Ho3. no difference in the six combinations of regions and positions.

Ho4. no difference in the six colonies within the six combinations of regions and positions.

5.1. Characterization of environmental gradient of macro-environments

Localities represent the range of environments in which *Electra pilosa* was observed growing on *Fucus serratus*. Neither *F. serratus* nor *E. pilosa* was observed in areas of an extremely stable water column with little tidal flow (e.g., restricted and closed heads of sea lochs), or on high-energy coasts exposed to the open ocean. Although not exhaustive, the localities selected for the present study do represent the breadth of environments within the occupied range of *E. pilosa* in western Scotland.

Localities along the Kintyre Peninsula (Fig. 2) included the following. ClaFer: Claonaig Ferry (55° 45.04′ N, 5° 23.33 W), west shore of Kilbrannan Sound, south of the dock of the ferry to the Isle of Arran. GroPor: Grogport (55° 38.71′ N, 5° 28.78 W), west shore of Kilbrannan Sound, near car park north of the village of Grogport. BalDun: Ballochagair Dun (55° 29.48′ N, 5° 30.76 W), bedrock shoreline ~1 km south of Ugadale, due east of fort (dun) ruins. Localities in the northern region of western Scotland (Fig. 2) included the following. ArdBro: Ardcharnich Loch Broom (57° 50.18′ N, 5° 04.48 W), East Shore of Loch Broom ~1 mi. south of Ardcharnich. UllCar: Ullapool Caravan Park (57° 53.67′ N, 5° 10.01 W) on the west edge of town, opening to Loch Broom. RhuLig: Rhue Light House (57° 55.41′ N, 5° 13.11 W), bedrock at the southern end of a beach at the opening of Loch Broom.

5.2. Sampling protocol (environmental gradient)

Specimens were collected during low neap tide. Individual fronds of *Fucus serratus* bearing colonies of *Electra pilosa* were cut free and placed in a storage bag with sea water. In the field, fronds with colonies were washed in three baths for about 1 min each of fresh water, diluted bleach solution (2% to remove delicate epibionts), and fresh water rinse. Moist fronds with colonies were kept cool with ice until they could be soaked in 70% ETOH for 24 h, drained for transport and then archived in 70% ethanol for subsequent measurement and analysis. This procedure retained soft parts of the colony such as cuticle, opercula and unmineralized spines.

6. Materials and methods: Data Set-4, macro-environments (western Scotland)

Principal component analysis was undertaken using Data Set-4, which comprised all of the data in Data Sets 1, 2 and 3, plus two more localities, Kenfer, IonFer (Fig. 2) and the addition of the appropriate number of specimens of specimens and measurements from *Electra pilosa* colonies grown under controlled laboratory conditions (Hageman et al., 2009) equivalent to an eleventh locality.

Coefficients of Variation (CV) were calculated for each character at each locality and compared among localities (macro-environments) in order to compare the relative amount of variation among localities.

6.1. Characterization of additional western Scotland macro-environments

The two additional field localities for Data Set-4 (Fig. 2) were KenFer: Kennacraig Ferry (55° 48.43′ N, 5° 28.99 W), south shore of West Lake Tarbert, to the northeast of the dock for the ferries to the Isle of Islay and Jura, and IonFer: Iona Ferry (56° 19.93′ N, 6° 23.39 W), bay southwest of the dock for the ferry to the Isle of Mull. The sampling protocol was as outlined in Section 5.2 for Data Set 3.

7. Results

7.1. Data Set-1, within Clachan Seil (meso- to micro-environments)

The significance level (from Uitenbroek, 2012) and percentage of variance accounted for by each of the five morphological variables in the four-level nested ANOVA for the meso- to micro-environment analysis [Data Set-1 (Clachan Seil, Fig. 3a)] are summarized in Table 2. Sum of Squares tables are provided in Supplementary Data 1.

7.1.1. Among stations and substations (within Clachan Seil)

There was no significant difference among the three stations (Fig. 3a, meso-environmental level IIa) for any of the five morphological characters (Table 2, fail to reject H_o^1), The percentage of overall variance accounted for by differences among stations was small (4.9%, Table 2). There was no significant difference among the four substations within stations (meso-environmental level IIb) for any of the five morphological characters (Table 2, fail to reject H_o^2). Among substations, the character zooecium area did account for a relatively large amount of the overall variance (13.8%) and approached significance ($0.1 < p < 0.05$).

Although not significant among either stations or substations (meso-environments), characters associated with "length" accounted for slightly more of the variance (6.1% to 9.0%) than did characters associated with "width" (2.0% to 2.8%, Table 2).

7.1.2. Among and within colonies (within Clachan Seil substations)

Variation among colonies, within substations, was significant for all characters (Table 2, reject H_o^3). Zooecium length displayed the highest level of significance ($p \leq 0.0001$) and zooecium width the least ($p < 0.01$). Among-colony variation accounted for an average of 24.2% of the total variation for each character (Table 2). Zooecium length was anomalous in accounting for a much greater (42.0%) proportion of the variance among colonies. The effect of this level is attributable either to micro-environmental level IIIa (Table 1), genotypic differences among colonies, or a combination of the two (see discussion 8.1.1).

For variation among patches within colonies (micro-environmental level IIIb), opesia length and zooecium area were highly significant (Table 2, reject H_o^4, $p \leq 0.0001$). Zooecium length was the only character that was not significant among patches within colonies. Among-colony variation accounted for an average of 12.2% of the total variation for each character (Table 2). Zooecium length was once again anomalous in accounting for a far lesser proportion (0.8%) of the variance among colonies.

Table 2
Summary of meso-environmental variation for Data Set-1 (Clachan Seil, Fig. 3a) Supplementary Data 1) for four level nested ANOVA. Percentage of variance accounted for by each factor (within each locality) and significance level. Variance components for each factor sum to 100%. The average of all five characters for each locality is based on the arcsine transform of each and therefore the back-transformed values do not sum to 100% (Clachan Seil, Fig 3a, Supplementary Data 1).

Character	Station	$Substation_{(Station)}$	$Colony_{(Substation)}$	$Patch_{(Colony)}$	Residual
	(I, II, III)	(1, 2, 3)	(1, 2, 3)	(a, b)	
	%var p(H_o^1)	%var p(H_o^2)	%var p(H_o^3)	%var p(H_o^4)	%var
Opesia width, OW	2.7% ns	2.0% ns	17.2%**	13.0%**	65.2%
Zooecium width, ZW	2.5% ns	2.8% ns	15.1%*	15.6%***	64.0%
Opesia length, OL	8.0% ns	7.7% ns	27.2%**	22.0%****	35.0%
Zooecium length, ZL	6.1% ns	9.0% ns	42.0%****	0.8% ns	42.1%
Zooecium area, ZA	6.6% ns	13.8% ~	22.2%**	17.1%****	40.3%
Average	4.9%	6.4%	24.2%	12.2%	49.3%

ns, not significant; ~, approaching significance $p \leq 0.1$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

7.1.3. Residual (Clachan Seil)

The average residual variation (variance not accounted for by the model at any of the levels Table 2) for Clachan Seil was 49.3%, and ranged from 35.0% to 65.2%. Characters associated with "width" had residual variance (64.0% to 65.2%) which was greater than characters associated with "length" (35.0% to 42.1%).

7.2. Data Set-2, within Ronachan Point (meso- to micro-environments)

The significance level and percentage of variance accounted for by each of the five morphologic variables in the four-level nested ANOVA for the meso- to micro-environments Data Set-2 (Ronachan Point, Fig. 3b) are summarized in Table 2. Sum of Squares tables are provided in Supplementary Data 2.

7.2.1. Among positions and stations (within Ronachan Point)

With the exception of zooecium length, there was no significant difference between the two positions (exposed vs. protected, meso-environmental level IIb) for morphological characters (Table 3, fail to reject H_o^1). Zooecium length was significant between positions (reject H_o^1, $p < 0.01$). The overall average variance accounted for by differences between positions was 2.9% (Table 3). Zooecium length is an example of a character for which a relatively small amount (2.6%) of the total variation was systematic and therefore significantly different between positions on the same boulder (exposed vs. protected).

There was no significant difference among the four stations nested within position (meso-environmental level IIa) for any of the five morphological characters (Table 3, fail to reject H_o^2), and the percentage of overall variance accounted for by differences among stations was accordingly small for all characters (0.2% to 4.1%, Table 3).

7.2.2. Among- and within-colonies (within Ronachan Point stations)

Variation among colonies, within stations, was significant for all characters except zooecium length (Table 3, reject H_o^2). Variation among colonies accounted for 24.2% to 27.1% of the total variation for (Table 3), except for zooecium length (7.1%). As for Data Set-1, the effect associated with this level either is micro-environmental level IIIa, genotypic differences among colonies, or a combination of the two.

Variation among patches within colonies (micro-environmental level IIIb, Table 1) was highly significant for all five characters (Table 3, reject H_o^3, $p \leq 0.0001$, 31.5% to 45.5% of the total variance), with zooecium length accounting for the most variance among patches within colonies.

7.2.3. Residual (Ronachan Point)

The average residual variation (Table 3) for Ronachan Point was 36.1% and ranged from 32.0% to 45.2%. Values for residual variation were less for Ronachan Point than for Clachan Seil for each character and level (Table 3). Characters associated with "width" had residual variance of 32.0% to 34.5%, which was less than characters associated with "length" at 40.3% to 42.5%. This relationship is the same as that observed in Data Set-1, Clachan Seil (Table 2).

7.2.4. Principal component analysis of meso-environmental variation (ClaSei and RonPoi)

Patterns of morphological variation (overlap in morpho-space of zooecia among stations and substations, with differentiation of zooecia into groups among and within colonies) are evident in scatter plots of PCA scores (Supplemental Data 1 and 2) for zooecium on principal component axes based on the five morphometric characters for both Data Set-1 (ClaSei) and Data Set-2 (RonPoi).

Scatter plots of PCA scores visually illustrate the ANOVA results for both Data Sets showing that, with few exceptions, the greatest percentage and significance of morphological variation is at low levels (among colonies at local sites and within colonies). The latter accounted for about 42% of the total observed variance, whereas potential sources of meso-environmental variation (among local sites within a macro-environment) were not significant and accounted for only about 8% of the total variation (Tables 2 and 3).

Table 3
Summary of meso-environmental variation for Ronachan Point (Fig. 3b, Data Set-2, Supplementary Data 2) for nested ANOVA. Percentage of variance accounted for by each factor (within each locality) and significance level. Variance components for each factor sum to 100%. The average of all five characters for each locality is based on the arcsine transform of each and therefore the back-transformed values do not sum to 100%. Exp. = exposed, Prot. = protected; other abbreviations as for Table 2.

Character	Position	$Station_{[Position]}$	$Colony_{(Station)}$	$Patch_{(Colony)}$	Residual
	(Exp., Prot.)	(I, II, III, IV)	(1, 2, 3, 4)	(a, b)	
	%var p(H_o^1)	%var p(H_o^2)	%var p(H_o^3)	%var p(H_o^4)	%var
Opesia width, OW	3.3% ns	4.1% ns	27.1%**	33.6%****	32.0%
Zooecium width, ZW	2.8% ns	3.2% ns	26.0%**	33.5%****	34.5%
Opesia length, OL	3.2% ns	0.9% ns	24.2%**	31.5%****	40.3%
Zooecium length, ZL	2.6% **	2.2% ns	7.1% ns	45.5%****	42.5%
Zooecium area, ZA	2.7% ns	0.2% ns	25.5%*	39.9%****	31.7%
Average	2.9%	1.8%	21.3%	36.7%	36.1%

7.3. Data Set-3 (environmental gradient) among and within macro-environments

The significance level and percentage of variance accounted for by each of the five morphologic variables in the three-level crossed and then nested ANOVA for the macro- to meso-environments, Data Set-3 (Environmental Gradient) are summarized in Table 4. Sum of Squares tables are provided in Supplementary Data 3.

7.3.1. Among regions and positions (environmental gradient)

Differences among and within the six localities (macro-environments) are mixed in their level of significance among the five morphological characters by both region (north vs. south) and position (inner-protected to outer-exposed). For the factor Region, opesia length (OL) displayed the only significant difference between north and south (Table 4, reject H_o^1 at $p < 0.01$), yet region accounted for only 6.2% of the total variation for opesia length (average of all characters for region was 2.7%, Table 4).

For the factor Position (inner, middle, outer) characters associated with "width" (OW, ZW) were not significant (Table 4). Opesia length was highly significant among positions (Table 4, reject H_o^2 at $p < 0.0001$) and zooecium length and area also were significant (Table 4, reject H_o^2 at $p < 0.01$). The percentage of variance for Position accounted for by each variable reflects their level of significance (Table 4, "width" ~3%; "length-area" ~13%).

None of the morphological characters were significant for the interaction of Region × Position (fail to reject H_o^3), but this interaction did account for an average of 2.7% of the total variation (Table 4), which was the same as for the factor region.

7.3.2. Among colonies (environmental gradient)

Variation among colonies, within the region × position interaction, was highly significant for all five characters (Table 4, reject H_o^3 at $p \leq 0.0001$), with variation among colonies accounting for an average of 22.7% of the total variation (Table 4).

7.3.3. Residual (environmental gradient)

Residual variation, among zooecia within colonies, was greatest for characters associated with "width" (70.4% to 75.4%, Table 4). The residual was the least for opesia length (46.6%) and intermediate for zooecium length and area (~61%).

7.4. Data Set-4 (Western Scotland), among and within macro-environments

7.4.1. Coefficient of variation, Data Set-4 (Western Scotland)

The average coefficient of variation (CV) for observations within each locality ($n = 11$, including the specimens grown under controlled laboratory conditions) was similar among all localities ($8.4 \leq CV \leq 13.6$; Table 5). Localities could be separated into three groups based on the magnitude of variation across all characters: those with the greatest variation (CV = 13.6, ClaFer, KenFer, ClaSei), localities with an intermediate level of variation ($9.7 \leq CV \leq 11.2$, KenFer, BalDun, RonPoi, UllCar, ArdBro, GroPor, IonFer), and those with the least variation ($CV \leq 8.5$, RhuLig and GatLab).

Across all localities, characters associated with "width" were more variable (CV = 2.6), relative to characters associated with "length" (CV = 1.6 to 1.8 (Table 5)).

Table 5
Macro-environmental variation comparison of coefficients of variation (CV) for each character from each locality (Data Set-4) Fig. 2, GatLab = laboratory culture. Localities are sorted by average CV, calculated across all five morphologic characters. OW = opesia width, ZW = zooecium width, OL = opesia length, ZL = zooecium length, ZA – zooecium area, Ave. = average, Stdev = standard deviation.

Locality	Coefficient of variation						
	OW	ZW	OL	ZL	ZA	Ave.	Std.
ClaSei	13.6	11.2	11.0	12.1	20.4	13.6	3.9
ClaFer	16.2	14.6	10.2	9.4	17.7	13.6	3.7
KenFer	14.9	13.9	12.0	10.5	16.7	13.6	2.4
BalDun	10.5	8.8	13.6	10.9	12.2	11.2	1.8
RonPoi	11.4	10.7	9.9	9.0	12.3	10.6	1.3
UllCar	11.5	10.9	9.9	7.9	12.6	10.6	1.8
ArdBro	11.1	10.4	9.6	7.3	12.8	10.2	2.0
GroPor	10.6	9.5	8.7	6.6	12.9	9.7	2.3
IonFer	10.3	7.8	11.6	8.2	10.5	9.7	1.6
RhuLig	8.2	8.0	9.3	6.8	10.2	8.5	1.3
GatLab	7.8	6.1	7.5	9.8	10.8	8.4	1.9
ave.	11.5	10.2	10.3	9.0	13.5	10.9	
stdev.	2.6	2.6	1.7	1.8	3.3	1.9	

7.4.2. Principal component analysis, Data Set-4 (Western Scotland)

A scatter plot of localities (including the controlled, laboratory-grown specimens) using the first two principal component scores of the standardized data for the five morphometric characters (Fig. 4) shows clinal variation across both axes. Loading coefficients (Supplementary Data 4) show that PCA-one, accounting for 65.6% of the total variance, corresponds to the overall size of the five characters. Localities with smaller zooecia included GatLab, BalDun, GroPor, RhuLig, and those with overall larger zooecia are from ClaFer, ArdBro and ClaSei (Fig. 4). PCA-two, accounting for 24.8% of the total variance, corresponds to the shape of zooecia and opesia (long-narrow vs. short-wide, Fig. 4, Supplementary Data 4).

A scatter plot of scores on PCA-three and -four (Fig. 5) shows a spread of localities on PCA-three (6.2% of total variance), which reflects an inverse of the relative lengths of opesia to zooecium (e.g. modules with the largest opesia:zooecium length ratio plot to the far right, Fig. 5) (Supplementary Data 4). PCA-four (2.1% of total variance) differentiates the specimens grown under controlled laboratory conditions (GatLab), and to a lesser extent those from Iona Ferry from the other localities (Fig. 5). Those from GatLab had exceptionally wide opesia and long zooecia but overall were small, whereas those from IonFer had a larger overall zooecium size (Fig. 5).

Table 4
Meso- to micro-environmental variation summary of p-values and percent variance accounted for each factor for three-way (crossed and nested) ANOVA for Data Set 3 Environmental Gradients (Fig. 2). Abbreviations as for Table 2.

Character	Region	Position	Reg. × Pos.	$Colony_{[Reg \times Pos]}$	Residual
	(N, S)	(I, M, O)	(1–12)	(1–6)	
	%var $p(H_o^1)$	%var $p(H_o^2)$	%var $p(H_o^3)$	%var $p(H_o^4)$	%var
Opesia width, OW	2.8% ns	3.2% ns	3.4% ns	20.1%****	70.4%
Zooecium width, ZW	1.6% ns	2.8% ns	2.2% ns	18.0%****	75.4%
Opesia length, OL	6.2%**	17.2%****	3.2% ns	26.9%****	46.6%
Zooecium length, ZW	1.8% ns	10.7%**	2.6% ns	23.4%****	61.4%
Zooecium area, ZA	1.8% ns	9.6%**	2.3% ns	25.5%****	60.8%
Average	2.7%	7.9%	2.7%	22.7%	63.2%

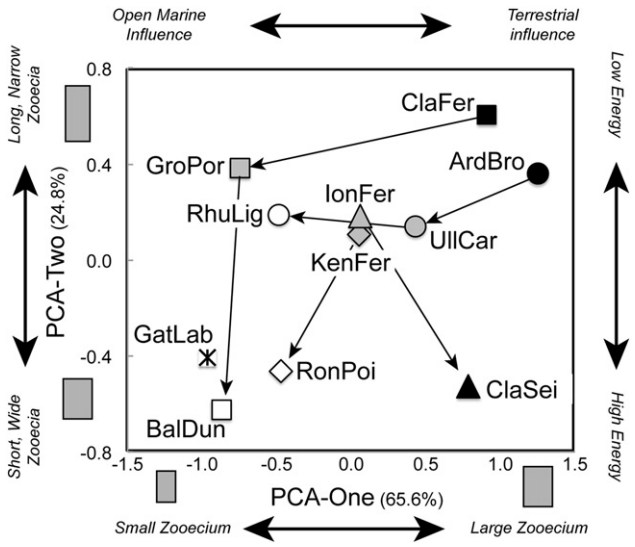

Fig. 4. Scatter plot for principal components one and two for average specimens of *Electra pilosa* among ten western Scotland localities plus those cultured in the laboratory. First two axes account for 90.2% (65.2 + 25.0) of total variance. Solid symbols represent more restricted localities (terrestrial influence) and white symbols are more exposed localities (open marine), with gray symbols at intermediate conditions. Absolute values of PCA loading coefficients for axes one and two are summarized for morphological features — PCA-Axis 1 = zooecium size (large to right, small to left), PCA Axis-Two = zooecium shape (long-narrow toward top, short wide toward bottom).

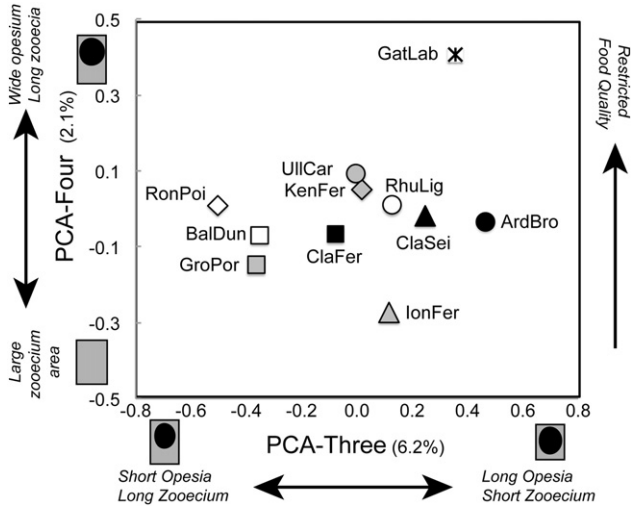

Fig. 5. Scatter plot for principal components one and two for average specimens of *Electra pilosa* among ten western Scotland localities plus those cultured in the laboratory. First two axes account for 90.2% (65.2 + 25.0) of total variance. Solid symbols represent more restricted localities (terrestrial influence) and white symbols are more exposed localities (open marine), with gray symbols at intermediate conditions. Absolute values of PCA loading coefficients for axes one and two are summarized for morphological features — PCA-Axis 1 = zooecium size (large to right, small to left), PCA Axis-Two = zooecium shape (long-narrow toward top, short wide toward bottom).

7.5. Summary of results across data sets

Based on the analysis of three data sets at different hierarchies of environmental variation, the greatest percentage of variance and level of significance was associated with variation at low levels, within and among colonies (Table 6). Relatively little of the total variance was associated with meso-environmental variation, and factors associated with this level were only once found to be significant (Table 6). Relatively little of the total variance was associated with macro-environmental factors, and this factor was significant only at extremes of environmental conditions (Table 6).

Characters associated with "length" (OL, ZL) were most affected by macro-environmental effects (Tables 4 and 6, high energy = short). The character zooecium length (ZL) was also most affected by the position of the colony (exposed vs. protected) on either side of a subtidal boulder (Tables 3 and 6, high energy = short).

8. Discussion

8.1. Genotypic signal in preservable skeletal morphology

Across all analyses, the second most significant source of variation (effect) for all five morphological characters was that among colonies (within meso-environments, Table 1, level IIIa). At this scale colonies were collected on the same or adjacent algal frond, ~2 cm to 2 m apart, and all within an environmental setting that cannot be differentiated into obvious, distinct microenvironments in either a modern or geologic setting. The source of the among-colony effect is due to (1) micro-environmental differences of unknown origin; (2) genotypic differences among colonies; or (3) a combination of the two. The present experimental design cannot allow for direct partitioning of variation between micro-environments (level IIIa) vs. genotypes; however, a number of lines of evidence suggest that this factor primarily reflects genetic differences among colonies.

In studies of other specimens of *Electra pilosa* grown under a variety of controlled laboratory conditions, with clonal replicates, the magnitude of the among-genotype effect (separated from among micro-environment and microenvironment × clonal replicate interaction) was 34.0% (Cheetham et al., 1994, Fig. 1, for *Stylopoma*), 26.5% (Hageman et al., 1999, for *E. pilosa*), and 27.7% (Hageman et al., 2002, for *E. pilosa*). These values are of the same magnitude as the among-colony (undifferentiated) variance components of the present study (28.2%, 22.6%, and 26.4% of Data Sets 1–3 respectively). Haplotype characterization (16s rDNA) of *E. pilosa* colonies within and among localities supports the expectation of detectable, distinctive genotypes among colonies at any one locality (Nikulina, 2008b).

There is no reason to believe that the magnitude of variation among the colony genotypes would be significantly less than those of colonies of the same species. Therefore, although some of the undifferentiated among-colony variation in this study may be due to meso-environmental differences (undetected within a macro-environment), a large part of the total observed morphological variation in all data analyzed here likely was due to genotypic differences among colonies (Tables 3–5).

Conclusion: Genetic differences among colonies account for a much greater amount of morphological variation than do macro- and meso-environmental effects (Table 1).

Implication: A very large portion of skeletal, morphological variation is due to genotypic control (in fossils).

If the importance of genetically controlled morphological variation among colonies is confirmed, this would reinforce previous findings in multiple studies of observable genetic control over zooecial scale morphology, regardless of the degree of environmental variation (Hageman et al., 2009).

8.2. Absence of "hidden" meso-environmental effects

Electra pilosa colonies typically have an area of several square centimeters but comprise multiple individual modules on a millimeter scale. This begs the question "How far away is the next bryozoan environment?" and has implications for understanding morphological variability in all aspects of its application. At the scale of an undifferentiated single environmental setting – in terms of substratum, wave/current energy, likelihood of sedimentation and turbidity (which are important to this suspension-feeder) – systematic (nonrandom) variation might arise from unobserved factors.

Table 6
Summary of the distribution of variance among hierarchical environmental levels based on the analysis of Data Sets 1–3. OW = opesia width, ZW = zooecium width, OL = opesia length, ZL = zooecium length, ZA — zooecium area.

Hierarchical level	Features	% of variance by level	Patterns of significance	Morphologic response
Macro-environmental ($I_{a\&b}$) $10°–10^2$ km^2 Regions (north–south) Positions (inner–mid.–outer)	Distinguishable facies or geographic setting	0% to 10% (7.5%)	Significant at extremes	OL, ZL
Meso-environmental ($II_{a\&b}$) $10°–10^2$ m^2 Stations Substations	Indistinguishable within macro-environment	0% to 5% (2.5%)	Rarely significant	ZL (one case)
Among nearby colonies $10^1–10^2$ cm^2 within stations & substations	Genotypic ± micro-environmental (*undifferentiated*)	20% to 40% (30%)	Usually significant	
Patches (positions) $10°–10^1$ mm^2 within colonies	Micro-environmental spatial and, or temporal (life history)	20% to 40% (30%)	Usually	ZW
Rows & columns (nearest neighbor packing) within patches	Micro-environmental spatial and, or temporal (life history)	5% to 15% (10%)	Always significant	Column (OW, ZW)
Residual 10^0 mm^2 zooecia within patch	Micro-environmental spatial and, or temporal (life history)	15% to 25% (20%)	–	–

However, none of the five morphological characters here showed significant levels of variation associated with meso-environments level-IIa, Table 1 (Tables 3–4, ClaSei and RonPoi). That is, within an environmental setting that appears to be homogeneous (and could not be subdivided using criteria from the geologic record), there is no reason to expect significant morphologic variation caused by "hidden" environmental sources over 10^1 to 10^2 m.

Conclusion: Within macro-environments there is no reason to expect significant, systematic morphological influences from undetected environmental sources.

Implication 1: In paleontological studies, when collecting specimens, even from a time-averaged fossil assemblage, if the setting appears to be a single micro-environment, one would not expect "hidden" meso-environmental effects.

Implication 2: In modern studies, the appropriate scale of replicates in growth panel or "common garden" studies (e.g., Vail and Tranter, 1981; Cheetham et al., 1994; Herrera et al., 1996; Lombardi et al., 2011) is the treatment and not the entire experiment. If one were to deploy an experimental frame accommodating multiple settlement panels for recruitment of colonies in contrasting environmental settings, one would not expect significant morphological differences either at the scale of replicate panels within the frame or at the among-frame scale with arrays set adjacent to one another. In a common garden setting, the meso-environmental variation (10° to 10^2 m) is not expected a priori to be significant for zooecial morphology.

In one case (Zooecium length, ZL, between positions of exposed vs. protected boulder side, RonPoi) there was significance, $p \leq 0.01$, at the meso-environmental level-IIb. However, even in this case, the Position effect accounted for only a small amount (2.6%) of the total variance. We would not preclude the possible relevance of such meso- or micro-environmental factors in other environmental settings, such as colonies on an algal holdfast being subject to rather different micro-environmental conditions compared to colonies located toward the distal margins of the lamina of the alga. But as in the case observed here, these factors are potentially both observable and testable.

8.3. Detectable macro-environmental effects (environmental gradient)

At extremes of their occurrence – range limited by exposure to high energy waves (RonPoi, RhuLig) vs. protected, calm water with terrestrial influence near the head of sea lochs, (ArdBro, ClaFer) – subtle, but systematic (nonrandom) morphological differences can be detected (Tables 2–3, Fig. 4). These trends are apparent in two independent environmental clines separated by 150 km (north ArdBro to RhuLig and south ClaFer to BalDun) (Fig. 4). A gradient in zooecium size is associated with the environmental distribution of colonies ranging from protected areas (larger zooecia) to those in more open coastal marine settings (smaller zooecia) (Fig. 4, PCA-one).

A relationship also is apparent between zooecium shape and macro-environmental extremes, i.e., colonies from low energy settings (long and narrow zooecia) versus higher energy settings (short and wide zooecia) (Fig. 4, PCA-two). Morphological variation within each gradient (~50 km) from protected/low energy to open coast/higher energy environments was of greater magnitude and significance than variation between the two sites separated by about 150 km.

The causal factor(s) of these morphological trends could not be tested directly from data presented here, but on the basis of other studies (Jebram, 1973, 1975, 1980; Okamura, 1984, 1987; Best and Thorpe, 1994; Riisgård and Goldson, 1997; Hermansen et al., 2001; Hageman et al., 2009), we suggest that nutrient levels and wave/current energy likely play an important role. The colonies with the largest zooecia are from locations proximal to sea loch entrances (ClaFer and ArdBro, Figs. 2, 4A). A third location with large zooecia is the tidal rapid at Clachan Seil (ClaSei). In contrast, colonies grown under controlled laboratory conditions (GatLab) with an optimal diet of *Rhinomonas* sp. monoculture 10,000 cells $(\mu l)^{-1}$, have some of the smallest zooecia in this study, suggesting that nutrients (both primary production and perhaps proximity to terrestrial organic and inorganic inputs) play a greater role in determining zooecium size than does wave energy.

Okamura and Partridge (1999), in studying variable flow rates associated with *Membranipora membranacea* encrusting algal fronds, showed that slower currents result in elongate, nearly rectangular zooecia, whereas faster flows result in shorter, more hexagonal, zooecia often possessing sinuous walls. Similar results are seen for shape in *Electra pilosa* (Fig. 4, PCA-two), long-narrow = lower energy, short-wide = higher energy, maintaining straight walls in both conditions. Although flow rate affected zooecium shape for *M. membranacea*, it did not affect zooecium area (Okamura and Partridge, 1999). Likewise in this study, zooecium size (area) does not correspond directly to current energy (Fig. 4, PCA-one).

The shape of zooecia does appear to be related to the wave energy in the water column. Colonies from more protected settings have longer and narrower zooecia, regardless of their size (ClaFer, ArdBro, GroPor, Fig. 4, PCA-two), than do colonies growing in more wave-exposed locations (BalDun, RonPoi, Figs. 2, 4). Short/wide zooecia also are present in colonies from ClaSei (Figs. 2, 4), which are subject to diurnally intense bi-directional tidal currents but consistently negligible wave action.

A further environmental factor that can affect zooecium size is water temperature (Okamura and Bishop, 1988; O'Dea and Okamura, 1999; Okamura et al., 2011). Within a colony, zooecia that form under warmer water conditions will be smaller, with size and temperature differences inversely correlated (O'Dea and Okamura, 2000a, 2000b). In the present

study, the seasonal range of water temperature (~min. 7° to max. 14°), is sufficient to expect within-colony zooecium size variation (O'Dea and Okamura, 2000b; Okamura et al., 2011) but temperature gradients within and among environments at any given time probably are insufficient to account for the majority of morphologic variation. The average temperature difference between northern and southern localities is about 0.5° (www.seatemperature.org and www.metoffice.gov.uk). The temperature gradient from the inner-protected sites to outer-exposed sites is about 1 °C in the south and 0.5° in the north.

Conclusion: Morphological extremes observed in this study are representative of the natural range of environments occupied by *E. pilosa* in western Scotland. Zooecial morphology follows a continuum within the range of environments where colonies can survive. The observed limits of colony survivability (algal substratum availability) were reached before the limits of any observable "zooecial viability" were crossed in a morphological threshold.

Implications: Morphological species concepts are not compromised in the case of *E. pilosa* by macro-environmental variation at its environmental extremes.

8.4. Ecophenotypic variation in solitary organisms: within-colony variation

In order to directly study ecophenotypic (environmentally induced) variation in a solitary animal, be it invertebrate or vertebrate, a population of clones is needed. Thus, any morphological variation observed among multiple individuals with identical genotypes can be attributed to differences in their known life histories (environment). Although laboratory cloning techniques are becoming more routine, naturally clonal organisms, such as bryozoans, do allow for direct partitioning of morphological variance into its non-genetic, within-genotype variance.

Within-colony variation typically is large for Bryozoa (30% to 60%, Cheetham et al., 1994, Fig. 1; Hageman et al., 1999). In this study, the within-colony variation (Patch + Residual) was comparable at 61.5%, 63.2% and 72.8%. A portion of the within-colony variation can be accounted for by the position of zooecia within the colony (Patch). In this study 12.2% of ClaSei and 36.7% of RonPoi variation resulted from position within the colony (microenvironment level-IIIb, Table 1). These values also are comparable to those observed in *E. pilosa* specimens grown under controlled laboratory conditions (minimized environmental differences), whereby position within colonies accounted for about 30% of total variance (Hageman et al., 2002). The unexplained residual variation was comparable among all Data sets at 21.1–27.1%. Under careful experimental design, allowance for rows and columns (nearest neighbor positions) within patches of zooecia result in a further 10–12% of variation to be accounted for by positional effects (Hageman et al., 2002), thereby reducing the residual to <20%.

Here, variation within colonies (micro-environmental scale of 0.5 to 5 cm between patches of zooecia from separate parts of the same colony) was significant for all cases except one (Tables 2 and 3, $Patch_{[Colony]}$), and consistently accounted for a large portion (13% to 45%) of the total variance (Tables 2 and 3).

Conclusion: A large portion of within-colony (non-genetic) variation is due to micro-environmental variation — either spatially along the substratum, or temporally as conditions varied over the growth history of the colony (spatiotemporal).

Implication: In an assemblage of organisms (time-averaged fossil or modern), a large part of the morphological variance is likely due to differences in life history (micro-environment level-IIIa). The magnitude of these effects is expected to be much greater than systematic (but undetected) variation at the meso-environmental, or even macro-environmental levels.

Conclusion: The magnitudes of the residual variance (not accounted for by the model) are comparable among studies of different scales (macro-environmental Data Set 4, meso-environmental Data Sets 1 & 2, or laboratory setting with minimal environmental variation (Hageman et al., 1999, 2002)).

Implication: The variance not accounted for by the hierarchical models of environmental variation from different scales is a result of independent variation within a colony and not undetected macro- or meso-environmental variation.

Implication: In an assemblage (fossil or modern) of solitary organisms much more of the morphological variation among individuals is due to differences in life history of the individual rather than to systematic macro- or meso-environmental effects; but life-history effects do not obscure the significant genotypic effects.

8.5. Specific morphological characters associated with environmental levels

The functional explanation for the association of the shape of the zooecium and opesia with macro-environments remains unclear, but predictive patterns invite future study. Long, narrow zooecia were characteristic of lower energy environments (with the exception of the laboratory-reared specimens) and short and wide zooecia were typical of higher energy, wave- and current-dominated environmental settings. Variation in opesia size (length and width) was associated with the macro-environmental gradient from protected- to wave-exposed environments (Table 3, Fig. 4a). Opesia were shorter and the zooecium longer at more exposed sites (Fig. 4b). Smaller, shorter zooecia (Fig. 6) are apparently a response to higher energy environment (RhuLig, BalDun, RonPoi), or nutritional limitation (food monocultures for GatLab colonies). Larger and wider zooecia are present in calm water column settings (ArdBro, ClaFer, ClaSei, Fig. 6).

8.6. Electra species diagnoses, species concepts and the role of morphometrics

The morphometric characters employed here are routinely reported in systematic descriptions of *Electra pilosa* and related species (e.g. Winston and Hayward, 2012; Nikulina et al., 2013a, 2013b). In some cases the absolute size of the feature is diagnostic at the species level, but more typically it is the relationships among the characters (e.g. shape of opesia or size of the opesia opening relative to total zooecium length) that are more important for differentiating between and among species of *Electra* (e.g. Nikulina, 2007, 2008b; Winston and Hayward, 2012; Nikulina et al., 2013a).

It is important to recognize that although morphologic characters varied in this study, they did not cross a threshold of shape change that would invite even speculation of assignment to another new or existing species within *Electra*, which are now robustly supported with molecular phylogenies (Nikulina et al., 2007; Nikulina, 2008b; Nikulina et al., 2013b). More importantly, the characters that are used in modern systematics for species differentiation, e.g., colony budding patterns, zooid shape, opesia size and shape, gymnocyst size (Winston and Hayward, 2012; Nikulina et al., 2013a, 2013b) were invariant at the scale of species differentiation for all specimens in this study. These features are essentially invariant over the environmental range that characterizes the distribution of the species within Scotland.

Conclusion: Morphometric features can be used as a proxy for a detectable portion of genetic differences at the level of the individual (genotype) to species or even genus.

Implications: Morphology is useful to track evolution but, in this case, zooecium-level morphology is correlative with the other discrete characters that are use to define and identify the species.

9. Broader implications for study of micro-evolution and speciation from the fossil record

We acknowledge that before the present results can be generalized to hypothesis level, there is a need to test these models more fully by their application to a wide variety of taxa and environments. Nonetheless, we believe that the implications for the treatment and applicability of fossils to the study of evolutionary biology, and particularly

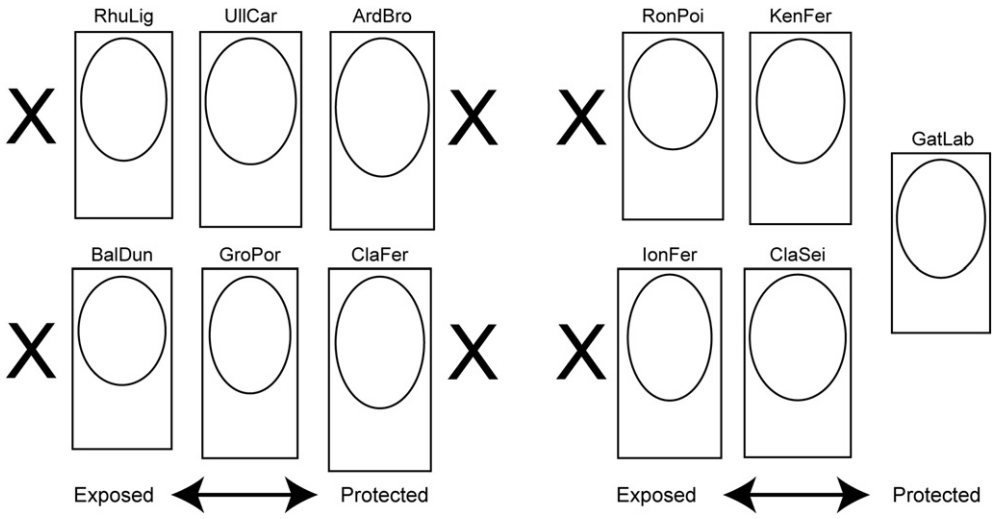

Fig. 6. Range of zooecium and opesia morphology (to scale) induced by Macro-environmental variation (among localities). "X" denotes limits of the distribution of *Electra pilosa* along environmental gradients (Fig. 2).

microevolution, are not trivial. This may be particularly pertinent to questions regarding the place of "morpho-taxa" concepts in interpreting rates of genetic evolution.

If:

1. The two greatest sources of morphological variation are genotypic and individual life history; *and*
2. Macro-environmental effects are detectable, but never obscure the morphological variation resulting from the two main sources in magnitude or significance; *and*
3. Factors of potentially unidentified meso-environmental environmental influences are non-significant or nearly so, *then*
4. Morphologic variation in the fossil record does represent a significant, measurable amount of genetic variation and can be used as a meaningful proxy for evolution of the genotype.

The impact of environmental variation on morphology in bryozoans is converging on some predictable ranges (Fig. 7). Additional studies on other bryozoan groups and environmental conditions will strengthen our understanding of these patterns and their appropriate applications. In many ways, these results may appear to confirm the intuitively obvious; that is, a great deal of morphological variation can be accounted for by small-scale spatiotemporal variation in life history (e.g., Reznick et al., 1990). The important contribution of this study is the clarification that neither the sizable life history effects (spatiotemporal micro-environment), nor macro-environmental effects at their extremes, obscure the among-colony genotypic effects on morphology. Furthermore, the results suggest that undetected/cryptic meso-environmental effects are minimal and are not expected to confound direct interpretation of genotypic effects on morphological variation.

The next challenge is to constrain these patterns of variation with their attributable sources to solitary organisms (Fig. 7). Although this is not possible in a single experimental study, the values are not unknowable. We can start with direct extrapolation from the observations shown here (Fig. 7) as a working hypothesis, which can be tested with controlled experimental designs. The goal is to document the relationships between genotypes, the environment and morphology in a way that allows for predictive models that can be incorporated into the study of microevolution and speciation in the fossil record.

10. Summary

(1) Micro-environmental variation (within-colony, mm- to cm-scale): the most significant source of morphological variation (~60% of total) for *Electra pilosa* colonies growing on algal substrata from ten sublittoral localities in western Scotland was attributable to within-colony, micro-environmental effects. Within-colony variation can be further partitioned by the spatiotemporal position within the colony (~30%), local packing arrangement of the individual module (~20%), and the Life History of the individual module. These results are commensurate with other studies of partitioned morphological variance in bryozoans.

(2) Among-colony variation (among-colony, cm- to m-scale): variation among colonies within a common meso-environment (cm to m-scale) was highly significant and accounted for a large proportion (~30%) of the total variance. Although experimental design did not allow for direct partitioning of environmental vs. genotypic effects, the magnitude of variation (~30%), and level of significance, was directly comparable to results from laboratory-grown *E. pilosa* (clonal replicates of multiple genotypes grown under shared and controlled environmental conditions). Based on the absence (~0% variance explained) of additional levels of variation that could be attributed to environmental sources, among-colony variation in this study was also strongly associated with a genotypic effect. The implication is that a significant genotypic effect is detectable in the preservable skeleton, and will be so for fossils.

(3) Meso-environmental variation (m- to km-scale): meso-environmental effects had minimal significance and accounted for the least proportion of morphological variation (~2.5%). Systematic (nonrandom) variation was minimal among colonies collected within a local environmental setting that did not display discernible environmental differences based on sedimentological or biological facies. Thus, within an apparently homogeneous meso-environment, one would not expect systematic, undetected environmental variation over the scale of meters to kilometers. Furthermore, at the resolution of detectable environments within the rock record based on careful, detailed analysis, "hidden" non-random variation due to environmental effects directly attributable to unknown sources within meso-environments are not pervasive, and should not be expected a priori.

If and when systematic morphological variation is observed at the Meso-environmental level, one should actively seek – and, with a degree of confidence, expect to identify – the environmental factor(s) affecting morphology. Any hypothetical explanation that meso-environmental sources of morphologic variation are pervasive, confounding, unresolvable and undetectable must be defended with independent, empirical data.

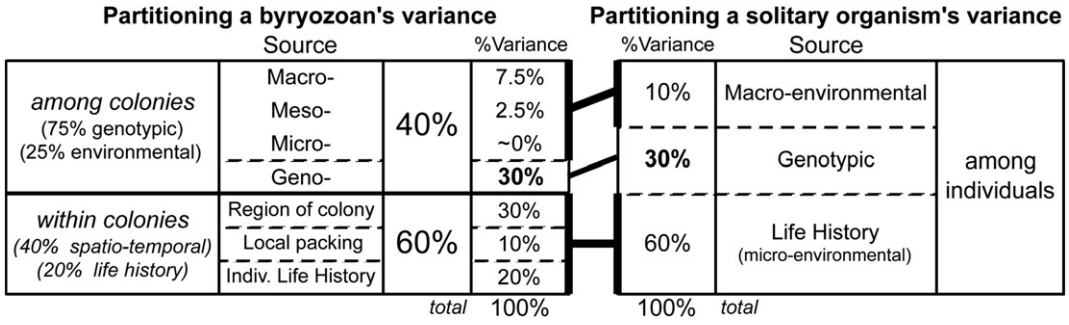

Fig. 7. Partitioning morphologic variation into sources, with the percentage of variation accounted for by each effect (after, Hageman et al., 2002; Hageman and Sawyer, 2006; Hageman et al., 2011). Variance percentages for individuals organisms derived from multiple bryozoan observations summarized here.

(4) Macro-environmental variation: extremes of macro-environments (sheltered inshore vs. wave-exposed coast) induced significant morphological variation (~7.5% of total). Variation in the size of modules (zooecia) among environments probably was due to a combination of wave energy and nutrient supply variations. Temperature may have induced variation within colonies, but temperature differences among localities of the same environment (e.g., among inner protected localities ± 0.5° at any one time) were insufficient to account for module size differences among localities. Macro-environmental condition was more important than absolute geographic distance in controlling morphology. Thus, colonies from the same macro-environment – but separated by 100s of km – were more similar morphologically than colonies from different macro-environments separated by 10s of km.

(6) Ecophenotypic variation and species concepts: morphological characters used to differentiate *E. pilosa* from other species in the genus were invariate in all specimens. The limits of morphologic variation for the module size and shape characters studied correspond to the limits of environmental tolerance of the species. Thus, for *E. pilosa*, environmental factors do not confound species identification.

(7) Pooling sources of environmental variation and extrapolation to solitary/unitary organisms: if conclusions are extrapolated to evolutionary studies of non-colonial animals, the most significant sources of variation are 1) life history-related and micro-environmental, 2) genotypic, 3) macro-environmental [which typically is interpretable in the geologic record], and finally 4) meso-environmental. This latter typically is not detectable, and plays a minimal role in the observed morphological variation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.palaeo.2014.01.015. These data include Google map of the most important areas described in this article.

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